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XXI CICLO

## Novel pathways induced by melatonin on leukocytes: possible pharmacological and inflammatory perspectives

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*Alla mia famiglia*

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# ABBREVIATIONS

AD        Alzheimer's Disease
AIF          Apoptosis inducing factor
Apaf-1   Apoptotic Protease-Actvating Factor-1
Bcl-2        B-cell leukemia/lymphoma-2
BH        Bcl-2 Homology Domain
BIR       Baculoviral IAP Repeat
BSA      Albumina di siero bovina
BSO      L-butionina-(S,R)-sulfoximina
CAD      Caspase-Activated DNAse
CARD   Caspase Recruitment Domain
CCE      Capacitative $CA^{2+}$ Entry
CAPE   Caffeic acid phenethyl ester
Cyt c     Citocromo c
DAG     diacilglicerolo
DD        Death Domain
DED      Death Effector Domain
DHCFDA Diclorodiidrofluoresceina diacetato
DISC    Death Inducing Segnaling Complex
DMSO  Dimetil sulfossido
DTT      Ditiotretolo
EBV      Epstein Barr Virus
ECL      Enhanced chemiluminescence
EGTA   Etilene glicol-bis (β-aminoetil etere)
FADD   Fas-Associated Deathe Domain
FCS      Serum Fetal Calf
GAPDH Gliceraldeide-3-fosfato-deidrogenasi
GSH      Glutatione ridotto
GSSG    Glutatione ossidato
$H_{42}$        Hoechst 33342
HBSS    Hank's balanced salt solution
HETE    Hydroxyeicosatetraenoic acid
HIOMT  Idrossindolo-O-metiltranferasi
IAPs     Proteine inibitrici di apoptosi

IP        Ioduro di Propidio
$IP_3$      Inositolo 1,4,5-trifosfato
LOX     Lipoossigenasi
L-NAMEN$^G$-nitro-l-arginina metilestere
MAPK   Mitogen-Activated Protein Kinase
Mel      Melatonina
MT$\Delta\psi$   Potenziale di membrana mitocondriale
MPTP    1-metil-4-fenil-1,2,3,6-tetraidropirina
MTR     Mito Tracker Red
NCCE    Non-Capacitative $Ca^{2+}$ Entry
Neo      Neomicina
NO       ossido nitrico
NOS     NO Sintetasi
PAF      Platelet Activating Factor
PARP    poli(ADP-ribosio)polimerasi
PBS     Phosphatate Saline Buffer
PD       Parkinson's Disease
PHA     fitoemmagglutinina
$PI_3K$     fosfatidilinositide-3'-OH chinasi
$PIP_2$    fosfatidilinositolo-3,4,5-trifosfato
PKB     Protein chinasi B
PKG     Protein chinasi G
$PLA_2$    fosfolipasi $A_2$
PLC     fosfolipasi C
PMC    puromicina
PS       fosfatidilserina
PTP     Permeability Transition Pore
PUFAs   Polyunsaturated Fatty Acids
ROS     Reactive Oxygen Species
SD       Deviazione Standard
SMAC    Second Mitochondrial Activator of Caspases
SNAP    S-nitroso-N-acetilpenicillamina
SOD     superossido dismutasi
TGF     Tumor Growth Factor
TLC     Cromatografia su strato sottile
TNF     Tumor Necrosis Factor
TRADD  TNF-Associated Death Domain
VP16    Etoposide

# ABSTRACT

Melatonin, in addition to its main role as regulator of circadian rhythms, has recently been shown to modulate immune functions by controlling the behaviour of leukocytes, which are indeed able to synthesize melatonin and possess the specific high affinity (1nM) plasma membrane receptors (MT1 and MT2). Great interest is receiving the ability of melatonin to contrast apoptosis, a well accepted fact whose mechanisms however are still quite controversial. In this study, we analyze the mechanisms involved in the anti-apoptotic effect of melatonin in normal and tumor leukocytes. We have shown that this effect is due to two different, cooperating mechanisms, involving two primary targets melatonin directly interacts with, i.e., MT1/MT2 receptors; and calmodulin, a known melatonin low affinity (63uM) target. Receptor engagement and calmodulin binding give rise to two independent signal transduction pathways, consisting of a canonical MT1/MT2 receptor mediated signal transduction (involving G protein and phospholipase C) on the one side, and calmodulin/phospholipase A2 (a known calmodulin interactor)/5-lipoxygenase (LOX) activation culminating in 5-HETE production, on the other. These pathways converge into melatonin anti-apoptotic effect at the mitochondrial level, preventing the activation of Bax, the key trigger of the intrinsic apoptotic pathway. The novelty of this finding is that Bax is maintained within mitochondria in an anti-apoptotic state. Indeed, melatonin causes the translocation of Bcl-2 to mitochondria, which directly binds to Bax inhibiting its activation/dimerization. The anti-apoptotic effect is completely abrogated if one or the other pathway is inhibited. The necessity of the low affinity calmodulin binding explains the requirement of high melatonin doses (>100uM). The involvement of 5-LOX in the anti-apoptotic effect of melatonin is particularly intriguing since, the recruitment of a key enzyme of the inflammatory response may shed new lights on the role melatonin plays in the regulation of the immune response. Moreover, LOX activation implies a burst of free radicals that immediately (<1min) and strongly (up to 15folds) follows melatonin administration, peaking at 2hrs to go back to normal values at 6hrs. This is a biological pro-oxidant effect that co-exists with, and contrasts, the well known chemical radical scavenging ability of the melatonin molecule.

# INTRODUCTION

# 1. Apoptosis: cell death signaling

There are generally two principal mechanisms of cell death; necrosis and apoptosis. Necrotic death and apoptotic death are characterized by biochemical and morphological differences. Necrosis is accidental and not well regulated. Cell undergoing necrosis swell, due to increased osmotic pressure, and will lyse with release of cellular contents into the surroundings, which might induce inflammation (1).

Conversely, apoptosis is a regulated physiological process. Cell committing apoptotic cellular suicide rapidly shrinks and loses their normal intracellular contacts. Morphological changes associated with apoptosis include blebbing of the plasma membrane, nuclear condensation, DNA fragmentation, and segregation of the cell into a number of apoptotic bodies that are phagocytosed by macrophages (2). Apoptosis is involved in cell maintenance; development, tumor regression, cell-mediated immunity. It occurs during certain pathological states (ischemia-reperfusion damage, infarction, neurodegenerative diseases and viral or chemical toxicity) and can be triggered by activation of certain death receptors on the plasma membrane receptor (extrinsic pathway) or by cellular stress (intrinsic pathway) (3).

The mitochondrial or intrinsic pathway and the trans-membrane or extrinsic pathway are the two principal pathways leading to apoptosis both of which converge on the activation of caspases, a cysteinyl aspartate proteases that cleave regulatory and structural molecules, coordinating the efficient dismantling of doomed cells (**Fig.1**).

Caspases are divided in two groups; initiator and effector caspases. The recruitment and activation of the initiator caspases by adaptor proteins causes subsequent activation of downstream (effector) caspases. The initiator caspase for the mitochondrial pathway is caspase-9 (4, 5), whereas the initiator caspases for the extrinsic pathway are caspase-8 and -10 (4). Both pathways share effector caspases-3 -6 and -7 (6, 7).

*The extrinsic pathway*

Caspase activation by the extrinsic pathway involves the binding of extracellular death ligands, such as FasL or tumor necrosis factor-α (TNFα) to transmembrane death receptors (8). Engagement of death

receptors with their ligands provokes the recruitment of adaptor proteins, such as the Fas-associated death domain protein (FADD) which recruit and aggregate procaspase-8 promoting its autoptocessing and activation (9). Active caspase-8 then proteolytically processes and activates caspase-3 and -7, provoking further caspase activation events that culminate in substrate proteolysis and cell death (10).

In some cells, most notably hepatocytes, extrinsic pathway can crosstalk with the intrinsic pathway through caspase-8-mediated proteolysis of the BH3-only protein Bid, generating a truncated form of Bid (t-Bid) which can promote mitochondrial cytocrome c release and assembly the apoptosome (11, 12).

*The intrinsic pathway*

In the intrinsic pathway, diverse stimuli that provoke cell stress or damage tipically activate one or more intracellular sensors which are responsible to mitochondrial outer membrane permeabilization (MOMP) (13).

The mechanisms responsible for MOMP during apoptosis remain controversial, although it is clear that many proteins can inhibit or prevent MOMP by local effects on mitochondrial membranes. In general, two classes of mechanism have been described and each may function under different circumstances: those in which the inner mitochondrial membrane participates (14), and those involving only the outer membrane (15) (**Fig.2**).

Permeabilization of the outer mitochondrial membrane induces the release of pro-apoptotic molecules from the mitochondrial intermembrane space, such as cytochrome c, Smac/DIABLO, HtrA2/Omi, which induce caspase activation and apoptosis inducing factor (AIF) and endonuclease G which directly induce nuclear modification (16).

In the cytosol cytochrome c, in presence of ATP, induces the oligomerization of apoptosis protease activating factor-1 (Apaf-1), thus promoting caspase 9 activation and allowing apoptosis to take place (17).

The release of Smac/DIABLO and HtrA2/Omi neutralizes the inhibitory effects of IAPs proteins on caspase-3, -7 and -9 (18).
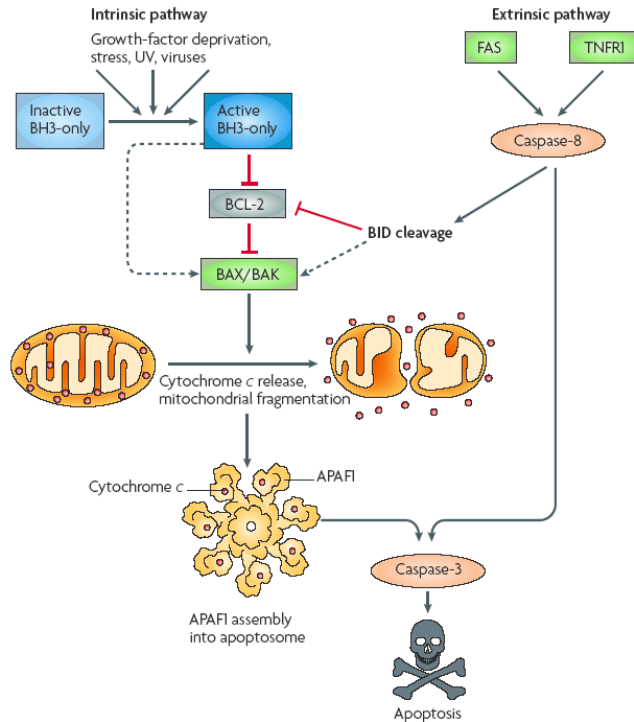
**Fig.1    Apoptotic pathways.** Apoptosis can be induced by cell surface receptors, such as Fas and tumour necrosis factor receptor-1 (TNFR1) (extrinsic pathway, right), or by various genotoxic agents, metabolic insults or transcriptional cues (intrinsic pathway, left). The intrinsic pathway starts with BH3-only protein induction or post-translational activation, which results in the inactivation of some BCL-2 family members. This relieves inhibition of BAX and BAK activation, which in turn promotes apoptosis. Some BH3-only proteins, such as BIM and PUMA, may also be able to activate BAX and/or BAK (as shown by the dotted line). Once activated, BAX and BAK promote cytochrome *c* release and mitochondrial fission, which leads to the activation of APAF1 into an apoptosome and activates caspase-9 to activate caspase-3. Caspases in turn cleave a series of substrates, activate DNases and orchestrate the demolition of the cell. The extrinsic pathway can bypass the mitochondrial step and activate caspase-8 directly, which leads to caspase-3 activation and cell demolition. The BCL-2 family regulates the intrinsic pathway and can modulate the extrinsic pathway when cleavage of BID communicates between the two pathways (3).
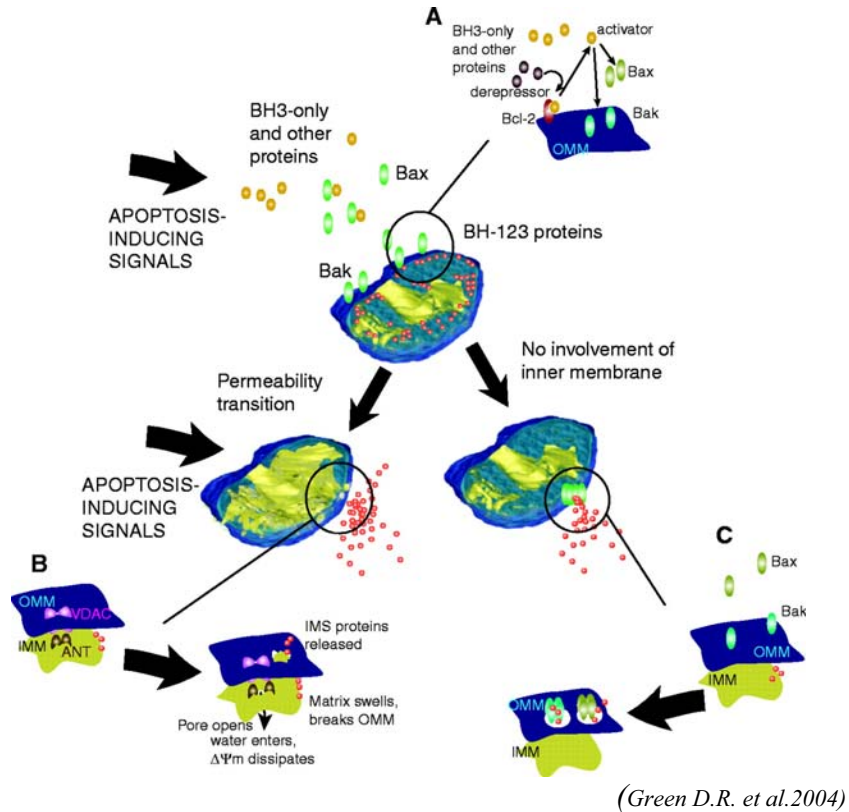
**Fig. 2  Mechanisms for  MOMP during  apoptosis.** (**A**) Signals for the induction of apoptosis (top) engage the activities of a subgroup of pro-apoptotic, BH3-only members of the Bcl-2 protein family and other proteins, which in turn activate the pro-apoptotic, BH123 proteins Bax and Bak to oligomerize and insert into the outer mitochondrial membrane (OMM). Other BH3-only proteins can act indirectly by releasing the first subgroup of BH123-activators from the anti-apoptotic Bcl-2 family proteins that sequester them. The BH123 proteins engage either of the two mechanisms that follow, perhaps depending on cell type or other conditions. (**B**) In PT-dependent MOMP, apoptosis-inducing signals act directly or indirectly to open the putative PT pore. This is composed of ANT or other proteins in the inner mitochondrial membrane (IMM) and is associated with VDAC and perhaps other proteins in the OMM. Opening the pore allows water to enter the matrix and ions to equilibrate, dissipating $\Delta\Psi$m at least transiently. The matrix swells, rupturing the OMM to release proteins of the mitochondrial intermembrane space (IMS). (**C**) In PT-independent MOMP, BH123 proteins, perhaps with other proteins, cause the formation of pores in the OMM through which IMS proteins are released (13).

## 2.  The Bcl-2 protein family: modulators of cell death

The Bcl-2 family proteins are critical death regulators that reside immediately upstream of mitochondria and consist of both anti- and pro-apoptotic members. The anti-apoptotic members function to block MOMP, whereas the various pro-apoptotic members promote it (19).

*Structure and function*

Bcl-2 family members posses conserved α–helices with sequence conservation clustered in Bcl-2 homology (BH) domains. Anti-apoptotic members exhibit the homology in all segments BH1 to 4, while pro-apoptotic molecules lack sequence conservation of the first α–helical BH4 domain and can be subdivided into "multi-domain" and "BH3-only" proteins (**fig. 3**) (20). Multi-domain pro-apoptotic members such as Bax and Bak display sequence conservation in BH1-3 domains. BH3-only members display sequence conservation only in the BH3 region.

Bcl-2 family members have classically been grouped into three classes, according to their structure and function:

- the pro-survival proteins, Bcl-2  Bcl-XL, Bcl-W, A1, Mcl1 (myeloid-cell leukaemia sequence 1)
- the pro-apoptotic Bax/Bak-like proteins, Bax,BaK, Bokand Bcl-XS
- the pro-apoptotic BH3-only proteins, Bad, Bik, Bid, Hrk, Bim, Noxa, Puma, Bmf

The two subfamilies of pro-apoptotic members are functionally distinct. BH3-only proteins act as a sensor of cellular damage and initiate the death process (21). The multi-domain protein Bax and Bak probably act downstream of the BH3-only proteins (22).

*Conformational changes during apoptosis*

Both pro- and anti-apoptotic Bcl-2 family members undergo conformational changes during apoptosis.
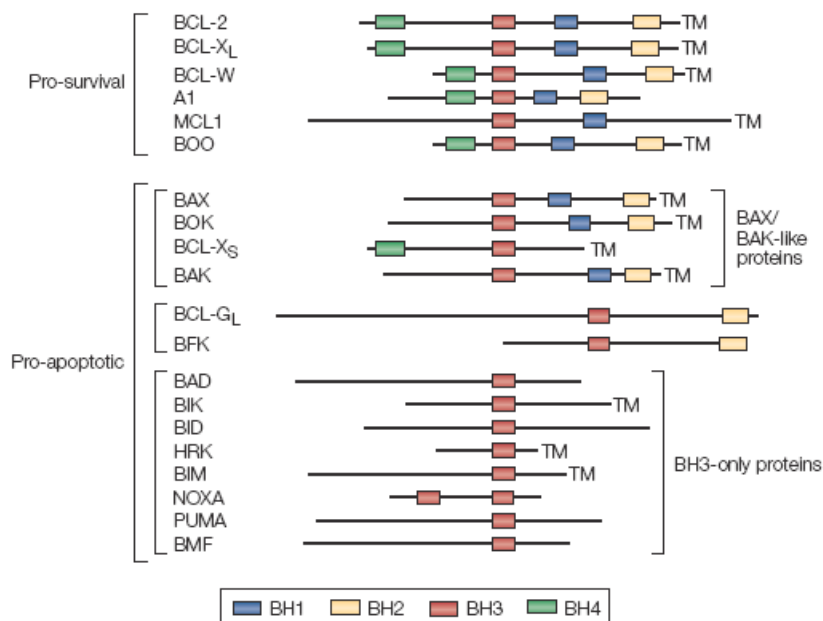
**Fig.3 The Bcl-2 family members** A diagrammatic representation of the mammalian B-cell lymphoma 2 (BCL-2) family is shown (20).

For example, Bax, in healthy cells, is predominantly a soluble monomeric protein in the cytosol (23). In response to apoptotic stimuli (24) or oxidative stress (25), it is activated and translocates to mitochondria where Bax changes conformation to reveal a hidden epitope in its N-terminus (26), forms oligomeric complexes (24), possibly with BAK, and inserts into the mitochondrial membrane triggering permeabilization of the outer mitochondrial membrane (27).

Bcl-2 also changes conformation during apoptosis owing to binding of BH3-only proteins (28). In healthy cells Bcl-2 is associated with the membranes of various organelles including endoplasmatic reticulum, mitochondria and nuclei (29). Bcl-2 may bind BAX and BAK after they insert into membranes and further inhibit Bax or Bak oligomerization to promote cell survival (30). However, BH3-only proteins can bind with

high affinity to pro-survival Bcl-2 proteins by promoting a change in the conformation of Bcl-2 and trigger apoptosis (28). It has been reported that at least some of BH3-only proteins, in particular Bid and Bim, can bind not only to pro-survival Bcl-2 members but also to Bax and/or Bak (31, 32). It has been postulated that Bim and Bid are directly initiator of apoptosis, whereas Bad and Bik sensitize cells to death stimuli by reducing the level of "free" pro-survival Bcl-2 proteins, binding to their as a "depressor" (33)
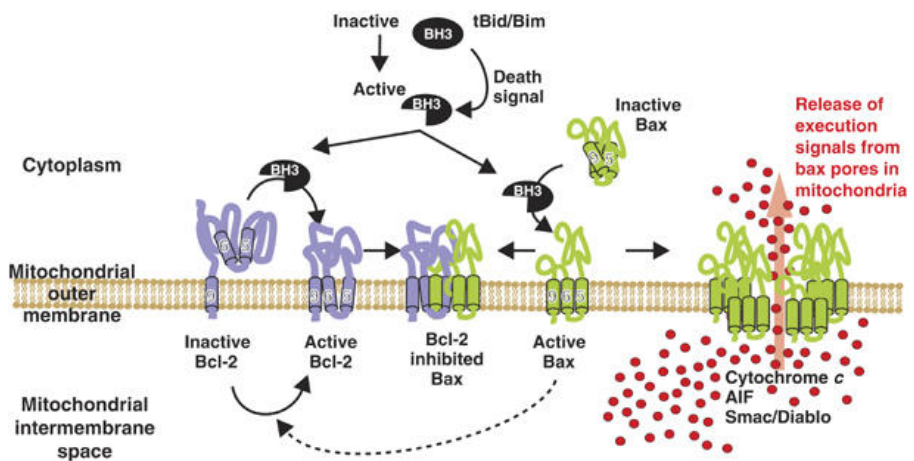


(Dlugosz PJ et al. 2006)

**Fig.4 Conformational change of Bax and Bcl-2.** Conformationally changed Bcl-2 inhibits membrane-bound activated Bax. In dividing cells, Bcl-2 is constitutively bound to intracellular membranes by a carboxyl-terminal tail-anchor sequence including α-helix 9. This form of Bcl-2 is inactive for preventing Bax oligomerization, but is probably a homodimer with other antiapoptosis functions, and Bax is an inactive monomer located in the cytoplasm or loosely bound to mitochondria. Death signals activate BH3-only proteins (tBid, Bim), which cause changes in the membrane conformation of both Bax and Bcl-2: Bax translocates and inserts into organellar membrane and Bcl-2 changes conformation such that cysteine 158 (α-helices 5 and 6) are inserted into the membrane (28).

*Altering mitochondria*

Bcl-2 family members interact with mitochondria either constitutively or on induction of apoptosis by their impact on the OMM (19).

The OMM becomes permeable to soluble intermembrane space proteins when Bax oligomer (maybe a tetramer) form a pore (24) large enough for the release of apoptogenic factors such as cytochrome c (34) and apoptosis-inducing factor (AIF) (35) into the cytoplasm, which activates the downstream executional phase of apoptosis (**fig.5A**) (36). Anti-apoptotic proteins could either prevent formation of the Bax pore or modify the structure of the pore such that it would be unable to allow the efflux of mitochondrial proteins (28). Furthermore, the BH3-only protein Bid can sinergized with Bax to cause cytochrome c release by activating Bax or by preventing anti-apoptotic Bcl-2 proteins from inhibiting Bax and Bak (19). Alternatively, Bax was proposed to modulate opening of resident mitochondrial channels such as VDAC and to stimulate their opening (**fig. 5B**) (36, 37).
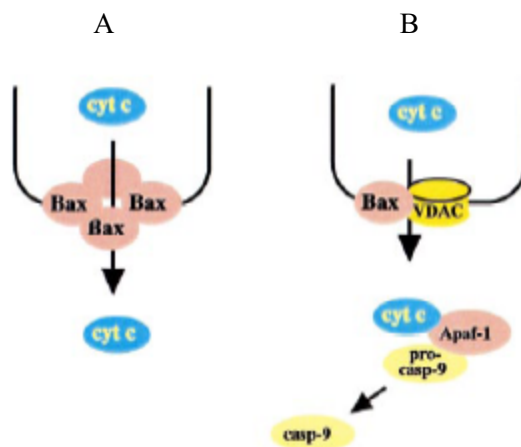


(Tsujimoto Y. and Shimizu S., 1999)

**Fig. 5. Mechanisms for cytocrome *c* release during apoptosis.** Three mechanisms are proposed by which cytochrome *c* is released during apoptosis: (A) a channel formed by pro-apoptotic Bcl-2 family members such as Bax, (B) a novel channel formed by VDAC and pro-apoptotic Bcl-2 family members such as Bax. Molecular nature of the novel VDAC-Bax channel is not determined but might be a composite channel. Once cytochrome *c* is in the cytoplasm, it activates caspase(s) (36).

*Regulation of apoptosis by other proteins*

Among the transcriptional mediators of the various signal transduction pathways that have been shown to regulate lymphocyte apoptosis, Rel/NF-kappaB transcription factors have emerged as key regulators of B cell survival during their differentiation and in their activation by antigens or mitogens (38).

NF-kB proteins comprise a family of transcription factors involved in the regulation of a variety of physiological aspects of immune and inflammatory response (39). These proteins bind a common DNA sequence motif, the kB-box (40), as dimmers, and directly regulate gene transcription involved in cellular proliferation, activation and apoptosis (38). NF-kB is present in the cytosol in a inactive form where it is retained through its interaction with inhibitory IkB proteins. Activation of NF-kB dimmers is the result of IKK-mediated, phosphorylation-induced, degradation of the IkB inhibitors which enables NF-kB dimer to enter the nucleus and activate specific target gene expression (41).

NF-kB generally mediates cell survival signals by regulating the expression of anti-apoptotic proteins, such as Bcl-2 family members (42) and caspase inhibitors IAPs (43). Cells that express constitutively activated NF-kB are resistant to various chemotherapeutic agents (44). Indeed, the activation of NF-kB increases resistance to apoptosis induced by chemotherapy or radiation therapy.

# 3. Oxidative stress and apoptosis

Oxidative stress can be defined as the imbalance between cellular oxidant species production and antioxidant capability. Reactive oxygen species (ROS) are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis (45).

Oxidative stress has been evoked as a mediator of intracellular apoptotic signalling. Indeed, it was observed that many treatments that directly or indirectly induce oxidative stress were capable of inducing apoptosis (46).

*Origin and consequences of oxidative stress*

Free radicals such as ROS are produced by a variety of biochemical reactions (enzyme system) and cellular functions (mitochondria metabolism) (47).

Mitochondria are the major intracellular source of ROS, which are mainly generated at Complex I and III of the respiratory chain. Significant fractions (approximately 2%) of oxygen are converted to the superoxide radical. hydroperoxyl radical and its reactive metabolites (ROS) in and around mitochondria (48). Although ROS have been known to impair a wide variety of biological molecules including lipids, proteins and DNA, thereby causing various diseases, they also play critical roles in the maintenance of aerobic life (49).

Oxidative damage to DNA causes modification of the purine and pyrimidine bases, the deoxyribose backbone, single and double strand breaks, as well as cross-links to other molecules. DNA modifications are potentially mutagenic, contributing to cancer, premature ageing and neurodegenerative diseases (50).

ROS can directly modify signalling proteins through different modifications, for example by nitrosylation (51), carbonylation (52), di-sulphide bond formation (25) and glutathionylation (53). These modifications modulate a protein's activity and several recent papers have demonstrated their importance in cell signalling events, especially those involved in cell death/survival. Protein modifications mediated by oxidative stress can modulate apoptosis, either through specific protein modifications resulting in regulation of signalling

pathways, or through a general increase in oxidised proteins resulting in reduced cellular function (54).

Oxidative stress is able to regulate and to induce the transcription of several genes responsible of survival and apoptosis through its activity on localization and activation of the main redox sensible transcription factors such as NF-kB and it is possible to observe either the up-regulation of death proteins or survival proteins (55).

ROS formation and stimulation of lipid peroxidation in mitochondria can lead to suppression of mitochondrial metabolism. Lipid peroxides affect vital mitochondrial functions, such as respiration and oxidative phosphorylation, inner membrane barrier properties, maintenance of mitochondrial membrane potential ($\Delta\psi$), and mitochondrial $Ca^{2+}$ buffering capacity (56-58).

Mitochondrial lipid peroxidation products can impair the barrier function of membranes by interacting either directly with the protein and/or indirectly with the lipid moieties of the membrane (59) .

A potentially deleterious effect of ROS production in mitochondria is facilitation of $Ca^{2+}$-dependent mitochondrial permeability transition (MPT), which plays a key role in certain modes of cell death. In addition to ATP production in aerobic cells, mitochondria play a crucial role in the regulation of intracellular $Ca^{2+}$ homeostasis. Mitochondria can take up and retain $Ca^{2+}$; however, the retention capacity is limited. If the accumulated $Ca^{2+}$ exceeds a certain threshold-concentration, it is subsequently released from the mitochondria by the opening of a proteinaceous channel, commonly known as the permeability transition pore (PTP). The efflux of $Ca^{2+}$ along with other matrix constituents disrupts the solute homeostasis, and causes drastic changes in mitochondrial ultrastructure and functional activity (60).

However, to protect cells from oxidative insult, the cell contain an elaborate defense system to detoxify ROS and repair ROS-induced damage.

*Antioxidant defence system*

Antioxidants are classified in two main groups: enzymatic antioxidant agents and non- enzymatic antioxidant agents.

Enzymatic antioxidants include superoxide dismutase (SOD), glutatihione peroxidase (GPx) and catalase (CAT). SOD are localized to the cytosol and mitochondria and function to reduce superoxide anion to hydrogen peroxide which can be converted to water by catalase or glutathione peroxidase (61).

Non- enzymatic antioxidant such as vitamin E (62), vitamin C (63), beta-carotene (64), glutathione (GSH) (65), melatonin (66) and coenzyme Q (67), function to quench ROS.

Oxidative stress builds up in a cell when balance between production of ROS and antioxidant defense system is lost.

*Glutathione: the main intracellular antioxidant*

Reduced glutathione (L-*γ*-glutamyl-L-cysteinyl-glycine, GSH) is the prevalent low-molecular-weight thiol in mammalian cells. It is formed in a two-step enzymatic process including, first, the formation of *γ*-glutamylcysteine from glutamate and cysteine, by the activity of the *γ*-glutamylcysteine synthetase; and second, the formation of GSH by the activity of GSH sythetase which uses *γ*-glutamylcysteine and glycine as substrates. While its synthesis and metabolism occur intracellularly, its catabolism occurs extracellularly by a series of enzymatic and plasma membrane transport steps. Glutathione metabolism and transport participates in many cellular reactions including: antioxidant defence of the cell, drug detoxification and cell signalling (involved in the regulation of gene expression, apoptosis and cell proliferation) (68). GSH/GSSG ratio is normally closely regulated. Disruption of this ratio is involved in several cellular reactions involved in signal transduction and cell cycle regulation under conditions of oxidative stress. Importantly, GSH is required for the maintenance of the thiol redox status of the cell, protection against oxidative stress, detoxification of exogenous reactive metals, storage and transport of cysteine.

The intracellular GSH concentration regulates also the trigger of apoptotic signalling. In instances of apoptosis induced by non-oxidative agents, GSH may be part of the progression, rather than the triggering of the apoptotic signalling, being actively extruded by specific carriers in the reduced form (69). This GSH efflux occurs through a non-oxidative mechanism, but it can passively promote an oxidative stress, lowering the cells defence against radical oxygen species (70, 71).

This apoptotic GSH extrusion occurs through specific carriers and is required for commitment to apoptosis, since preventing extrusion, the induction of apoptosis is blocked. This implies that GSH extrusion is a necessary step in order to trigger the downstream events of apptosis.

Recently, we have found that the depletion of GSH is necessary and sufficient to induce cytochrome c release via Bax dimerization and translocation to mitochondria even in the absence of apoptosis (25).

A recent study in our lab shows that GSH depletion up-regulates Bcl-2 which exerts their pro-survival effects and lack of chemo sensitisation by BSO (72).

Interestingly Bcl-2 and GSH are both anti-apoptotic molecules and both exert their anti-apoptotic effect by radical scavenging mechanism.

*Role of lipoxyganase*

There are also pro-oxidative enzymes whose activity leads to the generation of free radicals such as lipoxygenase. Lipoxygenases are a family of ubiquitously expressed non-heme iron-containig enzyme that oxidizes the unsatured fatty acid arachidonate to bioactive hydroperoxides and other metabolites (73). In mammals, most work has been done on 5-lipoxygenase, which is found primarily in polymorphonuclear leukocytes, macrophages and mast cells, where it plays a central role in cellular leukotriene synthesis. The 5- LOX pathway was discovered in the late 1970s by Borgeat and Samuelsson (74) and (75). This iron-containing enzyme catalyzes two reactions. The first is the introduction of a molecule of oxygen to the 5-position of Arachidonic acid (AA) to give the intermediate 5$S$-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HpETE) (Fig.7). This is immediately followed by the rearrangement of 5-HpETE, a highly unstable compound that is enzymatically converted to leukotriene (LT) (76). Not all of the 5-HpETE formed by 5-LO is converted to LT, as a substantial amount is released from the enzyme and converted by peroxidase(s) to 5-HETE (**Fig.6**) (76). Indeed, mammalian cells can reduce the lipid hydroperoxides (LOOH) to the less toxic hydroxides (LOH), in a reaction requiring glutathione (GSH) and catalyzed by glutathione peroxidase (77).

$$LOOH + 2GSH \rightarrow LOH + GSSG + H_2O$$
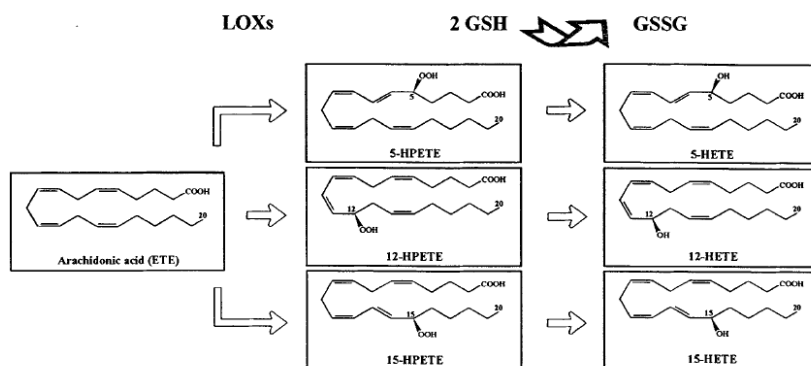


( *Maccarrone M. et al. 2001*)

**Fig. 6. Hydroperoxides generated by different lipoxygenases (LOXs) with arachidonic.** The hydroperoxides can be reduced to the corresponding hydroxides by cellular glutathione peroxidases, which concomitantly convert reduced glutathione (GSH) to its oxidized form (GSSG)) (76).

The synthesis of 5-LO products is tightly regulated. Release of the substrate AA from cellular lipids is dependent on the $Ca^{2+}$-induced translocation of cytosolic phospholipase $A_2$ (cPLA$_2$) to membranes and phosphorylation of the enzyme by MAP kinase. Similarly, elevation of cytosolic $Ca^{2+}$ levels is required to induce 5-LO translocation to the nuclear membrane, where it acts on AA that has been released by cPLA$_2$ and then bound by the nuclear membrane accessory protein 5-LO activating protein (FLAP). Both 5-LO and FLAP are required for the synthesis of LTs and 5-HETE by intact cells and these two proteins are normally co-expressed. LOX products have been shown to induce PCD in human T cells (78) ,neutrophils, (79) PC12h cells (80) and Jurkat cells

(81).Therefore, it is not surprising that activation of lipoxygenases has been associated to programmed death of different cells and tissues, induced by different, unrelated stimuli (76). Activation of different LOX isozymes has been shown to be associated to an early phase of apoptosis, triggered by several, unrelated stimuli. Consistently, LOXs and the hydro(pero)xides generated by their activity have been shown to induce programmed cell death in different cellular models, through a series of events including an immediate and sustained rise in cytosolic calcium, followed by mitochondrial uncoupling and cytochrome $c$ release (76).

# 4. Melatonin

*N*-acetyl-5-methoxytryptamine (melatonin) is a derivative of the essential amino acid tryptophan. It was first isolated from the bovine pineal gland and structurally identified by Lerner and colleagues in 1958 (82).

The biosynthetic pathway of pineal melatonin (**fig.7**) has been studied thoroughly. L-Tryptophan is taken up from the circulation and converted to serotonin (5-HT) by tryptophan hydroxylase. 5-HT is metabolized by the rate-limiting enzyme arylalkylamine N-acetyltransferase (AA-NAT) to N-acetyl-5-hydroxytryptamine, and in turn by hydroxyindole-o-methyltransferase to melatonin (83).

When melatonin was first isolated, it was considered as an exclusive hormone to the pineal gland (83), however, in the last few years melatonin synthesis has also been documented to occur in the retinas (84), gastrointestinal tract (85, 86), some bone marrow cells (87, 88), peripheral lymphocytes (89), skin (90), and possibly in many other cells as well (91-93). These organs reportedly contain the enzymatic machinery necessary for melatonin production. It remains unknown whether melatonin synthesized by extra pineal organs is the source of baseline melatonin levels in the blood. Modern endocrinology has shown that many hormones exhibit various additional actions which would not be called 'hormonal' in the strict classical sense. This can comprise biosynthesis at non-endocrine sites as well as paracrine or autocrine effects. Many of these substances are communication molecules in a broad sense, playing additional roles as neurotransmitters, neuromodulators, and other types of local regulators. Many features of melatonin distinguish it from that of the classical hormones. Although its synthesis occurs in a number of cells, the release of melatonin, at least in large amounts, seems to be restricted to the pineal gland. Certainly, surgical removal of the pineal gland eliminates the nocturnal increase in blood melatonin concentrations (94).

The mechanisms of melatonin release remain enigmatic, although it is usually assumed that it is a consequence of simple diffusion of the indoleamine out of the pinealocytes. While a similar secretory process for melatonin may exist in other cells where it is produced, in these tissues its actions are confined to the immediate vicinity of its cells of production, and significant amounts typically do not escape into the

systemic circulation. In these organs, melatonin has autocrine, intracrine, and pararine actions (95, 96).

At least in the pineal gland and retina, melatonin production is light:dark dependent with highest levels of the indoleamine being generated during the dark phase of the light/dark cycle.

Besides its endogenous production, it is also ingested in the diet since melatonin is present in plants including edible foodstuffs (97, 98) . Consumption of foodstuffs containing melatonin is followed by its absorption into the blood (94).

Melatonin exerts some of its biological effects through specific, high-affinity, pertussis-toxin-sensitive, $G_i$ protein-coupled receptors (99-101). Membrane G-protein-coupled melatonin receptor MT1 was cloned and characterized by Ebisawa et al.(102). Subsequently, MT2 and Mel 1c receptors have also been identified, the former mainly differing from MT1 in terms of the tissues in which it is expressed, while Mel 1c is not found in mammals (101). Melatonin also has been tentatively shown to activate a nuclear orphan receptor belonging to the retinoid Z receptor $\beta$ and $\alpha$ (RZR $\beta$ and $\alpha$) family. Melatonin acts on ROR$\alpha$ receptor repressing the expression of the 5-lipoxygenase gene (103). Based on structure-activity relationships of ligands and their receptors, many agonists and antagonists to the melatonin receptors have been synthesized and their bioactivities have been tested (104).

Several intracellular pathways are regulated by melatonin (**fig.8**) (105). There are several features of the effects of melatonin, which are mediated by high-affinity G protein-linked receptors. These include effectiveness at the range of $10^{-11}$ to $10^{-8}$ M concentrations, similar rank orders of binding to the melatonin receptor and in most cases sensitivity to PTX. Because the receptors are located on plasma membrane, melatonin regulates the function of the cell through intracellular second messengers. For example, in many tissues, melatonin has been found to decrease intracellular concentration of cAMP (106-108). Melatonin effects on other second messengers such as $[Ca^{2+}]_i$, cGMP, diacylglycerol, protein kinase C, or arachidonic acid have been described in the neonatal rat pituitary cells and in SCN (109-119). The results from Mayo et al. (113) suggest that melatonin regulation of antioxidant enzymes is receptor-mediated, thereby most likely implicating the MT1/MT2 receptors via second messengers such as cAMP, phospholipase C or intracellular calcium concentration. In addition,

binding of melatonin to membrane receptors could stimulate MAP kinase cascades thereby activating several transcription factors (114). The possibility exists that RZR/ROR receptors could also mediate melatonin effects on antioxidative enzymes as suggested by the results of Pablos et al. (115); if so, the pathways involved in their regulation obviously remain unknown. One possibility may relate to MT1/MT2 melatonin binding that, through second messengers and phosphorylation cascades, activates RZR/ROR as reported by Ram et al. (116). Another possibility by which melatonin may regulate RZR/ROR receptors would be via modulation of the calcium/calmodulin signalling pathway, either by changing intracellular calcium concentrations by binding to MT1/MT2 receptors (117), or by direct binding to calmodulin (118). The calcium/calmodulin signalling pathway has been reported to regulate transcriptional activity of RZR/ROR receptors via CaM kinases (119).
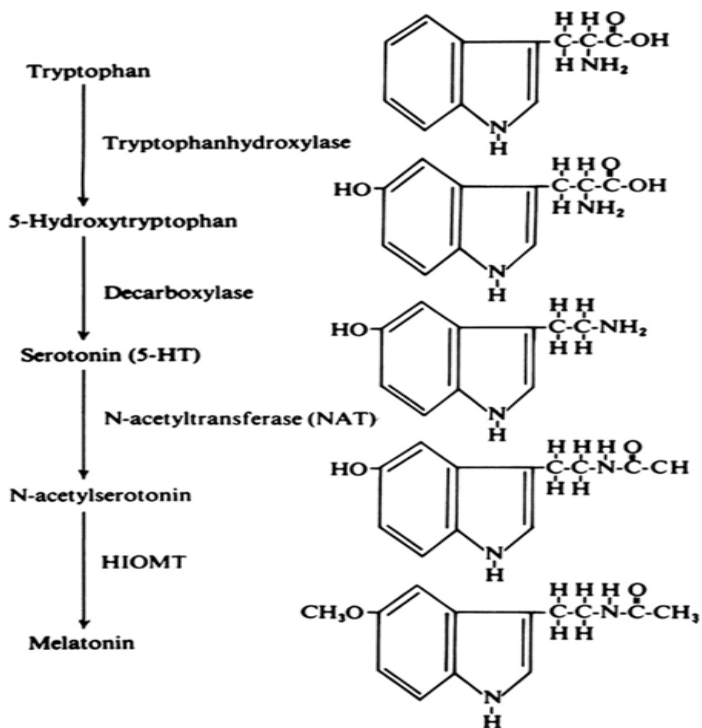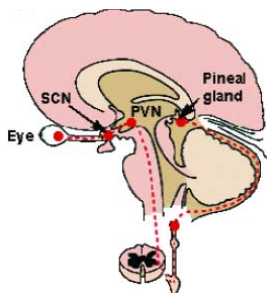
**Fig. 7 The biosynthetic pathway of pineal melatonin** (83).
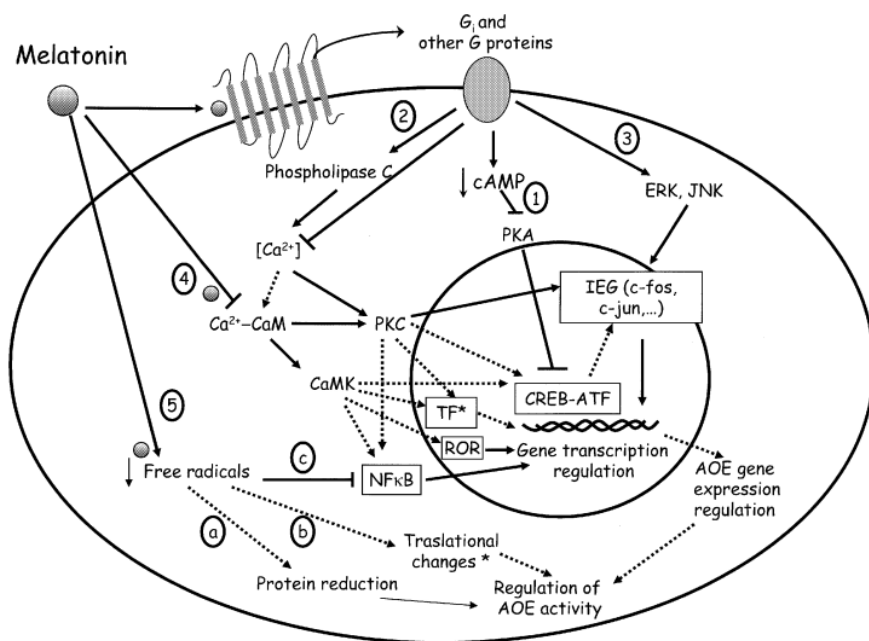
*(Rodriguez C. et al. 2004)*

**Fig. 8**. **Hypothetical pathways involved in melatonin biological effects** (105).

*Melatonin and oxidative stress*

The ability of melatonin to scavenge free radicals is undoubtedly an important property in its protection against oxidative stress. The discovery that melatonin is effective in antioxidative defence is related to the finding that under both *in vitro* and *in vivo* conditions this molecule directly scavenges the highly toxic $^\bullet OH$ to form cyclic 3-hydroxymelatonin (3-OHM), a stable metabolite of melatonin (120-127). Besides $^\bullet OH$, several other reactive species are also scavenged by melatonin. Compared with $^\bullet OH$, these agents are considered to be less toxic and less involved in the damage caused by ionizing radiation. However, several are involved in radical chain reactions that directly or indirectly influence cellular damage. Since ionizing radiation causes lipid damage as well (128-132) and because $LOO^\bullet$ propagates the radical chain reaction in the lipid environment, the reported ability of melatonin to scavenge $LOO^\bullet$ (133-137) is of understandable interest. One report even claims that melatonin is more effective in scavenging $LOO^\bullet$ than is vitamin E (133), which is regarded as a premier lipid antioxidant. Although the superiority of melatonin to vitamin E in scavenging $LOO^\bullet$ has not been confirmed (136), the efficacy of melatonin as a lipid antioxidant, regardless of the mechanisms involved, is not questioned (138, 139).

Melatonin has also been shown to quench singlet oxygen ($^1O_2$) (140), a high energy form of $O_2$ that exhibits high toxicity at the molecular level. Also the peroxynitrite anion ($ONOO^-$), the highly destructive product of the interaction between the superoxide anion radical ($O_2^{\bullet-}$) and nitric oxide ($NO^\bullet$), is scavenged by melatonin (141, 142). There is also evidence that melatonin directly neutralizes NO (143). Melatonin plays an important role also on the activities of enzymes involved in antioxidative defence such as, SOD, CAT, GSH-Px, glutathione reductase (GSH-Rd), and glucose-6-phosphate dehydrogenase (G6PD). The influence of melatonin on the activities of each of these enzymes showed that the indole stimulated the activity of each of them (138, 144, 145). By increasing the activities of antioxidant enzymes, melatonin reduces the number of free radicals or ROS generated and increases the production of molecules protecting against oxidative stress. SOD dismutases $O_2^{\bullet-}$ to $H_2O_2$, decreasing the amount of $O_2^{\bullet-}$ and the formation of $ONOO^-$. Melatonin increases tissue mRNA levels of two isoforms

(manganese/copper SOD) of this enzyme (146,147). Thus, melatonin may decrease the quantity of $O_2^{\cdot-}$ in two ways, directly by stimulating SOD and indirectly when the melatonyl cation radical scavenges it.

Extensive studies have been conducted on melatonin's influence on glutathione (GSH) metabolism. GSH is a well-known antioxidant. Melatonin stimulates the activity of GSH-Px, which transforms $H_2O_2$ to $O_2$ (148-152). In this process GSH is oxidized, thus forming oxidized glutathione (GSSG). The reduced form, GSH, is replenished by the action of the enzyme GSH-Rd, the activity of which is also stimulated by melatonin (151, 152). In general, the ability of melatonin to increase the metabolism of GSH, an effective antioxidant, is well documented.

Besides the antioxidative enzymes mentioned above, the activity of one pro-oxidative enzyme (i.e., nitric oxide synthase (NOS); lipoxygenase (LOX), is also altered by melatonin. Melatonin has been found to inhibit the activity of NOS (153-155), the enzyme catalyzing the formation of $NO^{\cdot}$. Melatonin, by inhibiting NOS activity, decreases the formation of $NO^{\cdot}$ and the product of its interaction with $O_2^{\cdot-}$, $ONOO^-$. LOX have been shown to be inhibited by melatonin, both at physiological and pharmacological levels. Reduction in the activities of these enzymes would function in reducing oxidative stress by lowering the production of reactive toxicants (156).

In spite of the clear chemical anti-oxidant action of melatonin, many reports indicate a surprising pro-oxidant activity that may occur within the cells (157-159). detected as the increase in fluorescence of oxidation-sensitive intracellular probes (namely dihydrorhodamine (160), dichlorofluorescein (161), dihydroethidium (162). The pro-radical activity of melatonin has been reported on a set of cells, most of which of tumor origin, which proved positive with very few exceptions (157-159). Intriguingly, even though intracellular free thiols are decreased by melatonin (163), frank oxidative stress such as lipid peroxidation seems not to occur (157). The issue is still unclear, many perplexities arising from the paradox that an anti-oxidant molecule may behave as a pro-oxidant.

*Melatonin and apoptosis*

Special interest is receiving the ability of melatonin of modulating apoptosis (164). Literature divide the role of melatonin in modulating apoptosis into three categories: first, the role of melatonin in inhibiting apoptosis in immune cells (165, 166); second, the role of melatonin in preventing neuronal cell death (167, 168) and finally, the role of melatonin in increasing apoptotic cell death in cancer cells (169) . The mechanism whereby melatonin regulates the apoptotic program however is a question still to be answered.

Physiologically coordinate apoptosis is a critical goal of homeostasis, and a failure to proper control leads to hyper- or hypo-proliferative disorders (170). In addition, apoptosis plays a critical role in the inflammatory and immune responses, by regulating the maturation rate of B and T cells, and maintaining/contrasting viability of cells engaged in the inflammatory sites (171, 172). Interestingly, recent evidences demonstrate an unforeseen role of melatonin in the control of immune and inflammatory responses, showing that leucocytes possess all the enzymatic machinery necessary to synthesize melatonin from tryptophane (173), and the proper receptors (174), thus being an autonomous compartment as far as melatonin responses are concerned. Accordingly, many evidences demonstrate that control of leukocyte apoptosis is among the most striking non-neurological roles of melatonin (175, 176).

Indeed, several findings document a role for melatonin in modulating experimentally induced apoptosis by a variety of agents. The indoleamine inhibits apoptosis in immune cells (165, 166) , peripheral tissues (177, 178) and prevents neuronal cell death in models of Parkinsonism (167, 168), Alzheimer's disease (179, 180) and ischemia–reperfusion injury (181, 182). The mechanism by which melatonin reduces apoptosis seems to be related to its antioxidant and free radical scavenging properties (177-182). However, recently, a new mechanism has revealed that the anti-apoptotic effects of melatonin may be explained by a direct interaction with the MTP (183). The recent discovery that mitochondria are a target for melatonin opened a new perspective to understand the mechanism of action of this indoleamine (184). Melatonin has a direct role in mitochondrial homeostasis (185-187), which may explain the protective effect of this molecule in diseases

such as Parkinson's disease, Alzheimer's disease, epilepsy, aging, ischemia–reperfusion and sepsis, all of which have mitochondrial dysfunction as a primary or secondary cause of the condition (188).

Studies in peripheral tissues have suggested that melatonin inhibits apoptotic processes via its antioxidant properties. For example, melatonin protects against cyclosporin A-induced hemolysis in human erythrocytes because of depuration resulting from $O_2^-$ produced by mitochondria (189). Melatonin is also highly protective against mitochondrial ROS-induced cardiotoxicity resulting from doxorubicin treatment. In this study, pre-treatment with melatonin prevented the release of lactate dehydrogenase and restored membrane potential (190).

Many lines of evidence indicate an anti-apoptotic effect of melatonin on thymic cells. The methoxyindole reduces DNA fragmentation induced by glucocorticoids in cultured thymocytes (165). A reduction in glucocorticoid-receptor mRNA levels in the intact thymus as well as in cultured thymocytes that were treated with melatonin seem to be the most likely mechanism whereby melatonin inhibits glucocorticoid-induced cell death (166). Other studies reported that melatonin inhibits DNA fragmentation and the release of cytochrome c from mitochondria of mouse thymocytes treated with dexamethasone. Melatonin may act by inhibiting the mitochondrial pathway, presumably through the regulation of Bax protein levels (191), although melatonin was ineffective per se on this parameter.

Interestingly, pro-apoptotic effects of melatonin have been noted in a number of tumor cell lines (169). In MCF-7 breast tumor cell studies conducted in the absence of exogenous steroid hormones, treatment with melatonin produced a 64% reduction in the cellular ATP levels through a membrane receptor-modulated pathway (192). These findings in tumor cells are in contrast to the described actions of melatonin in normal cells and suggest melatonin's potential use in killing cancer cells while preserving the function of normal cells.

# METHODS

These are the principal methods that I use in my PhD project:

*Cell culture*

U937 are human tumor monocytes stabilized from a histiocytic lymphoma; Jurkat are a human leukemic T-cell line; E2R are an Epstein-Barr virus (EBV) positive B-cell line obtained from Burkitt's lymphoma. Cells were cultured in RPMI medium supplemented with 10% FCS. Cells were routinely checked for absence of mycoplasm by using a mycoplasm detection kit (Mycoalert TM., Cambrex Bio Science Milano, Italy). The experiments were performed on cells in the logarithmic phase of growth under condition of $\geq$ 98% viability, as assessed by trypan blue exclusion. Peripheral blood mononuclear leukocytes (PBML) were isolated from heparinized blood samples of healthy individuals by collection with Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. Red cells were removed by hypotonic lysis. PBML were then plated in RPMI 1640 plus 10% human AB serum in culture flasks (pretreated with the same human AB serum) to promote monocytes adhesion. After 2hrs, floating cells (lymphocytes) were washed out, spinned down and separately plated in RPMI 1640 plus 10% hi-FCS. Adherent monocytes were washed 3 times with RPMI to remove residual lymphocytes, detached by cell scraping, and resuspended in RPMI 1640 plus 10% hi-FCS. Treatments (see below) were performed at 20-24hrs post-separation. Cell viability in both fractions was >98% as assessed by trypan blue exclusion test. Purity of the enriched fractions was controlled by labelling (20 min at room temperature) with cell-type specific antibodies: FITC-conjugated-anti-CD45 (from Becton-Dickinson) for lymphocytes; and PE-conjugated-anti-CD14 (from Becton-Dickinson) for monocytes. Cells were then washed with PBS and labelling evaluated. Both monocyte and lymphocyte fractions were >95% pure.

*Induction and evaluation of apoptosis*

Apoptosis was induced either with the protein synthesis inhibitor puromycin (PMC, 10 μg/ml), with 50 μM etoposide (VP16) or with 1 mM $H_2O_2$. Apoptosis was evaluated after 4 h of PMC or VP16

continuous treatment, or after 1 h incubation with $H_2O_2$ followed by 6 h of recovery in fresh medium (± melatonin). Apoptosis was quantified as previously described (193). Briefly, cells were stained with the Hoechst 33342; cells with nuclear apoptotic morphology, detected using a fluorescence microscope, were counted (at least 300 cells in at least 3 independent fields) and the fraction of apoptotic cells among total cells was evaluated. PMC, VP16, $H_2O_2$ were purchased from SIGMA Chemical Co (St Louis, MO), Hoechst 33342 from Calbiochem.

*Cell cycle analysis*

   U937 cells ($2x10^6$) were washed with 1% PBS and fixed in 65% ice cold ethanol for 15 min. Cells were then stained with 5ug/ml propidium iodide in RNase solution for 15 min at RT. Samples were processed for flow cytometry in a FACScan Becton&Dickinson. Cell cycle was analyzed with Cylchred software.

*Analysisn of ROS*

   Cells were loaded with 10 uM dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes), 2 uM dihydrorhodamine (DHR, Molecular Probes) or 5 uM dihydroethidium (DHE, Molecular Probes) by incubation at 37° for 30 min after melatonin treatments. These probes are non-fluorescent cell-permeable compounds; once inside the cell, they are de-esterified and turn fluorescent upon oxidation, fluorescence being proportional to ROS production. Analyses were performed by flow cytometry using FACScan Becton&Dickinson.

*Immunofluorescence analysis*

   U937 cells were fixed with 4% paraformaldehyde. Fixation was stopped by adding the same volume of 50 mM $NH_4Cl$ in PBS for 20min. cells were washed twice in PBS and incubated with 2 ug/ml anti Bax mouse monoclonal antibody 6A7 (BD Biosciciences Pharmingen, San Diego, CA, USA,) or anti Bax mouse polyclonal antibody D21: sc6236 (Santa Cruz Biotechnology, Santa Cruz, CA, USA,) or anti Bcl-2 mouse monoclonal (Oncogene, San Diego, CA, USA; Ab #0P60,) in a solution of PBS, 1% BSA, 0.05% saponin for 45

min followed for 45 minutes at room temperature. Then cells were washed with PBS and incubated with 10 ug/ml of of TRIC- or FITC-conjugated secondary antibodies (Dako, Glostrup, Denmark) in PBS, 0.05 % saponin for 30 min. Nuclei were visualized by staining with Hoechst 33342. In order to study the mitochondrial localization of Bcl-2 protein, U937 were stained, before the paraformaldehyde fixation, with MitoTrackerRed (Molecular Probes, Eugene, OR, USA) for 20 minutes at 37°C. Images were captured with a Nikon Eclipse TE 200 microscope. The merge and the analysis were performed with Adobe Photoshop software.

*Subcellular fractionation and immunoblot assay*

Western blot analysis of cytochrome c: cytosolic fraction from U937 cells were isolated by a quick cell lysis method with digitonin (194). Under vortexing, lysis buffer (9.4 ug digitonin/$10^6$ cells, 500mm sucrose in PBS: $NaH_2PO_4$ 2mM, $Na_2HPO_4$ 16 mM, NaCl 150 mM , pH 7.6) was added to a cell suspension of $5x10^6$ cells in PBS (1mM DTT and protease inhibitor cocktail, SIGMA P 8340, were added just prior to use). Heavy organelles and cell debris were pelletted for 60 seconds at 14000g at 4°C. The 30ug of protein of cytosolic fraction (supernatant) was analysed for cytochrome C by 12%SD-Page and immunoblotting (PharMingen, San Diego, CA, USA). The specific protein was identified using the ECL system by Amersham (Piscataway, NJ, USA) with HRP-conjugated secondary antibody (Dako).

Western blot analysis of Bax: to obtain mitochondrial and cytosolic extracts for the determination of Bax localisation, the subcellular fractionation procedure was performed as described (195), with minor modification. Briefly, sample of $20x10^6$ cells were washed with ice cold PBS (all the following procedures were performed at +4°C) and resuspended in 5x volume of hypotonic buffer (10mM NaCl, 1.5mM MgCl2, 10mM Tris-HCl, pH 7.5, 1mM DTT, 1mM PMSF and protease inhibitor cocktail, see above). The cells were incubated on ice until they completed swelling, and then dounced with the B pestle (Kontes Glass Company, Vineland, NY, USA) until 90% of cells were Trypan Blue positive. A volume of 2.5x MS buffer (525mM mannitol, 175mM sucrose, 12.5mM Tris-HCL pH 7.5, 2.5mM EDTA pH7.5, 1mM DTT, 1mM PMSF and protease inhibitor cocktail) was quickly added to the

lysate to achieve 1 x MS. The mixture was subjected to centrifugation at 1300g. The resulting supernatant was centrifuged two more times to completely remove the nuclei and unlysed whole cells. The supernatant was then subjected to centrifugation at 10000 g to isolate mitochondria. The pellet (mitochondrial fraction) was re-suspended in hypotonic buffer containing 0.5% Triton x-100 (Calbiochem) and rotated for 30 min at +4°C.

The resulting fractions were placed in loading buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 5 % beta-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue) for Western blot analysis.

For samples separated in non-reducing gels, the same procedure as above was followed; except that cells were lysed in non-reducing hypotonic buffer (DTT was omitted), and the sample buffer was modified by omission of beta-mercaptoethanol. For western blot analysis, 50ug of cytosolic or mitochondrial protein extracts were loaded into a 12% standard SDS-PAGE and blotted to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The filter was incubated for the immunoreactions with the Bax polyclonal antibody (Santa Cruz, D21).

Western Blot analysis of Bcl-2 protein levels: to obtain mitochondrial and cytosolic extract, for the detection of Bcl-2 cellular localization, we perform the same procedure as above was followed for Bax. Whole cell extracts were prepared with M-PER Mammalian Protein Extract Reagent (Pierce, Rockford, IL, USA); briefly, $10 \times 10^6$ U937 were washed in PBS and the pellet was resuspended in 400ul of the solution containing protease inhibitor, vortexed horizontally for 15 minutes at 4°C and then centrifugated at 10000g for 15 minutes. 5ug of the supernatant were loaded into a 12% standard SDS-PAGE and blotted to nitrocellulose membrane. The filter was incubated for the immunoreactions with the Bcl-2 monoclonal antibody (Calbiochem).

Western Blot bands were quantified by image analysis with NIH Image 1.4 software. Following values were normalised against Ponceau red staining of the nitrocellulose filter.

*siRNA transfection*

2ug of siRNA Bcl-2 (Dharmacon, Lafayette, CO, USA) were transfected into $10^6$ cells using kit V from AMAXA (Cologne, Germany) according to the manufacturer's protocol and program V-001 an

AMAXA's nucleofactor device. Treatments were performed 48hours after transfection. 1ug of enhanced green fluorescent protein expression vector (pmaxGFPTM) was co-transfected with the siRNA; percent of transfected cells based on the GFP-green signal were measured by flow cytometer and selected by microscope for the in situ apoptosis analysis.

# GUIDE TO THE RESULTS

**This thesis is based on the following publications and manuscripts**:

**Paper I)**
**Radogna F**, Paternoster L, Albertini MC, Accorsi A, Cerella C, D'Alessio M, De Nicola M, Nuccitelli S, Magrini A, Bergamaschi A, Ghibelli L. Melatonin as an apoptosis antagonist. Ann N Y Acad Sci. 2006;1090:226-33.

**Paper II)**
**Radogna F**, Paternoster L, Albertini MC, Cerella C, Accorsi A, Bucchini A, Spadoni G, Diamantini G, Tarzia G, De Nicola M, D'Alessio M, Ghibelli L. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res. 2007; 43:154-62.

**Paper III)**
**Radogna F**, Cristofanon S, Paternoster L, D'Alessio M, De Nicola M, Cerella C, Dicato M, Diederich M, Ghibelli L. Melatonin antagonizes the intrinsic pathway of apoptosis via mitochondrial targeting of Bcl-2. J Pineal Res. 2008; 44:316-25.

**Paper IV)**
Albertini MC, **Radogna F**, Accorsi A, Uguccioni F, Paternoster L, Cerella C, De Nicola M, D'Alessio M, Bergamaschi A, Magrini A, Ghibelli L.Intracellular pro-oxidant activity of melatonin deprives U937 cells of reduced glutathione without affecting glutathione peroxidase activity. Ann N Y Acad Sci. 2006 ;1091:10-6.

**Paper V)**
Paternoster L., **Radogna F.** , Accorsi A., Albertini M.C.; Gualandi G., Ghibelli L. Melatonin as a modulator of apoptosis in B lymphoma cells. (Ann N Y Acad Sci; 2009 in press)

**Paper VI)**

**<u>Radogna F.</u>** , Paternoster L., De Nicola M., Cerella C , Ammendola S., Bedini A. , Tarzia G., Aquilano K., Ciriolo MR ,Ghibelli L. Rapid and transient stimulation of intracellular reactive oxygen species by melatonin in normal and tumor leukocytes. (Submitted )

**Paper VII)**

**<u>Radogna F</u>**., Sestili P. °, Martinelli C. °, Paolillo M. °, Paternoster L.\*, Albertini M.C. \*, Accorsi A. \*, Gualandi G. ^, and Ghibelli L. Lipoxygenase-mediated pro-radical effect of melatonin via stimulation of arachidonic acid metabolism. (Submitted)

**Manuscript VIII)**

**<u>Radogna F .</u>**, De Nicola M. , Cerella C., Albertini M.C., Accorsi A., Ghibelli L. Melatonin antagonizes apoptosis by concurrent stimulations of high and low affinity targets. (In preparation)

**Paper IX)**

Cristofanon S., Uguccioni F., Cerella C., **<u>Radogna F</u>**., Dicato M., Ghibelli L., Diederich M. Intracellular pro-oxidant activity of melatonin induces a serviva pathway involving NF-kB activation. ( Ann N Y Acad Sci; 2009 in press)

**Paper X)**

**<u>Radogna F.</u>**, Nuccitelli S., Mengoni F., Ghibelli L. Neuroprotection by melatonin on astrocytoma cell death. (Ann N Y Acad Sci; 2009 in press)

The goal of my PHD project was to understand the role of melatonin on apoptosis and investigate the mechanisms through which melatonin controls apoptosis among leukocytes, exploring a possible pharmacological and inflammatory potential role. Indeed, many features of melatonin distinguish it from that of the classical hormones. Recently increasing evidences showed that many non-neuroendocrine tissues produce, and respond to, melatonin; this is especially true for the white blood cells compartment, in which melatonin seems to play a role in controlling their number and functioning. Indeed leucocytes possess all the enzymatic machinery necessary to synthesize melatonin from tryptophan as well as the proper receptors. This proposes melatonin as a possible modulator of the inflammatory/immune. Thus, it is very important to understand how melatonin controls apoptosis in cells of immune/inflammatory relevance.

In the last few years, the ability of melatonin to modulate apoptosis is receiving increased attention; however, the mechanisms involved are still quite controversial. The potential use of melatonin as a pharmaceutical agent derives in part from early observations showing that at physiological concentrations melatonin may inhibit tumor growth in animal models (169) and proliferation of tumor cells in vitro (192) This led researchers to postulate a possible action of melatonin as a pro-apoptotic agent in cancer cells (159), with possible pharmaceutical use. Melatonin also increases the efficacy of some specific chemotherapic drugs on cancer cells (176), being directly pro-apoptotic in many cancer cells while functioning as an anti-apoptotic agent in normal cells (166, 169). It is important to understand the mechanisms through which melatonin modifies apoptosis. In particular, one of the first questions is to understand whether melatonin's anti-apoptotic effect derives from its ability to engage specific receptors, thereby triggering a signal transduction pathway, or whether melatonin's radical scavenging properties may interfere with apoptosis.

Starting from this background, we have focused our research around three goals:

a) to understand the mechanisms through which melatonin controls apoptosis

b) to understand the mechanisms through which melatonin modulate ROS

c) to understand if a relationship exists between these two different melatonin ability.

First of all, with the goal to clarify the effects of melatonin on apoptosis, we investigated both the possible pro-apoptotic or anti-apoptotic effect in U937 human monocytic cells , known to possess MT1 melatonin receptors ( **paper I**). Our results indicate that melatonin does not exert a pro-apoptotic effect in U937; these are tumor cells, thus this result breaks the rule of the pro-apoptotic effect of melatonin on tumor cells. Then, we evaluated a possible anti-apoptotic effect. Melatonin significantly reduces apoptosis induced by two different types of inducers: physiological and cell damaging. Thus, melatonin exerts a similar protective effect on apoptosis induced via stimulation of two independent pathways, the physiological (or extrinsic) pathway and stress-induced (or intrinsic) pathway. The doses required for the anti-apoptotic effect ($\geq$100uM) in U937 are apparently not compatible with receptor stimulation (receptor affinity <1nM). We have shown in **paper I** that this is due, at least in part, to a masking by serum proteins, present in culture medium in large concentration, which stick to melatonin lowering the actual concentration available for cell entry. Thus, in the absence of fetal calf serum (FCS), melatonin is more effective in its anti-apoptotic effect at all the doses tested. However, the doses are still too high to indicate MT1/MT2 receptor involvement; thus, we cannot rule out other possible explanations, such as melatonin protection from apoptosis is due to its antioxidant ability. For this reason, we explored in detail the mechanism involved in melatonin antiapoptotic effect.

This is described in **paper II**. We focused our attention on the intrinsic pathway of apoptosis; we showed that melatonin efficiently contrasts apoptosis induced by a set of stressing agents (puromycin, etoposide, hydrogen peroxide) which directly or indirectly elicit oxidations (fig.1).
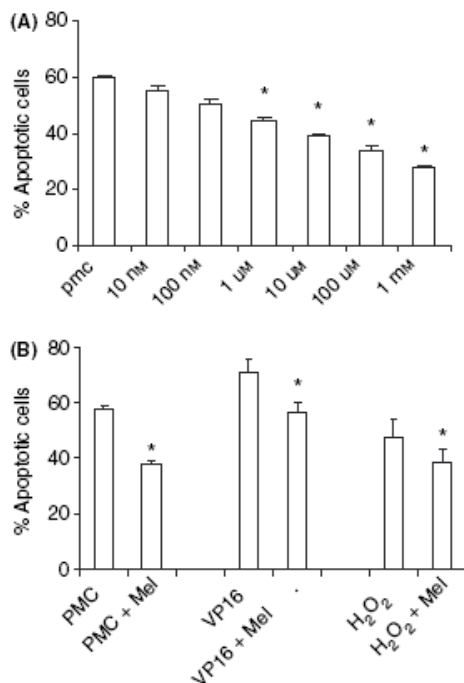
**Fig.1. Melatonin antagonizes apoptosis.**

To understand whether melatonin's anti-apoptotic effect may depend on receptor interaction or antioxidant ability we exploit a panel of melatonin synthetic analogs with different radical scavenging abilities and affinities for melatonin plasma membrane receptors (for details see Table I, Paper II) to test whether the anti-apoptotic activity may parallel the analogs' ability of engaging receptors or to scavenge radicals. The antioxidant ability of the compounds was then tested. To control a wide range of oxidative phenomena, the inhibition by melatonin vs. the analogs was tested in three different oxidative systems, namely a colorimetric method based on the stable free radical diphenyl-p-

picrylhydrazyl (DPPH), a colorimentric method based on the stable free radical 2,2'-azinobis [3-ethylbenzthiazoline-sulfonic acid] (ABTS) and an enzymatic/luminometric method based on the xanthine/xanthine oxidase system. The three assays reveal different antioxidant abilities of the analogs depending on the oxidative system examined; notably, none of them coincides with the extent of affinity.

We then compared the radical scavenging vs. anti-apoptotic ability of the analogs. We probed the anti-apoptotic effect of these analogs on U937 induced to apoptosis by puromycin and we found that the anti-apoptotic ability of the analogs parallels with receptor affinity, being instead unrelated to any of the four antioxidant activities. This suggests that the signal transduction elicited by melatonin receptor stimulation is the mechanism through which melatonin exerts its anti-apoptotic effect. To explore the possible role of receptor stimulation in the anti-apoptotic effect of melatonin, the different steps of melatonin-induced signal transduction were examined. Luzindole is an important tool in melatonin research, since it specifically inhibits G protein activation by a melatonin-engaged $MT_1$ or $MT_2$ receptor. Luzindole inhibits melatonin (fig.2 panel A) and 2-Br-MLT (fig.2 panel B) anti-apoptotic effects. The specificity of luzindole emphasizes the importance of these findings. Further evidence that receptor-initiated signal transduction is required for apoptosis antagonism comes from the finding that pertussis toxin, a specific inhibitor of the alpha subunit of heterotrimeric G proteins, which inhibits melatonin-activated signal transduction (94), completely reverses melatonin anti-apoptotic effect (fig.2). With these results, we demonstrate that melatonin antagonizes apoptosis via MT1/MT2 receptor interaction.
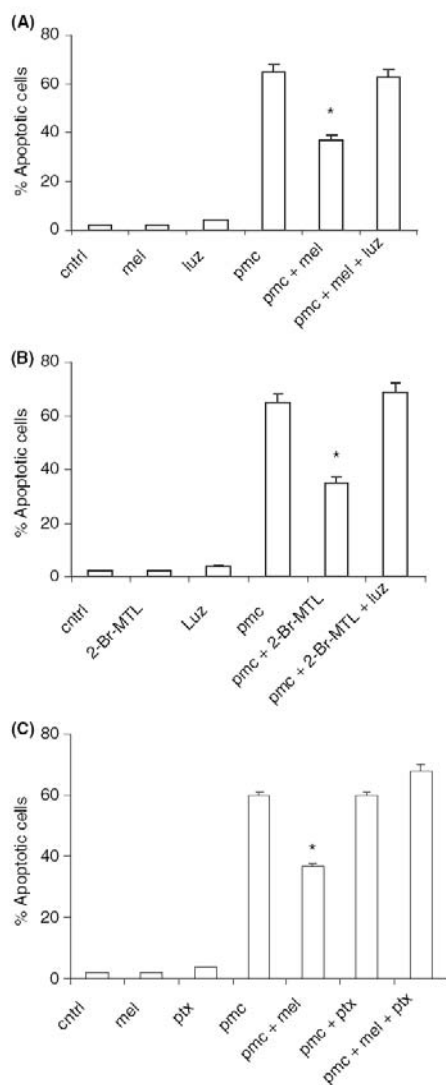
**Fig.2. Melatonin anti-apoptotic effect is sensitive to luzindole and pertussis toxin.**

To understand which signal transduction pathway is triggered by MT1/MT2 engagemetn by melatonin to give the anti-apoptotic effect, we measured the levels of inosytol-3-phosphate (IP3), which may be produced as part of the MT1/MT2 signal transduction, after melatonin challenge. Melatonin increases [IP$_3$], with a peak at 1 min. This is a required step for melatonin anti-apoptotic effect, which is indeed reversed by inhibiting PLC with two different inhibitors, namely neomycin, which protects PLC substrate PIP$_2$, and U7322, which inhibits the activation of PLC by the upstream G protein melatonin revert its anti-apoptotic effect. (Fig. 3). This indicates that the anti-apoptotic effect of melatonin requires this particular pathway. The PLC pathway may possibly contrasts apoptosis by promoting Ca$^{2+}$ influx: indeed, inhibition of Ca$^{2+}$ influx was able to revert melatonin anti-apoptotic effect.

This study provides strong evidence to show that melatonin inhibits apoptosis via engagement of MT1/MT2. However, it remains that the doses of melatonin required to contrast apoptosis are much higher than those necessary to efficiently engage receptors. As I will show later, this will be explained by showing that eliciting MT1/MT2 signal transduction is necessary but not sufficient to contrast apoptosis.
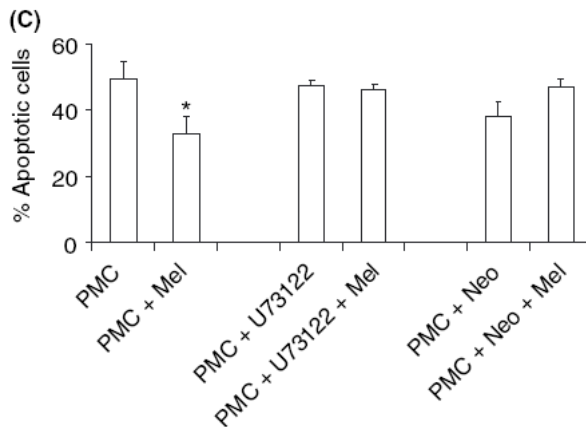


**Fig. 3. Phospholipase C is required for melatonin anti-apoptotic effect.**

In order to explore the mechanism through which the signal transduction elicited by MT1/MT2 receptors leads to stress-induced apoptosis antagonism we focused our attention on the intrinsic apoptotic signal which is triggered by PMC; and checked whether melatonin may impair the intrinsic apoptotic pathway. Therefore, we analyzed the effects of melatonin on earlier events of apoptosis on U937 cells (**paper III)**, going backwards from events of the execution phase (nuclear vesiculation), up to caspase 3 (the borderline between the commitment and execution phase), up to the commitment events, i.e., caspase 9 activation, cytochrome c release and up to Bax translocation.

We found that melatonin, though allowing mitochondrial translocation of the pro-apoptotic protein Bax, impairs Bax activation and dimerization.

First, we performed a bi-parametrical analysis at the single cell level analyzing Bax localization and nuclear vesiculation on the same cells. As shown in figure 4A, the dotted pattern of Bax is present in cells in apoptosis treated with puromycin, as shown by the Hoechst DNA staining, that points out the apoptotic nuclear vesiculation. But in the presence of melatonin, some cells with the dotted Bax pattern display a regular rounded nucleus, suggesting an abortive apoptotic pathway.

To control if Bax is present in its active or inactive form in the mitochondria of viable cells, we analyzed whether the Bax-positive dots correspond to an activated Bax. To this purpose, we made use of an anti-Bax antibody that is specific for the activated form, recognizing the N-terminus, buried in the latent, but exposed in the active, form. Interestingly, the active form of Bax is present only in frankly apoptotic cells, independently of the presence of melatonin (figure 4B). Thus, the translocation of Bax revealed in viable, melatonin-treated cells by the regular antibody, is not accompanied by activation since the activation-specific antibody fails to detect it (figure 4C). This means that in the presence of melatonin, the translocation of Bax to mitochondria is not necessarily accompanied by its activation.

In order to confirm this finding, we analyzed another parameter of Bax activation, i.e., the formation of disulfide bridges among Bax cysteines, which may be revealed by a western blot analysis performed under denaturing, but non-reducing, conditions. As shown in figure 4D, the detection of slowly migrating bands upon puromycin treatment is impaired by melatonin, confirming that melatonin interferes with apoptosis acting at the level of Bax activation.

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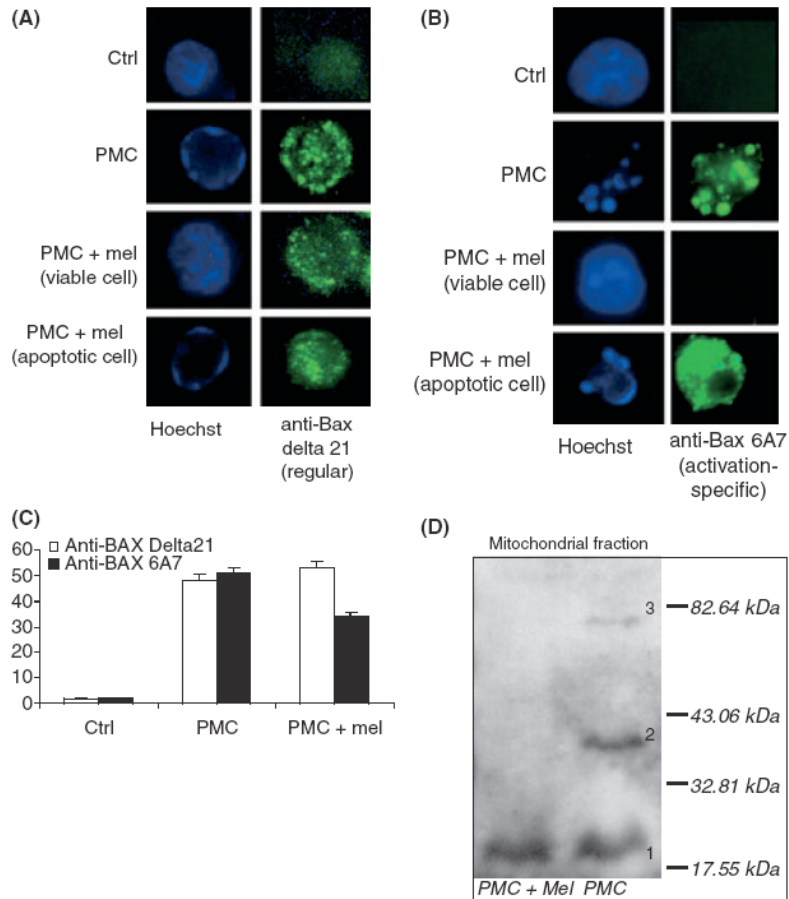
**Fig.4 Melatonin affects Bax activation and dimerization.** Panel A and B show the bi-parametric analysis performed by double-labelling cells with Hoechst (to visualize the nucleus) vs. delta21 anti-Bax antibody (recognizing both active and inactive form of Bax, panel A); and Hoechst vs. anti-Bax 6A7 antibody (recognizing only the active form of Bax, panel B). Panel C shows the extent of cells with the dotted pattern was evaluated by counting 300 cells in at least 10 randomly selected fields: the % fractions of Bax-dotted cells is reported on top of each treatment. Panel D shows the western blot of the mitochondrial fraction of cell lysates as indicated, prepared and run under denaturing but non-reducing conditions; Bax-specific bands are labelled from 1 to 3 according to migration, and are compatible with monomeric (1), dimeric (2) and polymeric (3) forms.

The downstream apoptotic events, i.e., cytochrome c release, caspase 9 and 3 activation and nuclear vesiculation are equally impaired, indicating that melatonin interferes with Bax activation within mitochondria. One of the most frequent means of impairing Bax function is the physical sequestration by the cognate Bcl-2. Thus, in order to understand whether Bcl-2 may be the mediator of melatonin anti-apoptotic effect, we silenced its expression or action. First, we transfected small interfering RNAs to down-regulate Bcl-2 expression. Figure 5B shows that the down-regulation of Bcl-2 *per se* induces a basal level of apoptosis. Upon puromycin treatment, melatonin exerts an anti-apoptotic effect in un-transfected cells, as expected, whereas it is ineffective on transfected, Bcl-2-negative cells. This result demonstrates that the presence of Bcl-2 is required for melatonin anti-apoptotic effect. In fact, we can even see that under these circumstances a possible (i.e, below significance so far) pro-apoptotic effect of melatonin seems to be unmasked.

The requirement of Bcl-2 for melatonin anti-apoptotic effect was confirmed by interference with Bcl-2 action via the use of 10uM of the small-molecule inhibitor targeted at the BH3 binding pockets in Bcl-2, NSC345600, i.e., the domain that interacts with Bax (and the other family members), and is responsible for the anti-apoptotic action of Bcl-2. Also in this instance, as shown in figure 5C, melatonin failed to exert any anti-apoptotic effect when Bcl-2 is inhibited.
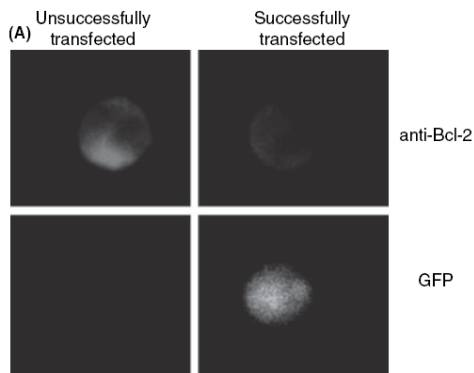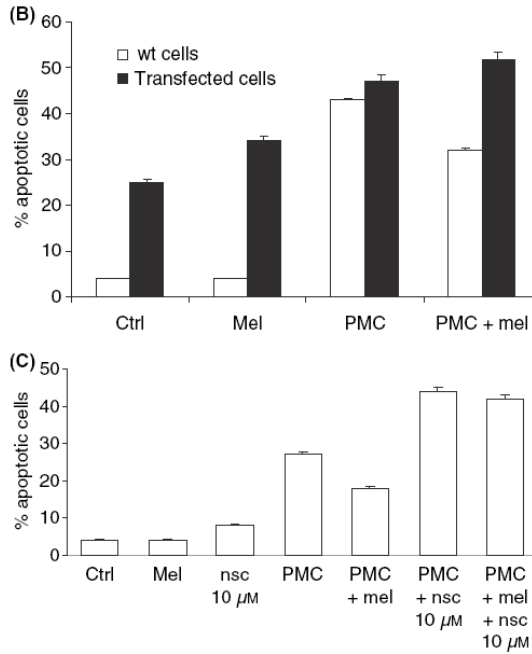
**Fig. 5 Bcl-2 is required for melatonin anti-apoptotic effect.** Panel A shows the different green vs. red staining of cells nucleofected with specific Bcl-2 siRNA and Green Fluorescence Protein (GFP), and immuno-stained with TRIC-conjugated anti-Bcl-2 antibodies: left, a cell not expressing GFP, with a strong signal for Bcl-2, unsuccessfully transfected; right: a cell expressing GFP, with a weak Bcl-2 signal; successfully transfected (transfection efficiency = 50%, not shown). Panel B shows the extent of apoptosis induced by puromycin in the presence/absence of melatonin, among the successfully vs. unsuccessfully transfected cells. Panel C shows a similar experiment performed on cells pre-treated for 4 hours with 10uM NSC345600, inhibitor of Bcl-2 activity .

Thus, once established the involvement of Bcl-2 in the anti-apoptotic activity of melatonin, we explored the mechanism through which this occurs i.e., via up-regulation or by other mechanisms. We performed a western blot analysis on total cell lysates; we found that the levels of Bcl-2 do not change at 3 or 5 hrs of melatonin, as shown in figure 6A, (an up-regulation was found only at 24 hrs), showing that short term exposure to melatonin treatment does not affect Bcl-2 protein level. Instead, melatonin causes Bcl-2 translocation from cytosol to mitochondria; we performed a cell fractionation followed by a western blot of the cytosolic and mitochondrial fractions and we found that melatonin strongly re-localizes Bcl-2 from the cytosolic (plus microsomal) fraction to mitochondria (figure 6B). To double-check the translocation, we performed an immuno-fluorescence analysis; Bcl-2 appears diffusely distributed in the cytoplasm in control cells, whereas upon melatonin treatments, Bcl-2 assumes a bright dotted localization (figure 6C). The dotted pattern was identified as mitochondrial by merging the Bcl-2 spots with mitochondrial staining with the dye Mitotracker Red (MTR), which identifies mitochondria due to active trans-membrane potential (figure 6D, see merge). Thus, melatonin causes the translocation of Bcl-2 to mitochondria, which is a conceivable mechanism for inhibiting Bax activation at the mitochondrial level.

As we had previously shown, melatonin antagonizes apoptosis via interaction with the MT1/MT2 melatonin receptors. Thus, in order to evaluate the role of the signal transduction elicited by MT1/MT2 receptor stimulation on Bcl-2 re-localization, we analyzed whether luzindole, which specifically antagonizes melatonin binding/activation of MT1/MT2 receptors, is able to counteract melatonin-induced re-localization of Bcl-2. As shown in figure 6E, in the presence of luzindole melatonin is no longer able to re-localize Bcl-2. To confirm the involvement of the signal transduction, we analyzed a downstream step, of a more generalized nature, i.e., activation of phospholipase C (PLC), which may be contrasted by the inhibitor U73122. Also in the presence of U73122, Bcl-2 is unmoved by melatonin. These findings show that the signal transduction elicited by MT1/MT2 receptor stimulation by melatonin triggers a set of events culminating with Bcl-2 re-localization.
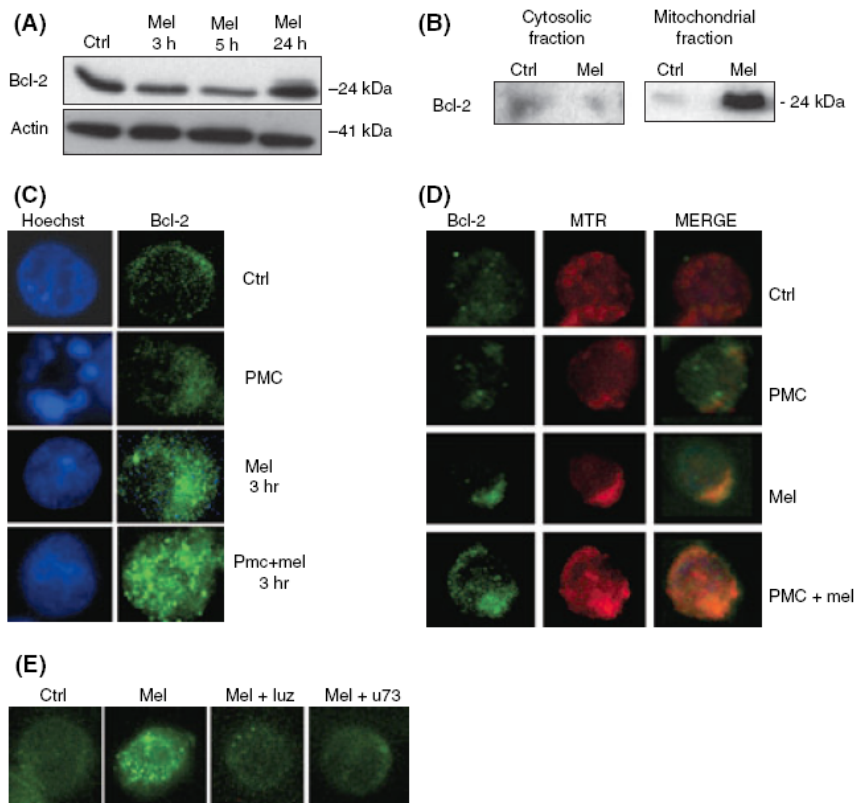
**Fig. 6 Melatonin induces the re-localization of Bcl-2 to mitochondria via MT1/MT2 receptor interaction.** Panel A shows the western blot analysis of the time course of Bcl-2 after melatonin treatment. Actin was used as a loading control in the bottom; Panel B shows the western blot analysis of Bcl-2 in the cytoplasmic vs. mitochondrial fraction of cells untreated or treated for 3hrs with melatonin; loading control was performed with Ponceau red staining of the nitrocellulose filter. Panel C and D show the immuno-fluorescence analysis with anti-Bcl-2 antibodies of cells treated as specified, counterstained with Hoechst to delineate nuclear status (panel C) and mitotracker red to label active mitochondria (panel D). The merging between the mitochondrial and Bcl-2 pattern is shown in the right column of panel D. Panel E shows the pattern of Bcl-2, as revealed by immuno-fluorescence with anti-Bcl-2 antibodies, in cells treated with melatonin and its receptor antagonist luzindole or with the phospholipae C inhibitor, U73122.

In various experimental models of tissue damage, it was reported a protective role of melatonin due to its ability of reducing oxidative stress. The protection against oxidative stress is due to the ability of melatonin to scavenge free radicals and to increase the activity of many antioxidant enzymes. Therefore, to understand whether melatonin's anti-apoptotic effect may depend on radical scavenging, we examined the effect of melatonin on ROS generation.

Surprisingly, we have shown **(paper IV**) that in U937 melatonin acts as a pro-oxidant, inducing a time dependent increase of intracellular ROS and promoting glutathione depletion. The activity of glutathione peroxidase is not modified by melatonin treatment as it does occur in other experimental models. Since apoptosis is very often dependent on oxidations, it has been hypothesized that the pro-radical effect of melatonin may be limited to tumor cells, thus providing a rationale to the selective pro-apoptotic effect on tumor cells (159).

To explore whether these correlations may apply to lymphocytic cells, we examined in **paper V** the effects of melatonin on the apoptotic process and intracellular ROS production on a set of lymphocytes, namely normal lymphocytes, and a couple of tumor lymphocytes, the Burkitt's lymphoma Epstein-Barr virus (EBV)-negative cells, BL41, and the same cells EBV-converted, E2r, that are resistant to apoptosis though having increased endogenous cellular levels of peroxides compared to their parental EBV negative cell line. We found that melatonin promotes ROS production on all these cells. Melatonin protects from apoptosis BL41 in the same guise as normal lymphocytes, whereas E2r are unaffected. These results show that ROS production is not limited to tumor lymphocytes, nor it is involved in apoptosis promotion; that melatonin does not promote apoptosis in tumor lymphocytes, but EBV inhibits melatonin anti-apoptotic effect; and that the anti-apoptotic effect of melatonin does not depend on melatonin's well known chemical antioxidant properties.

In order to explore the origin and the consequence of melatonin-induced ROS we evaluated the role of two intracellular melatonin binding sites (namely MT1/MT2 plasma membrane receptor and calmodulin) and the melatonin-induced oxidative status. We have demonstrated in **paper VI** that on U937 this pro-radical effect, evaluated as the increment of dichlorofluorescein (DCHF) signal, is immediate (<1min) and transient (up to 5-6 hrs) (fig. 7). Melatonin equally elicits its pro-radical effect on a set of normal or tumor leukocytes (fig. 8).
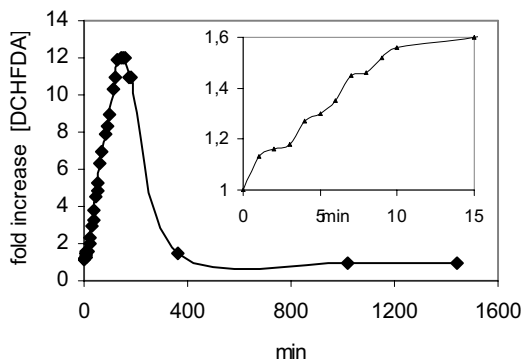
**Fig.7 Immediate and transient stimulation of intracellular reactive oxygen species by melatonin.** Flow cytometric analysis of ROS measured with DCHFDA for 24 h of treatment with 1mM melatonin; the insert magnifies the first 15 min.
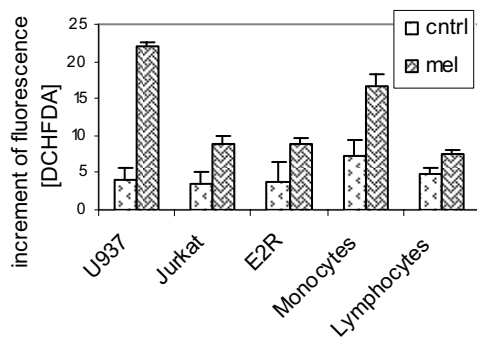


**Fig.8 The pro-radical effect of melatonin is general among leukocytes**. Flow cytometric analysis of ROS measured with DCHFDA after 3 h of 1 mM melatonin treatment in normal or tumor haematopoietic cells.

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Intriguingly, ROS production does not lead to a frank oxidative stress, as shown by absence of protein carbonylation, by maintenance of free thiols, preservation of viability and regular proliferation rate.

As far as the origin of melatonin-produced ROS, we showed that it is independent from MT1/MT2 receptor interaction; indeed, ROS production requires micromolar (as opposed to nanomolar) doses of melatonin and is not contrasted by the specific MT1/MT2 antagonist luzindole nor it is mimicked by a set of MT1/MT2 high affinity melatonin analogues. Instead, chlorpromazine, the calmodulin inhibitor shown to prevent melatonin-calmodulin interaction, also prevents melatonin pro-radical effect, suggesting that the low affinity binding to calmodulin (in the micromolar range) may be the event that promotes melatonin ROS production. We investigated the possible mechanism leading from calmodulin binding to ROS production

It was recently shown that, in addition to ROS detection, DCHF is a direct substrate of the pro-oxidant enzyme lipoxygenases (LOX), and that DCHF fluorescence is a function of LOX activity. Thus, we analyzed (**paper VII)** the effect of two additional, LOX-independent oxidation-sensitive probes, namely dihydrorhodamine (DHR) and dihydroethidium (DHE). Melatonin increases the fluorescence of these additional probes (fig. 9 A), confirming the notion of melatonin as an intracellular pro-oxidant agent. However, the extent of DCHF fluorescence is much higher than DHR and DHE, the different sensitivity to DCHF vs. DHR and DHE being possibly diagnostic for LOX activity. We demonstrated (**paper VII**) that melatonin-induced ROS are produced by lipoxygenase (LOX), since they are prevented by a set of LOX inhibitors (fig. 9 B) and are accompanied by increase of the 5-LOX product 5-HETE.

To understand the mechanism of LOX activation by melatonin, we explored whether it could derive from an increased availability of its substrate AA. Fig. 10A shows that 1mM melatonin determines a strong release of AA; this is a very early event, which peaks at 60min. A dose-effect experiment (fig. 10B) reveals that stimulation requires at least 10 uM melatonin, being significant at 1 mM. When comparing ROS production and AA release, it becomes evident that the two phenomena are correlated, occurring with the same kinetics and requiring the same doses of melatonin.

**A**

**B**
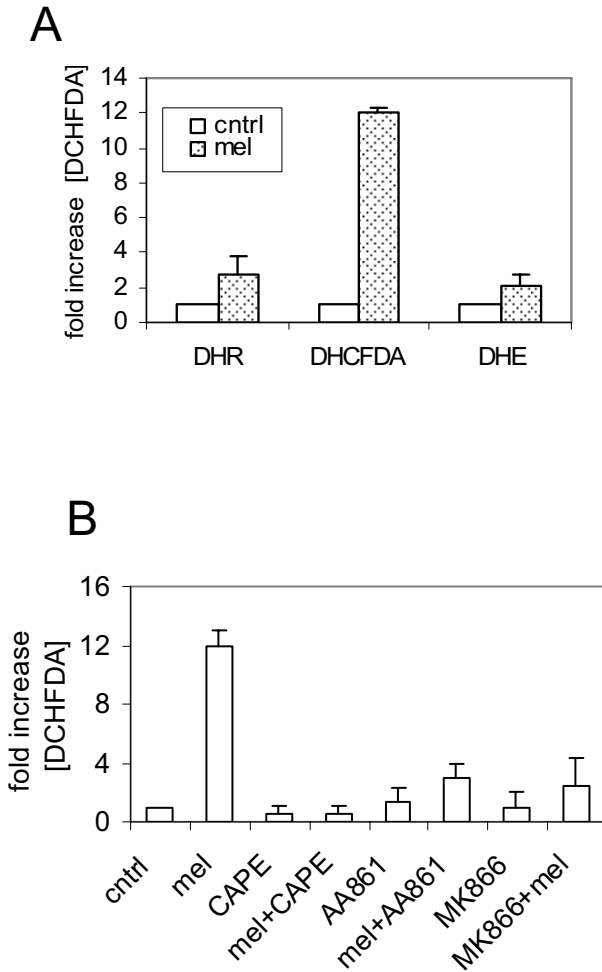
**Fig. 9 Melatonin produces reactive oxygen species by stimulating lipoxygenas.** A)it is shown the flow cytometric analysis of ROS measured with three different redox-sensitive probes, DHR, DCFDA or DHE after 3 h of 1 mM melatonin treatments. B) U937 cells were pre-treated for 30 min with either the general LOX inhibitor CAPE, or the specific 5-LOX inhibitor AA861, or the FLAP inhibitor MK866; 1 mM melatonin was then added. ROS were evaluated with DCFDA at 3 h of melatonin. Results indicate differences with respect to control posed =1 and are the average of 3 independent experiment +/- SD.
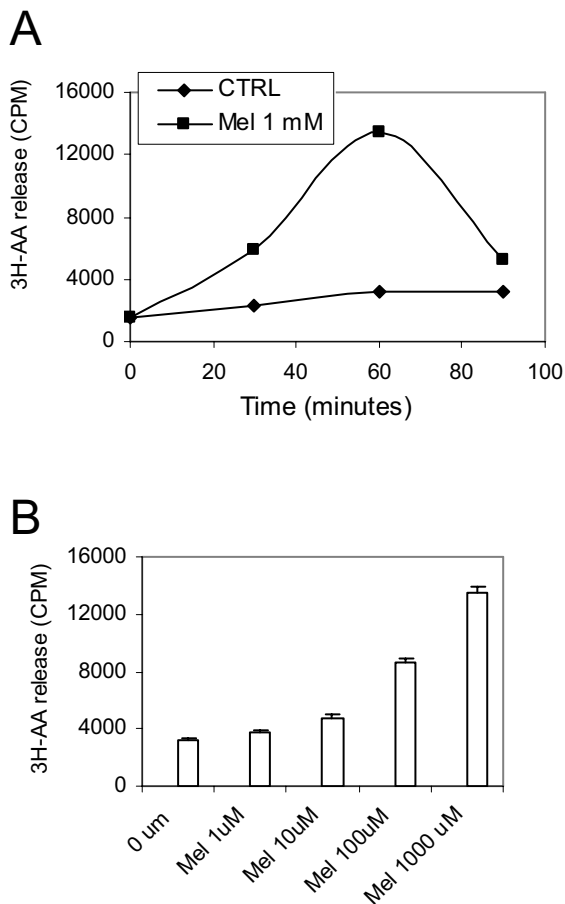
**Fig. 10  Melatonin stimulates the release of AA.**
   (A) shows the time course of extracellular release of [3H]-AA (see Methods) for 90 minutes of treatment with 1 mM melatonin; 3 experiments have been performed with similar results; 2 uM ionomicin was used as a positive control (11,514±312). (B) shows the measurement of extracellular release of [3H]-AA (see Methods) after 1 hr of treatment with different doses of melatonin.

Arachidonic acid is mainly released by phospholipase A2. To understand whether arachidonic acid is released by melatonin through PLA2, and in case by which isoform, we analyzed the effect exerted by different inhibitors of PLA2 on ROS production and arachidonic acid release. We found that melatonin-produced ROS are abrogated by the general PLA2 inhibitor brophenacil bromide (BPB) as well as by bromoenol lactone (BEL), an inhibitor of the Ca2+-independent PLA2, indicating that this isoform of PLA2 is involved (fig. 11A). Melatonin-induced arachidonic acid release was also abrogated by BEL (fig. 11B), supporting the involvement of the Ca2+-independent form of PLA2 (iPLA2), whereas it resulted insensitive to the Ca2+-dependent PLA2 inhibitor arachidonyl-trifluoromethyl ketone (AACOCF3), indicating that the latter isoform is not implicated. Overall, these results indicate that arachidonic acid is liberated by melatonin through PLA2, and specifically by Ca2+-independent PLA2. Since arachidonic acid is a substrate of LOX, its release should be an upstream event with respect to ROS production; thus, we expect that inhibitors of LOX prevent ROS but not melatonin-induced arachidonic acid. Indeed, LOX inhibition only prevents ROS, indicating that PLA2 is upstream with respect to LOX, as occurs in many signalling pathways. As the next step, we explored the mechanism through which melatonin could activate iPLA2. It is known that this isoform may bind to calmodulin, thus being sequestered in the cytosol; its liberation leads to membrane translocation and activation (196). But also melatonin binds to calmodulin (118), and we have shown that this interaction may initiate the pro-radical activity of melatonin. All this evidence strongly pushed us examining whether also AA release (in addition to the already shown ROS production) may depend on calmodulin-melatonin interaction. Chlorpromazine, the inhibitor of melatonin-calmodulin interaction, inhibits both ROS and arachidonic acid production, thus possibly placing calmodulin at the origin of a melatonin-induced pro-radical pathway. As already mentioned, Ca2+-independent PLA2 is the isoform that binds to calmodulin: our results are compatible with PLA2 being liberated by melatonin from a steady-state calmodulin sequestration, thus initiating an arachidonate signal transduction. We hypothesize the existence of a pathway where melatonin binds to calmodulin, thus inducing the release of sequestered Ca2+-independent PLA2; this is thus free to move to membranes and release high doses of AA; in turn, liberated AA feeds 5-LOX to produce free radicals and 5-HETE.

## A
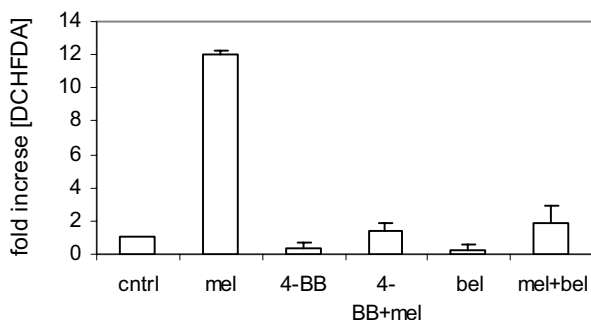


## B
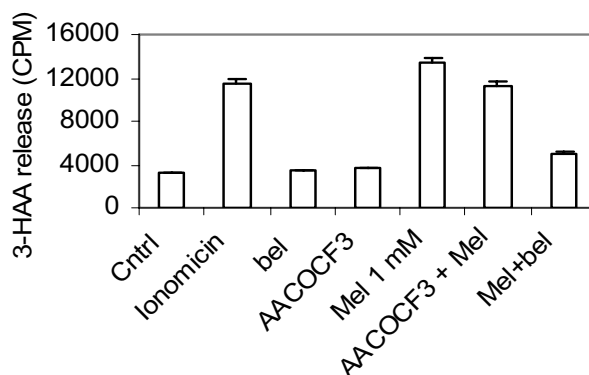


**Fig. 11 Ca2+-independent PLA2 mediates melatonin-induced AA release and ROS production.** (A) U937 cells were pre-treated for 30 min with either the general PLA2 4-Brophenacil Bromide (BPB) and a specific Ca2+-independent inhibitor Bromoenol Lactone (bel), 1mM melatonin was then added. ROS were evaluated with DCFHDA at 3 h of melatonin.(B) shows the measurement of extracellular release of [3H]-AA (see Methods) after a pre-treatment for 30 min with bel or the cytosolic (Ca2+-dependent) PLA2 (cPLA2) inhibitor Arachidonyltrifluoromethyl ketone (AACOCF3), and then 1 hr of 1mM melatonin treatment. Ionomicin was used as a positive control. Results are the average of 3 independent experiment +/- SD.

We have shown that the anti-apoptotic effect of melatonin requires MT1/MT2 plasma membrane receptor stimulation, but that the doses necesary for contrasting apoptosis are 10000 times higher. To solve this apparent paradox, we investigated first whether melatonin is able to engage receptors at nM concentrations in our cell system. Thus, we measured the IP3 intracellular concentration at different melatonin doses; we have found (**manuscript VIII)** that melatonin is able to induce a canonical stimulation of IP3 signal at nM doses. Since MT1/MT2 receptor engagement is the mechanism through which melatonin contrasts apoptosis, it is not clear why the doses of melatonin required to counteract apoptosis on U937 are so much higher than those necessary to efficiently engage receptors. we reasoned that MT1/MT2 pathway might be necessary but not sufficient for apoptosis antagonism, and that an additional target to which melatonin binds with lower affinity such as calmodulin may be required. Thus, we analyzed whether apoptosis antagonism might require the independent stimulation of an additional, lower affinity target.

It is evident that melatonin doses required for LOX activation (>10uM) are similar to those required for apoptosis antagonism (>1uM): we explored whether calmodulin, or one of the molecular determinants placed downstream to it in the signal transduction pathway proposed in paper VII, may be required for melatonin anti-apoptotic effect.

In **manuscript VIII,** we demonstrate that LOX activation, and specifically the LOX products 5-HETE is also required for successful anti-apoptotic effect. First, we demonstrated that a generic LOX inhibitor CAPE and the 5-LOX inhibitor AA861 reverted melatonin anti-apoptotic effect on all cell types examined, indicating 5-LOX involvement in melatonin anti-apoptotic effect. This finding suggests that lipoxygenase metabolism cooperates with MT1/MT2 signalling for apoptosis antagonism. Second, the exogenous addition of 5-HETE, restores melatonin anti-apoptotic effect abolished by LOX inhibition (fig.12 A). In particular, the exogenous addition of 5-HETE, bypassing the requirement for LOX activation, lowers the concentrations of melatonin necessary for apoptosis antagonism down to those required for IP3 receptor stimulation. Indeed, ss shown in panel 12 B, 5-HETE allows melatonin anti-apoptotic effect to occur at nM concentrations.

Then we analyze which role LOX (5-HETE) play in the anti-apoptotic effect.

The first piece of data is that melatonin by itself induces a rapid and substantial re-localization of Bax to mitochondria, in the absence of any apoptogenic stimuli. This is surprising since mitochondrial re-localization of Bax is generally an active, pro-apoptotic event, whereas melatonin is an anti-apoptotic agent. Moreover, we had shown (see paper III) that impairment of Bax activation is the very target of melatonin anti-apoptotic effect.

We analyzed how melatonin sends Bax to mitochondria, and especially if requires MT1/MT2 receptor engagements and/or LOX activation (HETE); and whether this may be a novel anti-apoptotic event, being in fact a sequestration rather than an activation.

We demonstrated that Bax mitochondrial translocation requires both HETE production and Bcl-2. Indeed, immuno-fluorescence analysis shows that in the presence of the 5-LOX inhibitor AA861, melatonin is no longer able to re-localized Bax in the mitochondria. However, down-regulating Bcl-2, melatonin is no more able to re-localize Bax to mitochondria (fig.13), suggesting that Bcl-2 is involved in Bax translocation.

Thus, melatonin-induced Bax translocation from cytosol to mitochondria requires the simultaneous presence of two different effectors: HETE (via the calmoduli/LOX pathway) on the one side, and Bcl-2 relocalization (via the MT1/MT2 receptor/G-protein pathway) on the other. We demonstrated, through an immuno-precipitation assay, that melatonin promotes the direct physical interaction between Bax and Bcl-2 on a total cell lysate. This suggests that Bcl-2 may target Bax in the cytosol, sequester it by co-localization in mitochondria.

Thus, melatonin inhibits apoptosis through a surprisingly complex cooperation between receptor-induced signal transduction pathway and LOX activation, by directly hitting two different, primary targets (MT1/MT2 and calmodulin). These pathways converge into melatonin antiapoptotic effect at the mitochondrial (or pre-mitochondrial) level, preventing the activation of Bax, the key trigger of the intrinsic apoptotic pathway.
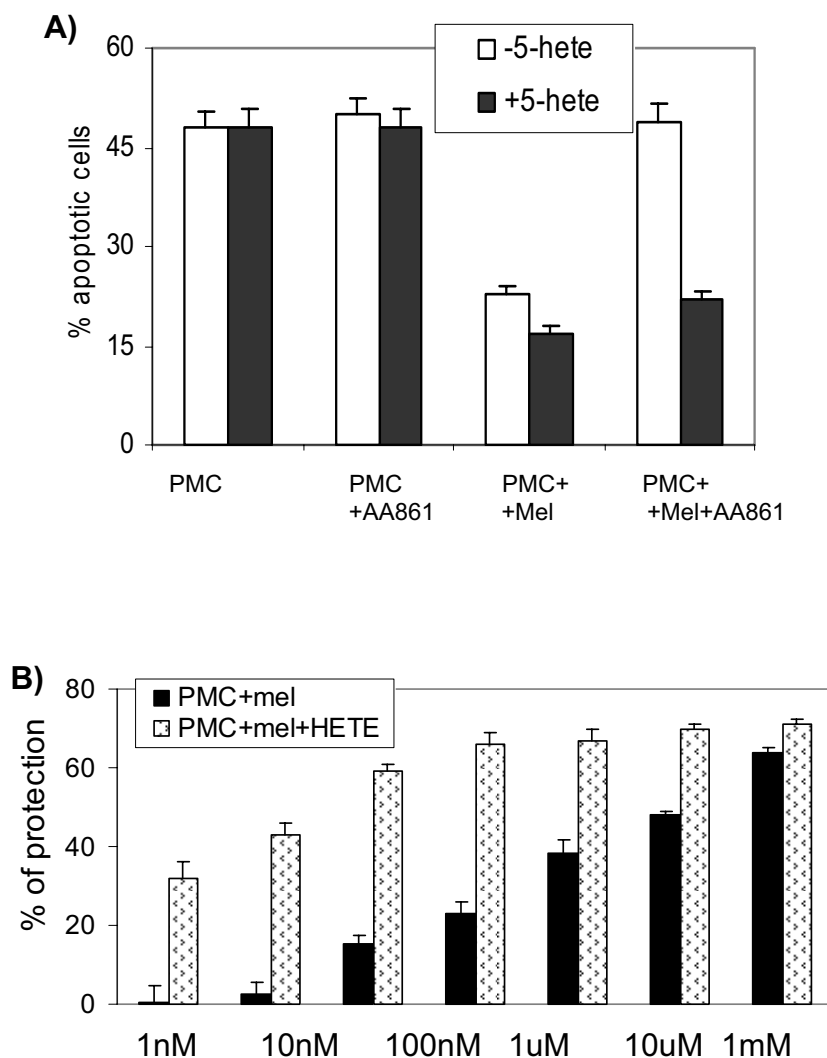
**Fig. 12 Lipoxygenase metabolism cooperates with MT1/MT2 signaling for apoptosis antagonism.** (A) U937 cells were pre-treated for 2 hrs with 5-hete and 30 min with a 5-LOX inhibitor AA861 before 1mM melatonin treatment; and then apoptosis was induced with PMC 10ug/ml. b) U937 cells were pre-treated for 2 hrs with 5-hete before 1mM melatonin treatment; and then apoptosis was induced with PMC 10ug/ml.
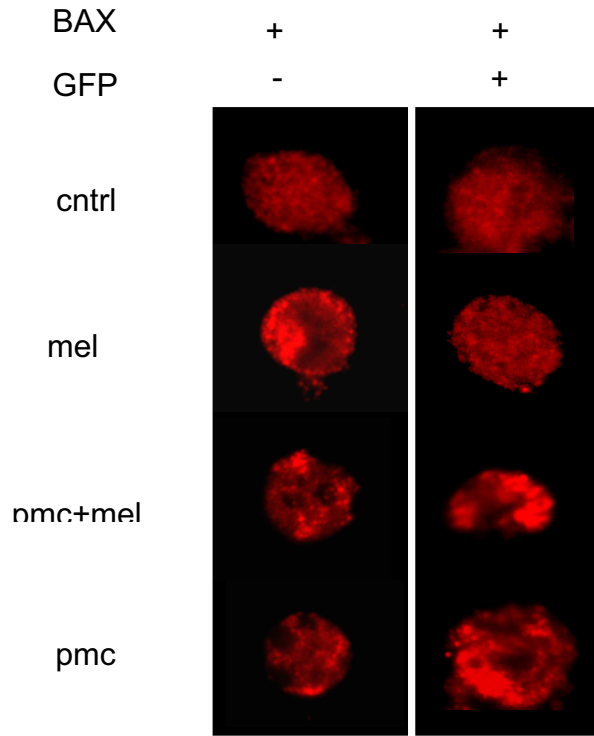
**Fig.13 Bax mitochondrial traslocation requires Bcl-2 involvement.** U937 cells were nucleofected with specific Bcl-2 siRNA and Green Fluorescence Protein (GFP), and immuno-stained with TRIC-conjugated anti-Bax antibodies. Immunofluorescence shows the pattern of Bax among the successfully vs. unsuccessfully transfected cells.

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The novelty of this finding is that Bax is maintained within mitochondria in a anti-apoptotic form. Indeed, melatonin causes the translocation of Bcl-2 to mitochondria, inhibiting Bax activation/dimerization at the mitochondrial level trough a direct binding with Bcl-2.

In paper III we showed that Bcl-2 is transactivated by melatonon at longer incubation times, i.e., 24hrs; this confirms previous findings in the literature. Since this might be an additional mechanism through which melatonin contrasts apoptosis via Bcl-2 in leukocytes, we explored whether an additional Bcl-2-related pro-survival pathway may exist. To this purpose, we considered NF-kB. In **paper IX,** we demonstrate that melatonin promotes a pro-survival activation of NF-kB. Melatonin promotes the activation of NF-kappaB, the transcptition factor often involved in pro-survival pathways, via the canonical pathway (i.e., p50/p65). In this pathway, activation implies degradation of the sequestering protein IkB and nuclear translocation of NF-kB subunits p50/p65. We showed that over-expression of dominant negative IkB, which prevents NF-kB activation, not only prevented melatonin antiapoptotic effect but even transformed melatonin in a pro-apoptotic molecule. The timing of NF-kappaB activation overlaps with the timing of ROS generation by melatonin. The results indicate a possible involvement in survival pathways of melatonin generated ROS.

We demonstrated that melatonin is required to induce a pro-survival NF-kB pathway in order to allow cells to survive to the same melatonin treatment. This might imply that melatonin could be per se a potent stress agent affecting cell viability, in those cells where such survival pathway cannot be promoted. Thus, the effect of melatonin on Bcl-2 re-localization and on NF-kB activation might belong to two different overlapping pro-survival pathways.

We extended our research on non-hematopoietic cells, by analyzing the effect on a human astrocytoma cell line. Thus, with the goal to clarify the therapeutic potential of melatonin in neurodegenerative disease and neurological disorder, we investigated the role of melatonin on apoptosis of astrocytoma.

In **paper X**, we describe the neuro-protective potential of melatonin, showing that melatonin significantly reduces damage-induced apoptosis in astrocytoma cells. However, the mechanism of protection is different with respect to that shown on U937 (and conceivably all the other

leukocytes examined), since it does not involve MT1/MT2 or lipoxyegenase; likewise, Ca2+ influx is not involved. Intriguingly, inhibition of PLC with neomycin reverses melatonin protection, suggesting that a PLC-dependent signal transduction, different from that triggered by MT1/MT2, may be involved in the anti-apoptotic pathway of melatonin. Thus it is very important to consider that melatonin may exert very different effects depending on the cell type under study, and that even if the end result is similar, it may take place according to different mechanisms. In astrocytoma, antagonism of apoptosis occurs in a lipoxygenase-independent, MT1/MT2 receptor-independent way, but with the possible involvement of the PLC pathway. We cannot rule out other possible explanations; indeed, neomycin may have other cellular targets, and other means of PLC inhibition will help to identify more clearly the role of PLC. Further investigations are under study to elucidate the mechanism of this effect, since the identification of the mechanism of melatonin protective effect may contribute to generate novel strategies for treating CNS injuries.

# DISCUSSION

The aim of my thesis was to investigate the role of melatonin on apoptosis of leukocytes and to understand the mechanisms involved, with the goal to explore a possible melatonin pharmacological and inflammatory perspective.

In particular, the first goal of my PhD work was to understand whether melatonin's anti-apoptotic effect derives from its ability to engage specific receptors, thereby triggering a signal transduction pathway, or whether melatonin's radical scavenging properties may interfere with apoptosis.

In this investigation, with the goal to clarify and to exploit the melatonin potential therapeutic role the mechanisms involved on melatonin anti-apoptotic effects have been highlighted.

Early observations showed that at physiological concentrations melatonin inhibits tumor growth in animal models (169) and proliferation of tumor cells in vitro (192) leading researchers to postulate a possible action of melatonin as a pro-apoptotic agent in cancer cells (159), with very important pharmaceutical use in anti-cancer treatments. Melatonin is believed to increase the efficacy of some specific chemotherapic drugs on cancer cells (176), being directly pro-apoptotic in many cancer cells while functioning as an anti-apoptotic agent in normal cells (166, 169). Even though such effects have been reported in several review articles, few original studies reported it. Also our results failed to confirm them, demonstrating instead an anti-apoptotic effect on all the cells examined. Indeed melatonin significantly reduces apoptosis on normal and tumor leukocytes induced by two different types of inducers: physiological and cell-damaging. It is an established notion that some types of apoptosis, especially those induced by cell damage and occurring through the so-called intrinsic (previously referred to as mitochondrial) pathway, imply redox imbalance or even direct oxidative stress. Thus, it was reasonable to hypothesize that the well known radical-scavenging properties of melatonin was the mechanism through which melatonin exerted its anti-apoptotic activity.

Even though it is not an openly accepted notion, recent data from the literature (159) report that in cancer cells melatonin is a producer of intracellular radicals. Indeed, on a set of cells most of which of tumor origin, it has been demonstrated a surprising pro-radical activity of melatonin (159). This pro-radical effect was confirmed also in our system. We have shown that both normal and tumor white blood cells react to melatonin producing free radicals: thus, at least for leukocytes,

the paradigm that melatonin only elicits a pro-radical effect on tumor cells is not applicable (**paper VI**).

The notion that a well-known anti-oxidant such as melatonin may behave as a pro-oxidant, is an (apparent) contradiction that still needs to be explained. Anti-oxidants may often exert pro-oxidant activities according to their concentrations and/or the surrounding milieus, as shown for many endogenous or exogenous compounds, such as tocopherol or ascorbate (197); this is probably related with the reactivity of each redox couple. In the case of melatonin, conceivably the pro-radical effect is not a direct chemical pro-oxidant ability of melatonin (which was never demonstrated in vitro), but a cellular reaction. The effect is so rapid, that it cannot be ascribed to an exhaustion of cell anti-oxidant defences, which in fact are unaffected in our system in the first 24 h of melatonin treatment (**paper IV**)

Intriguingly, we report that two markers of oxidation are differently affected by melatonin: protein carbonylation is decreased (anti-oxidant effect), whereas free thiols (glutathione) are unaffected. This may be explained by the notion that the scavenging efficiency of melatonin depends on the type of radical involved thus, the chain of oxidations primed by melatonin may be differently scavenged by melatonin itself, thus allowing only part of radical signalling to actually take place.

The apparent contradiction that a well known anti-oxidant such as melatonin may behave as a pro-oxidant, may be more easily accepted by the finding that the pro-radical effect is not a direct chemical pro-oxidant ability of melatonin, but the result of the stimulation of a signalling pathway (via calmodulin-PLA2-5-lipoxygenase), thus implying that the biological pro-radical effect and the chemical radical scavenging activity of melatonin may actually co-exist. Indeed, we show (**paper VII**) that ROS production is independent of stimulation of the plasma membrane MT1-MT2 receptors. Instead, we present evidences that the pro-radical activity may derive from the activation of LOX, by many orders of evidences: first, it is prevented by all of the LOX inhibitors tested (CAPE, AA861, MK866); second, is marked by a transient increase in the intracellular levels of 5-HETE; third, the differential stimulation that melatonin exerts on the probe DCF over the DHR or DHE (12 fold vs. 2-3 fold): DCFH is a direct substrate for the enzyme, and on this basis, a fluorescence-based enzyme assay of human 5-lipoxygenase has been developed in *in vitro* systems (198); also in *in vivo* systems, a similar correlation with LOX expression and DCF over DHE fluorescence was

recently reported (199). Thus, our results suggest that DCF fluorescence increase by melatonin is partly due to oxidation by ROS, and partly to be ascribed with direct interaction with LOX. The ability of melatonin to elicit the activation of LOX provides a possible mechanism to explain why the pro-radical effect of melatonin does not create any toxicity to U937 cells. Indeed 5-LOX has been shown to supply pro-survival signals to many cells (198, 200) in tumor cells, LOX has been implicated in chemo-resistance (200); we have reported that 5-LOX expression is required to maintain viability of EBV-converted B lymphocytes, its inhibition being a rapid and efficient mean of killing EBV+ cells (198); all these findings have suggested the notion that LOX may play a role in facilitating tumor progression. We have recently shown that freshly explanted blood monocytes require LOX-derived signals and ROS to maximize in vitro maturation and viability by triggering over-expression of Bcl-2 (201), suggesting that LOX may control survival also in normal cells.

We found that a large amount of AA is released upon melatonin treatment thus possibly being made accessible to LOX. The major responsible for the liberation of AA from the phospholipids bi-layer is PLA2, which exists in various isoforms that are differently activated and are sensitive to different inhibitors (202). In our system, the inhibitors pattern that modulates melatonin-induced AA release and ROS production indicates that the form of PLA2 involved is the $Ca^{2+}$-independent. This was a very important point in this study, because it is known that this isoform can be bound and sequestered by calmodulin, and that its release is an activator event (196). This provides a possible interpretation of the mechanism through which melatonin may activate PLA2 and LOX, since calmodulin is one of the low affinity targets of melatonin. The melatonin/calmodulin binding was found sensitive to the calmodulin inhibitor chlorpromazine but not calmidazolium (203); the specific action of these inhibitors led the authors to suggest that melatonin binds to the calmodulin domain interacting with protein partners. We explored the possibility that melatonin pro-radical effect may be actually triggered upon binding to calmodulin, and presented two evidences in support. First, melatonin binds to calmodulin at concentrations (Kd=63uM) (203) compatible with the melatonin concentrations required for the pro-radical effect and the AA release. Second, our analysis showed that also AA release and ROS production are sensitive to chlorpromazine but insensitive to calmidazolium. These

evidences suggest for the first time a possible function for the high-dose calmodulin binding. We hypothesize the existence of a pathway where melatonin binds to calmodulin, thus inducing the release of sequestered Ca2+-independent PLA2; this is thus free to move to membranes and release high doses of AA; in turn, liberated AA feeds 5-LOX to produce free radicals and 5-HETE (see fig. 1).
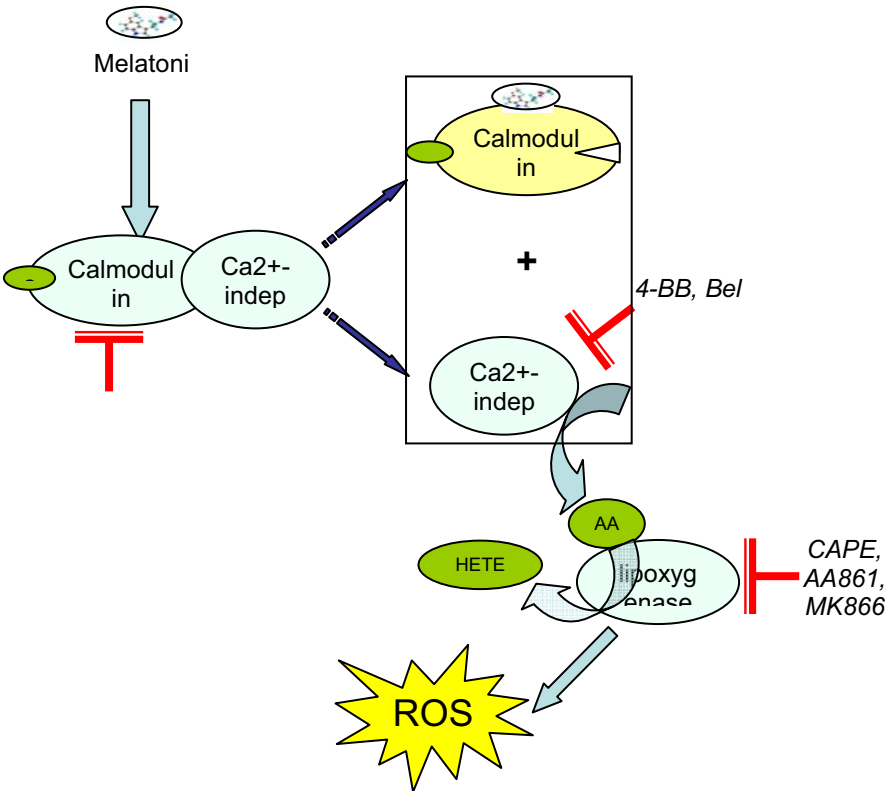


**Fig. 6. Melatonin-induced pro-radical pathway: a hypothesis.** Melatonin might bind to calmodulin, thus inducing the release of sequestered Ca2+-independent PLA2; this is thus free to move to membranes and release high doses of AA; in turn, liberated AA feeds 5-LOX to produce free radicals and 5-HETE. The inhibitors used, as well as the products enhanced by melatonin (HETE, ROS, AA), which allowed proposing this model, are indicated.

These findings open a new important important avenue for the pharmacological use of melatonin on inflammation. In particular, it has been shown that melatonin down-regulates, or inactivates, the pro-inflammatory enzymes PLA2 (204) and LOX (156). In this context, the activation of the same enzymes, LOX and PLA2 that we report here, may seem a paradox. However, we have shown that the melatonin-induced increases of ROS, 5-HETE and AA are all transient phenomena. This is consistent with single-hit LOX stimulation: indeed, LOX participates to the early phases of the inflammatory response, since leukotrienes promote diapedesis by acting as vessel permeabilizing agents and chemo-attractants. In the absence of further signals, LOX effects are of short duration, and extinguish within 2-3 h: this coincides with the time required for the decrease of DCF signal and AA after melatonin treatment. A conciliation between our results with those available in the literature suggests that melatonin may transiently activate LOX via calmodulin binding, while working for its silencing via other targets/mechanisms; an important implication of this reasoning would be that melatonin might facilitate promoting the inflammatory response, at the same time limiting it by providing to its resolution via down-regulation of LOX (or PLA2), thus helping avoiding complications of chronic inflammation. Indeed, if LOX is required for a correct inflammatory response, its altered/prolonged activation may favor chronic inflammation and the onset of extremely serious diseases such as atherosclerosis, tumor progression, infarction (205).

We provide strong evidence that the signal transduction elicited by melatonin receptor stimulation is the mechanism through which melatonin exerts its anti-apoptotic effect in U937 monocytes. Among all the described evidences spanning from the use of melatonin analogs to the identification of signal transduction steps, the key indications come from the effects exerted by luzindole, which specifically antagonizes MT1/MT2 receptor thus emphasizing the importance of these findings.

MT1 receptor mediates the melatonin-induced inhibition of PGE2 in lymphocytes (206). Both PLC and adenylcyclase pathways have been shown to be involved in melatonin-induced signal transduction; interestingly, under different conditions (i.e., cell type), $IP_3$ and cAMP, products of the two pathways, respectively, can be reciprocally activated/repressed by melatonin (207). We found that $IP_3$ is transiently induced by melatonin, and that inhibition of PLC is sufficient to reverse melatonin anti-apoptotic effect. This indicates that the anti-apoptotic

effect of melatonin requires this particular pathway. The PLC pathway may possibly counteract apoptosis by promoting $Ca^{2+}$ influx; indeed, inhibition of $Ca^{2+}$ influx was able to reverse melatonin's anti-apoptotic effect. An increase in cytosolic $Ca^{2+}$ was originally thought of as a canonical pro-apoptotic signal [48], but it is now well demonstrated that $Ca^{2+}$ influx may have also a pro-survival role (208). We demonstrated that Bcl-2 is required for the anti-apoptotic effect of melatonin, since its silencing, or the inhibition with BH3-mimetic synthetic small proteins, abrogate melatonin anti-apoptotic effect.

As luzindole, an antagonist of melatonin MT1/MT2 receptors, reverts the ability of melatonin to re-localize Bcl-2 to mitochondria, and to protect from apoptosis, we conclude that the signal transduction elicited by interaction with MT1/MT2 is responsible for such an event. Interestingly, this signal transduction involves phospholipase C (PLC), since its inhibitor U73122 also reverts melatonin-induced Bcl-2 re-localization. Bcl-2 behaviour/localization, and particularly mitochondrial targeting , are controlled by a very complex phosphorylation pattern on multiple aminoacid residues (20). We hypothesize that a specific kinase may be activated/disactivated as a result of MT1/MT2-elicited signal transduction. Indeed, our preliminary data suggest that protein kinase C, which is known to phosphorylate Bcl-2, and to be a downstream target of PLC, may be involved in melatonin-induced Bcl-2 re-localization.

In spite of the many evidences we provide that melatonin antagonizes apoptosis via receptor interaction, it is perplexing that the doses of melatonin required to counteract apoptosis are so much higher than those necessary to efficiently engage receptors. We explored whether the $MT_1$/$MT_2$ pathway might be necessary but not sufficient for apoptosis antagonism, and an additional target to which melatonin binds with lower affinity (e.g., calmodulin, kd=63uM) (204) may be required. To address this apparent paradox, we analyzed whether apoptosis antagonism might require the independent stimulation of an additional, lower affinity target. We showed that melatonin activates 5-lipoxygenase in a MT1/MT2-independent fashion, at the same doses required for apoptosis antagonism. We report that melatonin anti-apoptotic effect is abolished by the inhibition of 5-lipoxygenase, and restored by the addition of its product 5-HETE, thus indicating that lipoxygenase metabolism cooperates with MT1/MT2 signalling for apoptosis antagonism. In the presence of exogenously added 5-HETE, i.e., bypassing the bottleneck of lipoxygenase requirement, melatonin

becomes able to antagonize apoptosis at nanomolar doses, demonstrating that the independent and simultaneous interaction with high (MT1/MT2) and low (lipoxygenase) affinity targets are required for, and converge into, the anti-apoptotic effect. These pathways converge into melatonin anti-apoptotic effect at the mitochondrial level possibly promoting the sequestration in mitochondria of Bax, the key trigger of the intrinsic apoptotic pathway. We demonstrated that Bax mitochondrial localization is abolished in presence of 5-LOX inhibitor AA861 and, down-regulating Bcl-2. These suggest that Bax mitochondrial translocation requires both HETE production via LOX activation and MT1/MT2 receptor Bcl-2 re-localization.

The novelty of these findings is that maintainance of Bax within mitochondria is an anti- apoptotic event. Indeed, melatonin causes the translocation of Bcl-2 to mitochondria, inhibiting Bax activation at mitochondrial level trough a direct binding with Bcl-2, blocking cytocrome c release, inducing in this way a pro-survival pathway (see fig.2).

It is known that mitochondria are one of the most important sites where Bcl-2 exerts its anti-apoptotic functions, especially as far as inhibition of Bax activity is concerned. It is known since long time that the balance between Bax and Bcl-2 determines the propensity of cells to respond with apoptosis or survival to a given insult (23). The molecular mechanisms through which this occurs have been clarified, consisting on the sequestration of Bax, which thus becomes unable to exert its pore forming action on the mitochondrial membrane and release cytochrome c. Sequestration may occur either directly, via the BH3 domain (22) or by complex interplays with the cognate proteins belonging to the sub-family of BH3-only proteins, whose behaviour is influenced by Bcl-2 concentration/localization (30). Thus, we think that Bcl-2 re-localization to mitochondria may favour its Bax-contrasting activity even in the absence of an up-regulation.

Bax activation is a complex phenomenon that requires multiple steps, whose order is still a matter of debate. The exposure of the N-terminus of Bax occurs concomitantly with the release of the mitochondrial apoptotic factors (cytochrome c or SMAC/diablo), being in fact a synonimous of Bax activation (26); however, it is still discussed whether the conformational change occurs prior to, or after, translocation to mitochondria (26). Our results show that, in the presence of melatonin, Bax is induced to translocate in the absence of N-term exposure,

suggesting that this latter event occurs after translocation. However, we have to consider that the melatonin-induced Bax translocation is an anti-apoptotic, rather than a pro-apoptotic event, and conceivably its mechanism may completely diverge from what has been described so far. The finding that melatonin promotes the physical interaction between Bax and Bcl-2, suggests a novel mechanism of Bax inactivation, i.e., the mitochondrial sequestration by Bcl-2.

We have recently shown that oxidative alterations cause Bax disulfide dimerization, proposing that the resulting conformational changes are sufficient for translocation to mitochondria (25). Strangely enough, cells induced to apoptosis after melatonin pre-treatment show the presence of a Bax monomer, suggesting that Bcl-2 prevents Bax dimerization within the mitochondrial membrane, through a direct binding with Bcl-2, thus blocking cytocrome c release and promoting survival.

We demonstrated that melatonin induces an additional pro-survival pathway involving NF-kB. The effect of melatonin on Bcl-2 re-localization and on NF-kB activation might belong to two different overlapping pro-survival pathways within leukemia cells. Indeed, the two pathways are temporally separated: Bcl-2 up-regulation occurs at >6 hrs of melatonin treatment, whereas Bcl-2 translocation is already detectable at 2-3hrs.

In conclusion, this is the first report showing:

a) a melatonin anti-apoptotic effect requiring stimulation of high (nanomolar) affinity plasma membrane receptors (MT1/MT2) and additional lower (micromolar) affinity targets, calmodulin.

b) a melatonin pro-oxidant effect that co-exists with, and contrasts, the well known chemical radical scavenging ability, which involves a direct bind to calmodulin, and pro-inflammatory enzyme activation 5-LOX.

c) Bcl-2 mitochondrial re-localization and sequestration of Bax, through a direct binding, suggesting a novel mechanism of Bax inactivation.

These findings open a new important avenue for the study of the role of melatonin in inflammation underlining unexpected potential role of melatonin as a regulator of homeostasis of immune and inflammatory response, controlling the balance between life/ death of leukocytes within the organisms.
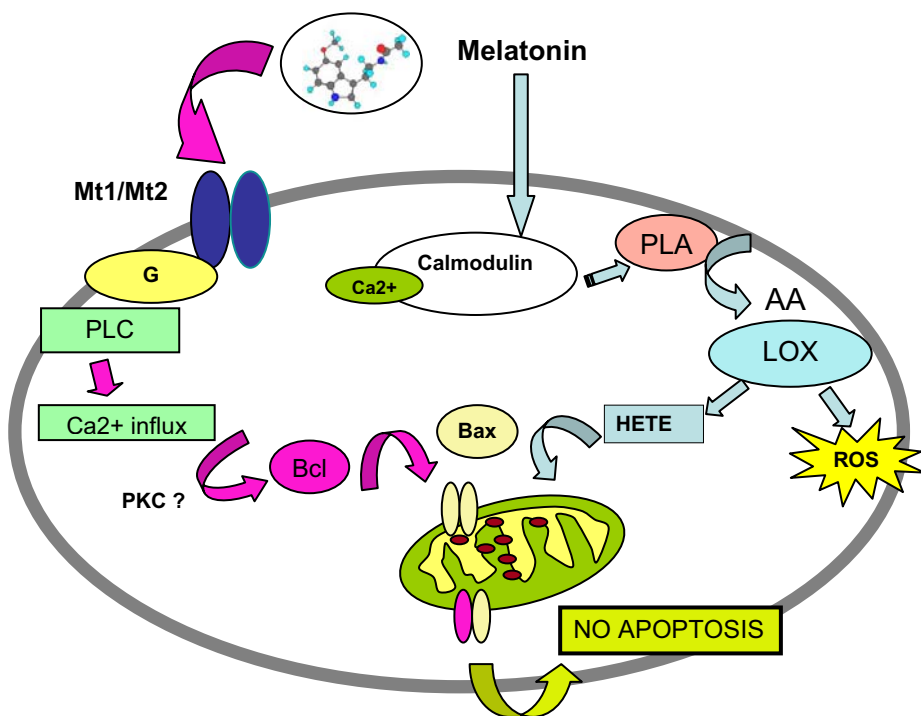
**Fig.2 Melatonin inhibits apoptosis through a double cooperating pathways, receptor-induced signal transduction pathway and calmodulin-LOX pathway**. These pathways converge into melatonin anti-apoptotic effect at the mitochondrial level, preventing the activation of Bax, the key trigger of the intrinsic apoptotic pathway. Melatonin causes the translocation of Bcl-2 to mitochondria, which directly binds to Bax inhibiting its activation/dimerization and blocks cytochrome c release (red dots).

# REFERENCES

1. Hetts S.W. To die or not to die: an overview of apoptosis and its role in disease. (1998) JAMA 28;279:300-7. Review.

2. KERR, J. F., A. H. WYLLIE, and A. R. CURRIE. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. (1972) .Br. J. Cancer. 26: 239-257.

3. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. (2008) Nat Rev Mol Cell Biol.. 9:47-59. Review

4. Green DR, Amarante-Mendes GP.The point of no return: mitochondria, caspases, and the commitment to cell death. Results Probl Cell Differ. 1998;24:45-61. Review

5. Bergeron L, Perez GI, Macdonald G, Shi L, Sun Y, Jurisicova A, Varmuza S, Latham KE, Flaws JA, Salter JC, Hara H, Moskowitz MA, Li E, Greenberg A, Tilly JL, Yuan J. Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev. 1998 May 1;12(9):1304-14.

6. Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase.Cell. 1995 Jun 2;81(5):801-9.

7. Thornberry NA. Caspases: key mediators of apoptosis. Chem Biol. 1998 May;5(5):R97-103. Review.

8. Ashkenazi A.Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer. 2002 Jun;2(6):420-30. Review

9. Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. (1995) Cell. 19;81(4):505-12.

10. Salvesen GS. Caspase 8: igniting the death machine. Structure. 1999 Oct 15;7(10):R225-9. Review.

11. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. (1998) Cell. 21;94(4):491-501.

12. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell. 1998. 94(4):481-90.

13. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004 Jul 30;305(5684):626-9. Review.

14. Mattson MP, Kroemer G. Mitochondria in cell death: novel targets for neuroprotection and cardioprotection. Trends Mol Med. 2003 May;9(5):196-205.

15. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ.Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science. 2001 Apr 27;292(5517):727-30.

16. Ravagnan L, Roumier T, Kroemer G.Mitochondria, the killer organelles and their weapons.J Cell Physiol. 2002 Aug;192(2):131-7. Review.

17. Saleh A, Srinivasula SM, Acharya S, Fishel R, Alnemri ES.Cytochrome c and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation.J Biol Chem. 1999 Jun 18;274(25):17941-5.

18. Vaux DL, Silke J.HtrA2/Omi, a sheep in wolf's clothing. Cell. 2003 Oct 31;115(3):251-3.

19. Lucken-Ardjomande S, Martinou JC. Regulation of Bcl-2 proteins and of the permeability of the outer mitochondrial membrane.C R Biol. 2005 Jul;328(7):616-31. Review.

20. Strasser A. The role of BH3-only proteins in the immune system. Nat Rev Immunol. 2005 Mar;5(3):189-200. Review

21. Labi V, Erlacher M, Kiessling S, Villunger A. BH3-only proteins in cell death initiation, malignant disease and anticancer therapy. Cell Death Differ. 2006 Aug;13(8):1325-38. Epub 2006 Apr 28. Review.

22. Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR, Thompson CB.The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell. 2000 Dec;6(6):1389-99.

23. Hsu YT, Wolter KG, Youle RJ. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A. 1997 Apr 15;94(8):3668-72.

24. Antonsson B, Montessuit S, Sanchez B, Martinou JC. Bax is present as a high molecular weight oligomer/complex in the mitochondrial membrane of apoptotic cells. J Biol Chem. 2001 Apr 13;276(15):11615-23. Epub 2001

25. D'Alessio M, De Nicola M, Coppola S, Gualandi G, Pugliese L, Cerella C, Cristofanon S, Civitareale P, Ciriolo MR, Bergamaschi A, Magrini A, Ghibelli L. Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis.FASEB J. 2005 Sep;19(11):1504-6. Epub 2005 Jun 21.

26. Nechushtan A, Smith CL, Hsu YT, Youle RJ.Conformation of the Bax C-terminus regulates subcellular location and cell death. EMBO J. 1999 May 4;18(9):2330-41.

27. Roucou X, Antonsson B, Martinou JC. Involvement of mitochondria in apoptosis. Cardiol Clin. 2001 Feb;19(1):45-55.

28. Dlugosz PJ, Billen LP, Annis MG, Zhu W, Zhang Z, Lin J, Leber B, Andrews DW.Bcl-2 changes conformation to inhibit Bax oligomerization. EMBO J. 2006 Jun 7;25(11):2287-96. Epub 2006 Apr 27.

29. Kim PK, Annis MG, Dlugosz PJ, Leber B, Andrews DW (2004) During apoptosis bcl-2 changes membrane topology at both the endoplasmic reticulum and mitochondria. Mol Cell 14: 523–529

30. Tan C, Dlugosz PJ, Peng J, Zhang Z, Lapolla SM, Plafker SM, Andrews DW, Lin J.Auto-activation of the apoptosis protein Bax increases mitochondrial membrane permeability and is inhibited by Bcl-2. J Biol Chem. 2006 May 26;281(21):14764-75. Epub 2006 Mar 29.

31. Wang K, Yin XM, Chao DT, Milliman CL, Korsmeyer SJ.BID: a novel BH3 domain-only death agonist. Genes Dev. 1996 Nov 15;10(22):2859-69.

32. Marani M, Tenev T, Hancock D, Downward J, Lemoine NR. Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. Mol Cell Biol. 2002 Jun;22(11):3577-89.

33. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ.Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell. 2002;2(3):183-92.

34. Scorrano L. and Korsmeyer S.J. Mechanisms of cytochrome c release by proapoptotic Bcl-2 family members. Biochem. Biophys. Res. Comm. (2003) 304:437-444.

35. Lorenzo HK, Susin SA, Penninger J, Kroemer G.Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell Death Differ. 1999 Jun;6(6):516-24. Review

36. Tsujimoto Y, Shimizu S.Bcl-2 family: life-or-death switch. FEBS Lett. 2000 Jan 21;466(1):6-10. Review.

37. Tsujimoto Y, Shimizu S.VDAC regulation by the Bcl-2 family of proteins. Cell Death Differ. 2000 Dec;7(12):1174-81. Review.

38. Gilmore TD.Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. 2006 Oct 30;25(51):6680-4. Review

39. Atreya I, Atreya R, Neurath MF.NF-kappaB in inflammatory bowel disease. J Intern Med. 2008 Jun;263(6):591-6. Review.

40. Sen R, Baltimore D.Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell. 1986 Dec 26;47(6):921-8.

41. Keutgens A, Robert I, Viatour P, Chariot A. Deregulated NF-kappaB activity in haematological malignancies. Biochem Pharmacol. 2006 Oct 30;72(9):1069-80.

42. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X.Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol. 1999;15:269-90. Review.

43. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC.IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J. 1998 Apr 15;17(8):2215-23

44. Paillard F.Induction of apoptosis with I-kappaB, the inhibitor of NF-kappaB. Hum Gene Ther. 1999 Jan 1;10(1):1-3. Review. No abstract available.

45. Yao H, Guo L, Jiang BH, Luo J, Shi X.Oxidative stress and chromium(VI) carcinogenesis.J Environ Pathol Toxicol Oncol. 2008;27(2):77-88. Review.

46. Ortona E, Margutti P, Matarrese P, Franconi F, Malorni W.Redox state, cell death and autoimmune diseases: a gender perspective. Autoimmun Rev. 2008 Jul;7(7):579-84. Epub 2008 Jul 1. Review

47. Andreyev AY, Kushnareva YE, Starkov AA (2005) Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc) 70:200–214

48. Fariss MW, Chan CB, Patel M, van Houten B, Orrenius S (2005) Role of mitochondria in toxic oxidative stress. Mol Interven 5:98–114

49. Orrenius S, Gogvadze V, Zhivotovsky B Mitochondrial oxidative stress: implications for cell death. Annu Rev Pharmacol Toxicol. 2007;47:143-83. Review.

50. Bohr VA (2002) Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. Free Radic Biol Med 32:804–812

51. Eu JP, Liu L, Zeng M, Stamler JS. An apoptotic model for nitrosative stress.Biochemistry. 2000 Feb 8;39(5):1040-7.

52. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J. 1997 May 15;324 ( Pt 1):1-18. Review.

53. Chrestensen CA, Starke DW, Mieyal JJ.Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. J Biol Chem. 2000. 25;275(34):2655665

54. England K, Cotter TG. Direct oxidative modifications of signalling proteins in mammalian cells and their effects on apoptosis. Redox Rep. 2005;10(5):237-45. Review

55. Gloire G, Legrand-Poels S, Piette J.NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol. 2006 Nov 30;72(11):1493-505. Epub 2006 Apr 27. Review

56. Zhang Y, Marcillat O, Giulivi C, Ernster L, Davies KJ (1990) The oxidative inactivation of mitochondrial electron transport chain components and ATPase. J Biol Chem 265:16330–16336

57. Albano E, Bellomo G, Parola M, Carini R, Dianzani MU (1991) Stimulation of lipid peroxidation increases the intracellular calcium content of isolated hepatocytes. Biochim Biophys Acta 1091:310–316

58. Bacon BR, O'Neill R, Britton RS (1993) Hepatic mitochondrial energy production in rats with chronic iron overload. Gastroenterology 105:1134–1140

59. Chen JJ, Bertrand H, Yu BP (1995) Inhibition of adenine nucleotide translocator by lipid peroxidation products. Free Radic Biol Med 19:583–590

60. Orrenius S, Zhivotovsky B, Nicotera P (2003) Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 4:552–565

61. Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tell G, Lungarella G, Colombo R, Rossi R, Milzani A.Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. Mass Spectrom Rev. 2005 Jan-Feb;24(1):55-99

62. Clarke MW, Burnett JR, Croft KD Vitamin E in human health and disease.Crit Rev Clin Lab Sci. 2008;45(5):417-50. Review

63. Panda AK, Ramarao SV, Raju MV, Chatterjee RN. Effect of dietary supplementation with vitamins E and C on production performance, immune responses and antioxidant status of White Leghorn layers under tropical summer conditions. Br Poult Sci. 2008 Sep;49(5):592-9.

64. Black HS, Gerguis J. Modulation of dietary vitamins E and C fails to ameliorate b-carotene exacerbation of UV carcinogenesis in mice.Nutr Cancer. 2003;45(1):36-45.

65. Hashimoto K, Takasaki W, Yamoto T, Manabe S, Sato I, Tsuda S. Effect of glutathione (GSH) depletion on DNA damage and blood chemistry in aged and young rats. J Toxicol Sci. 2008 Oct;33(4):421-9.

66. Buyukokuroglu ME, Cemek M, Yurumez Y, Yavuz Y, Aslan A. Antioxidative role of melatonin in organophosphate toxicity in rats. Cell Biol Toxicol. 2008 Apr;24(2):151-8. Epub 2007 Sep 2.

67. Mancini A, Leone E, Festa R, Grande G, Di Donna V, De Marinis L, Pontecorvi A, Tacchino RM, Littarru GP, Silvestrini A, Meucci E.Evaluation of antioxidant systems (coenzyme Q10 and total antioxidant capacity) in morbid obesity before and after biliopancreatic diversion. Metabolism. 2008 Oct;57(10):1384

68. R. Franco ; O. J. Schoneveld ; A. Pappa ; M. I. Panayiotidis. The central role of glutathione in the pathophysiology of human diseases. Arch Physiol Biochem. 2007 Oct-Dec;113(4-5):234-58. Review

69. Ghibelli L, Fanelli C, Rotilio G, Lafavia E, Coppola S, Colussi C, Civitareale P, Ciriolo MR.Rescue of cells from apoptosis by inhibition of active GSH extrusion. FASEB J. 1998 Apr;12(6):479-86.

70. Ghibelli L, Coppola S, Rotilio G, Lafavia E, Maresca V, Ciriolo MR.Non-oxidative loss of glutathione in apoptosis via GSH extrusion. Biochem Biophys Res Commun. 1995 Nov 2;216(1):313-20.

71. D'Alessio M, Cerella C, De Nicola M, Bergamaschi A, Magrini A, Gualandi G, Alfonsi AM, Ghibelli LApoptotic GSH extrusion is associated with free radical generation. Ann N Y Acad Sci. 2003 Dec;1010:449-52.

72. D'Alessio M, Cerella C, Amici C, Pesce C, Coppola S, Fanelli C, De Nicola M, Cristofanon S, Clavarino G, Bergamaschi A, Magrini A, Gualandi G, Ghibelli L.Glutathione depletion up-regulates Bcl-2 in BSO-resistant cells. FASEB J. 2004 Oct;18(13):1609-11. Epub 2004 Aug 2.

73. Mei G, Di Venere A, Nicolai E, Angelucci CB, Ivanov I, Sabatucci A, Dainese E, Kuhn H, Maccarrone M.Structural properties of plant and mammalian lipoxygenases. Temperature-dependent conformational alterations and membrane binding ability. Biochemistry. 2008 Sep 2;47(35):9234-42. Epub 2008 Aug 12.

74. P. Borgeat and B. Samuelsson, Arachidonic acid metabolism in polymorphonuclear leukocytes: unstable intermediate in formation of dihydroxy acids. Proc Natl Acad Sci USA 76 (1979), pp. 3213–3217.

75. P. Borgeat, M. Hamberg and B. Samuelsson. Transformation of arachidonic acid and homo γ linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenasesJ Biol Chem 251 (1976), pp. 7816–7820.

76. Maccarrone M, Melino G, Finazzi-Agrò A.Lipoxygenases and their involvement in programmed cell death.Cell Death Differ. 2001 Aug;8(8):776-84. Review.

77. Schnurr K, Belkner J, Ursini F, Schewe T, Kuhn H (1996) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase controls the activity of the 15-lipoxygenase with complex substrates and preserves the specificity of the oxygenation products. J. Biol. Chem. 271: 4653̃4658.

78. Sandstrom PA, Pardi D, Tebbey PW, Dudek RW, Terrian DM, Folks TM, Buttke TM (1995) Lipid hydroperoxide-induced apoptosis: lack of inhibition by Bcl-2 over-expression. FEBS Lett. 365: 6̃670.

79. Hébert M-J, Takano T, Holthöfer H, Brady HR (1996) Sequential morphologic events during apoptosis of human neutrophils. Modulation by lipoxygenase-derived eicosanoids. J. Immunol. 157: 3105̃3115.

80. Aoshima H, Satoh T, Sakai N, Yamada M, Enokido Y, Ikeuchi T, Hatanaka H (1997) Generation of free radicals during lipid hydroperoxide-triggered apoptosis in PC12h cells. Biochim. Biophys. Acta 1345: 3̃542.

81. Liberles SD, Schreiber SL (2000) Apoptosis-inducing natural products found in utero during murine pregnancy. Chem. Biol. 7: 365̃372.

82. LERNER AB, CASE JD, MORI W, WRIGHT MR. Melatonin in peripheral nerve. Nature. 1959 Jun 27;183:1821

83. Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr Rev 12:151–180, 1991.[Medline]

84. Faillace MP, Cutrera R, Keller-Sarmento MI, Rosenstein RE. Evidence for local synthesis of melatonin in golden hamster retina. Neuroreport 6:2093–2095, 1995.[Medline]

85. Huether G, Poeggeler B, Reimer A, George A. Effect of tryptophan administration on circulating melatonin levels in checks and rats: evidence for stimulation of melatonin synthesis and release in the gastrointestinal tract. Life Sci 52:945–953, 1992.

86. Bubenik GA. Gastrointestinal melatonin: localization, function and clinical relevance. Dig Dis Sci 47:2336–2348, 2002.[Medline]

87. Tan DX, Manchester LC, Reiter RJ, Qi W, Zhang M, Weintraub ST, Cabrera J, Sainz RM, Mayo JC. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochim Biophys Acta 1472:206–214, 1999.[Medline]

88. Conti A, Conconi S, Hertens E, Skwarlo-Sonta K, Markowska M, Maestroni GJM. Evidence for melatonin synthesis in mouse and human bone marrow cells. J Pineal Res 28:193–202, 2000.[Medline]

89. Carrillo-Vico A, Calvo JR, Abreu P, Lardone PJ, Garcia-Morino S, Reiter RJ, Guerrero JM. Evidence of melatonin synthesis in human lymphocytes and its physiological significance: possible role as intracrine, autocrine and/or paracrine substance. FASEB J 18:537–539, 2004.[Abstract/Free Full Text]

90. Slominski A, Pisarchik A, Semak I, Sweatman T, Wortsman J, Szczesniewski A, Slugocki G, McNulty J, Kauser S, Tobin DI, Jing C, Johansson O. Serotoninergic and melatoninergic systems are fully expressed in human skin. FASEB J 16:896–898, 2002.[Abstract/Free Full Text]

91. Lopez-Gonzalez MA, Guerrero JM, Delgado F. Presence of the pineal hormone in rat cochlea: its variations with lighting conditions. Neurosci Lett 238:81–83, 1997.[Medline]

92. Abe M, Itoh MT, Miyata M, Shimizu K, Sumi Y. Circadian rhythm of serotonin N-acetyltransferase activity in rat lens. Exp Eye Res 70:805–808, 2000.[Medline]

93. Stefulj J, Hörtner M, Ghosh M, Schauenstein K, Rinner I, Wölfer A, Semmler J, Liebmann PM. Gene expression of the key enzymes of melatonin synthesis in extrapineal tissues of the rat. J Pineal Res 30:243–247, 2001.[Medline]

94. Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr Rev 12:151–180, 1991.

95. Carrillo-Vico A, Calvo JR, Abreu P, Lardone PJ, Garcia-Morino S, Reiter RJ, Guerrero JM. Evidence of melatonin synthesis in human lymphocytes and its

physiological significance: possible role as intracrine, autocrine and/or paracrine substance. FASEB J 18:537–539, 2004.

96.   Tan DX, Manchester LC, Hardeland R, Lopez-Burillo S, Mayo JC, Sainz RM, Reiter RJ. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. J Pineal Res 34:75–78, 2003.

97.   Hattori A, Migitaka H, Iigo M, Itoh M, Yamamoto K, Oktani-Kandro R, Hara M, Suzuki T, Reiter RJ. Identification of melatonin in plants: its effects on plasma melatonin levels and binding to melatonin receptors in vertebrates. Biochem Mol Biol Int 35:627–634, 1995.

98.   Reiter RJ, Tan DX, Burkhardt S, Manchester LC. Melatonin in plants. Nutr Rev 59:286–290, 2001

99.   REPPERT Sm, WEAVER Dr, EBISAWA T. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron 1994; 13:1177–1185.

100.  REPPERT SM, GODSON C, MAHLE CD et al. Molecular characterization of a second melatonin receptor expressed in human retina and brain: the Mel1b melatonin receptor. Proc Natl Acad Sci USA 1995; 92:8734–8738.

101.  REPPERT Sm, WEAVER Dr, CASSONE VM et al. Melatonin receptors are for the birds: molecular analysis of two receptor subtypes differentially expressed in chick brain. Neuron 1995; 15:1003–1015.

102.  Ebisawa T, Karne S, Lerner MR, Reppert SM. Expression cloning of a high-affinity melatonin receptor from Xenopus dermal melanophores. Proc Natl Acad Sci USA 1994; 91: 6133–6137.

103.  Steinhilber D, Brungs M, Werz O et al. The nuclear receptor for melatonin repress 5-lipoxygenase gene expression in human B lymphocytes. J Biol Chem 1995; 270: 7037–7040.

104.  Spadoni G, Balsamini C, Diamantini G et al. 2-N-acylaminoalkylindoles: design and quantitative structure-activity relationship studies leading to MT2-selective melatonin antagonists. J Med Chem 2001; 44: 2900–2912.

105.  Rodriguez C, Mayo JC, Sainz RM, Antolín I, Herrera F, Martín V, Reiter RJ. Regulation of antioxidant enzymes: a significant role for melatonin. Pineal Res. 2004 Jan;36(1):1-9. Review.

106.  Carlson, L. L., D. R. Weaver, And S. M. Reppert. Melatonin signal transduction in hamster brain: inhibition of adenylyl cyclase by a pertussis toxin-sensitive G protein. Endocrinology 125: 2670-2676, 1989

107.  Morgan, P. J., W. Lawson, G. Davidson, And H. E. Howell. Melatonin inhibits cyclic AMP in cultured ovine pars tuberalis cells. J. Mol. Endocrinol. 5: R3-R8, 1989

108. Vanecek, J., And L. Vollrath. Melatonin inhibits cyclic AMP and cyclic GMP accumulation in the rat pituitary. Brain Res. 505: 157-159, 1989

109. Mcarthur, A. J., A. E. Hunt, And M. U. Gillette. Melatonin action and signal transduction in the rat suprachiasmatic circadian clock: activation of protein kinase C at dusk and dawn. Endocrinology 138: 627-634, 1997

110. Vanecek, J., And D. C. Klein. Melatonin inhibits gonadotropin-releasing hormone-induced elevation of intracellular $Ca^{2+}$ in neonatal rat pituitary cells. Endocrinology 130: 701-707, 1992.

111. Vanecek, J., And L. Vollrath. Melatonin inhibits cyclic AMP and cyclic GMP accumulation in the rat pituitary. Brain Res. 505: 157-159, 1989.

112. Vanecek, J., And L. Vollrath. Melatonin modulates diacylglycerol and arachidonic acid metabolism in the anterior pituitary of immature rats. Neurosci. Lett. 110: 199-203, 1990.

113. Mayo JC, Sainz RM, Antolin I et al. Melatonin regulation of antioxidant enzyme gene expression. Cell Mol Life Sci 2002; 59: 1706–1713.

114. Chan A, Lai F, Lo R et al. Melatonin mt1 and MT2 receptors stimulate c-Jun N-terminal kinase via pertussis toxin-sensitive and -insensitive G proteins. Cell Signal 2002; 14: 249–257.

115. Pablos MI, Guerrero JM, Ortiz GG, Agapito MT, Reiter RJ. Both melatonin and putative nuclear melatonin receptor agonist CGP 52608 stimulate glutathione peroxidase and glutathione reductase activities in mouse brain in vivo. Neuroendocrinol Lett 1997; 18: 49–58.

116. Ram PT, Dai J, Yuan L et al. Involvement of the mt1 melatonin receptor in human breast cancer. Cancer Lett 2002; 179: 141–150

117. Vanecek J. Cellular mechanisms of melatonin action. Physiol Rev 1998; 78: 687–721. Vanecek J. Cellular mechanisms of melatonin action. Physiol Rev 1998; 78: 687–721.

118. Benitez-King G, Huerto-Delgadillo L, Anton-Tay F. Binding of ^{3}H-melatonin to calmodulin. Life Sci 1993; 53: 201–207

119. Kane CD, Means AR. Activation of orphan receptor-mediated transcription by $Ca^{2+}$/calmodulin-dependent protein kinase IV. EMBO J 2000; 19: 691–701.

120. Tan D-X, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. Melatonin: A potent, endogenous hydroxyl radical scavenger. Endocr J 1:57–60, 1993.

121. Poeggeler B, Reiter RJ, Hardeland R, Tan DX, Barlow-Walden LR. Melatonin and structurally related endogenous indoles act as potent electron donors and radical scavengers in vitro. Redox Rep 2:179–184, 1996.

122. Matuszak Z, Reszka KJ, Chignell CF. Reaction of melatonin and related indoles with hydroxyl radicals: EPR and spin trapping investigations. Free Radic Biol Med 23:367–372, 1997.[Medline]

123. Susa N, Ueno S, Furukawa Y, Ueda J, Sugiyama M. Potent protective effect of melatonin on chromium (VI) -induced DNA single-strand breaks, cytotoxicity, and lipid peroxidation in primary cultures of rat hepatocytes. Toxicol Appl Pharmacol 144:377–384, 1997.[Medline]

124. Li XJ, Zhang LM, Gu J, Zhang AZ, Sun FY. Melatonin decreases production of hydroxyl radical during cerebral ischemia-reperfusion. Chung Kuo Yao Li Hsueh Pao 18:394–396, 1997.

125. Tan DX, Manchester LC, Reiter RJ, Plummer BF, Hardies LJ, Weintraub ST, Vijayalaxmi Shepherd AMM. A novel melatonin metabolite, cyclic 3-hydroxymelatonin: A biomarker of in vivo hydroxyl radical generation. Biochem Biophys Res Commun 253:614–620, 1998.[Medline]

126. Stasica P, Ulanski P, Rosiak JM. Melatonin as a hydroxyl radical scavenger. J Pineal Res 25:65–66, 1998.[Medline]

127. Pähkla R, Zilmer M, Kullisaar T, Rägo L. Comparison of the antioxidant activity of melatonin and pinoline in vitro. J Pineal Res 24:96–101, 1998.[Medline]

128. Edwards JC, Chapman D, Cramp WA, Yatvin MB. The effects of ionizing radiation on biomembrane structure and function. Prog Biophys Mol Biol 43:71–93, 1984.

129. Verma SP, Sonwalker N. Structural changes in plasma membranes prepared from irradiated Chinese hamster V79 cells as revealed by Raman spectroscopy. Radiat Res 126:27–35, 1991.

130. Samuni AM, Barenholz Y, Crommelin DJA, Zuidam NJ. $\gamma$-Irradiation damage to liposomes differing in composition and their protection by nitroxides. Free Radic Biol Med 23:972–979, 1997.

131. Parasassi T, Giusti AM, Gratton E, Monaco E, Raimondi M, Ravagnan G, Sapora O. Evidence for an increase in water concentration in bilayers after oxidative damage of phospholipids induced by ionizing radiation. Int J Radiat Biol 65:329–334, 1994.

132. Parasassi T, Ravagnan G, Sapora O, Gratton E. Membrane oxidative damage induced by ionizing radiation detected by diphenylhexatriene fluorescence lifetime distributions. Int J Radiat Biol 61:791–796, 1992.

133. Pieri C, Marra M, Moroni F, Recchioni R, Marcheselli F. Melatonin: A peroxyl radical scavenger more effective than vitamin E. Life Sci 55:PL271–PL276, 1994.[Medline]

134. Pieri C, Moroni F, Marra M, Marcheselli F, Recchioni R. Melatonin is an efficient antioxidant. Arch Gerontol Geriatr 20:159–165, 1995.

135. Scaiano JC. Exploratory laser flash photolysis study of free radical reactions and magnetic field effects in melatonin chemistry. J Pineal Res 19:189–195, 1995.[Medline]

87

136. Marshall KA, Reiter RJ, Poeggeler B, Aruoma OI, Halliwell B. Evaluation of the antioxidant activity of melatonin in vitro. Free Radic Biol Med 21:307–315, 1996.[Medline]

137. Livrea MA, Tesoriere L, D'Arpa D, Morreale M. Reaction of melatonin with lipoperoxyl radicals in phospholipid bilayers. Free Radic Biol Med 23:706–711, 1997.[Medline]

138. Reiter RJ. Antioxidant actions of melatonin. Adv Pharmacol 38:103–117, 1997.

139. Reiter RJ, Tan DX, Cabrera J, D'Arpa D, Sainz RM, Mayo JC, Ramos S. The oxidant/antioxidant network: Role of melatonin. Biol Signals Recept 8:56–63, 1999.[

140. Cagnoli CM, Atabay C, Kharlamov E, Manev H. Melatonin protects neurons from singlet oxygen-induced apoptosis. J Pineal Res 18:222–226, 1995.

141. Gilad E, Cuzzocrea S, Zingarelli B, Salzman AL, Szabo C. Melatonin is a scavenger of peroxynitrite. Life Sci 60:PL169–PL174, 1997.

142. Cuzzocrea S, Zingarelli B, Gilad E, Hake P, Salzman AL, Szabo C. Protective effect of melatonin in carrageenan-induced models of local inflammation: Relationship to its inhibitory effect on nitric oxide production and its peroxynitrite scavenging activity. J Pineal Res 23:106–116, 1997.

143. Noda Y, Mori A, Liburdy R, Packer L. Melatonin and its precursors scavenge nitric oxide. J Pineal Res 27:159–163, 1999.

144. Reiter RJ. Oxidative processes and antioxidative defense mechanisms in the aging. FASEB J 9:526–533, 1995.

145. Reiter RJ. Oxidative damage in the central nervous system: Protection by melatonin. Prog Neurobiol 56:359–384, 1998.

146. Antolin I, Rodriguez C, Sainz RM, Mayo JC, Uria H, Kotler ML, Rodriguez-Colunga MJ, Tolivia D, Menendez-Pelaez A. Neurohormone melatonin prevents cell damage: Effect on gene expression for antioxidant enzymes. FASEB J 10:882–890, 1996.

147. Kotler M, Rodriguez C, Sainz RM, Antolin I, Menendez-Pelaez A. Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. J Pineal Res 24:83–89, 1998.

148. Barlow-Walden LR, Reiter RJ, Abe M, Pablos M, Menendez-Pelaez A, Chen LD, Poeggeler B. Melatonin stimulates brain glutathione peroxidase activity. Neurochem Int 26:497–502, 1995.

149. Pablos MI, Chuang JI, Reiter RJ, Ortiz GG, Daniels WMU, Sewerynek E, Melchiorri D, Poeggeler B. Time course of melatonin-induced increase in glutathione peroxidase activity in chick tissues. Biol Signals 4:325–330, 1995.

150. Pablos MI, Guerrero JM, Ortiz GG, Agapito MT, Reiter RJ. Both melatonin and a putative nuclear melatonin receptor agonist CGP 52608 stimulate glutathione

peroxidase and glutathione reductase activities in mouse brain in vivo. Neuroendocrinol Lett 18:49–58, 1997.

151. Pablos MI, Reiter RJ, Ortiz GG, Agapito MT, Chuang JI, Sewerynek E. Rhythms of glutathione peroxidase and glutathione reductase in the brain of chick and their inhibition by light. Neurochem Int 32:69–75, 1998.

152. Sewerynek E, Reiter RJ, Melchiorri D, Ortiz GG, Lewinski A. Oxidative damage in the liver induced by ischemia-reperfusion: Protection by melatonin. Hepatogastroenterology 43:898–903, 1996.

153. Bettahi I, Pozo D, Osuna C, Reiter RJ, Acuna-Castroviejo D, Guerrero JM. Physiological concentrations of melatonin inhibit nitric oxide synthase activity in the rat hypothalamus. J Pineal Res 20:205–210, 1996.

154. Guerrero JM, Reiter RJ, Ortiz GG, Pablos MI, Sewerynek E, Chuang JI. Melatonin prevents increases in neural nitric oxide and cycle GMP production after transient brain ischemia and reperfusion in the Mongolian gerbil (Meriones unguiculatus). J Pineal Res 23:24–31, 1997.

155. Pozo D, Reiter RJ, Calvo JM, Guerrero JM. Inhibition of cerebellar nitric oxide synthase and cyclic GMP production by melatonin via complex formation with calmodulin. J Cell Biochem 65:430–442, 1997.

156. Steinhilber, D., Brungs, M., Werz, O., Wiesenberg, I., Danielsson, C., Kahlen, J.-P., Nayeri, S., Schräder, M., Carlberg, C. (1995) The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes J. Biol. Chem. 270,7037-7040.

157. Büyükavci, M., Ozdemir, O., Buck, S., Stout, M., Ravindranath, Y., Savaşan, S. 2006. Melatonin cytotoxicity in human leukemia cells: relation with its pro-oxidant effect. Fundam Clin Pharmacol.; 20:73-79.

158. Medina-Navarro, R. Duran-Reyes, G., Hicks, J.J. 1999. Pro-oxidating properties of melatonin in the in vitro interaction with the singlet oxygen. Endocr Res. 25; 263-280.

159. Wölfler, A., Caluba, H.C., Abuja, P.M., Dohr, G., Schauenstein, K., Liebmann, P.M. 2001. Prooxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. FEBS Lett. 502; 127-131.

160. Pieri, C., Recchioni, R., Moroni, F., Marcheselli, F., Marra, M., Marinoni, S., Di Primio, R. 1998. Melatonin regulates the respiratory burst of human neutrophils and their depolarization. J Pineal Res. 24; 43-49.

161. Osseni, R.A., Rat, P., Bogdan, A., Warnet, J.M., Touitou, Y. 2000. Evidence of prooxidant and antioxidant action of melatonin on human liver cell line HepG2. Life Sci 15; 68:387-399.

162. D'agostino, D.P., Putnam, R.W., Dean, J.B. 2007. Superoxide (*O2- ) production in CA1 neurons of rat hippocampal slices exposed to graded levels of oxygen. J Neurophysiol. 98; 1030-1041.

163. Eskiocak, S., Tutunculer, F., Basaran, U.N., Taskiran, A., Cakir, E. 2007. The effect of melatonin on protein oxidation and nitric oxide in the brain tissue of hypoxic neonatal rats. Brain Dev. 29; 19-24.

164. Sainz Rm, Mayo Jc, Rodriguez C, et al. Melatonin and cell death: differential actions on apoptosis in normal and cancer cells. Cell Mol Life Sci. 2003; **60**:1407-1426. Review

165. Sainz RM, Mayo JC, Uria H et al. The pineal neurohormone melatonin prevents in vivo and in vitro apoptosis in thymocytes. J Pineal Res 1995; 19: 178–188.

166. Sainz RM, Mayo JC, Reiter RJ, Antolin I, Esteban MM, Rodriguez C. Melatonin regulates glucocorticoid receptor: an answer to its antiapoptotic action in thymus. FASEB J 1999; 13: 1547–1556.

167. Mayo JC, Sainz RM, Uria H, Antolin I, Esteban MM, Rodriguez C. Melatonin prevents apoptosis induced by 6-hydroxydopamine in neuronal cells: implications for Parkinson's disease. J Pineal Res 1998; 24: 179–192.

168. Chuang JI, Chen TH. Effect of melatonin on temporal changes of reactive oxygen species and glutathione after MPP+ treatment in human astrocytoma U373MG cells. J Pineal Res 2004; 36: 117–125.

169. Sainz RM, Mayo JC, Rodriguez C, Tan DX, Lopez-Burillo S, Reiter RJ. Melatonin and cell death: differential actions on apoptosis in normal and cancer cells. Cell Mol Life Sci 2003; 60: 1407–1426.

170. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science. 1995; 267:1456-62. Review.

171. Wesche-Soldato De, Swan Rz, Chung Cs, Ayala A. The apoptotic pathway as a therapeutic target in sepsis. Curr Drug Targets. 2007; 8:493-500. Review.

172. Bolitho P, Voskoboinik I, Trapani Ja, Smyth Mj. Apoptosis induced by the lymphocyte effector molecule perforin. Curr Opin Immunol. 2007; 19:339-347. Review.

173. Cubero J, Valero V, Narciso D, et al. L-tryptophan administered orally at night modifies the melatonin plasma levels, phagocytosis and oxidative metabolism of ringdove (Streptopelia roseogrisea) heterophils. Mol Cell Biochem. 2006; 293 :79-85.

174. Carrillo-Vico A, Reiter Rj, Lardone Pj, et al. The modulatory role of melatonin on immune responsiveness. Curr Opin Investig Drugs. 2006; 7:423-431. Review.

175. Lu L, Osmond Dg. Apoptosis and its modulation during B lymphopoiesis in mouse bone marrow. Immunol Rev. 2000;175:158-174. Review.

176. Miller Sc, Pandi-Perumal Sr, Esquifino AI, et al. The role of melatonin in immuno-enhancement: potential application in cancer. Int J Exp Pathol. 2006 Apr; 87:81-87. Review.

177. Nava M, Romero F, Quiroz Y, Parra G, Bonet L, Rodriguez-Iturbe B. Melatonin attenuates acute renal failure and oxidative stress induced by mercuric chloride in rats. Am J Physiol Renal Physiol 2000; 279: F910–F918.

178. Meki AR, Abdel-Ghaffar SK, EL-Gibaly I. Aflatoxin B1 induces apoptosis in rat liver: protective effect of melatonin. Neuroendocrinol Lett 2001; 22: 417–426.

179. Pappolla MA, Sos M, Omar RA et al. Melatonin prevents death of neuroblastoma cells exposed to the Alzheimer amyloid peptide. J Neurosci 1997; 17: 1683–1690.

180. Lahiri DK, Chen D, GE YW, Bondy SC, Sharman EH. Dietary supplementation with melatonin reduces levels of amyloid beta-peptides in the murine cerebral cortex. J Pineal Res 2004; 36: 224–231.

181. Cheung RT. The utility of melatonin in reducing cerebral damage resulting from ischemia and reperfusion. J Pineal Res 2003; 34: 153–160.

182. Pei Z, Cheung RT. Melatonin protects SHSY5Y neuronal cells but not cultured astrocytes from ischemia due to oxygen and glucose deprivation. J Pineal Res 2003; 34: 194–201.

183. Andrabi SA, Sayeed I, Siemen D, Wolf G, Horn TF. Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism responsible for anti-apoptotic effects of melatonin. FASEB J 2004; 18: 869–871.

184. Acuña-Castroviejo D, Escames G, Carazo A, Leon J, Khaldy H, Reiter RJ. Melatonin, mitochondrial homeostasis and mitochondrial-related diseases. Curr Top Med Chem 2002; 2: 133–151.

185. Martin M, Macias M, Escames G, Leon J, Acuña-Castroviejo D. Melatonin but not vitamins C and E maintains glutathione homeostasis in t-butyl hydroperoxide-induced mitochondrial oxidative stress. FASEB J 2000; 14: 1677–1679.

186. Martin M, Macias M, Escames G et al. Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. J Pineal Res 2000; 28: 242–248.

187. Martin M, Macias M, Leon J, Escames G, Khaldy H, Acuña-Castroviejo D. Melatonin increases the activity of the oxidative phosphorylation enzymes and the production of ATP in rat brain and liver mitochondria. Int J Biochem Cell Biol 2002; 34: 348–357.

188. Acuña-Castroviejo D, Martin M, Macias M et al. Melatonin, mitochondria, and cellular bioenergetics. J Pineal Res 2001; 30: 65–74.

189. Kwak CS, Mun KC, Suh SI. Production of oxygen free radicals and of hemolysis by cyclosporine. Transplant Proc 2002; 34: 2654–2655.

190. Xu M, Ashraf M. Melatonin protection against lethal myocyte injury induced by doxorubicin as reflected by effects on mitochondrial membrane potential. J Mol Cell Cardiol 2002; 3: 75–79.

191. Hoijman E, Rocha Viegas L, Keller Sarmiento MI, Rosenstein RE, Pecci A. Involvement of Bax protein in the prevention of glucocorticoid-induced thymocytes apoptosis by melatonin. Endocrinology 2004; 145: 418–425.

192. Scott AE, Cosma GN, Frank M, Wells RL, Gardner HS, Jr Disruption of mitochondrial respiration by melatonin in MCF-7 cells. Toxicol Appl Pharmacol 2001; 171: 149–156.

193. Ghibelli L, Coppola S, Fanelli C, Et Al.. Glutathione depletion causes cytochrome c release even in the absence of cell commitment to apoptosis. FASEB Journal 1999; 13: 2031-2036.

194. Single B., Leist M., And Nicotera P. Simultaneus release of adenylate kinase and cytochrome c in cell death. Cell Death and Differentiation, 5, 1001-1003, 1998.

195. d'alessio M, De Nicola M, Coppola S, et al. Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis. FASEB J. 2005; 19:1504-1506.

196. Jenkins, C.M., Wolf, M.J., Mancuso, D.J., Gross, R.W. 2001. Identification of the calmodulin-binding domain of recombinant calcium-independent phospholipase A2beta. implications for structure and function. J Biol Chem. 276; 7129-35.

197. Maellaro E, Bello BD, Comporti M. 1996. Protection by ascorbate against apoptosis of thymocytes: implications of ascorbate-induced nonlethal oxidative stress and poly(ADP-ribosyl)ation. Exp Cell Res. 226; 105-113.

198. Belfiore, M.C., Natoni, A., Barzellotti, R., Merendino, N., Pessina, G., Ghibelli, L., Gualandi, G. 2007. Involvement of 5-lipoxygenase in survival of Epstein-Barr virus (EBV)-converted B lymphoma cells. Cancer Lett. 254; 236-243.

199. Pufahl, R.A., Kasten, T.P., Hills, R., Gierse, J.K., Reitz, B.A., Weinberg, R.A., Masferrer, J.L. 2007. Development of a fluorescence-based enzyme assay of human 5-lipoxygenase. Anal Biochem. 364; 204-212.

200. Zhou, G.X., Ding, X.L., Huang, J.F., Zhang, H., Wu, S.B. 2007. Suppression of 5-lipoxygenase gene is involved in triptolide-induced apoptosis in pancreatic tumor cell lines. Biochim Biophys Acta. 1770; 1021-1027.

201. Cristofanon, S., Nuccitelli, S., D'Alessio, M., Dicato, M., Diederich, M., Ghibelli, L. 2008.Oxidation-dependent maturation and survival of explanted blood monocytes via Bcl-2 up-regulation. Biochem Pharmacol.

202. Balsinde, J., Balboa, M.A., Insel, P.A., Dennis, E.A. 1999. Regulation and inhibition of phospholipase A2. Annu Rev Pharmacol Toxicol. 39; 175-189.

203. Romero, M.P., García-Pergañeda, A., Guerrero, J.M., Osuna, C. 1998. Membrane-bound in Xenopus laevis oocytes as a novel binding site for melatonin. FASEB J. 12; 1401-1408.

204. Li, B., Zhang, H., Akbar, M., Kim, H.Y. 2000. Negative regulation of cytosolic phospholipase A(2) by melatonin in the rat pineal gland. Biochem J. 351;709-716.

205. Pidgeon, G.P., Lysaght, J., Krishnamoorthy, S., Reynolds, J.V., O'Byrne, K., Nie, D., Honn, K.V. 2007. Lipoxygenase metabolism: roles in tumor progression and survival. Cancer Metastasis Rev. 26; 503-524. Review.

206. Carrillo-Vico A, Garcia-Maurino S, Calvo Jr, Guerrero Jm. Melatonin counteracts the inhibitory effect of PGE2 on IL-2 production in human lymphocytes via its membrane mt1 receptor. FASEB J. 2003; 17:755-757.

207. Markowska M, Mrozkowiak A, Pawlak J And Skwarl-Sonta K. Intracellular second messengers in melatonin signal transduction in chicken splenocytes in vitro. J. Pineal Res. 2004; 37:207-212.

208. Lampe Pa, Cornbrooks Eb, Juhasz A, Et Al., Suppression of programmed neuronal death by a thapsigargin-induced $Ca^{2+}$ influx. J Neurobiol 1995;26:205-212.

# PAPERS AND MANUSCRIPTS

# PAPER I

*Melatonin as an apoptosis antagonist*

# Melatonin as an Apoptosis Antagonist

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**ABSTRACT: The pineal hormone melatonin (Mel), in addition to having a well-established role as a regulator of circadian rhythms, modulates non-neural compartments by acting on specific plasma membrane receptors (MT1/MT2) present in many different cell types. Mel plays immunomodulatory roles and is an oncostatic and antiproliferative agent; this led to the widespread belief that Mel may induce or potentiate apoptosis on tumor cells, even though no clear indications have been presented so far. Here we report that Mel is not apoptogenic on U937 human monocytic cells, which are known to possess MT1 receptors at the times (up to 48 h) and doses (up to 1 mM) tested. Mel does not even potentiate apoptosis, but instead, significantly reduces apoptosis induced by both cell-damaging agents (intrinsic pathway) and physiological means (extrinsic pathway). The doses required for the antiapoptotic effect ($\geq$100 μM) are apparently not compatible with receptor stimulation (receptor affinity $<$1 nM). However, receptor involvement cannot be ruled out, because we discovered that the actual Mel concentration active on cells was lower than the nominal one because of sequestration by fetal calf serum (FCS). Accordingly, in FCS-free conditions, Mel doses required for a significant antiapoptotic effect are much lower.**

**KEYWORDS: melatonin; apoptosis; receptor engagement**

## INTRODUCTION

It has been historically known that the pineal gland is the major source of melatonin (Mel) in vertebrates. Mel plays a central role in fine-tuning circadian

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rhythms in vertebrate physiology. Its biosynthesis from L-tryptophan, which is converted to serotonin and then to Mel in a two-step pathway, involves *N*-acetylation and *O*-methylation.[1]

Mel, in addition to playing a main role as regulator of circadian rhythms, has recently been shown to modulate immune functions by controlling the behavior of white blood cells (WBCs), which are indeed able to synthesize Mel and possess the specific high affinity (MT1 and MT2, see below). Mel acts as an intracrine, autocrine, or paracrine substance in the immune system.[2]

In the blood stream, Mel binds (sticks) to plasma proteins[3]; it is not clear whether this may serve for Mel targeting, or as sequestration for lowering its blood levels, or for storage, or even for modulating serum protein functions. It is emerging that Mel is involved in many regulatory functions of the cells, possibly through receptor engagement, that is, it modulates the immune response,[2] regulates the apoptotic response on a number of cell types,[4] and regulates signal transduction reactions.[5] As a radical scavenger, Mel may reduce tissue destruction during inflammatory reactions,[6] and Mel also protects both polyunsatured fatty acid from oxidation and nuclear DNA from damage induced by carcinogens and ionizing irradiation.[7] The effects of Mel are mediated by the specific high-affinity receptors localized on the plasma membrane of target cells and coupled to GTP-binding protein. On the basis of the molecular structure, three subtypes of the Mel receptor have been described: $Mel_{1A}$ or mt1 (expressed in mammalian and bird brain), $Mel_{1B}$ or mt2 (expressed mainly in mammalian retina), $Mel_{1C}$ or mt3 (found in amphibian melanophores, brain, and retina, and also in bird and fish brain).[8] The dissociation constant ($K_d$) of the Mel receptors is in the range of 20–200 pM.[9] Mel receptors regulate several second messengers: cAMP, cGMP, diacylglycerol, inositol trisphosphate, arachidonic acid, and intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$).[5] Nevertheless, some intracellular actions of Mel are independent of any receptor interaction, and may possibly be the consequence of its radical scavenger ability.[7]

It is known that Mel is able to exert a consistent effect on tumor metabolism; in particular, it seems to have an oncostatic action, and several studies have reported that Mel can control tumor growth and inhibit cells proliferation. Mel has been observed exerting strong effects on apoptosis: indeed, Mel has been assumed to exert proapoptotic effects,[10] though no evidence for this has ever been reported. Instead, the recent literature shows a clear antiapoptotic effect on many normal and tumor cells, both at physiological and pharmacological doses.[11] The confusion created by such opposite beliefs is further increased by the contradictory reports on its mechanism of action, which are still being debated as to whether the effects on apoptosis are receptor-mediated or instead depend on its radical scavenger ability. It is a very important target of pharmacologic research to understand Mel's effects at the cellular level in general, and on apoptosis in particular, to be able to exploit Mel's therapeutic potential.

# MATERIALS AND METHODS

## *Cell Culture*

For this study, we used the human promonocytic leukemia cell line U937.

Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine, and antibiotics (penicillin, streptomycin). The cells were kept in a controlled atmosphere (5% $CO_2$) incubator at 37°C.

## *FCS Denaturation*

FCS was incubated at 90°C for 1 h and then added to the RPMI 1640 medium.

## *Treatment with Mel*

Mel was used at the final concentration of 1 mM, unless otherwise specified, and added to the culture medium 1 h before apoptosis induction.

## *Induction of Apoptosis*

(1) *By nonoxidative agents:* Apoptosis was induced by the protein synthesis inhibitor puromycin (PMC, 10 μg/mL)
(2) *By physiological agents:* U937 cells were treated with anti-Fas, # 05–201 (FAS, 50 ng/mL).

## *Identification and Quantification of Apoptotic Cells*

Nuclear morphology of control and treated cells was analyzed by fluorescence microscopy after staining with Hoechst 33342; apoptotic cells were characterized by nuclear condensation of chromatin and/or nuclear fragmentation. Apoptosis was evaluated among the Hoechst-stained cells by counting at least 300 cells in at least three randomly selected fields.

## *Estimation of Mel Concentration in Cell Extract*

U937 cells were suspended at a concentration of $7 \times 10^5$ cell/mL in the presence of 0, 0.5, 1.0, and 2 mM Mel (final concentrations), in an RPMI

medium containing or not FCS. Aliquots of $10 \times 10^6$ cells were collected after 4 h of incubation at 37 °C and washed two times with PBS, and suspended in 1 mL of Na phosphate buffer (0.04 M, pH 6.8). Five hundred microliters of Na tetraborate buffer (0.1 M, pH 9.0) and 1 mL of chloroform were added to the pellet and the suspension was vortexed for 3 min and centrifuged at 800 rpm for 5 min. The water phase was discarded, and the organic phase was mixed with 5 mL of borate buffer, vortexed for 3 min, and centrifuged at 800 rpm for 5 min. The water phase was discarded again and the remaining organic phase was dried under nitrogen and finally suspended in chloroform (100 µL). Thirty microliters were loaded onto silica gel 60 thin layer chromatographic (TLC) plates with fluorescent indicator (Merck Sharp & Dohme GMBH, Haar, Germany). A standard Mel solution (85 µM) was prepared and amounts of 70 and 210 µg were loaded onto the TLC plates to localize Mel and quantify its concentration in the cell extracts. This was carried out by UV irradiation of the plates, image acquisition through a CCD camera and by using the Gel Doc 1000/2000 gel documentation system (Quantity One software package for image analysis). The data are expressed in linear intensity units. Quantity One can quantify and analyze the spots through a light/radiation detector that converts signals from TLC samples into digital data. Quantity One displays the digital data on the computer screen in the form of gray-scale images.

## RESULTS

### *Mel Reduces Apoptosis in U937 Cells*

U937 human monocytic cells are known to possess MT1 Mel receptors,[12] thus possibly being an ideal system for investigating the effects of Mel on apoptosis.

First of all, we observed that Mel exerts no apoptogenic effects within 48 h of incubation and up to 1 mM doses (not shown). Next, we investigated Mel's effects on stress-induced apoptosis. FIGURE 1A shows that Mel is able to reduce PMC-induced apoptosis in a dose-dependent fashion.

Mel was discovered exerting antiapoptotic effects also on physiologically induced apoptosis, such as that induced by Fas, as shown in FIGURE 1B. This suggests that the Mel antiapoptotic effect involves both the intrinsic and the extrinsic pathways. Thus, from this first set of results, we can conclude that Mel is able to protect U937 cells from apoptosis.

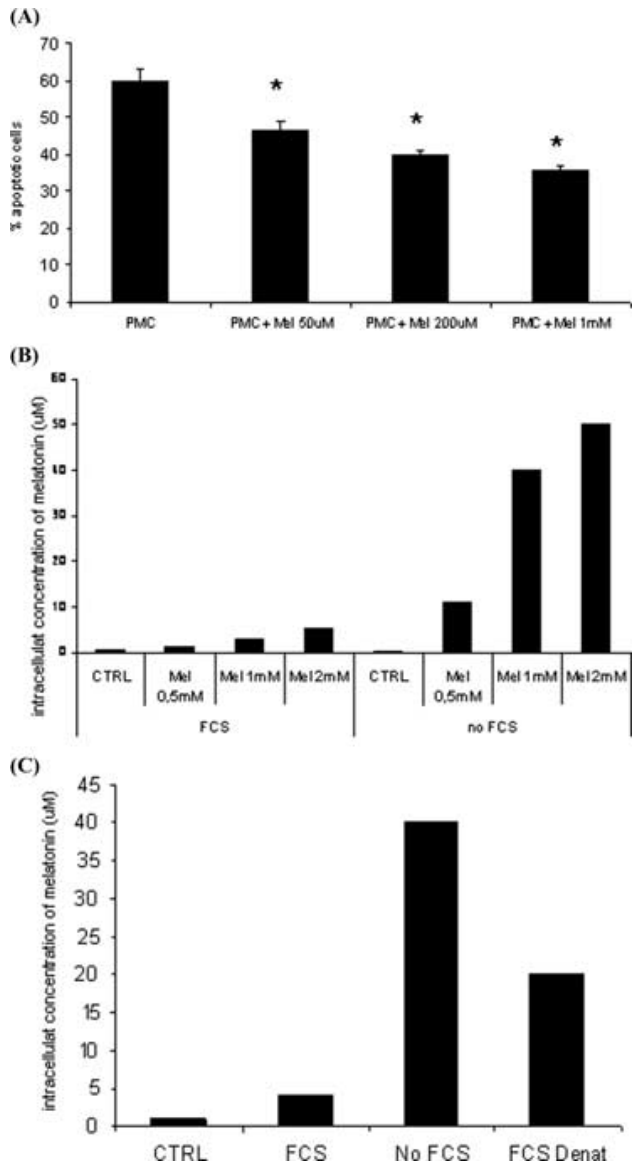### *Serum Proteins Sequester Mel in Cellular Culture Medium*

We did not find significant protection for doses <200 µM. This seems incompatible with Mel's receptor-engaging function. However, the actual
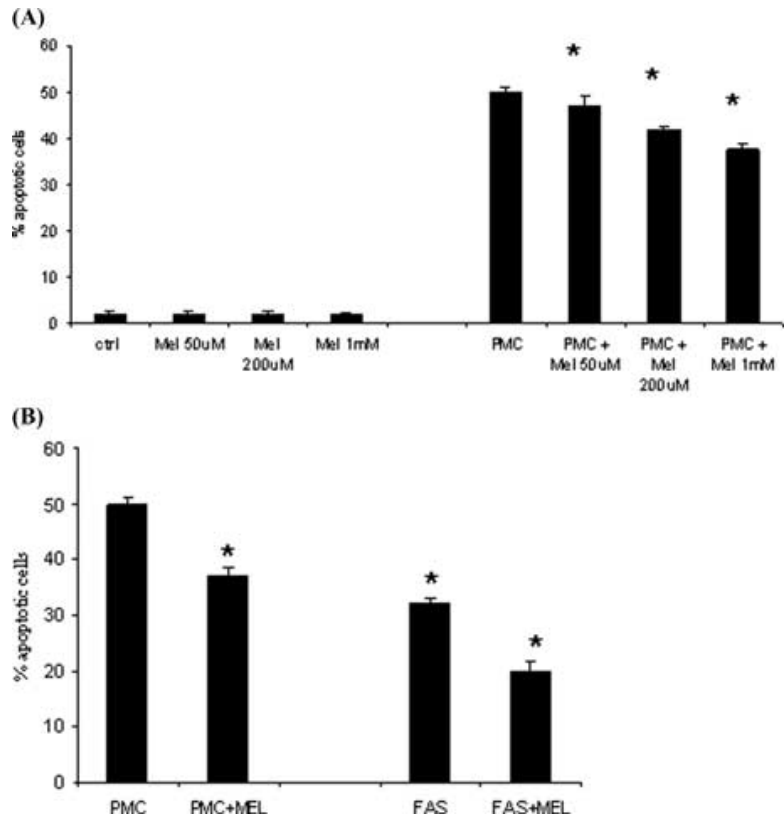
**FIGURE 1.** Mel antagonizes apoptosis. (**A**) Dose-effect of Mel. U937 cells were incubated in complete medium (presence of 10% FCS) with different doses of Mel for 24 h (*left*); or for 1 h, followed by puromycin (PMC, *right*); apoptosis was evaluated at 24 h of Mel (*left*) or 3 h of PMC (*right*). Results are the average of at least five independent experiments ±SD. Apoptosis reduction at 1 mM Mel is highly significant ($P < 0.05$). (**B**) Mel protects cells from apoptosis induced by both cell-damaging agents (*intrinsic pathway*) and physiological means (*extrinsic pathway*). Apoptosis was estimated at 3 h of PMC, and Fas overnight. Results are the average of at least three independent experiments ±SD. Apoptosis reduction by Fas is significant ($P < 0.05$).

dose able to reach cells may possibly be lower. Indeed, it is known that Mel sticks to protein present in plasma, thus possibly lowering concentration of active Mel. Thus, we performed the same dose–response experiment shown in FIGURE 1A in the absence of FCS: FIGURE 2A shows that indeed in this case, Mel is more effective in its antiapoptotic effect at all the corresponding doses tested.

The TLC analysis of intracellular Mel concentration after 4 h of incubation with the different Mel doses shows that FCS causes a decrease in cellular uptake of about 20-fold (FIG. 2C), thus suggesting that the actual Mel dose in

**FIGURE 2.** Serum proteins sequester Mel in cellular culture medium. (**A**) A concentration of 50 μM protects U937 cells from apoptosis in culture medium lacking in serum. Cells were treated with PMC ± the different doses of Mel in the absence of serum. Results are the average of at three independent experiments ±SD. Apoptosis reduction at all Mel doses is highly significant ($P < 0.05$). (**B**) Serum impairs Mel uptake. Intracellular concentrations of Mel were measured by TLC after 4 h of incubation and are the average of two independent experiments. (**C**) Denatured FCS gave an intracellular uptake that is about five-fold that observed in the presence of regular FCS. Intracellular concentrations of Mel were measured by TLC after 4 h of incubation and are the average of two independent experiments.

the experiments carried out under standard conditions (10% FCS) is much lower than the nominal one. The presence of partially denatured FCS gave intermediate results, with an intracellular uptake that is about five-fold that observed in the presence of regular FCS, suggesting that the ability of sequestering Mel is partially lost by denaturation.

## DISCUSSION

With the goal to clarify the effects of Mel on apoptosis, we investigated both the possible proapoptotic or antiapoptotic effect in U937 human monocytic cells, known to possess MT1 Mel receptors,[12] by evaluating the extent of apoptosis on cells treated, respectively, with Mel alone, or Mel plus a direct apoptogenic agent.

Our results confirm that a Mel proapoptotic effect does not exist *per se*, even though such an effect has been reported in several reviews. However, Mel significantly reduces apoptosis induced by two different means: physiological and cell damaging. Thus, the antagonistic action of Mel toward apoptosis may occur via stimulation of two independent pathways, the physiological (or extrinsic) pathway and the stress-induced (or intrinsic) pathway. Thus, this study supports recent studies that have described an antiapoptotic role of Mel[13]; however, the mechanism involved remains to be understood, that is, whether Mel's antiapoptotic effect is generated by receptor stimulation or by Mel's radical scavenger ability.

In malignant cells, such as the human breast cancer cell line MCF-7, micromolar concentrations of Mel have been reported to increase the extent of induced apoptosis.[14]

The doses required for the antiapoptotic effect ($\geq 100$ $\mu$M) in U937 are apparently not compatible with receptor stimulation (receptor affinity $<1$ nM). We have shown here that this is due, at least in part, to a masking by serum proteins, present in culture medium in large concentration, which stick to Mel,[3] lowering the actual concentration available for cell entry. Thus, in the absence of FCS, Mel is more effective in its antiapoptotic effect at all the doses tested. The TLC analysis of intracellular Mel concentration shows that FCS causes a decrease in cellular uptake of about 20-fold, thus suggesting that the actual Mel dose in the experiments carried on in standard conditions (10% FCS) is much lower than the nominal one.

This is, however, not enough to explain the involvement of receptor engagement; thus, we cannot rule out other possible explanations, such as the role of Mel as an antioxidant in comparing apoptosis. Some groups indeed reported protection against programmed cell death in neuronal cells by the antioxidant activity of Mel.[15] Alternatively, protection may occur through receptor stimulation, but this might require an additional Mel effect in order to achieve the antiapoptotic effect. In conclusion, the antiapoptotic effect of Mel has been

demonstrated. However, further investigations are required to elucidate the mechanism of this effect and to what extent receptor engagement and pro-oxidant activity of Mel are involved in its antiapoptotic effect.

## REFERENCES

1. REITER, R.J. 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr. Rev. **12:** 151–180.
2. CARRILLO-VICO, A., J.R. CALVO, P. ABREU, *et al*. 2004. Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possibile role as intracrine, autocrine, and/or paracrine substance. FASEB J. **18:** 537–539.
3. GILAD, E. & N. ZISAPEL. 1995. High-affinity binding of melatonin to hemoglobin. Biochem. Mol. Med. **56:** 115–120.
4. SAINZ, R.M., J.C. MAYO, C. RODRIGUEZ, *et al*. 2003. Melatonin and cell death: differential actions on apoptosis in normal and cancer cells. Cell Mol. Life Sci. **60:** 1407–1426.
5. VANEK, J. 1998. Cellular mechanism of melatonin action. Physiol. Rev. **78:** 687–721.
6. REITER, R.J., J.R. CALVO, M. KARBOWNIK, *et al*. 2000. Melatonin and its relation to the immune system and inflammation. Ann. N. Y. Acad. Sci. **917:** 376–386.
7. REITER, R.J., D.X. TAN, C. OSUNA, *et al*. 2000. Action of melatonin in the reduction of oxidative stress. J. Biomed. Sci. **7:** 444–458.
8. DUBOCOVICH, M.L. & M. MARKOWSKA. 2005. Functional MT1and MT2 melatonin receptor in mammals. Endocrine **27:** 101–110.
9. DUBOCOVICH, M.L. 1991. Pharmacological characterization of melatonin binding sites. Adv. Pineal Res. **5:** 167–173.
10. WOLFLER, A., H.C. CALUBA, P.M. ABUJA, *et al*. 2001. Pro-oxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. FEBS Lett. **502:** 127–131.
11. SAINZ, R.M., J.C. MAYO, R.J. REITER, *et al*. 1999. Melatonin regulates glucocorticoid receptor: an answer to its antiapoptotic action in thymus. FASEB J. **13:** 1547–1556.
12. GARCIA-MAURINO, S., M.G. GONZALEZ-HABA, J.R. CALVO, *et al*. 1998. Involvement of nuclear binding sites for melatonin in the regulation of IL-2 and IL-6 production by human blood mononuclear cells. J. Immunol. **92:** 76–84.
13. JUKNAT, A.A., M. DEL VALLE, A. MENDEZ, *et al*. 2005. Melatonin prevents hydrogen peroxide-induced Bax expression in cultured rat astrocytes. J. Pineal Res. **38:** 84–92.
14. ECK, K.M., L. YUAN, L. DUFFY, *et al*. 1998. A sequential treatment regimen with melatonin and all-trans retinoic acid induces apoptosis in MCF-7 tumour cells. Br. J. Cancer **77:** 2129–2137.
15. POST, A., F. HOLSBOER & C. BEHL. 1998. Induction of NF-kappaB activity during haloperidol-induced oxidative toxicity in clonal hippocampal cells: suppression of NF-kappaB and neuroprotection by antioxidants. J. Neurosci. **18:** 8236–8246.

# PAPER II

*Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells*

# Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells

Abstract: Among the non-neurological functions of melatonin, much attention is being directed to the ability of melatonin to modulate the immune system, whose cells possess melatonin-specific receptors and biosynthetic enzymes. Melatonin controls cell behaviour by eliciting specific signal transduction actions after its interaction with plasma membrane receptors ($MT_1$, $MT_2$); additionally, melatonin potently neutralizes free radicals. Melatonin regulates immune cell loss by antagonizing apoptosis. A major unsolved question is whether this is due to receptor involvement, or to radical scavenging considering that apoptosis is often dependent on oxidative alterations. Here, we provide evidence that on U937 monocytic cells, apoptosis is antagonized by melatonin by receptor interaction rather than by radical scavenging. First, melatonin and a set of synthetic analogues prevented apoptosis in a manner that is proportional to their affinity for plasma membrane receptors but not to their antioxidant ability. Secondly, melatonin's antiapoptotic effect required key signal transduction events including G protein, phospholipase C and $Ca^{2+}$ influx and, more important, it is sensitive to the specific melatonin receptor antagonist luzindole.

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## Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is biosynthesized by a multistep enzymatic reaction chain from tryptophane [1] in pinealocytes [2] and leucocytes [3]. After its release, melatonin exerts its functions in the extracellular space, where it acts as a free radical scavenger [4] behaving in fact as an antioxidant [5], such as during ischaemic/reperfusion injuries [6]; and after its interaction with its specific receptors, present in the cell nucleus [7] and on the plasma membrane [8]. Melatonin is a lipophilic molecule that freely crosses cell membranes and enters cells [9], where it has been reported to alter redox balance, i.e. by increasing glutathione levels [10] and via radical scavenging [11]. Melatonin's action as a regulator of circadian rhythms [12] occurs in a hormone-like fashion through its direct effects on target cells; also, its newly discovered action as a regulator of immune functions [3] may derive from an autocrine activity. The potential use of melatonin as a pharmaceutical agent derives in part from early observations showing that at physiological concentrations melatonin may inhibit tumour growth in animal models [13] and

proliferation of tumour cells in vitro [14]. This led researchers to postulate a possible action of melatonin as a pro-apoptotic agent in cancer cells [15], with possible pharmaceutical use. Melatonin also increases the efficacy of some specific chemotherapic drugs on cancer cells [16], being directly pro-apoptotic in many cancer cells while functioning as an antiapoptotic agent in normal cells [17].

The findings summarized above explain the mechanism through which melatonin controls immune cells, which are carefully regulated by apoptosis, and may open novel avenues for pharmacological interventions on immune deregulations. Thus, it is important to understand the mechanisms through which melatonin modifies apoptosis. In particular, one of the first questions is whether melatonin's antiapoptotic effect derives from its ability to engage specific receptors, thereby triggering a signal transduction pathway whose second messengers interfere with apoptosis or elicit a survival pathway, or whether melatonin's radical scavenging properties may interfere with apoptosis, a process that is known to be controlled by the intracellular redox environment [18].

Herein, we analysed the mechanism of melatonin's antiapoptotic effect. In this study, we examined the possible

role of receptor interaction and the subsequent signal transduction process during the antiapoptotic effect of melatonin using a twofold approach: (i) the use of synthetic analogues with different radical scavenging abilities and affinities for the melatonin plasma membrane receptors, to test whether the antiapoptotic activity parallels the analogues' ability of engaging receptors or to scavenge radicals and (ii) an analysis of signal transduction events known to be triggered by melatonin receptor interaction, to determine if inhibition of these events may disturb melatonin's antiapoptotic effects.

## Materials and methods

### Cell culture

U937 cells were cultured in RPMI medium supplemented with 10% FCS [17]. Cells are routinely check for absence of mycoplasm by using a mycoplasm detection kit (Myco-alert™, Cambrex Bio Science, Milano, Italy). The experiments were performed on cells in the logarithmic phase of growth under condition of ≥98% viability, as assessed by trypan blue exclusion.

### Induction and evaluation of apoptosis

Apoptosis was induced either with the protein synthesis inhibitor puromycin (PMC, 10 $\mu$g/mL), with 50 $\mu$M etoposide (VP16) or with 1 m$M$ $H_2O_2$. Apoptosis was evaluated after 4 hr of PMC or VP16 continuous treatment, or after 1 hr incubation with $H_2O_2$ followed by 6 hr of recovery in fresh medium ($\pm$melatonin). Apoptosis was quantified as previously described [19]. Briefly, cells were stained with the Hoechst 33342; cells with nuclear apoptotic morphology, detected using a fluorescence microscope, were counted (at least 300 cells in at least three independent fields) and the fraction of apoptotic cells among total cells was evaluated. PMC, VP16, $H_2O_2$ were purchased from Sigma Chemical Co. (St Louis, MO, USA), Hoechst 33342 from Calbiochem (La Jolla, CA, USA).

### Melatonin and analogues

Melatonin was purchased from Sigma; 2-bromomelatonin (2-Br-MLT) [20], 6-methoxy-*N*-acetyltryptamine (6-OMe-aT) [21] and *N*-[2-(5-methoxy-1*H*-indol-2-yl)ethyl]aceta-mide (2-AEtI) [22] were synthesized as previously described, and used at the concentration of 1 m$M$ unless otherwise specified. For the experiments, melatonin and analogues were added 1 hr prior to apoptosis induction.

### Other treatments

Phospholipase C (PLC) inhibition was achieved when neomycin sulphate (NEO; 1 m$M$) and U73122 (10 $\mu$M) were added 30 min before treatments. Extracellular $Ca^{2+}$ influx was inhibited by incubating the cells with ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA; 650 $\mu$M) for 15 min before other treatments. G-protein inhibition was achieved when pertussis toxin (PTX; 200 n$M$) was added 24 hr before other treatments. Melatonin

receptor ($MT_1/MT_2$) antagonism was achieved using 2-benzyl-*N*-acetyltryptamine (luzindole 50 $\mu$M), which was added 30 min before other treatments.

### Melatonin and analogues antioxidant potency assays

Antioxidant power was assessed by three different methods. The data obtained were then used to calculate the concentration of a compound required to inhibit the reference oxidant by 50% ($IC_{50}$). (i) The colorimetric method based on the stable free radical diphenyl-*p*-picrylhydrazyl (DPPH) was performed according to Mellors and Tappel [23]. (ii) The colorimetric method based on the stable free radical 2,2′-azinobis [3-ethylbenzthiazoline-sulfonic acid] (ABTS) was carried out as indicated by Poeggeler et al. [24]. (iii) The enzymatic/luminometric method based on the xanthine/xanthine oxidase system was performed according to Sud'ina et al. [25].

### $IP_3$ assay

After the treatments, U937 cells were collected, washed and resuspended at $6 \times 10^6$ cells/mL in RPMI medium. About 200 $\mu$L of 60% ice-cold perchloric acid were added to the cell suspension. The cell suspension was then centrifuged and aliquots of the supernatant were neutralized with 10 $M$ KOH for inositol-1,4,5-trisphosphate ($IP_3$) assay. $IP_3$ was assayed using a commercially available kit [D-myo-inositol-1,4,5-trisphosphate ($IP_3$) [$^3$H]; Biotrak Assay System, Amersham Biosciences, Piscataway, NJ, USA]. The assay is based on competition between unlabelled and tritium-labelled $IP_3$ (the tracer) in the standard or samples, for binding to a binding protein prepared from bovine adrenal cortex. The bound $IP_3$ is separated from free $IP_3$ by centrifugation leaving the bound fraction adherent to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled $IP_3$ in the sample to be determined by interpolation from a standard curve. The data represent the mean values of five experiments as indicated in figure legends.

### $Ca^{2+}$ measurements

#### Fluo3 staining

Cells were washed and resuspended at the concentration of $1 \times 10^7$/mL in Hank's balanced salt solution (HBSS) + 1 mg/mL glucose + 650 $\mu$M $CaCl_2$ and loaded with 1 $\mu$M Fluo3-AM, in the dark at room temperature [26, 27]. After 40 min of incubation, Fluo3 was washed-out and cells postincubated in HBSS + 1 mg/mL glucose + 650 $\mu$M $CaCl_2$ in the dark at room temperature at the concentration of $2 \times 10^6$/mL for 20 min before analysis for complete Fluo3 de-esterification.

#### Flow cytometric analysis

Measurements of cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) was performed on a FACScalibur (Becton Dickinson, San Jose, CA, USA), tuned at 488 nm, using the standard FL1 photomultiplier (bandpass 530 nm, bandwidth 30 nm). Data were recorded for further analysis with CELL QUEST

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software (Becton Dickinson, Franklin Lakes, NJ, USA). The mean fluorescence value was determined by counting 10,000 cells. Fluorescence values were converted in $[Ca^{2+}]$ values according to the equation of Grynkiewicz (Fluo3 $K_d = 430$ nM; $F_{max}$ was measured in the presence of $\geq 10$ $\mu$M ionomycin and 650 $\mu$M $CaCl_2$) [28]. The addition of the reagents in the appropriate sequence was manually performed in the same cuvette for each sample. The flow was temporarily stopped to allow the addition of the reagents, and immediately restarted.

### Measurements of cytosolic calcium levels

$[Ca^{2+}]_c$ upon addition of melatonin or its analogues was measured in cells kept (i) in the absence of free $Ca^{2+}$ and (ii) in the presence of 650 $\mu$M $CaCl_2$.

### Statistical analysis

Statistical analyses were performed using Student's $t$-test for unpaired data and $P$-values $< 0.05$ were considered significant. Data are presented as mean $\pm$ S.E.M.

## Results

U937 human monocytic cells are known to possess $MT_1$ melatonin receptors [29], thus being an ideal system for investigating the mechanisms of the antiapoptotic effect of melatonin. First of all, we analysed whether melatonin might exert any toxic or apoptogenic effect on exponentially growing U937 cells. However, doses up to 1 mM, for incubation times reaching 48 hr, failed to exert apoptosis or toxicity (not shown).

We then evaluated the antiapoptotic effect. Fig. 1A shows that melatonin reduces apoptosis induced by the protein synthesis inhibitor PMC in a dose-dependent fashion, protection being highly significant for the 1 mM dose. Melatonin exerted a similar protective effect on apoptosis induced by two additional stressing agents, acting with different mechanisms, namely the chemotherapic drug VP16, an inhibitor of topoisomerase II, and $H_2O_2$, as illustrated in Fig. 1B.

To understand whether melatonin's antiapoptotic role may depend on receptor interaction or antioxidant ability, we used a panel of melatonin synthetic analogues (Fig. 2A). These analogues [30] possess different affinities and intrinsic activities for the melatonin plasma membrane receptors: 2-Br-MLT is an $MT_1/MT_2$ melatonin receptor agonist with affinity higher than melatonin; 6-OMe-aT is a partial agonist characterized by low melatonin receptor affinity and 2-AEtI is a melatonin antagonist that binds melatonin receptors with poor affinity (for details see Table 1).

The antioxidant ability of the compounds was then tested. To control a wide range of oxidative phenomena, the inhibition by melatonin versus the analogues was tested in three different oxidative systems, namely a colorimetric method based on the stable free radical DPPH [23], a colorimentric method based on the stable free radical ABTS [24] and an enzymatic/luminometric method based on the xanthine/xanthine oxidase system [25]. As illustrated in Fig. 2B, the three assays reveal different antioxidant
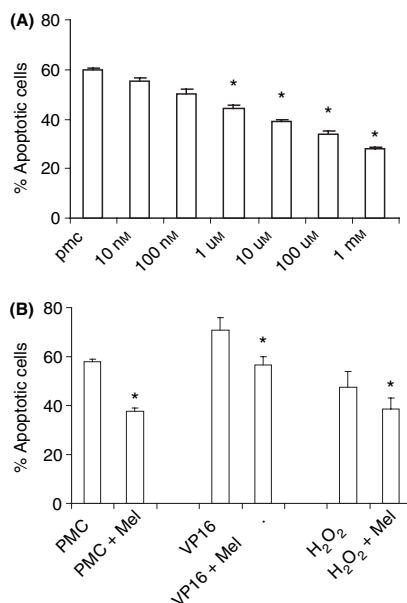


Fig. 1. Melatonin antagonizes apoptosis. (A) U937 cells were incubated with different doses of melatonin and induced to apoptosis with puromycin (PMC; 10 μg/mL) in complete medium. Apoptosis was evaluated at 3 hr. Results are the average of at least three independent experiments (±S.E.M.). The reduction of apoptosis is significant starting from 100 nM ($P < 0.05$)*. (B) Melatonin 1 mM protects cells from apoptosis induced with different apoptogenic agents. Apoptosis was estimated at 3 hr of PMC 10 μg/mL, at 4 hr of VP16 50 μM, and at 6 hr of recovery after $H_2O_2$ 1 mM removal (see Materials and methods). Results are the average of at least three independent experiments (±S.E.M.). Apoptosis reduction of VP16 and $H_2O_2$ is significant ($P < 0.05$)*.

abilities of the analogues depending on the oxidative system examined; notably, none of them coincides with the extent of affinity.

We probed the antiapoptotic effect of these compounds on U937 induced to undergo apoptosis by PMC Fig. 2C shows that the antiapoptotic ability of the compounds parallels with receptor affinity, being unrelated to any of the four antioxidant activities. Indeed, 2-Br-MLT, the analogue with the highest receptor affinity is also the most effective antiapoptotic agent, whereas the partial agonist 6-OMe-aT, and the weak antagonist (2-AEtI), are ineffective. These results suggest a relationship between melatonin receptor engagement and antiapoptotic action.

To explore the possible role of receptor stimulation in the antiapoptotic effect of melatonin, the different steps of melatonin-induced signal transduction were examined. First, competition experiments between melatonin and the analogues were performed. 2-AEtI, as a weak antagonist, was used in combination with melatonin or 2-Br-MLT (a strong agonist), to check whether it might inhibit melatonin's (or 2-Br-MLT) antiapoptotic effect. As illustrated in Fig. 3, the antagonist abolished the effect of
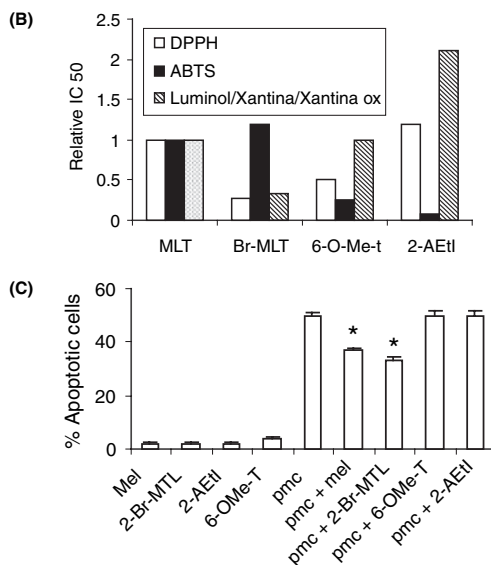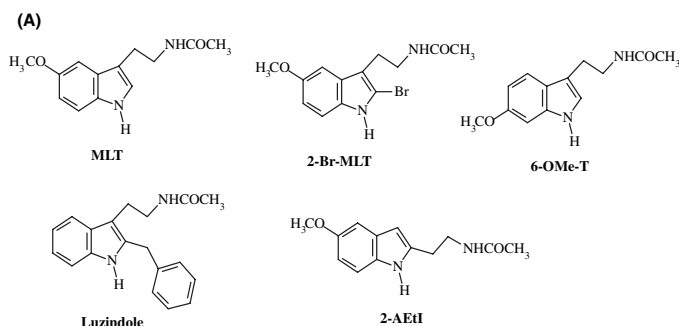
**(A)**



**(B)**



*Fig. 2.* Melatonin analogues antagonizes apoptosis according to $MT_1/MT_2$ receptor affinity rather than antioxidant ability. (A) Chemical structures of $MT_1/MT_2$ melatonin receptor ligands used in this study. (B) Antioxidant activity of melatonin and its structural isomers was quantified with DPPH, ABTS+, luminol/xanthine/xanthine oxidase assays as described in Materials and methods. The $IC_{50}$ values for melatonin were as follows: DPPH: 30 mM; ABTS+: 130 mM and luminol/xanthine/xanthine oxidase: 4 mM. For the analogues, the $IC_{50}$ values are presented for each assay as a fraction of that of melatonin posed = 1. (C) U937 cells were incubated with melatonin or analogues for 1 hr before induction of apoptosis. We measured the extent of apoptosis at 3 hr of puromycin 10 $\mu$g/mL. Results are the average of at least three independent experiments ($\pm$S.E.M.). Apoptosis reduction at 1 mM doses of melatonin and 1 mM of 2-bromomelatonin (2-Br-MLT) is significant ($P < 0.05$)*.

**(C)**



Table 1. Binding affinities of melatonin ligands for the human $MT_1$ and $MT_2$ melatonin receptors

| Compounds | $pK_i$ $MT_1$ | Rel affin[a] | $pK_i$ $MT_2$ | Rel affin[a] | Activity | Refs |
|---|---|---|---|---|---|---|
| Melatonin | 9.63 | 1 | 9.43 | 1 | A | (i) |
| 2-Br-MLT | 10.54 | 0.12 | 9.94 | 0.31 | A | (i) |
| 6-OMe-aT | 5.94 | 4898 | 6.24 | 1549 | PA | (ii) |
| 2-AEtI | 4.79 | 69,184 | 5.03 | 25,118 | ANT | (iii) |

[a]Relative affinity ($IC_{50}$ compound/$IC_{50}$ MLT).
A, agonist; PA, partial agonist; ANT, antagonist; 2-Br-MLT, 2-bromomelatonin; 6-OMe-aT, 6-methoxy-N-acetyltryptamine; 2-AEtI, N-[2-(5-methoxy-1H-indol-2-yl)ethyl]acetamide.

melatonin (panel A) and 2-Br-MLT (panel B) at equimolar doses. The poor partial agonist, here used as a negative control, was ineffective. This supports the hypothesis that the antiapoptotic effect of melatonin is mediated by membrane receptor stimulation.

Luzindole is an important tool in melatonin research, as it specifically inhibits G protein activation by a melatonin-engaged $MT_1$ or $MT_2$ receptor [31]. Fig. 4 shows that luzindole inhibits melatonin (panel A) and

2-Br-MLT (panel B) antiapoptotic effects. The specificity of luzindole emphasizes the importance of these findings. Further evidence that receptor-initiated signal transduction is required for apoptosis antagonism comes from the finding that pertussis toxin, a specific inhibitor of the $\alpha$-subunit of heterotrimeric G proteins, which inhibits melatonin-activated signal transduction [8], completely reverses melatonin antiapoptotic effect (panel C).
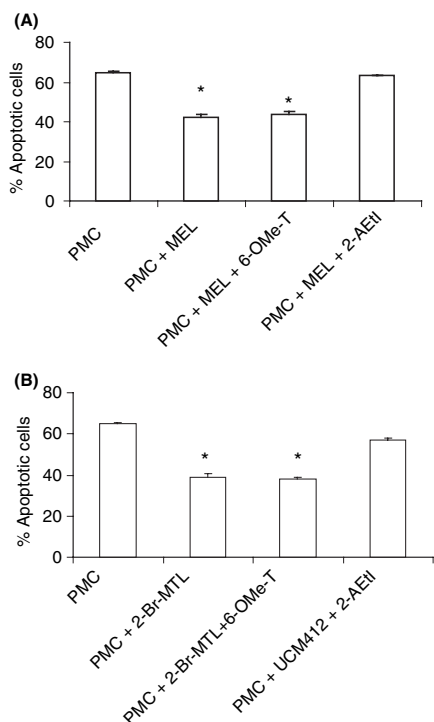
**157**

**(A)**

**(B)**

Fig. 3. Receptor antagonist abolished the effect of melatonin and 2-bromomelatonin (2-Br-MLT). 6-Methoxy-*N*-acetyltryptamine (a low-affinity partial agonist) 1 mM and *N*-[2-(5-methoxy-1*H*-indol-2-yl)ethyl]acetamide (antagonist) 1 mM were added 30 min before incubation with melatonin (A) or 2-Br-MLT (B), and then apoptosis was induced with puromycin 10 μg/mL. Results are the average of at least three independent experiments (±S.E.M.). Apoptosis reduction at 1 mM doses of melatonin and 1 mM of 2-Br-MLT is significant (*P* < 0.05)*.

Activation of the two high-affinity G-protein-coupled receptors (MT$_1$ and MT$_2$) stimulates the plasma membrane PLC to process phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into diacylglycerol (DAG) and IP$_3$ [32]; increased [IP$_3$] by melatonin has been reported [33]. We observed that also in our system melatonin caused [IP$_3$] increase, with a peak at 1 min (Fig. 5A), showing PLC activation. The potent agonist 2-Br-MLT induces a stronger and earlier IP$_3$ increase, peaking at 10 s (Fig. 5B). To understand whether this PLC activation plays a role in melatonin antiapoptotic effect, we inhibited PLC with two different inhibitors, namely neomycin, which protects PLC substrate PIP$_2$, and U7322, which inhibits the activation of PLC by the upstream G protein. Both inhibitors reversed melatonin's antiapoptotic effect (Fig. 5C), showing that PLC is part of the antiapoptotic action of melatonin.

IP$_3$ stimulates the release of Ca$^{2+}$ from the endoplasmic reticulum (ER) into the cytosol, thus increasing cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]). This can be detected as a Ca$^{2+}$ transient in cells loaded with fluorescent Ca$^{2+}$ dyes



**(A)**

**(B)**

**(C)**
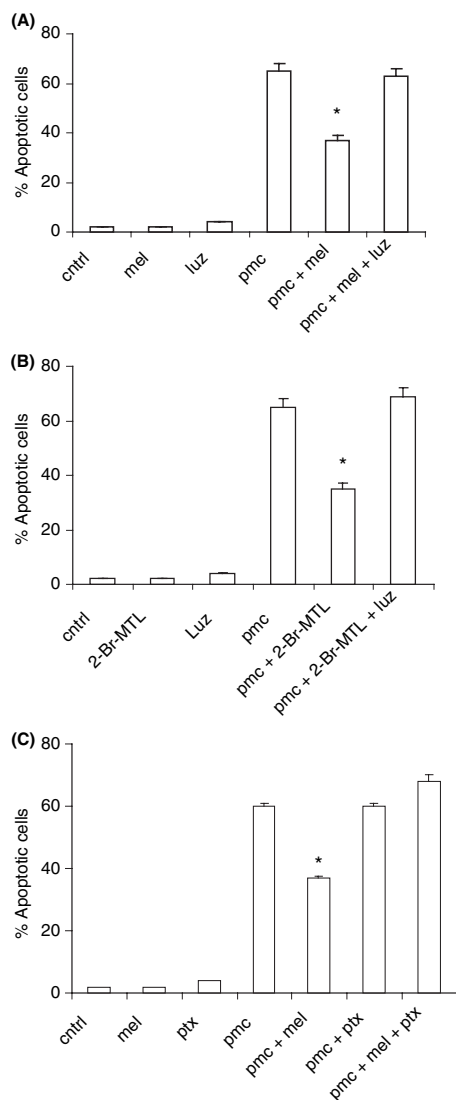
Fig. 4. Melatonin antiapoptotic effect is sensitive to luzindole and pertussis toxin. Luzindole 50 μM was added 30 min before incubation with melatonin 1 mM (A) or 2-bromomelatonin (2-Br-MLT) 1 mM (B), and then apoptosis was induced with puromycin (PMC) 10 μg/mL. Results are the average of at least three independent experiments (±S.E.M.). Apoptosis reduction at 1 mM doses of Mel and 1 mM of 2-Br-MLT is significant (*P* < 0.05)*. (C) Pertussis toxin was added 12 hr before incubation with melatonin, and then apoptosis was induced with PMC 10 μg/mL. Results are the average of at least three independent experiments (±S.E.M.). Apoptosis reduction at 1 mM doses of melatonin is significant (*P* < 0.05)*.

localized in the cytosol (see Materials and Methods). This transient [Ca$^{2+}$]$_c$ is accompanied and reinforced by ER refilling due to stimulation of Ca$^{2+}$ influx from the
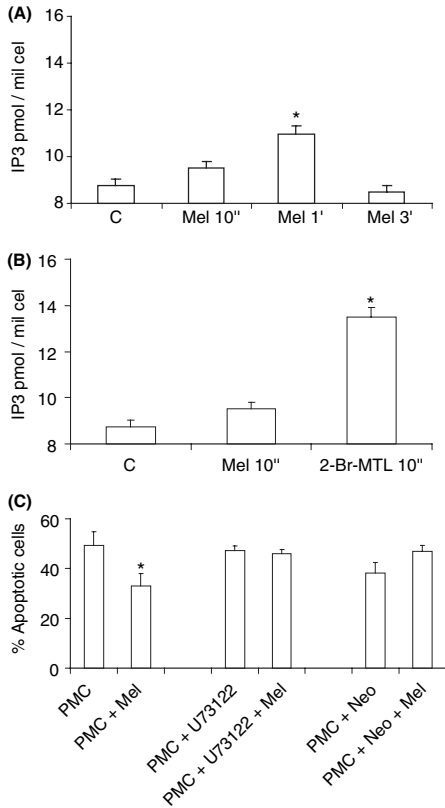
*Fig. 5.* Phospholipase C is required for melatonin antiapoptotic effect. U937 cells were incubated with melatonin 1 mM (A) or 2-bromomelatonin (2-Br-MLT) 1 mM (B) and $IP_3$ intracellular concentration was measured (see Materials and methods). Values are the average of four measurement in at least three independent experiments ($\pm$S.E.M.). (C) Neomycin 1 mM or U73122 10 $\mu$M were added 15 min before incubation with melatonin, and then apoptosis was induced with puromycin 10 $\mu$g/mL. Results are the average of at least three independent experiments ($\pm$S.E.M.). Apoptosis reduction at 1 mM doses of melatonin is significant ($P < 0.05$)*.

extracellular environment (capacitative influx). Melatonin was shown to mobilize $Ca^{2+}$ [34]. Fig. 6 shows a melatonin-dependent increase in $[Ca^{2+}]_c$, in the absence (panel A) or presence (panel B) of external $Ca^{2+}$. In U937 melatonin is able to promote $Ca^{2+}$ transients, which are higher in the presence of extracellular $Ca^{2+}$, which is compatible with the superimposition of a capacitative $Ca^{2+}$ influx. If this $Ca^{2+}$ mobilization is a cell response to melatonin receptor interaction, 2-Br-MLT, the melatonin analogue with increased receptor affinity, should promote even higher $Ca^{2+}$ transients. Indeed, this is what we found, as illustrated in Fig. 6C.

In some instances of receptor stimulation, the capacitative $Ca^{2+}$ influx plays a crucial role in carrying on signal

transduction [35]. To understand whether the melatonin-induced $Ca^{2+}$ influx is required for melatonin antiapoptotic effects, we inhibited $Ca^{2+}$ influx by extracellular $Ca^{2+}$ chelation with EGTA and checked whether melatonin would still exert its antiapoptotic action. Fig. 6D shows that in this instance melatonin is no longer able to reduce apoptosis.

## Discussion

It is an established notion that some types of apoptosis, especially those induced by cell damage and occurring through the so-called intrinsic (previously refereed to as mitochondrial) pathway, imply redox imbalance or even direct oxidative stress [36–38]. Thus, it was reasonable to hypothesize that the radical-scavenging properties of melatonin was the mechanism through which melatonin exerted its antiapoptotic activity. An important point that favoured this hypothesis over the melatonin receptor-induced signal transduction was the observation that the concentrations of melatonin required for radical scavenging fitted with those required for its antiapoptotic action. Also, in the present study, the melatonin concentrations required for scavenging a set of different radicals is in the range of 4–130 $\mu$M, i.e. similar to that required for a significant antiapoptotic effect of melatonin. However, we added a novel tool here, i.e. a set of melatonin analogues to compare radical scavenging versus antiapoptotic ability. This comparison uncouples the antiapoptotic effect of melatonin from its radical scavenging ability, as the ability to scavenge three different types of radicals by each analogue does not correspond to a similar antiapoptotic effect; in addition, recent data from the literature [39] report that in cancer cells melatonin is a producer of intracellular radicals.

We provide strong evidence that the signal transduction elicited by melatonin receptor stimulation is the mechanism through which melatonin exerts its antiapoptotic effect in U937 monocytes: (i) in the set of melatonin analogues, the extent of the apoptosis antagonistic action corresponds to the $MT_1/MT_2$ receptor affinity and (ii) inhibition of the receptor-induced signal transduction steps, including the specific effect of luzindole, abolished the antiapoptotic action.

In spite of the many evidence we provide here (some of which, namely, the reversion of the antiapoptotic effect by luzindole, by pertussis toxin and by the $MT_1/MT_2$ antagonist 2-AEtI are convincing) that melatonin antagonizes apoptosis via receptor interaction, it is not clear why the doses of melatonin required to counteract apoptosis are so much higher than those necessary to efficiently engage receptors. A possible explanation comes from a recent observation we made [40] of a masking effect by serum proteins, present in culture medium in large concentration, which are known to stick to melatonin [41] lowering the actual concentration available for cell entry. However, it cannot be excluded that the $MT_1/MT_2$ pathway might be necessary but not sufficient for apoptosis antagonism, and an additional target to which melatonin binds with lower affinity (e.g. calmodulin, Kd = 63 $\mu$M [42]) may be required. The problem of the doses is anyway complicated by the presence of melatonin endogenously synthesized by
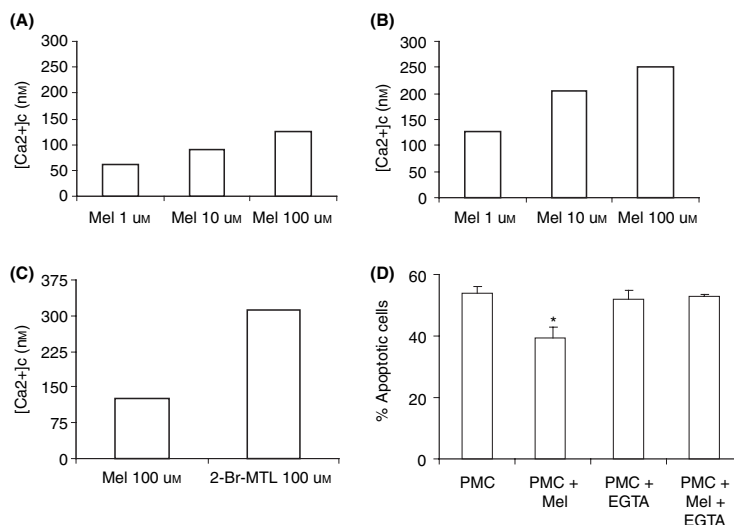
*Fig. 6.* Melatonin-dependent $Ca^{2+}$ influx is required for melatonin antiapoptotic effect. Melatonin stimulates $Ca^{2+}$ fluxes. Intracellular calcium concentrations were measured upon addition of the different doses of melatonin in the absence (A) or presence (B) of extracellular $Ca^{2+}$ (650 $\mu$M). Delta is calculated as the difference between the highest value of $[Ca^{2+}]_i$ and the basal level (110 $\pm$ 12 nM). It is shown one of two experiments performed with similar results; (C) the extent of melatonin-induced $Ca^{2+}$ fluxes in the absence of extracellular $Ca^{2+}$ were compared with those induced by the potent agonist 2-bromomelatonin (2-Br-MLT). It is shown one of two experiments performed with similar results; (D) EGTA reverts the antiapoptotic effects of melatonin. Cells were treated with EGTA 650 $\mu$M, 15 min before incubation with melatonin 1 mM. Apoptosis was then induced with puromycin 10 $\mu$g/mL. Results are the average of at least three independent experiments ($\pm$S.E.M.). Apoptosis reduction at 1 mM doses of melatonin is significant ($P < 0.05$)*.

the cells. Indeed, we observed by thin layer chromatography that U937 produce melatonin [40]. This ability is shared by many haematopoietic cells [43, 44]; interestingly, this may create extremely high bone marrow melatonin concentrations [45], which may provide unexpected physiological significance to the often-reported experimental effects of high melatonin doses.

Although the two high-affinity G-protein-coupled receptors are quite well characterized from the chemical point of view [8], the specific functional responses in target tissues linked to each melatonin are still unclear and many contradictions easily come up in the literature. $MT_1$ receptor mediates the melatonin-induced inhibition of $PGE_2$ in lymphocytes [46]. Both PLC and adenylcyclase pathways have been shown to be involved in melatonin-induced signal transduction; interestingly, under different conditions (i.e. cell type), $IP_3$ and cAMP, products of the two pathways, respectively, can be reciprocally activated/repressed by melatonin [47]. We found that $IP_3$ is transiently induced by melatonin, and that inhibition of PLC is sufficient to reverse melatonin antiapoptotic effect. This indicates that the antiapoptotic effect of melatonin requires this particular pathway. The PLC pathway may possibly counteract apoptosis by promoting $Ca^{2+}$ influx; indeed, inhibition of $Ca^{2+}$ influx was able to reverse melatonin's antiapoptotic effect. An increase in cytosolic $Ca^{2+}$ was originally thought of as a canonical pro-apoptotic signal [48], but it is now well demonstrated that $Ca^{2+}$ influx may have also a pro-survival role [49, 50]. It will be interesting to explore how the G-protein/PLC pathway leads to apoptosis antagonism. The literature offers a wide range of events to explore, such as the activation of protein kinase C as a response to the increase in $[Ca^{2+}]_c$ and DAG (the breakdown products of PLC) concentrations; interestingly, phosphorylation of key proteins controllers of apoptosis is receiving a renewed burst of interest in the recent months.

## Acknowledgement

## References

1. REITER RJ. Melatonin: the chemical expression of darkness. Mol Cell Endocrinol 1991; **79**:C153–C158.
2. REITER RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr Rev 1991; **12**:151–160.
3. CARRILLO-VICO A, CALVO JR, ABREU P et al. Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possibile role as intracrine, autocrine, and/or paracrine substance. FASEB J 2004; **18**:537–539.
4. TAN DX, MANCHESTER LC, TERRON MP, FLORES LJ, REITER RJ. One molecule, many derivatives: a never ending interacton of melatonin with reactive oxygen and nitrogen species. J Pineal Res 2007; **42**:28–42.
5. REITER RJ, TAN DX, OSUNA C, GITTO E. Action of melatonin in the reduction of oxidative stress. J Biomed Sci 2000; **7**:444–458.

6. REITER RJ, TAN DX, LEON J, KILIC U, KILIC E. When melatonin gets on your nerves: its beneficial actions in experimental models of stroke. Exp Biol Med 2005; **230**:104–117.

7. GUERRERO JM, POZO D, GARCIA-MAURINO S et al. Involvement of nuclear receptors in the enhanced IL-2 production by melatonin in Jurkat cells. Ann N Y Acad Sci 2000; **917**:397–403.

8. DUBOCOVICH ML, MARKOWSKA M. Functional $MT_1$ and $MT_2$ melatonin receptor in mammals. Endocrine 2005; **27**:101–110.

9. MENENDEZ-PELAEZ A, POEGGELER B, REITER RJ et al. Nuclear localization of melatonin in different tissues: immunocytochemical and radioimmunoassay evidence. J Cell Biochem 1993; **53**:373–382.

10. URATA Y, HONMA S, GOTO S. et al. Melatonin induces gamma-glutamylcysteine synthetase mediated by activator protei-I in human vascular endothelial cells. Free Radic Biol Med 1999; **27**:838–847.

11. REITER RJ, TAN DX, POEGGELER B et al. Melatonin as a free radical scavenger: implications for aging and age-related diseases. Ann N Y Acad Sci 1994; **719**:1–12.

12. MALPAUX B, MIGAUD M, TRICOIRE H, CHEMINEAU P. Biology of mammalian photoperiodism and the critical role of the pineal gland and melatonin. J Biol Rhythms 2001; **16**:336–347.

13. BLASK DE, SAUER LA, DAUCHY RT. Melatonin as a chronobiotic/anti-cancer agent: cellular, biochemical and molecular mechanisms of action and their implications for circadian-based cancer therapy. Curr Top Med Chem 2002; **2**:113–132.

14. HILL S, BLASK DE. Effects of the pineal hormone melatonin on the proliferation and morphological characteristics of human breast cancer cells (MCF-7) in culture. Cancer Res 1998; **48**:6121–6126.

15. WOLFLER A, CALUBA HC, ABUJA PM et al. Pro-oxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. FEBS Lett 2001; **502**:127–131.

16. REITER RJ, TAN DX, SAINZ RM et al. Melatonin: reducing the toxicity and increasing the efficacy of drugs. J Pharm Pharmacol 2002; **54**:1299–12321.

17. SAINZ RM, MAYO JC, REITER RJ et al. Melatonin regulates glucocorticoid receptor: an answer to its antiapoptotic action in thymus. FASEB J 1999; **13**:1547–1556.

18. JOU MJ, PENG TI, REITER RJ et al. Visualization of the antioxidant effects of melatonin at the mitochondrial level during oxidative stress-induced at the mitochondrial level during oxidative stress-induced apoptosis of rat brain astrocytes. J Pineal Res 2004; **37**:55–70.

19. COLUSSI C, ALBERTINI MC, COPPOLA S et al. $H_2O_2$-induced block of glycolysis as an active Adp-ribosylation reaction protecting cells from apoptosis. FASEB J 2000; **14**:2266–2276.

20. DURANTI E, STANKOV B, SPADONI G et al. 2-Bromomelatonin: synthesis and characterization of a potent melatonin agonist. Life Sci 1992; **51**:479–485.

21. MOR M, RIVARA S, SILVA C et al. Melatonin receptor ligands: synthesis of new melatonin derivatives and comprehensive comparative molecular field analysis (CoMFA) study. J Med Chem 1998; **41**:3831–3844.

22. SPADONI G, BALSAMINI C, BEDINI A et al. 2-[N-acylamino ($C_1$-$C_3$)alkyl]indoles as $MT_1$ melatonin receptor partial agonists, antagonists and putative inverse agonists. J Med Chem 1998; **41**:3624–3634.

23. MELLORS A, TAPPEL AL. The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. J Biol Chem 1966; **241**:4353–4356.

24. POEGGELER B, REITER RJ, HARDEL R, TAN D-X, BARLOW-WALDEN LR. Melatonin and structurally-related, endogenous indoles act as potent electron donors and radical scavengers in vitro. Redox Report 1996; **2**:179–184.

25. SUD'INA GF, MIRZOEVA OK, PUSHKAREVA MA et al. Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. FEBS Lett 1993; **329**:21–24.

26. FANELLI C, COPPOLA S, BARONE R et al. Magnetic fields increase cell survival by inhibiting apoptosis via modulation of $Ca^{2+}$ influx. FASEB J 1999; **13**:95–102.

27. CERELLA C, D'ALESSIO M, DE NICOLA M et al. Cytosolic and endoplasmic reticulum $Ca^{2+}$ concentrations determine the extent and the morphological type of apoptosis, respectively. Ann N Y Acad Sci 2003; **1010**:74–77.

28. GRYNKIEWICZ G, POENIE M, TSIEN RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985; **260**:3440–3450.

29. GARCIA-MAURINO S, GONZALEZ-HABA MG, CALVO JR et al. Involvement of nuclear binding sites for melatonin in the regulation of IL-2 and IL-6 production by human blood mononuclear cells. J Neuroimmunol 1998; **92**:76–84.

30. GARCIA-MAURINO S, POZO D, CALVO JR. Correlation between nuclear melatonin receptor expression and enhanced cytokine production in human lymphocytic and monocytic cell lines. J Pineal Res 2000; **29**:129–137.

31. MOR M, SILVA C, VACONDIO F et al. Indole-based analogs of melatonin: in vitro antioxidant and cytoprotective activities. J Pineal Res 2004; **36**:95–102.

32. DUBOCOVICH ML. Luzindole (N-0774): a novel melatonin receptor antagonist. J Pharmacol Exp Ther 1998; **246**:902–910.

33. BACH AG, WOLGAST S, MUHLBAUER E, PESCHKE E. Melatonin stimulates inositol-1,4,5-triphosphathe and $Ca^{2+}$ release from INS1 insulinoma cells. J Pineal Res 2005; **39**:316–323.

34. MARKOWSKA M, MROZKOWIAK A, PAWLAK J, SKWARL-SONTA K. Intracellular second messengers in melatonin signal transduction in chicken splenocytes in vitro. J Pineal Res 2004; **37**:207–212.

35. VANACEK J. Cellular mechanism of melatonin action. Physiol Rev 1998; **78**:687–721.

36. D'ALESSIO M, DE NICOLA M, COPPOLA S et al. Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis. FASEB J 2005; **19**:1504–1506.

37. COPPOLA S, GHIBELLI L. GSH extrusion and and the mitochondrial pathway of apoptotic signalling. Biochem Soc Trans 2000; **28**:56–61.

38. GAO J, LIU X, RIGAS B. Nitric oxide-donating aspirin induces apoptosis in human colon cancer cells through induction of oxidative stress. Proc Natl Acad Sci U S A 2005; **102**:17207–17212.

39. WOFLER A, CALUBA HC, ABUJA PM et al. Prooxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. FEBS Lett 2001; **502**:127–131.

40. RADOGNA F, PATERNOSTER L, ALBERTINI MC et al. Melatonin as an apoptosis antagonist. Ann N Y Acad Sci 2006; **1090**:226–233.

41. GILAD E, ZISAPEL N. High-affinity binding of melatonin to hemoglobin. Biochem Mol Med 1995; **56**:115–120.

42. PAZ ROMERO M, GARCIA-PERGAÑEDA A, GUERRERO JM et al. Membrane-bound in *Xenopus laevis* oocytes as a novel binding site for melatonin. FASEB J 1998; **12**:1401–1408.

43. CONTI A, CONCONI S, HERTENS E et al. Evidence for melatonin synthesis in mouse and human bone marrow cells. J Pineal Res 2000; **28**:193–202.

44. CARRILLO-VICO A, LARDONE P, FERNANDEZ-SANTOS JM et al. Human lymphocyte-synthesized melatonin is involved in the regulation of the IL-2/IL-2 receptor system. J Clin Endocrinol Metab 2005; **90**:992–1000.

45. TAN DX, MANCHESTER LC, REITER RJ et al. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochim Biophys Acta 1999; **1472**:206–214.

46. CARRILLO-VICO A, GARCIA-MAURINO S, CALVO JR, GUERRERO JM. Melatonin counteracts the inhibitory effect of PGE2 on IL-2 production in human lymphocytes via its membrane mt1 receptor. FASEB J 2003; **17**:755–757.

47. MARKOWSKA M, MROZKOWIAK A, PAWLAK J, SKWARL-SONTA K. Intracellular second messengers in melatonin signal transduction in chicken splenocytes in vitro. J Pineal Res 2004; **37**:207–212.

48. MCCONKEY DJ, ORRENIUS S. The role of calcium in the regulation of apoptosis. J Leukoc Biol 1996; **59**:775–783.

49. LAMPE PA, CORNBROOKS EB, JUHASZ A et al. Suppression of programmed neuronal death by a thapsigargin-induced $Ca^{2+}$ influx. J Neurobiol 1995; **26**:205–212.

50. GOMMERMAN JL, BERGER SA. Protection from apoptosis by steel factor but not interleukin-3 is reversed through blockade of calcium influx. Blood 1998; **91**:1891–1900.

# PAPER III

*Melatonin antagonizes the intrinsic pathway of apoptosis via mitochondrial targeting of Bcl-2*

# Melatonin antagonizes the intrinsic pathway of apoptosis via mitochondrial targeting of Bcl-2

Abstract: We have recently shown that melatonin antagonizes damage-induced apoptosis by interaction with the MT-1/MT-2 plasma membrane receptors. Here, we show that melatonin interferes with the intrinsic pathway of apoptosis at the mitochondrial level. In response to an apoptogenic stimulus, melatonin allows mitochondrial translocation of the pro-apoptotic protein Bax, but it impairs its activation/dimerization The downstream apoptotic events, i.e. cytochrome *c* release, caspase 9 and 3 activation and nuclear vesiculation are equally impaired, indicating that melatonin interferes with Bax activation within mitochondria. Interestingly, we found that melatonin induces a strong re-localization of Bcl-2, the main Bax antagonist to mitochondria, suggesting that Bax activation may in fact be antagonized by Bcl-2 at the mitochondrial level. Indeed, we inhibit the melatonin anti-apoptotic effect (i) by silencing Bcl-2 with small interfering RNAs, or with small-molecular inhibitors targeted at the BH3 binding pocket in Bcl-2 (i.e. the one interacting with Bax); and (ii) by inhibiting melatonin-induced Bcl-2 mitochondrial re-localization with the MT1/MT2 receptor antagonist luzindole. This evidence provides a mechanism that may explain how melatonin through interaction with the MT1/MT2 receptors, elicits a pathway that interferes with the Bcl-2 family, thus modulating the cell life/death balance.

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## Introduction

Melatonin has recently gained much attention as a regulator of biological processes in addition to its effects on the circadian rythms. Melatonin acts on practically all cell types by binding specific receptors/interactors, such as the plasma membrane receptors MT1/MT2 [1], calmodulin [2], nuclear receptors [3], thereby eliciting specific, though not fully described, signal transduction pathways [4]. Furthermore, melatonin and its metabolites potently scavenges reactive oxygen species [5, 6], both at the extracellular and intracellular level, thus altering redox-sensitive events and preventing oxidative damage. The overlapping of these effects, combined with different cell sensitivities produce a variety of alterations in different cellular compartments that still need to be elucidated.

The ability of melatonin to modulate apoptosis is receiving increased attention [7]. Physiologically coordinated apoptosis is a critical goal of homeostasis, and a failure to properly control this process leads to hyper- or hypo-proliferative disorders [8]. In addition, apoptosis plays a critical role in the inflammatory and immune responses by regulating the maturation rate of B and T cells, and maintaining/contrasting viability of cells engaged in the inflammatory sites [9, 10]. Interestingly, recent evidences demonstrate an unforeseen role of melatonin in the control of immune and inflammatory

responses, showing that leucocytes possess all the enzymatic machinery necessary to synthesize melatonin from tryptophan [11] as well as the proper receptors MT1/MT2 [12], thus being an autonomous compartment as far as melatonin responses are concerned. Accordingly, much evidence demonstrates that control of leukocyte apoptosis is among the most striking non-neurological roles of melatonin [13, 14]. For this reason, it is important to understand how melatonin controls apoptosis in cells of immune/inflammatory relevance. Additionally, melatonin was found to be at extremely high concentrations in bone marrow [15], thus possibly accounting for the intriguing observation that higher levels of melatonin are required for apoptosis control than for neuro-endocrine functions.

Apoptosis is the result of the coordinated involution of cellular structures, achieved by protein–protein interaction and conformational changes through independent pathways. The best characterized of these is the intrinsic (or damage-induced, or mitochondrial) pathway and the extrinsic (or receptor-induced, or physiological) pathway [16, 17].

A survey of the literature shows that melatonin in leukocytes exerts mainly an anti-apoptotic role [18, 19], thus suggesting that it may support viability of cells engaged in active/acute responses. The mechanism through which this occurs has not been thoroughly investigated. However, some interesting regularities may be envisaged,

such as (i), the ability of melatonin to influence regulation of the Bcl-2 family [20, 21], and (ii) convincing evidence that mitochondria may be the site where melatonin exerts its anti-apoptotic action [22, 23]. Interestingly, the Bcl-2 proteins act primarily at the mitochondrial level. Mitochondria are also the site where the initial events of the intrinsic apoptotic (or mitochondrial) pathway take place [16]. These considerations suggest that a primary target of melatonin is anti-apoptotic action which may be the intrinsic pathway.

The intrinsic apoptotic pathway begins with environmental disturbances (oxidants, $Ca^{2+}$ rises, pH alterations) that directly or via specific kinases activate Bax, a pivotal molecule of the entire pathway [24]. Bax, the best known pro-apoptotic member of the Bcl-2 family is a cytosolic killer protein, kept in check by a tutoring protein. Upon apoptogenic stimuli, Bax undergoes conformational changes, such as exposure of the C and N terminal region (the latter differentially recognized by specific antibodies [25]), and oxidative dimerization through formation of inter-chain disulfide bonds between two Bax monomers [26]. These configurational changes allow translocation from the cytosol to the mitochondria and insertion into the outer mitochondrial membrane, where it forms a pore that allows the release of pro-apoptotic proteins [27], thus priming the caspase cascade. Bcl-2 and the other anti-apoptotic members of the family act primarily by preventing the formation of the mitochondrial pore by Bax (and the cognate Bak) by direct binding/sequestration through the conserved domain BH3 [28], thus acting at a very upstream event, i.e. during the commitment phase.

In experimental as well as naturally occurring induction of apoptosis, reactive oxygen species play a crucial role, especially inducing the intrinsic pathway [16]. In instances such as ischemia/riperfusion injuries, neuronal excitotoxicity, chronic inflammation [29, 30], i.e. where the oxidative conditions are the direct cause of cell death, melatonin was shown to counteract apoptosis by exerting its potent radical scavenging ability [31, 32].

We have recently shown that melatonin efficiently contrasts apoptosis induced by a set of agents [puromycin (PMC), etoposide, hydrogen peroxide] that trigger the intrinsic pathway [18]. As we had previously shown that these agents directly or indirectly elicit oxidation reactions [33, 34], we investigated whether the protective action of melatonin was because of its anti-oxidant properties or to other processes. To our surprise, we observed that the melatonin's apoptosis-antagonizing effect did not depend on radical scavenging, but instead it required an interaction with the plasma membrane MT1/MT2 receptors [18]. This poses the intriguing question as to the mechanism through which the signal transduction elicited by MT1/MT2 receptors may impair the intrinsic apoptotic signal.

In the present study, we analyze the molecular mechanisms through which the MT1/MT2-elicited pathway counteracts PMC-induced apoptosis in U937 cells demonstrating that melatonin interferes with apoptosis at the level of Bax activation. This is because of Bcl-2 which is re-localized by melatonin to mitochondria as a consequence of MT1/MT2 interaction.

## Materials and methods

### Cell culture

U937 are human tumour monocytes stabilized from a histiocytic lymphoma [34]. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cells were routinely checked for absence of mycoplasm by using a mycoplasm detection kit (Mycoalert TM, Cambrex Bio Science, Milano, Italy). The experiments were performed on cells in the logarithmic phase of growth under condition of ≥ 98% viability, as assessed by trypan blue exclusion.

### Induction and evaluation of apoptosis

Apoptosis was induced with the protein synthesis inhibitor PMC (10 $\mu$g/mL). Apoptosis was evaluated after 4 hr of continuous PMC treatment. Apoptosis was quantified as previously described [35]. Briefly, cells were stained with the Hoechst 33342. Cells with nuclear apoptotic morphology, detected using a fluorescence microscope were counted (at least 300 cells in at least three independent fields) and the fraction of apoptotic cells among total cells was evaluated. PMC was purchased from Sigma Chemical Co. (St Louis, MO, USA), Hoechst 33342 from Calbiochem (San Diego, CA, USA).

### Melatonin

Melatonin was purchased from Sigma Chemical Co. and used at the concentration of 1 mM. For the experiments, melatonin was added 1 hr prior to apoptosis induction.

### Other treatments

For phospholipase C (PLC) inhibition, U73122 (Sigma Chemical Co.) was used at 10 $\mu$M, and added 30 min before treatments. Melatonin action on $MT_1/MT_2$ receptors was antagonized by 2-benzyl-N-acetyltryptamine (luzindole) (Sigma Chemical Co.). Luzindole was added at the concentration of 50 $\mu$M, 30 min before other treatments.

### Immunofluorescence analysis

U937 cells were fixed with 4% paraformaldehyde. Fixation was stopped by adding the same volume of 50 mM $NH_4Cl$ in phosphate buffered saline (PBS) for 20 min. Cells were washed twice in PBS and incubated with 2 $\mu$g/mL antiBax mouse monoclonal antibody 6A7 (BD Biosciences Pharmingen, San Diego, CA, USA) or antiBax mouse polyclonal antibody D21 (sc6236; Santa Cruz Biotechnology, Santa Cruz, CA, USA,) or antiBcl-2 mouse monoclonal (Oncogene, San Diego, CA, USA; Ab #0P60,) in a solution of PBS, 1% bovine serum albumin (BSA) and 0.05% saponin, for 45 min at room temperature [36]. Then cells were washed with PBS and incubated with 10 $\mu$g/mL of tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Dako, Glostrup, Denmark) in PBS and 0.05% saponin for, 30 min. Nuclei were visualized by staining with

Hoechst 33342. In order to study the mitochondrial localization of Bcl-2 protein, U937 cells were stained before the paraformaldehyde fixation with MitoTrackerRed (Molecular Probes, Eugene, OR, USA) for 20 min at 37°C. Images were captured with a Nikon Eclipse TE 200 microscope. The merge and the analysis were performed with Adobe Photoshop software.

## Caspase activity

Specific caspase 3 and caspase 9 activity were measured by fluorigenic substrates (Calbiochem, San Diego, CA, USA), used according to the manufacturer's instructions as described [35]. For preparation of cell lysates, $10^7$ cells were collected by centrifugation and washed in 2x standard $Ca^{2+}$-free PBS The pellet was resuspended in 0.1 mL of lysis buffer (10 mM HEPES, pH 7.4; 50 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 40 mM sucrose) and subjected to 5 cycles of freeze/thawing. The supernatant was then kept at −80°C. For activity measurements, 10 μL lysates were added to 500 μL of the assay buffer [100 mM N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 10% sucrose, 10 mM DL-dithiothreitol (DTT), 500 μM ethyl-ene-diaminetetraacetate (EDTA), pH7.5] and brought to 30°C; the substrate was then added at 200 μM. For the different treatments, the values are expressed as arbitrary units obtained by monitoring the increase in fluorescence of treated samples with respect to untreated cells.

## Subcellular fractionation and immunoblot assay

For Western blot analysis of cytochrome *c*, cytosolic fraction from U937 cells was isolated by a quick cell lysis method with digitonin [37]. Under vortexing, lysis buffer (9.4 μg digitonin/$10^6$ cells, 500 mg sucrose in PBS: NaH₂PO₄ 2 mM, Na₂HPO₄ 16 mM, NaCl 150 mM, pH 7.6) was added to a cell suspension of $5 \times 10^6$ cells in PBS [1 mM DTT and protease inhibitor cocktail (SIGMA P 8340) were added just prior to use]. Heavy organelles and cell debris were pelletted for 60 s at 14,000 g at 4°C. Thirty micrograms of protein of cytosolic fraction (supernatant) were analyzed for cytochrome *c* by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting (PharMingen, San Diego, CA, USA). The specific protein was identified using the enhanced chemiluminescence (ECL) system by Amersham (Piscata-way, NJ, USA) with horseradish peroxidase (HRP)-conju-gated secondary antibody (Dako).

By Western blot analysis of Bax to obtain mitochon-drial and cytosolic extracts for the determination of Bax localization, the subcellular fractionation procedure was performed as described [26] with minor modification. Briefly, sample of $20 \times 10^6$ cells was washed with ice cold PBS (all the following procedures were performed at +4°C) and resuspended in 5x volume of hypotonic buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris–HCl, pH 7.5, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail, see above). The cells were incubated on ice until they completed swelling, and then dounced with the B pestle (Kontes Glass Company, Vineland, NY, USA) until 90% of cells were trypan blue

positive. A volume of 2.5x MS buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris–HCl pH 7.5, 2.5 mM EDTA pH7.5, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail) was quickly added to the lysate to achieve 1x MS buffer. The mixture was subjected to centrifugation at 1300 g. The resulting supernatant was centrifuged two more times to completely remove the nuclei and unlysed whole cells. The supernatant was then subjected to centrifugation at 10,000 g to isolate mito-chondria. The pellet (mitochondrial fraction) was resus-pended in hypotonic buffer containing 0.5% Triton x-100 (Calbiochem) and rotated for 30 min at +4°C.

The resulting fractions were placed in loading buffer [0.0625 M Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% beta-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue] for Western blot analysis.

For samples separated in nonreducing gels, the same procedure as above was followed except that cells were lysed in nonreducing hypotonic buffer (DTT was omitted), and the sample buffer was modified by omission of beta-mercaptoethanol. For Western blot analysis, 50 μg of cytosolic or mitochondrial protein extracts was loaded into a 12% standard SDS-PAGE and blotted to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The filter was incubated for the immunoreactions with the Bax polyclonal antibody (Santa Cruz Biotechnology, D21).

For Western blot analysis of Bcl-2 protein levels, we performed the same procedure as followed for Bax. Whole cell extracts were prepared with Mammalian Protein Extract Reagent (M-PER; Pierce, Rockford, IL, USA). Briefly, $10 \times 10^6$ U937 cells were washed in PBS and the pellet was resuspended in 400 μL of the solution containing protease inhibitor, vortexed horizontally for 15 min at 4°C and then centrifuged at 10,000 g for 15 min. Five micro-gram of the supernatant was loaded into a 12% standard SDS-PAGE and blotted to nitrocellulose membrane. The filter was incubated for the immunoreactions with the Bcl-2 monoclonal antibody (Calbiochem).

Western blot bands were quantified by image analysis with National Institute of Health (NIH) Image 1.4 software. Following values were normalized against Pon-ceau red staining of the nitrocellulose filter.

## siRNA transfection

Two microgram of siRNA Bcl-2 (Dharmacon, Lafayette, CO, USA) was transfected into $10^6$ cells using kit V from AMAXA (Cologne, Germany) according to the manufac-turer's protocol and program V-001, an AMAXA's nucleo-factor device. Treatments were performed 48 hr after transfection. One microgram of enhanced green fluorescent protein expression vector (pmaxGFPTM) was co-transfected with the siRNA, and the percentage of transfected cells based on the green fluorescent protein (GFP)-green signal was measured by flow cytometer and selected by micro-scope for the in situ apoptosis analysis.

## Bcl-2 activity inhibition

NSC345600 [38] was a gift of the Laboratoire d'Ingénierie Moléculaire et Biochimie Pharmacologique (Université

Paul Verlaine, Metz, France); it was used at 10 $\mu$M, 4 hr before the treatments.

### Statistical analysis

Statistical analysis were performed using Student's *t*-test for unpaired data and *P* values < 0.05 were considered significant. Data are presented as mean ± S.E.M.

## Results

We have previously shown that melatonin decreases the extent of apoptotic nuclear vesiculation induced by PMC, etoposide and hydrogen peroxide on U937 cells. Here, we want to establish where the apoptotic signal is interrupted by melatonin. For this purpose, we analyzed the effects of melatonin on the earlier events of the damage-induced, intrinsic apoptotic signalling by retracing back caspase 3 activation, activation of caspase 9, the initiator caspase of the intrinsic pathway and cytochrome *c* release.

Fig. 1 shows these events compared with nuclear vesiculation in cells treated with PMC in the presence/absence of melatonin (panels A–D). The impairment by melatonin on each of these events was then quantified (panel E), showing that these events are reduced in a similar way. This indicates that melatonin interferes with the intrinsic apoptotic pathway, upstream of cytochrome *c* release.

Cytochrome *c* is released in apoptosis by Bax, which is translocated from its cytosolic location, where it is dormant, to mitochondria and activated by disulfide homo-dimerization and by exposure of the N-terminal domain, thus allowing passage of cytochrome *c*. Thus, we performed a Western blot analysis of the mitochondrial fraction of U937 cells induced to apoptosis by PMC in the presence/absence of melatonin. Interestingly, melatonin does not affect the extent of PMC-induced Bax translocation (Fig. 2A).
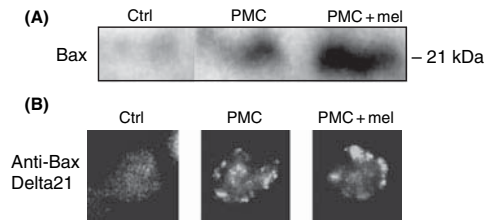
*Fig. 2*. Melatonin does not interfere with Bax translocation to mitochondria. (A) Western blot analysis of Bax with polyclonal antibody Delta21 on the mitochondrial fraction (purity was > 70% as assessed by anti-actin and cytochrome *c* oxidase antibody labelling, not shown) on U937 cells, untreated, or treated with puromycin (PMC, 3 hr) ± 1 hr pretreatment with 1 mM melatonin, showing that melatonin does not inhibit PMC-induced Bax translocation to mitochondria. (B) The pattern of Bax localization as assessed by immunofluorescence analysis with polyclonal antibody Delta21 on U937 (treatments as above); Bax may be visualized as a diffused staining pattern, or by bright dots.

This was further controlled by immunofluorescence analysis of Bax localization. Fig. 1 (Panel B, left) shows control cells with diffused Bax staining. Upon challenge with PMC, a fraction of cells acquires a dotted pattern of Bax (see a selected examples in the middle) that we have previously shown to coincide with mitochondria [26], thus demonstrating translocation. The extent and pattern of such translocation is not affected by melatonin in terms of image analysis (see a selected examples on the right panel). These results indicate that melatonin allows PMC-induced Bax translocation, suggesting that its interference with apoptosis must occur downstream.

As the step in between Bax translocation and cytochrome *c* release is the molecular activation of Bax, we investigated whether melatonin interferes with Bax activation. For this
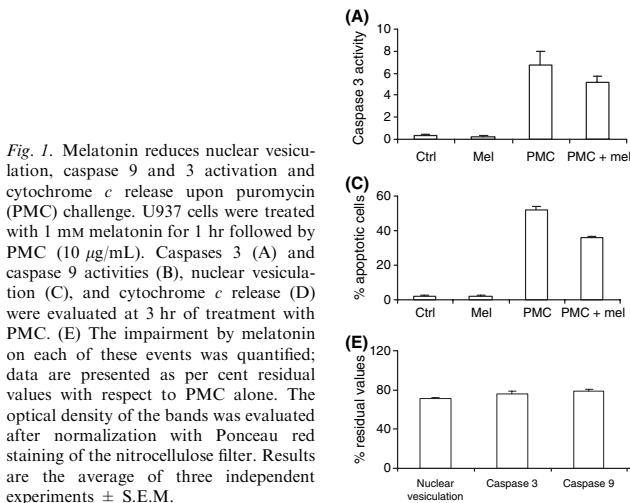
*Fig. 1.* Melatonin reduces nuclear vesiculation, caspase 9 and 3 activation and cytochrome *c* release upon puromycin (PMC) challenge. U937 cells were treated with 1 mM melatonin for 1 hr followed by PMC (10 $\mu$g/mL). Caspases 3 (A) and caspase 9 activities (B), nuclear vesiculation (C), and cytochrome *c* release (D) were evaluated at 3 hr of treatment with PMC. (E) The impairment by melatonin on each of these events was quantified; data are presented as per cent residual values with respect to PMC alone. The optical density of the bands was evaluated after normalization with Ponceau red staining of the nitrocellulose filter. Results are the average of three independent experiments ± S.E.M.

purpose, we performed three sets of experiments. First, we performed a bi-parametrical analysis at the single cell level analyzing Bax localization and nuclear vesiculation on the same cells. As shown in Fig. 3A, the dotted pattern of Bax is present in apoptotic cells (treated with PMC), as shown by the Hoechst DNA staining, that indicates the apoptotic nuclear vesiculation. But in the presence of melatonin, some cells with the dotted-Bax pattern displayed a regular rounded nucleus, suggesting an abortive apoptotic pathway.

To control if Bax is present in its active or inactive form in the mitochondria of viable cells, we analyzed whether the Bax-positive dots correspond to an activated Bax. For this purpose, we made use of an antiBax antibody that is specific for the activated form, recognizing the N-terminus buried in the latent form, but exposed in the active form. Interestingly, the active form of Bax is present only in apoptotic cells, independent of the presence of melatonin (Fig. 3B). Thus, the translocation of Bax revealed that viable melatonin-treated cells by the regular antibody is not accompanied by activation as the activation-specific antibody fails to detect it (Fig. 3C). This means that in the presence of melatonin, the translocation of Bax to mitochondria is not necessarily accompanied by its activation.

In order to confirm this finding, we analyzed another parameter of Bax activation, i.e. the formation of disulfide bridges among Bax cysteines, which may be revealed by a Western blot analysis performed under denaturing, but nonreducing conditions [26]. As shown in Fig. 3D, the detection of slowly migrating bands upon PMC treatment is impaired by melatonin, confirming that melatonin interferes with apoptosis acting at the level of Bax activation.
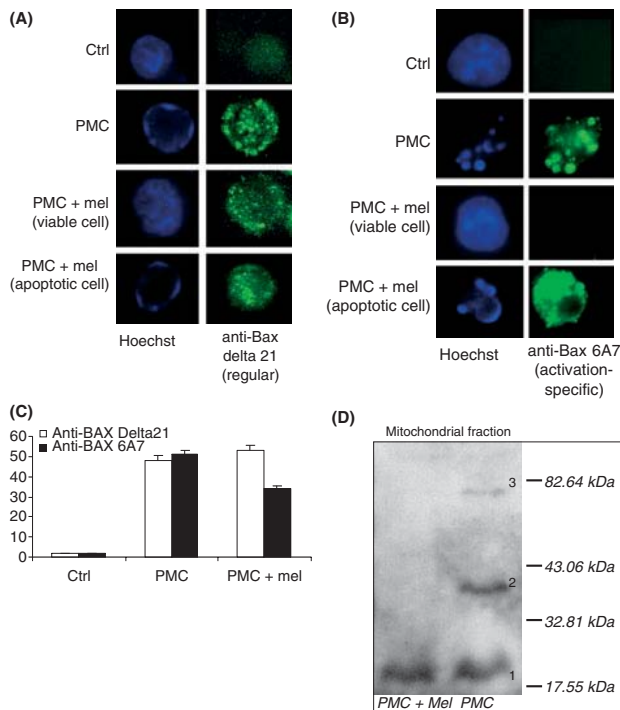


*Fig. 3.* Melatonin affects Bax activation and dimerization. Cells were pretreated with melatonin for 1 hr and then treated with puromycin (PMC) for 3 hr. Panel (A) and (B) show the bi-parametric analysis performed by double-labelling cells with Hoechst (to visualize the nucleus) versus Delta 21 antiBax antibody (recognizing both active and inactive form of Bax, panel A); and Hoechst versus antiBax 6A7 antibody (recognizing only the active form of Bax, panel B). Panel (A) shows that with PMC alone, all cells showing a dotted-Bax pattern are apoptotic, whereas in the presence of melatonin, a set of cells with a dotted-Bax pattern but a viable nucleus appear. Panel (B) shows that the Bax antibody that activated Bax, recognizes only cells that are apoptotic also in the presence of melatonin. More than three experiments have been performed with similar results. Panel (C) shows the extent of cells with the dotted pattern which was evaluated by counting 300 cells in at least 10 randomly selected fields: the per cent fractions of Bax-dotted cells is reported on top of each treatment. Results are the average of three independent experiments ± S.E.M. Panel (D) shows the Western blot of the mitochondrial fraction of cell lysates as indicated, prepared and run under denaturing but nonreducing conditions; Bax-specific bands are labelled from 1 to 3 according to migration, and are compatible with monomeric (1), dimeric (2) and polymeric (3) forms. More than three experiments have been performed with similar results.

One of the most frequent means of impairing Bax function is the physical sequestration by the cognate Bcl-2. Thus, in order to understand whether Bcl-2 may be the mediator of melatonin anti-apoptotic effect, we silenced its expression or action. First, we transfected small interfering RNAs to down-regulate Bcl-2 expression. The transfected cells were labelled by co-transfection with GFP, and Bcl-2 levels were monitored by immunostaining with TRITC-labelled antibodies. Detection of red versus green fluorescent by either flow cytometry showed a transfection efficiency of about 50% (i.e. fraction of GFP + cells, not shown). In all transfected cells, Bcl-2 was efficiently down-regulated, even though not totally, as shown by the bi-parametrical analysis of green versus red fluorescence at the fluorescence microscopy (Fig. 4A). This suggests that green fluorescence is a reliable marker for Bcl-2 status. The transfected cells were challenged to apoptosis by PMC in the presence/absence of melatonin, and then scored for apoptosis (nuclear vesiculation) by image analysis, separately evaluating successfully transfected (green) versus unsuccessfully-transfected cells.

Fig. 4B shows that the down-regulation of Bcl-2 per se induces a basal level of apoptosis. Upon PMC treatment, melatonin exerts an anti-apoptotic effect in unsuccessfully-transfected cells as expected, whereas it is ineffective on transfected Bcl-2-negative cells. This result demonstrates that the presence of Bcl-2 is required for melatonin's anti-apoptotic effect. In fact, we can even see that under these circumstances a possible pro-apoptotic effect of melatonin seems to be unmasked.

The requirement of Bcl-2 for melatonin's anti-apoptotic effect was confirmed by interference with Bcl-2 action via the use of 10 $\mu$M of small-molecule inhibitor targeted at the BH3-binding pockets in Bcl-2, NSC345600, i.e. the domain that interacts with Bax (and the other family members), and is responsible for the anti-apoptotic action of Bcl-2. Also in this instance, as shown in Fig. 4C, melatonin failed to exert any anti-apoptotic effect when Bcl-2 is inhibited. Overall, we may conclude that melatonin antagonizes apoptosis through Bcl-2.

The findings just described imply that melatonin acts on Bcl-2 to increase its anti-apoptotic (antiBax) functions. Thus, we explored the mechanism through which this occurs, i.e. via up-regulation or by other mechanisms. The increase of Bcl-2 levels occurred as a response to melatonin. Here, we need to stress that our experimental conditions include preincubation with melatonin for a short period, i.e. 1 hr, instead of the 24 hr used in most studies, exploring the effect of melatonin on apoptosis. To actually monitor if this time was enough for up-regulation of Bcl-2, we performed a Western blot analysis on total cell lysates; we found that the levels of Bcl-2 did not change at 3 or 5 hr of melatonin treatment as shown in Fig. 5A, whereas an up-regulation was found at 24 hr.

In search for an alternative mechanism, we performed a cell fractionation followed by a Western blot of the cytosolic and mitochondrial fractions; we found that melatonin strongly re-localizes Bcl-2 from the cytosolic (plus microsomal) fraction to mitochondria (Fig. 5B). To double-check the translocation, we performed an immuno-fluorescence analysis; Bcl-2 appears diffusely distributed in
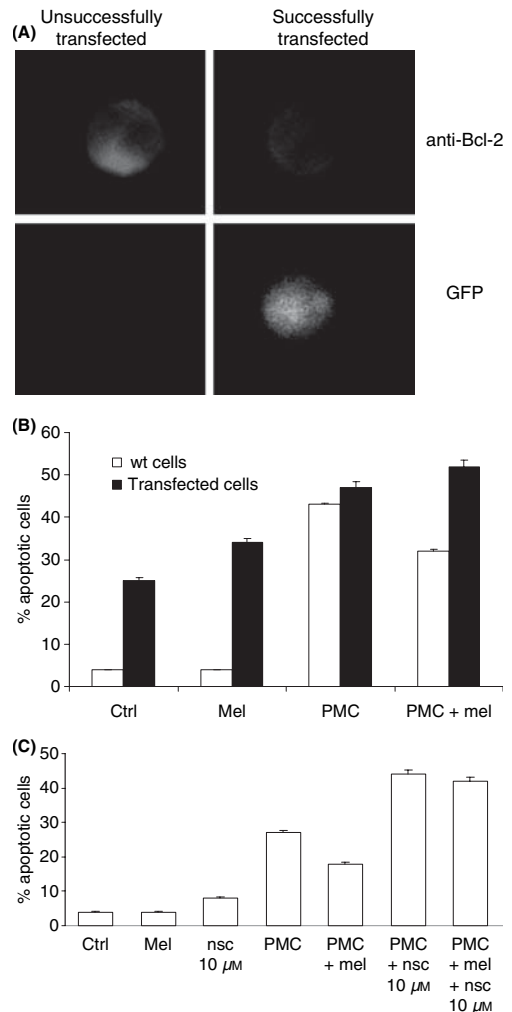
Fig. 4. Bcl-2 is required for melatonin's anti-apoptotic effect. Panel (A) shows the different green versus red staining of cells nucleofected with specific Bcl-2 siRNA and green fluorescence protein (GFP), and immuno-stained with TRITC-conjugated anti-Bcl-2 antibodies: left, a cell not expressing GFP, with a strong signal for Bcl-2, unsuccessfully transfected; right: a cell expressing GFP, with a weak Bcl-2 signal; successfully transfected (transfection efficiency = 50%, not shown). Panel (B) shows the extent of apoptosis induced by puromycin in the presence/absence of melatonin, among the successfully versus unsuccessfully transfected cells. Results show the fraction of cells with apoptotic nuclei, separately scored according to the presence/absence of green fluorescence, and are the average of three independent experiments ± S.E.M. Panel (C) shows a similar experiment performed on cells pretreated for 4 hr with 10 $\mu$M NSC345600, inhibitor of Bcl-2 activity (see Materials and methods). Results show the fraction of cells with apoptotic nuclei, and are the average of three independent experiments ± S.E.M.
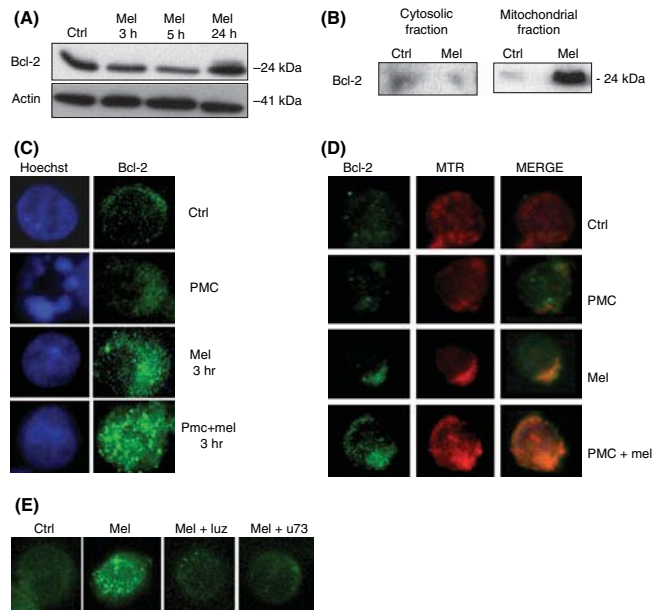
*Fig. 5.* Melatonin induces the re-localization of Bcl-2 to mitochondria via MT1/MT2 receptor interaction. Panel (A) shows the Western blot analysis of the time course of Bcl-2 after melatonin treatment. Actin was used as a loading control in the bottom; > 3 experiments have been performed with similar results. Panel (B) shows the Western blot analysis of Bcl-2 in the cytoplasmic versus mitochondrial fraction of cells untreated or treated for 3 hr with melatonin; loading control was performed with Ponceau red staining of the nitrocellulose filter. More than three experiments have been performed with similar results. Panel (C) and (D) show the immuno-fluorescence analysis with anti-Bcl-2 antibodies of cells treated as specified, counterstained with Hoechst to delineate nuclear status (panel C) and mitotracker red to label active mitochondria (panel D). The merging between the mitochondrial and Bcl-2 pattern is shown in the right column of panel (D). More than three experiments have been performed with similar results. Panel (E) shows the pattern of Bcl-2, as revealed by immuno-fluorescence with anti-Bcl-2 antibodies in cells treated with melatonin and its receptor antagonist luzindole or with the phospholipase C inhibitor, U73122. More than three experiments have been performed with similar results.

the cytoplasm in control cells, whereas upon melatonin treatments, Bcl-2 assumes a bright punctate localization (Fig. 5C). The melatonin-induced pattern is maintained after PMC treatment in cells maintaining viability (Fig. 5C), but is lost in apoptosis (not shown). The punctate pattern was identified as mitochondria by merging the Bcl-2 spots with mitochondrial staining with the dye Mitotracker Red (MTR), which identifies mitochondria because of active trans-membrane potential (Fig. 5D, see merge). Thus, melatonin causes the translocation of Bcl-2 to mitochondria, which is a conceivable mechanism for inhibiting Bax activation at the mitochondrial level.

As we had previously shown that melatonin antagonizes apoptosis via an interaction with the MT1/MT2 melatonin receptors, we evaluated the role of the signal transduction elicited by MT1/MT2 receptor stimulation on Bcl-2 re-localization. To this purpose, we analyzed whether luzindole, which specifically antagonizes melatonin binding/activation of MT1/MT2 receptors is able to counteract melatonin-induced re-localization of Bcl-2. As shown in Fig. 5E, in the presence of luzindole, melatonin is no longer able to re-localize Bcl-2. To confirm the involvement of the signal transduction, we analyzed a downstream step of a

more generalized nature, i.e. activation of phospholipase C (PLC), which may be contrasted by the inhibitor U73122. Also in the presence of U73122, the localization of Bcl-2 is not altered by melatonin. These findings show that the signal transduction elicited by MT1/MT2 receptor stimulation by melatonin triggers a set of events culminating with Bcl-2 re-localization.

## Discussion

In this study, we provide molecular evidence of the mechanism through which melatonin interferes with the intrinsic pathway of apoptosis in U937 cells. Our results suggest that melatonin leads part of the induced cells to apoptosis to abort the apoptotic signalling at the level of Bax activation indeed, the early events such as Bax translocation to mitochondria still occur with a frequency that is not altered by melatonin, but the downstream events are strongly reduced. As Bax activation is placed well before the final commitment to apoptosis, i.e. before caspases are activated and cells begin to degrade their structures, there is no reason why the cells rescued by melatonin should not expect a normal viability afterwards.

In fact, this is compatible with a physiologically programmed situation, with melatonin allowing prolonged lifespan to target cells, especially in areas, e.g. inflammatory spots where environmental anomalies may push towards a damage-induced apoptosis via the intrinsic pathway.

Many studies have shown that the anti-apoptotic effects of melatonin are accompanied by modulation of the expression of the Bcl-2 family genes, showing down-regulation of the pro-apoptotic cognate Bax, and/or up-regulation of the pro-survival Bcl-2 itself [39]. On one hand, repeated melatonin administrations have been shown to reduce Bax steady-state level [40]; additionally, melatonin can contrast Bax up-regulation because of specific apoptotic triggers, such as gluco-corticoids on thymocytes [23], or $H_2O_2$ on different cell types [31]. On the other hand, several studies report the up-regulation of Bcl-2 by melatonin [39]. If the mechanisms through which melatonin reduce Bax levels are unexplored, Bcl-2 up-regulation probably occurs via activation of the transcription factor nuclear factor-kappa B (NF-kappaB), even though the mechanisms of NF-kappaB activation, as well as the modalities of activation of the Bcl-2 promoter (a discussed NF-kappaB target) [41] remain unexplored. Overall, when melatonin behaves as an anti-apoptotic agent, it is able to differently regulate, and in some cases coordinate the expression of pro-and anti-apoptotic members of the Bcl-2 family, shifting the balance towards a cell-protective asset. Interestingly, when melatonin behaves as a pro-apoptotic agent, i.e. in many instances of tumour cells, it seems to regulate the same proteins, but in the opposite way, i.e. by increasing the Bax/Bcl-2 ratio, thus shifting the balance towards a pro-apoptotic equilibrium [42].

In the studies that report altered regulation of Bax/Bcl-2 expression, the experiments on apoptosis are performed after treatment with melatonin for prolonged periods, usually 24–48 hr. Also in our system, Bcl-2 is substantially up-regulated after 24 hr of incubation with melatonin (Fig. 5A) via activation of NF-kappaB, as suggested by preliminary evidence (S. Cristofanon et al., unpublished data), thus showing that our cellular model is in line with the systems used elsewhere.

However, the mechanism we describe in this study is of a different nature. Indeed, within the time frame of the experiments described in this study, i.e. 1 hr preincubation + 3 hr apoptogenic treatment, no up-regulation of Bcl-2 is detected (Fig. 5A, at 3 and 5 hr time points). To ensure that no new protein synthesis occurred, we chose PMC, a protein synthesis inhibitor [35] as the apoptogenic inducer, thus halting the cellular protein asset at the very moment of apoptosis induction, i.e. 1 hr of melatonin. Even in these conditions, Bcl-2 is required for the anti-apoptotic effect of melatonin since its silencing, or the inhibition with BH3-mimetic synthetic small proteins abrogates melatonin anti-apoptotic effect. Thus, the absence of Bcl-2 up-regulation allowed revealing of an additional mechanism through which melatonin may enhance the pro-survival role of Bcl-2, i.e. its re-localization to mitochondria.

This raises two questions: how does melatonin cause Bcl-2 re-localization; and how does mitochondrial Bcl-2 abort an intrinsic apoptotic signal?

Melatonin may theoretically interfere with the apoptotic signalling by scavenging free radicals, or by any of the signal transduction events triggered by its multiple direct targets within cells. As luzindole, an antagonist of melatonin MT1/MT2 receptors prevents the ability of melatonin to re-localize Bcl-2 to mitochondria, and to protect from apoptosis, we conclude that the signal transduction elicited by interaction with MT1/MT2 is responsible for such an event. It is known that Bcl-2 has multiple locations within cells, being either in the soluble or membrane-inserted form [43], the latter being mainly localized in the endoplasmic reticulum and mitochondrial membrane. Bcl-2 behaviour/localization, and particularly mitochondrial targeting [44] are controlled by a very complex phosphorylation pattern on multiple aminoacid residues [45]. It is conceivable that a specific kinase may be activated/deactivated as a result of MT1/MT2-elicited signal transduction. Interestingly, this signal transduction involves phospholipase C (PLC) as its inhibitor U73122 also reverts melatonin-induced Bcl-2 re-localization. It is tempting to speculate that protein kinase C which is known (i), to phosphorylate Bcl-2, and (ii) to be a downstream target of PLC activation may be involved in melatonin-induced Bcl-2 re-localization.

As far as the second question is concerned, it is known that mitochondria are one of the most important sites where Bcl-2 exerts its anti-apoptotic functions, especially as far as inhibition of Bax activity is concerned. It has been known for years that the balance between Bax and Bcl-2 determines the propensity of cells to respond to apoptosis or survival to a given insult [28, 46]. The molecular mechanisms through which this occurs have been clarified, consisting on the sequestration of Bax, which thus becomes unable to exert its pore-forming action on the mitochondrial membrane and to release cytochrome *c*. Sequestration may occur either directly via the BH3 domain [46] or by complex interplays with the cognate proteins belonging to the sub-family BH3-only proteins, whose behaviour is influenced by Bcl-2 concentration/localization [47]. Thus, we think that Bcl-2 re-localization to mitochondria may favour its Bax-contrasting activity even in the absence of up-regulation.

Bax activation is a complex phenomenon that requires multiple steps, whose order is still a matter of debate [26]. The exposure of the N-terminus of Bax occurs concomitantly, with the release of the mitochondrial apoptotic factors (cytochrome *c* or second mitochondria-derived activator of caspases (SMAC)/diablo) in fact, being a sign of Bax activation [48]; however, it is still discussed whether the conformational change occurs, prior to or after translocation to mitochondria [49]. Our results show that in the presence of melatonin, Bax is induced to translocate even in the absence of N-term exposure, suggesting that this latter event occurs after translocation. We have recently shown that oxidative alterations cause Bax disulfide dimerization, suggesting that the resulting conformational changes are sufficient for translocation to mitochondria [26]. The presence of a Bax monomer in cells induced to apoptosis in the presence of melatonin suggests that either Bcl-2 is able to break (reduce) a Bax dimer within the mitochondrial membrane, or that in the presence of melatonin, Bax translocation may take place without

dimerization, perhaps because of the radical scavenging ability of melatonin.

In conclusion, we uncovered in our system that the mechanism of apoptosis antagonism by melatonin share many critical features with evidence available in the specific literature. Indeed, mitochondria have been often shown as the site of action of melatonin for apoptosis inhibition [22], and the proteins of the Bcl-2 family have been very often recognized as the mediators of such effects. To our knowledge, the evidence so far available is of a circumstantial nature, and this is the first report showing a cause-effect relationship between Bcl-2 and melatonin anti-apoptotic effect via the use of siRNA and specific BH3-mimetics. It is interesting that melatonin has developed many independent strategies to achieve the same objective, i.e. to block the intrinsic pathway of apoptosis at the level of Bax activation, the pivotal event of the intrinsic apoptotic signalling. Indeed, melatonin is able to directly inhibit Bax production [31, 50], over-produce Bcl-2; its main antagonist [39], translocate Bcl-2 to the site where Bax really works, i.e. the mitochondria. Interestingly, these effects are achieved via different actions of melatonin, Bax/Bcl-2 levels being mostly altered via the radical-scavenging ability of melatonin, whereas the MT1/MT2 signal transduction seems responsible for Bcl-2 re-localization to mitochondria. The discovery of multiple converging and/or redundant signals, such as those triggered by melatonin to modulate Bax activation usually indicates that we are dealing with a very important biological function, i.e. it is important that a given effect be produced in one way or another to maintain/return to homeostasis. This underlines the potential general interest of the role of melatonin as a controller of the life/death of cells within the organisms.

## Acknowledgments

## References

1. DUBOCOVICH ML, MARKOWSKA M. Functional $MT_1$ and $MT_2$ melatonin receptor in mammals. Endocrine 2005; **27**:101–110.

2. BENITEZ-KING G. Melatonin as a cytoskeletal modulator: implications for cell physiology and disease. J Pineal Res 2006; **40**:1–9.

3. GUERRERO JM, POZO D, GARCIA-MAURINO S et al. Involvement of nuclear receptors in the enhanced IL-2 production by melatonin in Jurkat cells. Ann N Y Acad Sci 2000; **917**:397–403.

4. RODRIGUEZ C, MAYO JC, SAINZ RM et al. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 2004; **36**:1–9.

5. REITER RJ, TAN DX, PILAR TERRON M et al. Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. Acta Biochim Pol 2007; **54**:1–9.

6. TAN DX, MANCHESTER LC, TERRON MP et al. One molecule, many derivatives: a never ending interaction of melatonin with reactive oxygen and nitrogen species? J.Pineal Res 2007; **42**:28–42.

7. SAINZ RM, MAYO JC, RODRIGUEZ C et al. Melatonin and cell death: differential actions on apoptosis in normal and cancer cells. Cell Mol Life Sci 2003; **60**:1407–1426.

8. THOMPSON CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995; **267**:1456–1462.

9. WESCHE-SOLDATO DE, SWAN RZ, CHUNG CS, AYALA A. The apoptotic pathway as a therapeutic target in sepsis. Curr Drug Targets 2007; **8**:493–500.

10. BOLITHO P, VOSKOBOINIK I, TRAPANI JA, SMYTH MJ. Apoptosis induced by the lymphocyte effector molecule perforin. Curr Opin Immunol 2007; **19**:339–347.

11. CUBERO J, VALERO V, NARCISO D et al. L-tryptophan administered orally at night modifies the melatonin plasma levels, phagocytosis and oxidative metabolism of ringdove (Streptopelia roseogrisea) heterophils. Mol Cell Biochem 2006; **293**:79–85.

12. CARRILLO-VICO A, REITER RJ, LARDONE PJ et al. The modulatory role of melatonin on immune responsiveness. Curr Opin Investig Drugs 2006; **7**:423–431.

13. LU L, OSMOND DG. Apoptosis and its modulation during B lymphopoiesis in mouse bone marrow. Immunol Rev 2000; **175**:158–174.

14. MILLER SC, PANDI-PERUMAL SR, ESQUIFINO AI et al. The role of melatonin in immuno-enhancement: potential application in cancer. Int J Exp Pathol 2006; **87**:81–87.

15. TAN DX, MANCHESTER LC, REITER RJ et al. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochim Biophys Acta 1999; **1472**:206–214.

16. COPPOLA SL. GSH extrusion and the mitochondrial pathway of apoptotic signaling. Biochem Soc Trans 2000; **28**:56–61.

17. WYLLIE AH, KERR JFR, CURRIE AR. Cell death: the significance of apoptosis. Int Rev Cytol 1998; **68**:251–273.

18. RADOGNA F, PATERNOSTER L, ALBERTINI MC et al. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res 2007; **43**:154–162.

19. RADOGNA F, PATERNOSTER L, ALBERTINI MC et al. Melatonin as an apoptosis antagonist. Ann N Y Acad Sci 2006; **1090**:226–233.

20. ECK-ENRIQUEZ K, KIEFER TL, SPRIGGS LL, HILL SM. Pathways through which a regimen of melatonin and retinoic acid induces apoptosis in MCF-7 human breast cancer cells. Breast Cancer Res Treat 2000; **61**:229–239.

21. WEINREB O, MANDEL S, YOUDIM MB. cDNA gene expression profile homology of antioxidants and their antiapoptotic and proapoptotic activities in human neuroblastoma cells. FASEB J 2003; **17**:935–937.

22. LUCHETTI F, CANONICO B, CURCI R et al. Melatonin prevents apoptosis induced by UV-B treatment in U937 cell line. J Pineal Res 2006; **40**:158–167.

23. HOIJMAN E, ROCHA VIEGAS L, KELLER SARMIENTO MI et al. Involvement of Bax protein in the prevention of glucocorticoid-induced thymocytes apoptosis by melatonin. Endocrinology 2004; **145**:418–425.

24. GROSS A, JOCKEL J, WEI MC, KORSMEYER SJ. Enforced dimerization of Bax results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J 1998; **17**:3878–3885.

25. PEYERL FW, DAI S, MURPHY GA et al. Elucidation of some Bax conformational changes through crystallization of an antibody-peptide complex. Cell Death Differ 2007; **14**:447–452.

26. D'ALESSIO M, DE NICOLA M, COPPOLA S et al. Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis. FASEB J 2005; **19**:1504–1506.

27. NOURAINI S, SIX E, MATSUYAMA S et al. The putative pore-forming domain of Bax regulates mitochondrial localization and interaction with Bcl-xL. Mol Cell Biol 2000; **20**:1604–1615.

28. ANTONSSON B, CONTI F, CIAVATTA A et al. Inhibition of bax channel-forming activity by Bcl-2. Science 1997; **277**:370–372.

29. VALKO M, LEIBFRITZ D, MONCOL J et al. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; **39**:44–84.

30. RYTER SW, KIM HP, HOETZEL A et al. Mechanisms of cell death in oxidative stress. Antioxid Redox Signal 2007; **9**:49–89.

31. JUKNAT AA, MENDEZ MDEL V, QUAGLINO A et al. Melatonin prevents hydrogen peroxide-induced Bax expression in cultured rat astrocytes. J Pineal Res 2005; **38**:84–92.

32. MAYO JC, SAINZ RM, URIA H et al. Inhibition of cell proliferation: a mechanism likely to mediate the prevention of neuronal cell death by melatonin. J Pineal Res 1998; **25**:12–18.

33. GHIBELLI L, COPPOLA S, ROTILIO G et al. Non-oxidative loss of glutathione in apoptosis via GSH extrusion. Biochem Biophys Res Commun 1995; **216**:313–320.

34. GHIBELLI L, FANELLI C, ROTILIO G et al. Rescue of cells from apoptosis by inhibition of active GSH extrusion. FASEB J 1998; **12**:479–486.

35. GHIBELLI L, COPPOLA S, FANELLI C et al. Glutathione depletion causes cytochrome c release even in the absence of cell commitment to apoptosis. FASEB J 1999; **13**:2031–2036.

36. JUNGAS T., MOTTA I., DUEUX F. et al. Glutathione levels and bax activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrois transmembrane conductance regulator. Biol Chem 2003; **227**:27912–27918.

37. SINGLE B., LEIST M., NICOTERA P. Simultaneus release of adenylate kinase and cytochrome c in cell death. Cell Death Differ 1998; **5**:1001–1003.

38. ENYEDY IJ, LING Y, NACRO K et al. Discovery of small-molecule inhibitors of Bcl-2 through structure-based computer screening. J Med Chem 2001; **44**:4313–4324.

39. LING X, ZHANG LM, LU SD et al. Protective effect of melatonin on injuried cerebral neurons is associated with bcl-2 protein over-expression. Zhongguo Yao Li Xue Bao 1999; **20**:409–414.

40. BAYDAS G, REITER RJ, AKBULUT M et al. Melatonin inhibits neural apoptosis induced by homocysteine in hippocampus of rats via inhibition of cytochrome c translocation and caspase-3 activation and by regulating pro- and anti-apoptotic protein levels. Neuroscience 2005; **135**:879–886.

41. KURLAND JF, KODYM R, STORY MD et al. NF-kappaB1 (p50) homodimers contribute to transcription of the bcl-2 oncogene. J Biol Chem 2001; **276**:45380–45386.

42. RUBIO S, ESTEVEZ F, CABRERA J et al. Inhibition of proliferation and induction of apoptosis by melatonin in human myeloid HL-60 cells. J Pineal Res 2007; **42**:131–138.

43. HOCKENBERY D, NUNEZ G, MILLIMAN C et al. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990; **348**:334–336.

44. VERMA YK, GANGENAHALLI GU, SINGH VK et al. Cell death regulation by B-cell lymphoma protein. Apoptosis 2006; **11**:459–471.

45. DENG X, KORNBLAU SM, RUVOLO PP, MAY WS, JR. Regulation of Bcl2 phosphorylation and potential significance for leukemic cell chemoresistance. J Natl Cancer Inst Monogr 2001; **28**:30–37.

46. ROUCOU X, MARTINOU JC. Conformational change of Bax: a question of life or death. Cell Death Differ 2001; **8**:875–877.

47. ANTIGNANI A, YOULE RJ. How do Bax and Bak lead to permeabilization of the outer mitochondrial membrane? Curr Opin Cell Biol 2006; **18**:685–689.

48. ARNOULT D, GAUME B, KARBOWSKI M et al. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. EMBO J 2003; **22**:4385–4399.

49. MAKIN GW, CORFE BM, GRITHS GJ et al. Damage-induced Bax N-terminal change, translocation to mitochondria and formation of Bax dimers/complexes occur regardless of cell fate. EMBO J 2001; **20**:6306–6315.

50. CHETSAWANG B, PUTTHAPRASART C, PHANSUWAN-PUJITO P, GOVITRAPONG P. Melatonin protects against hydrogen peroxide-induced cell death signaling in SH-SY5Y cultured cells: involvement of nuclear factor kappa B, Bax and Bcl-2. J Pineal Res 2006; **41**:116–123.

# PAPER IV

*Intracellular pro-oxidant activity of melatonin deprives U937 cells of reduced glutathione peroxidise activity*

# Intracellular Pro-oxidant Activity of Melatonin Deprives U937 Cells of Reduced Glutathione without Affecting Glutathione Peroxidase Activity

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ABSTRACT: It was long believed that melatonin might counteract intracellular oxidative stress because it was shown to potentiate antioxidant endogenous defences, and to increase the activity of many antioxidant enzymes. However, it is now becoming evident that when radicals are measured within cells, melatonin increases, rather than decreasing, radical production. Herein we demonstrate a pro-oxidant effect of melatonin in U937 cells by showing an increase of intracellular oxidative species and a depletion of glutathione (GSH). The activity of glutathione peroxidase is not modified by melatonin treatment as it does occur in other experimental models.

KEYWORDS: melatonin; pro-oxidant; reduced glutathione; ROS; glutathione peroxidase.

## INTRODUCTION

The pineal hormone melatonin has been reported to be an effective free radical scavenger and antioxidant *in vitro* as well as *in vivo*.[1–8] Melatonin is believed to scavenge the highly toxic hydroxyl radical, the peroxynitrite anion, and possibly the peroxyl radical; secondly, it reportedly scavenges the superoxide anion and also quenches singlet oxygen.[3] The neurotoxicity

of a number of compounds like kainic acid,[9] haloperidol,[10] and the cellular toxicity of hydrogen peroxide[11] is inhibited by melatonin administration. In some cases, the accompanying decrease in reduced glutathione (GSH) levels are also prevented by melatonin administration. On basis of these findings, melatonin was considered to exert its physiological and pharmacological effects, at least partly, via its antioxidant activity.[12] However, authors observed only a limited antioxidant activity of melatonin in several systems.[13–16]

Besides promoting deleterious effects at high concentrations, reactive oxygen species (ROS) function as intracellular downstream messengers targeting specific proteins and genes.[17–19] For example, programmed cell death in lymphocytes is known to be influenced by alterations of the cellular redox state as well as by intracellular ROS formation.[20–25] Because little is known about the effects of melatonin on the intracellular redox state, even though melatonin can easily cross cell membranes because of its amphiphilicity,[26] we examined whether melatonin interferes with intracellular ROS production, GSH intracellular concentration, and GSH peroxidase (GSH-Px) activity in U937 cells.

## MATERIALS AND METHODS

### *Cell Cultures and Treatments*

Melatonin was kindly provided by the Institute of Medicinal Chemistry, University of Urbino. L-buthionine-[*S,R*]-sulfoximine (BSO) was purchased from Sigma-Aldrich (Milan, Italy). Dichlorodihydrofluorescine diacetate (DHCFDA) was a product from Invitrogen (San Giuliano Milanese, Italy).

U937 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (50 units/mL), and streptomycin (50 μg/mL) (Sera-Lab Ltd., Crawley Down, England), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY) gassed with an atmosphere of 95% air to 5% $CO_2$. Treatments were performed in 2 mL of prewarmed saline A (8.182 g/L NaCl, 0.372 g/L KCl, 0.336 g/L NaHCO3, and 0.9 g/L glucose) containing $1 \times 10^6$ cells.

### *Determination of ROS Formation in Cells*

Levels of ROS were measured in U937 cells incubated for 2 h and 6 h in the presence and in the absence of 1 mM melatonin. The fluorescent probe dichlorodihydrofluorescine diacetate (DHCFDA) was used to detect intracellular ROS because it flouresces after oxidation by ROS and reactive nitrogen species (NOS). Fluorescence was measured with a Dako Galaxy flow cytometer (490-nm and 520-nm wavelegths for excitation and emission, respectively).

The protocol indicates the use of aliquots of $2 \times 10^6$ cells to be stained with 10 μM DHCFDA for 20 min in the dark. Samples are then centrifuged and the pellet resuspended in phosphate-buffered saline solution (PBS) at $2 \times 10^6$ cells/mL to be analyzed.

### Determination of Intracellular GSH

Because GSH represents more than 90% of the nonprotein thiols, the latter will be referred to as GSH. After 2 h and 6 h incubation of U937 cells ($1 \times 10^6$ cells/mL) with melatonin (1 mM) or BSO (1 mM), cells were washed in PBS and used for the determination of cellular GSH concentration by the method described by Beutler.[27] In brief, cells were centrifuged and the pellet was then resuspended in 150 μL of a solution containing 1.67% metaphosphoric acid, 0.2% EDTA, 30% NaCl, kept in ice for 5 min, and centrifuged at 10,000 $\times g$ for 5 min. The GSH content was measured spectrophotometrically in the supernatant, at 412 nm, using 5,5′-dithiobis(2-nitrobenzoic acid) ($\varepsilon_{412} = 13{,}600$ M$^{-1}$ cm$^{-1}$).

### Determination of Intracellular Glutathione Peroxidase (GSH-Px) Activity

For GSH-Px activity cells were washed twice with saline A, resuspended in the same medium at a density of $1 \times 10^6$ cells/mL, and finally lysed with a sonicator. The resulting lysates were centrifuged for 5 min at 18,000 $\times g$ at 4 °C. GSH-Px activity was determined spectrophotometrically in the supernatant as already described.[28]

## RESULTS

In contrast to other antioxidant molecules which decrease intracellular ROS concentration, melatonin significantly increases intracellular ROS formation following 2 h and 6 h of incubation with U937 cells (FIG. 1). The data also indicate that at 2 h of incubation intracellular ROS are strongly increased (more than sevenfold) while after 6 h the variation is less consistent (about threefold).

The increase of oxidative species is accompanied by a depletion of U937 intracellular GSH concentration during melatonin treatment. In fact, at 2 h and at 6 h of incubation, intracellular GSH concentration is 73.3% and 65.5% of the control, respectively (FIG. 2). In the presence of BSO, an inhibitor of γ-glutamylcysteine synthetase, the decrease of the thiol is similar to that induced by melatonin, being 66.6% and 62% of the control at 2 h and 6 h, respectively (FIG. 2).

To assess whether the treatment of U937 cells with melatonin may also cause an increase of cellular antioxidant enzymes, we measured the activity of GSH-Px, which has been found augmented in rat brain tissue following "*in vivo*"
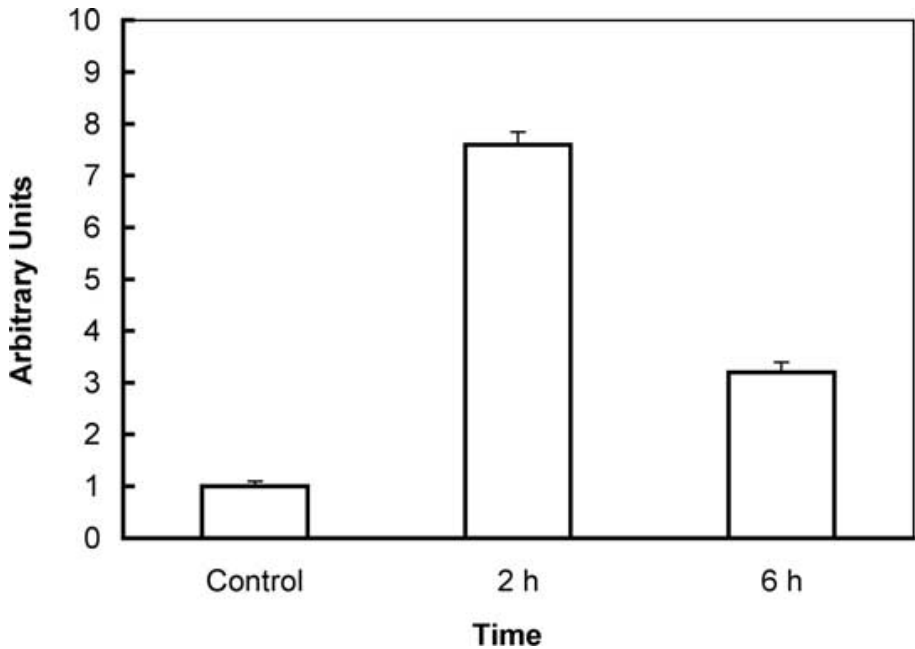
**FIGURE 1.** Evaluation of intracellular ROS concentration in U937 cells incubated with melatonin. Experimental conditions are reported in MATERIALS AND METHODS. ROS concentrations are expressed as arbitrary units of flourescence and are means $\pm$ SD ($n = 4$).
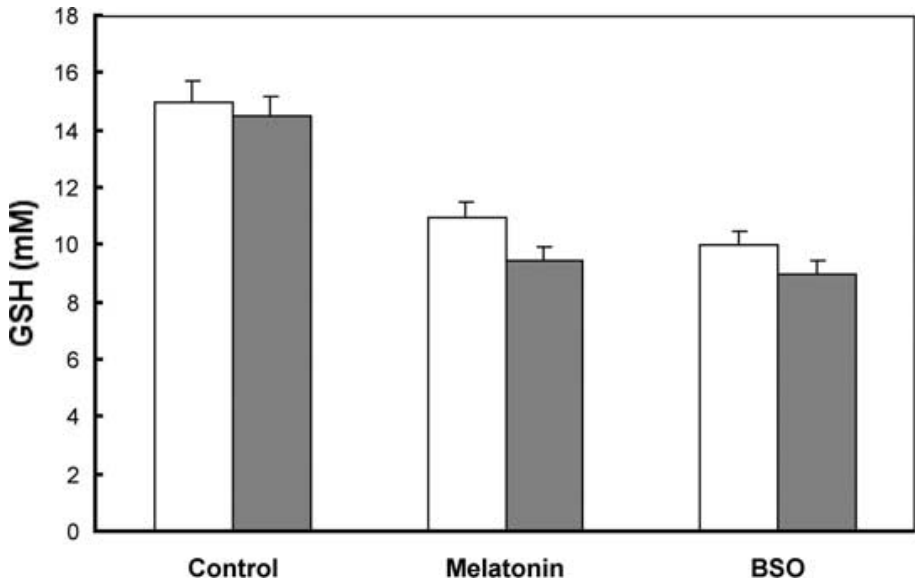


**FIGURE 2.** Measurement of intracellular GSH concentration in U937 cells incubated with melatonin and BSO for 2 h (white bars) and 6 h (dark bars). Experimental conditions are reported under "Materials and Methods." GSH concentrations are expressed as $\mu$mol/mL of cells and are means $\pm$ SD ($n = 4$).
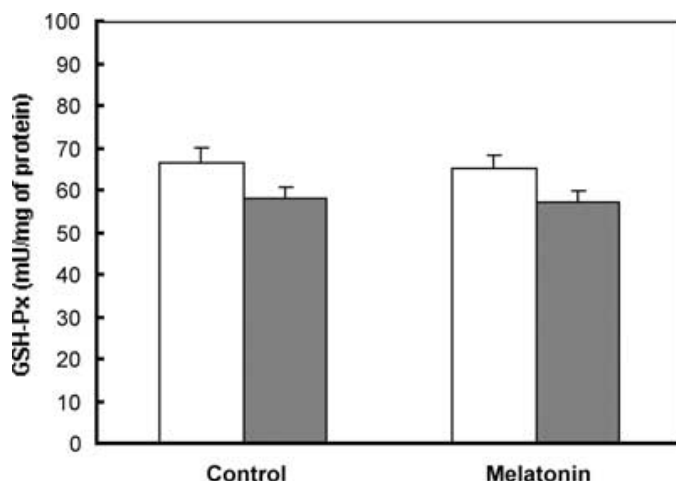
**FIGURE 3.** Evaluation of GSH-Px activity in U937 cells incubated with melatonin for 2 h (white bars) and 6 h (dark bars). Experimental conditions are reported under "Materials and Methods." GSH-Px activities are expressed as mU/mg of protein and are means $\pm$ SD ($n = 4$).

administration of melatonin.[28] Data reported in FIGURE 3 demonstrate that in our experimental conditions, U937 do not show any significantly increase of this enzyme at 2 h and 6 h of incubation.

## DISCUSSION

The antioxidant role of melatonin has been demonstrated *in vitro* (possibly through interaction with iron?). Its potential use as an antioxidant *in vivo* comes from evidence showing its ability to reduce radical production in oxidant-related pathological situations, where radicals are measured in the extracellular fluids.[1–8] It was long believed that melatonin might also counteract intracellular oxidative stress because it was shown to potentiate antioxidant endogenous defences, and to increase the activity of many antioxidant enzymes.[29] However, it is now becoming evident that when radicals are measured within cells, melatonin increases, rather than decreases, radical production,[30] even though it is still unknown which types of radicals are produced. Also completely unknown is the mechanism at the basis of this phenomenon, which may not necessarily involve direct oxidant activity of melatonin (or one of its catabolites), but the ability to elicit the activation of some cellular radical producing enzymes (e.g., lipoxygenases, cyclooxygenases, NO-synthase, NADPH-oxidase). This problem is particularly timely nowadays, because it is emerging that oxidative radicals, considered so far as causative agents of important human diseases, may also mediate resistance to apoptosis by triggering survival pathways.[31] Our results demonstrate a pro-oxidant effect of melatonin by inducing the

increase of intracellular ROS in U937. The increase of ROS is higher at 2 h than at 6 h, probably indicating that ROS production is transient. Intracellular GSH concentration decrease may reflect free radical formation in U937 cells treated with melatonin. Cell GSH-Px activity does not show any modification probably because, in our experimental conditions, U937 treated cells maintain sufficiently high levels of GSH to face the amounts of the formed radical species (note that after 6 h of incubation cells still contain more than 60% of control cell GSH, and the same is true for the positive control cells incubated with BSO). Experiments are in progress in our laboratory to investigate how melatonin may act on U937 cell metabolism and may directly or indirectly stimulate oxidative radical production.

## REFERENCES

1. MONTILLA, P.L., I.F. TUNEZ, C. MUNOZ DE AGUEDA, *et al*. 1998. Protective role of melatonin and retinol palmitate in oxidative stress and hyperlipidemic nephropathy induced by adriamycin in rats. J. Pineal Res. **25:** 86–93.
2. PRINC, F.G., A.G. MAXIT, C. CARDALDA, *et al*. 1998. *In vivo* protection by melatonin against delta-aminolevulinic acid-induced oxidative damage and its antioxidant effect on the activity of haem enzymes. J. Pineal Res. **24:** 1–8.
3. REITER, R.J. 1998. Oxidative damage in the central nervous system: protection by melatonin. Prog. Neurobiol. **56:** 359–384.
4. BEYER, C.E., J.D. STEKETEE & D. SAPHIER. 1998. Antioxidant properties of melatonin: an emerging mystery. Biochem. Pharmacol. **56:** 1265–1272.
5. RODRIGUEZ, C., J.C. MAYO, R.M. SAINZ, *et al*. 2004. Regulation of antioxidant enzymes: a significant role for melatonin. J. Pineal Res. **36:** 1–9.
6. MAYO, J.C., D.X. TAN, R.M. SAINZ, *et al*. 2003. Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: comparative effects of melatonin and other antioxidants. Biochim. Biophys. Acta **1620:** 139–150.
7. ALLEGRA, M., R.J. REITER, D-X. TAN, *et al*. 2003. The chemistry of melatonin's interaction with reactive species. J. Pineal Res. **34:** 1–10.
8. ORTEGA-GUTIERREZ, S., J.J. GARCIA, E. MARTINEZ-BALLARIN, *et al*. 2002. Melatonin improves deferoxamine antioxidant activity in protecting against lipid peroxidation caused by hydrogen peroxide in rat brain homogenates. Neurosci. Lett. **323:** 55–59.
9. FLOREANI, M., S.D. SKAPER, L. FACCI, *et al*. 1997. Melatonin maintains glutathion homeostasis in kainic acid exposed rat brain tissues. FASEB J. **11:** 1309–1315.
10. POST, A., F. HOLSBOER & C. BEHL. 1998. Induction of NF-kappa B activity during haloperidol-induced oxidative toxicity in clonal hippocampal cells. J. Neurosci. **18:** 8236–8246.
11. BALDWIN, W.S. & J.C. BARRETT. 1998. Melatonin attenuates hydrogen peroxide toxicity in MCF7 cells only at pharmacological concentrations. Biochem. Biophys. Res. Commun. **250:** 602–605.
12. REITER, R.J., C.S. OH & O. FUJIMORI. 1996. Melatonin: its intracellular and genomic actions. Trends Endocr. Metab. **7:** 22–27.

13. MARSHALL, K.A., R.J. REITER, B. POEGGELER, *et al*. 1996. Evaluation of the antioxidant activity of melatonin *in vitro*. Free Radic. Biol. Med. **21:** 307–315.

14. ABUJA, P.M., P.M. LIEBMANN, M. HAYN, *et al*. 1997. Antioxidant role of melatonin in lipid peroxidation of human LDL. FEBS Lett. **413:** 289–293.

15. WÖLFLER, A., P.M. ABUJA, K. SCHAUENSTEIN & P.M. LIEBMANN. 1999. N-acetylserotonin is a better extra- and intracellular antioxidant than melatonin. FEBS Lett. **449:** 206–210.

16. MONTALDO, C., E. CANNAS, T. DETTORI, *et al*. 2000. Lack of melatonin effect on hydrogen peroxide induced bronchoconstriction in isolated and perfused rat lung. Life Sci. **66:** PL339–PL344.

17. FINKEL, T. 1998. Oxygen radicals and signaling. Curr. Opin. Cell. Biol. **10:** 248–253.

18. ALLEN, R.G. & M. TRESINI. 2000. Oxidative stress and gene regulation. Free Radic. Biol. Med. **28:** 463–499.

19. FINKEL, T. 2000. Redox-dependent signal transduction. FEBS Lett. **476:** 52–54.

20. SATO, N., S. IWATA, K. NAKAMURA, *et al*. 1995. Thiol-mediated redox regulation of apoptosis. Possible roles of cellular thiols other than glutathione in T cell apoptosis. J. Immunol. **154:** 3194–3203.

21. WILLIAMS, M.S. & P.A. HENKART. 1996. Role of reactive oxygen intermediates in TCR-induced death of T cell blasts and hybridomas. J. Immunol. **157:** 2395–2402.

22. CHIBA, T., S. TAKAHASHI, N. SATO & K. KIKUCHI. 1996. Fas-mediated apoptosis is modulated by intracellular glutathione in human T cells. Eur. J. Immunol. **26:** 1164–1169.

23. BAUER, M.K.A., M. VOGT, M. LOS, *et al*. 1998. Role of reactive oxygen intermediates in activation-induced CD95 (APO-1/Fas) ligand expression. J. Biol. Chem. **273:** 8048–8055.

24. BANKI, K., E. HUTTER, N.J. GONCHOROFF & A. PERL. 1999. Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. J. Immunol. **162:** 1466–1479.

25. CLEMENT, M.V. & J. PERVAIZ. 1999. Reactive oxygen intermediates regulate cellular response to apoptotic stimuli: an hypothesis. Free Radic. Res. **30:** 241–252.

26. MENENDEZ-PELAEZ, A., B. POEGGELER, R.J. REITER, *et al*. 1995. Nuclear localization of melatonin in different mammalian tissues: immunocytochemical and radioimmunoassay evidence. J. Cell. Biochem. **53:** 373–382.

27. BEUTLER, E. 1984. Red Cell Metabolism: A Manual of Biochemical Methods: 131–134. Grune & Stratton. New York.

28. BARLOW-WALDEN, L.R., R.J. REITER, M. ABE, *et al*. 1995. Melatonin stimulates brain glutathione peroxidase activity. Neurochem. Int. **26:** 497–502.

29. ANTOLIN, I., C. RODRIGUEZ, R.M. SAINZ, *et al*. 1996. Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. FASEB J. **10:** 882–890.

30. WÖLFLER, A., H.C. CALUBA, P.M. ABUJA, *et al*. 2001. Prooxidant activity of melatonin promotes fas-induced cell death in uman leukemic Jurkat cells. FEBS Lett. **502:** 127–131.

31. SCHOEMAKER, M., J.E. ROS, M. HOMAN, *et al*. 2002. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes:NF-kB-regulated inhibitor of apoptosis protein 2 (c-IAP2) prevents apoptosis. J. Hepatol. **36:** 742–750.

# PAPER V

*Melatonin as a modulator of apoptosis in B lymphoma cells*

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**Melatonin as a modulator of apoptosis in B lymphoma cells**

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**Keywords**

Melatonin; apoptosis; reactive oxygen species; B lymphoma cell lines, Epstein Barr Virus.

**Abstract**

Melatonin is considered a promising anti-tumor agent, promoting apoptosis in tumor cells, and contrasting it in normal cells. The basis for this selectivity was proposed to rely on melatonin ability to stimulate ROS production on tumor cells. Here we investigate the effect of melatonin on three types of human lymphocytes, namely normal blood lymphocytes, BL41 Burkitt lymphoma, and the cognate EBV-converted E2r. We found that melatonin promotes ROS production on all these cells. Melatonin protects from apoptosis BL41 in the same guise as normal lymphocytes, whereas E2r are unaffected. These results show that ROS production is not limited to tumor lymphocytes, nor it is involved in apoptosis promotion; that melatonin does not promote apoptosis in tumor lymphocytes, but EBV inhibits melatonin antiapoptotic effect; and that the antiapoptotic effect of melatonin does not depend on melatonin's well known chemical antioxidant properties.

**Introduction**

Melatonin (or N-acetyl-5-methoxy-tryptamine, Mel), the major secretory product of the pineal gland,[1] is a lipophilic hormone widely distributed throughout the human body.[2] In addition to playing a role in the regulation of the circadian rhythms[3] and seasonal responses,[4] melatonin is involved in many different physiological processes. Recent reports indicate that melatonin is involved in the regulation of immune functions; [5,6] lymphoid cells are able to synthesize melatonin and to possess plasma membrane melatonin receptors. There are numerous studies showing that melatonin is involved in tumorigenesis [7,8]; it can control tumor growth, inhibit cell proliferation, regulate apoptosis and protect cell structures from harmful effects of ROS.[8-15] The complexity of melatonin's role is attributed to the multiple interactions with target cells, that include the engagement of MT1 and MT[16,17] plasma membrane and possibly nuclear receptors, the binding to intracellular proteins such as calmodulin and its antioxidant effects. Independently from receptor stimulation, melatonin can act as radical scavenger directly, by neutralizing reactive oxygen and nitrogen species (ROS and RNS)[18] or indirectly, by stimulating the intracellular antioxidant defenses and increasing the activity of detoxifying enzymes.[19]

Recently, it was reported the perplexing finding that melatonin stimulates intracellular production of reactive oxygen species (ROS) in tumor cells [19,20], via a still undisclosed mechanism.

Melatonin exerts a complex effect on cellular apoptosis; the present-day model implies a pro-apoptotic activity on tumor cells, and an anti-apoptotic activity on normal cells[11,13,21-24]. Since apoptosis is very often dependent on oxidations, it has been hypothesized that pro-radical effect of melatonin may be limited to tumor cells, thus providing a rationale to the selective pro-apoptotic effect on tumor cells[11].

To explore whether these correlations may apply to lymphocytic cells, which are known to possess MT1 plasma membrane receptors[25] in this work we examined the effects of melatonin on the apoptotic process and intracellular ROS production on a set of lymphocytes, namely normal lymphocytes, and a couple of tumor lymphocytes, the Burkitt's lymphoma Epstein-Barr virus (EBV)-negative cells, BL41, and the same cells EBV-converted, E2r, that are resistant to apoptosis though having increased endogenous cellular levels of peroxides compared to their parental EBV negative cell line.[26]

## Materials and Methods

### Cell Culture

BL41 is an EBV negative B-cell line obtained from a Burkitt's lymphoma carrying a mutant form of p53 gene. E2r clone was obtained after infection of parental BL41 cell line with a non-defective B95-8 EBV strain.[25] The cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin and kept in a controlled atmosphere (5% $CO_2$) incubator at 37°C. Experiments were performed at a concentration of $10^6$ cells/mL.

Peripheral blood mononuclear leukocytes (PBML) were isolated from heparinized blood samples of healthy individuals by collection with Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. Red cells were removed by hypotonic lysis. PBML were then plated in RPMI 1640 plus 10% human AB serum in culture flasks (pretreated with the same human AB serum) to promote monocytes adhesion. After 2hrs, floating cells (lymphocytes) were washed out, spinned down and separately plated in RPMI 1640 plus 10% hi-FCS.

### Induction and Detection of Apoptosis

Apoptosis was induced with 3 µg/mL puromycin (PMC), a protein synthesis inhibitor, that was kept throughout the experiments. Apoptosis was evaluated in terms of apoptotic nuclear morphology detectable by fluorescence microscopy on cells stained with the cell permeant DNA-specific dye Hoechst 33342. The fraction of cells with apoptotic nuclei among the total cell population was calculated by counting 100 per sample in at least three randomly selected microscopic fields; the results are expressed as percentage of apoptotic cells among the total cells counted. PMC was purchased from Sigma Chemical Co. (St. Louis, MO, USA); Hoechst 33342 from Calbiochem (San Diego, CA, USA).

### Treatment with Melatonin

Melatonin was used at the final concentration of 1 mM and added to the culture medium 1 h before apoptosis induction. Melatonin was purchased from Sigma-Aldrich (Milan, Italy).

### Analysis of Intracellular ROS

Levels of ROS were measured in BL41 and E2R cells in presence or in absence of 1 mM melatonin. Cells were loaded with 10 µM dichlorodihydrofluoresceindiacetate (DHCFDA) by incubation at 37 °C for 20 min. Cells were then washed and immediately analyzed using FACScan (Becton Dickinson, San Jose, CA, USA); data (10,000 events) were elaborated with the Cell Quest software.

DHCFDA was purchased from Molecular Probes.

### Results

After incubation with 1 mM melatonin for 1 h, we induced human peripheral blood lymphocytes, BL41 and E2R cells to apoptosis with 3 µg/mL PMC. As shown in FIGURE 1, melatonin was able to protect normal lymphocytes and BL41 from PMC-induced apoptosis, whereas it was ineffective in E2R cells.This indicates that the normal vs.tumorstatus of lymphocytesdoes not determinethe sensitivity to the apoptosis-modulating effect of melatonin, which instead seemsto be sensitive to the latentEBV infection.

We then analyzed the intracellular levels of ROS upon treatment with 1mM melatonin. FIGURE 2 shows that after 2hrs melatonin increases intracellular levels of reactive oxygen species in all lymphocytes, independently of their being normal or tumor; inthis case, the presence of EBV is notinfluent.

These results indicate that the effects of melatonin on apoptosis in lymphocytes, both normal or tumor, do not depend on its antioxidant and radical scavenging properties.

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## Discussion

The ability of melatonin to affect apoptosis is considered as a major mechanism of melatonin control of the immune system. For this reason, much attention is being posed on the mechanisms at the basis of the process. It is generally believed that the antiapoptotic effect of melatonin is due to its radical scavenger ability, considering that apoptosis is often dependent on oxidative stress[27]. We addressed this issue and demonstrated that melatonin contrasts apoptosis on U937 monocytic cells by interacting with MT1/MT2 receptor thus eliciting a G-protein, phosphliupase C mediated signal transduction[28,29]; interestingly, also B lymphocytes possess plasma membrane melatonin receptors MT1.[25] suggesting further analysis to assess how general this mechanism may be. The use of a set of synthetic melatonon analogs with different affinities for MT1/MT2 and different antioxidant ability[28] allowed also excluding that this latter feature may play a role in melatonin antiapoptotic effect. In support to this, the data shown in this study uncouple the anti-apoptotic effect from the role of melatonin in oxidations. In fact, melatonin behaves as a pro-oxidant, rather than as an anti-oxidant; nontheless, it may exert an anti-apoptotic action.

We show that melatonin is able to reduce stress-induced apoptosis in normal lymphocytes and in the lymphoma cells BL41. This confirms other reports that break the rule according to which melatonin would induce apoptosis on tumor cells and survival on normal cells. Interestingly, melatonin does not reduce apoptosis in E2R, suggesting that the latent infection with Epstein Barr virus might likely interfere with melatonin's action on apoptosis. E2r cells show an increased resistance to apoptosis compared to their parental cell line;[26] we can speculate that melatonin's antiapoptotic effects may coincide with the pro-survival action of EBV on these cells, thus resulting undetectable. The main mechanism of resistance to apoptosis of E2r is the hyperactivation of 5-lipoxygenase, and specifically its product 5-HETE[30], already described as a pro-survival agent responsible for the progression of several tumors[31]. Interestingly, we observed that melatonin stimulates the activity of lipoxygenase in U937 cells (Radogna et al., submitted), and that this may be an adjuvant mechanism for melatonin antiapoptotic effect (Radogna et al., in preparation).

In contrast with the general consensus about the anti-oxidant action of melatonin, we show that melatonin increases the intracellular production of reactive oxygen species in both normal and tumor lymphocytes (BL41 and E2R). This confirms other reports where melatonin has been found to exert prooxidant effects in some cell lines.[11,20,32] However, our study does not confirm the paradigma according to which melatonin is pro-oxidant and pro-apoptotic on tumor cells, whereas in normal cells it reduces apoptosis exerting its chemical antioxidant effects. In fact, we report that on the one side melatonin stimulates ROS on normal lymphocytes; on the other side, melatonin reduces apoptosis on tumor BL41; finally, the pro-oxidant ability is clearly uncoupled from a pro-apoptotic effect of melatonin, which never appears on the lymphocytes here examined.

Further investigations are required to elucidate the mechanism through which melatonin is able to increase ROS production, which might result from an increased activity of enzymes such as lypoxygenase, cyclooxygenase or be due to mitochondrial alterations, since mitochondria are the major source of intracellular ROS through the electron transport chain. ROS produce oxidative stress only at high and

deregulated levels, whereas physiological ROS production is rather a signaling event; it will be interesting to explore whether the increase in intracellular ROS induced by melatonin could play a role in a signal transduction pathway leading to cell survival.

**References**

1. Claustrat, B., J. Brun, G. Chazot. 2005. The basic physiology and pathophysiology of melatonin. Sleep Med. Rev. 9: 11–24

2. Reiter, R.J. 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr. Rev. 12: 151–80

3. Scheer, F.A., C.A. Czeisler. 2005. Melatonin, sleep and circadian rhythms. Sleep Med. Rev. 9: 5–9.

4. Morgan, P.J., P. Barrett, H.E. Howell, *et al*. 1994. Melatonin receptors: localization, molecular pharmacology and physiological significance. Neurochem. Int. 24 (2): 101-146.

5. Guerrero, J.M. & R.J. Reiter. 2002. Melatonin-immune system relationships. Curr. Topics Med. Chem. 2: 167-179.

6. Carrillo-Vico A., J.R. Calvo, P. Abreu, *et al*. 2004. Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance. FASEB J. 18 (3): 537-9.

7. Ram, P.T., J. Dai, L. Yuan, *et al*. 2002. Involvement of the mt1 melatonin receptor in human breast cancer. Cancer Lett. 179 (2): 141-150.

8. Xi, S.C., P.C. Tam, G.M. Brown, *et al*. 2000. Potential involvement of mt1 receptor and attenuated sex steroid-induced calcium influx in the direct anti-proliferative action of melatonin on androgen-responsive LNCaP human prostate cancer cells. J. Pineal Res. 29 (3): 172-183.

9. Reiter, R.J., D. Acuña-Castroviejo, D.X. Tan *et al*. 2001. Free radical-mediated molecular damage. Mechanisms for the protective actions of melatonin in the central nervous system. Ann. N. Y. Acad. Sci. 939: 200-215.

10. Reiter, R.J., D.X. Tan, C. Osuna *et al*. 2000. Actions of melatonin in the reduction of oxidative stress. J. Biomed. Sci. 7: 444-458.

11. Wölfler, A., H.C. Caluba, P.M. Abuja, *et al*. 2001. Prooxidant activity of melatonina promotes Fas-induced cell death in human leukemic Jurkat cells. FEBS Letters. 502: 127-13.

12. Osseni, R.A., P. Rat, A. Bogdan, *et al*. 2000. Evidence of prooxidant and antioxidant action of melatonin on human liver cell line HepG2. Life Sci. 68 (4): 387–399.

13. Sainz, R.M., J.C. Mayo, R.J. Reiter, *et al*. 2000. Melatonin regulates glucocorticoid receptor: an answer to its antiapoptotic action in thymus FASEB J. 13: 1547-1556.

14. Yu, Q., S.C. Miller & D.G. Osmond. 2000. Melatonin inhibits apoptosis during early B-cell development in mouse bone marrow. J. Pineal Res. 29: 86-93.

15. Uz, T., P. Giusti, D. Franceschini, *et al*. (1996) Protective effect of melatonin against hippocampal DNA damage induced by intraperitoneal administration of kainate to rats. Neuroscience. 73: 631-636.

16. Dubocovich M.L., M.A. Rivera-Bermudez, M.J. Gerdin, *et al*. 2003. Molecular pharmacology, regulation and function of mammalian melatonin receptors. Front. Biosci. 8: d1093–d1108

17. Vanecek, J. 1998. Cellular mechanisms of melatonin action. Physiol. Rev. 78: 687-721.

18. Reiter, R.J. 1998. Oxidative damage in the central nervous system: protection by melatonin. Prog. Neurobiol. 56: 359-384.

19. Mayo, J.C., R.M. Sainz, V. Martin, *et al*. 2003. Melatonin regulation of antioxidant enzyme gene expression. Cell. Mol. Life. Sci. 59: 1706-1713.

20. Barsacchi, R., C. Kusmic, E. Damiani, *et al*. 1998. Vitamin E consumption induced by oxidative stress in red blood cells is enhanced by melatonin and reduced by N-acetylserotonin. Free Rad. Biol. Med. 24: 1187-1192.

21. Eskiocak s, Tutunculer f, Basaran un et al. The effect of melatonin on protein oxidation and nitric oxide in the brain tissue of hypoxic neonatal rats. Brain Dev 2007; **29**:19-24.

22. RUBIO S, ESTEVEZ F, CABRERA J et al. Inhibition of proliferation and induction of apoptosis by melatonin in human myeloid HL-60 cells. J Pineal Res 2007; **42**:131-138.

23. Garcia-Santo S., Antolin I., Herrera F., et al. Melatonin induces apoptosis in human neuroblastoma cancer cells. J Pineal Res 2006; **41**:130–135.

24. Juknat A.A ,. Mendez mdel V., Quaglino A., et al. Melatonin prevents hydrogen peroxide-induced Bax expression in cultured rat astrocytes. J Pineal Res 2005; **38**:84–92.

25. Pozo, D., M. Delgado, J.M. Fernandez-Santos, *et al*. 1997. Expression of the Mt-1 melatonin receptor mRNA in T and B subsets of lymphocytes from rat thymus and spleen. FASEB J. 11 : 466-473.

26. Gualandi, G., L. Giselico, M. Carloni, *et al*. 2001. Enhancement of genetic instability in human B cells by Epstein-Barr virus latent infection. Mutagenesis. 16 : 203-208.

27. Ghibelli L, Fanelli C, Rotilio G, et al. 1998. Rescue of cells from apoptosis by inhibition of active GSH extrusion.FASEB J. 12:479-86.

28. Radogna, F., Paternoster, L., Albertini, M.C., Cerella, C., Accorsi, A., Bucchini, A., Spadoni, G., Diamantini, G., Tarzia, G., De Nicola, M., D'Alessio, M., Ghibelli, L. 2007. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res. 43;154-162.

29. Radogna F, Cristofanon S, Paternoster L, D'Alessio M, De Nicola M, Cerella C, Dicato M, Diederich M, Ghibelli L. Melatonin antagonizes the intrinsic pathway of apoptosis via mitochondrial targeting of Bcl-2. J Pineal Res. 2008 ;44:316-25

30. Belfiore, M.C., A. Natoni, R. Barzellotti, *et al*. 2007. Involvement of 5-lipoxygenase in survival of Epstein Barr virus (EBV)-converted B lymphoma cells. Cancer Lett. 254 (2): 236-243.

31. Romano M., Catalano A., Nutini M., *et al*. 2001. 5-lipoxygenase regulates malignant mesothelial cell survival: involvment of vascular endothelial growth factor. FASEB J. 15: 2326-2336.

32. Albertini M.C., F. Radogna, A. Accorsi, F. Uguccioni *et al*. 2006. Intracellular pro-oxidant activity of melatonin deprives U937 cells of reduced glutathione without affecting glutathione peroxidase activity. Ann N. Y. Acad. Sci. 1091: 10-6.

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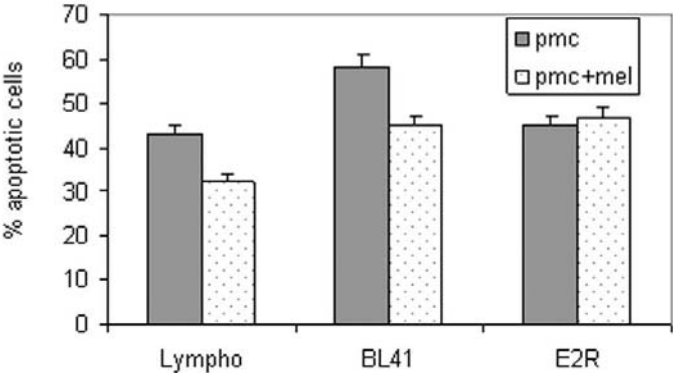54
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**Figure Legends**

**FIGURE 1. Melatonin Reduces Apoptosis in normal lymphocytes and BL41 cells, but not in E2R.**

Effects of melatonin on apoptosis in lymphocytes, in the parental B lymphoma cell line, BL41 and EBV-converted E2R. All cells line were incubated with 1 mM melatonin for 1 h and then induced to apoptosis by 3 µg/mL PMC. Apoptosis was measured at 3 h of apoptogenic treatment. Values are the average of at least three independent experiments ± SD. Melatonin protection from PMC-induced apoptosis is significant (P < 0.5).

**FIGURE 2. Melatonin increases intracellular ROS in both cell types, normal lymphocytes, BL41 and E2R.**

Effects of melatonin on intracellular ROS levels. After 3 h of treatment with 1 mM melatonin, cells were stained with DHCFDA and fluorescence intensity was measured by flow cytometric analysis. Basal fluorescence level were respectively: lymphocytes=4.7; BL41=0.8; E2R= 3.8 fluorescence arbitrary units. The data are the average of at least three independent experiments ± SD.

FIGURE 1. Melatonin Reduces Apoptosis in normal lymphocytes and BL41 cells, but not in E2R. Effects of melatonin on apoptosis in lymphocytes, in the parental B lymphoma cell line, BL41 and EBV-converted E2R. All cells line were incubated with 1 mM melatonin for 1 h and then induced to apoptosis by 3 µg/mL PMC. Apoptosis was measured at 3 h of apoptogenic treatment. Values are the average of at least three independent experiments ± SD. Melatonin protection from PMC-induced apoptosis is significant (P < 0.5).

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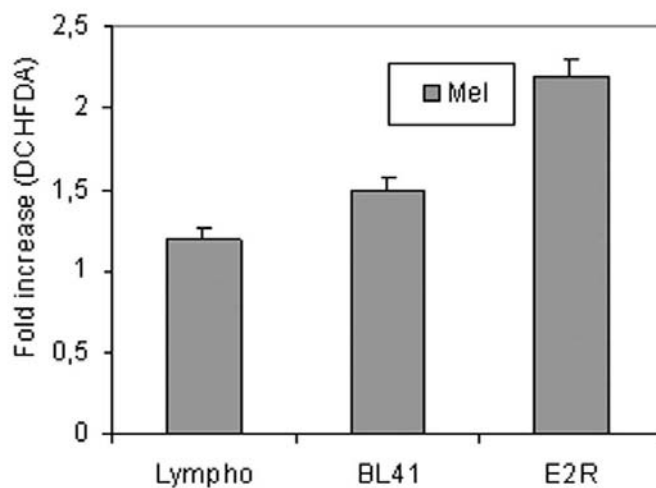
FIGURE 2. Melatonin increases intracellular ROS in both cell types, normal lymphocytes,  BL41 and E2R.
Effects of melatonin on intracellular ROS levels. After 3 h of treatment with 1 mM melatonin, cells were stained with DHCFDA and fluorescence intensity was measured by flow cytometric analysis. Basal fluorescence level were respectively:  lymphocytes=4.7; BL41=0.8; E2R= 3.8 fluorescence arbitrary units. The data are the average of at least three independent experiments ± SD.

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# PAPER VI

*Rapid and transient stimulation of intracellular reactive oxygen species by melatonin in normal and tumor leukocytes*

# RAPID AND TRANSIENT STIMULATION OF INTRACELLULAR REACTIVE OXYGEN SPECIES BY MELATONIN IN NORMAL AND TUMOR LEUKOCYTES

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**Running title**: Pro-radical effect of melatonin

## 1. Abstract

Melatonin is a modified tryptophan with potent biological activity, exerted by stimulation of specific plasma membrane (MT1/MT2) receptors, by lower affinity intracellular enzymatic targets (quinone reductase, calmodulin), or through its strong antioxidant ability. Scattered studies also report a perplexing pro-oxidant activity, showing that melatonin is able to stimulate production of intracellular reactive oxygen species (ROS). Here we show that on U937 human monocytes melatonin promotes intracellular ROS in a fast (<1 min) and transient (up to 5-6 h) way. Melatonin equally elicits its pro-radical effect on a set of normal or tumor leukocytes; intriguingly, ROS production does not lead to oxidative stress, as shown by absence of protein carbonylation, maintenance of free thiols, preservation of viability and regular proliferation rate. ROS production is independent from MT1/MT2 receptor interaction, since a) requires micromolar (as opposed to nanomolar) doses of melatonin; b) is not contrasted by the specific MT1/MT2 antagonist luzindole; c) is not mimicked by a set of MT1/MT2 high affinity melatonin analogues. Instead, chlorpromazine, the calmodulin inhibitor shown to prevent melatonin-calmodulin interaction, also prevents melatonin pro-radical effect, suggesting that the low affinity binding to calmodulin (in the micromolar range) may promote ROS production.

## 2. Introduction

Melatonin is a hormone principally produced and released by the pineal gland under the influence of the environmental light/dark cycle (Wurtman et al. 1964). It participates in several important physiological functions, including biological regulation of circadian rhythms, sleep, mood, reproduction and neuro-immuno-modulation. Exogenous melatonin has been used for treating a variety of circadian rhythm disorders, including jet-lag and insomnia. Melatonin appears to evoke its effects in humans through two subtypes of high affinity, 7-pass G protein-coupled plasma membrane receptors: MT1 (preferentially expressed in the brain), and MT2 (preferentially expressed in the retina) (Reppert et al, 1994; Lai et al., 2002). The two receptors are also widely expressed in non-neuroendocrine tissue (Carrillo-Vico et al. 2004; Yuan et al.2002). MT1/MT2 engagement triggers G-protein mediated signal transduction that can imply either the phospholipase C (Brydon et al. 1999) or the adenylate cyclase (Vanecek, 1998) pathways; the study of the different roles of the two receptor subtypes has been impeded so far by the lack of selective melatonin receptor agonists and antagonists. Melatonin also possesses high affinity binding for nuclear receptors ROR/RZR, which act as transcriptional activators (Wiesenberg et al. 1998); however, their role in melatonin signaling is still debated. In addition to regular receptors, melatonin binds at lower affinities two cytosolic enzymes, namely quinone reductase 2 (that coincides with the binding site formerly known as MT3 (Tan et al. 2007), and calmodulin (Benítez-King et al. 1993). It is presently unknown whether these binding may exert any physiological functions, since they require higher melatonin concentrations than those present in human blood. Melatonin is a powerful antioxidant, acting both as a direct radical scavenger (Reiter et al. 2007) and by stimulating production/activity of intracellular antioxidant enzymes (Rodriguez, et al. 2004); melatonin contrasts oxidative stress in tissues (Baydas et al. 2007) as well as within cells (Bongiovanni et al. 2007), ameliorating tissue homeostasis in oxidative related pathologies (Clapp-Lilly etal. 2001). The mechanism through which melatonin stimulates antioxidant enzymes is unclear: generally speaking, the increase of antioxidant defense is a cell response to oxidative stress, and the ability of an anti-oxidant such as melatonin to promote antioxidant defense remains an intriguing issue (Rodriguez, et al. 2004).

Possibly, the phenomenon may be related to the surprising recent findings indicating that melatonin is able to produce intracellular reactive oxygen species (ROS) (Albertini et al. 2006; Büyükavci et al.2006; Medina-Navarro et al. 1999), detected as the increase in

fluorescence of oxidation-sensitive intracellular probes (namely dihydrorhodamine (Pieri et al. 1998), dichlorofluorescein (Osseni et al. 2000), dihydroethidium (D'agostino et al. 2007). The pro-radical activity of melatonin has been reported on a set of cells, most of which of tumor origin, which proved positive with very few exceptions (Albertini et al. 2006; Büyükavci et al.2006; Medina-Navarro et al. 1999; Wolfler et al.2001). Intriguingly, even though intracellular free thiols are decreased by melatonin (Eskiocak et al. 2007), frank oxidative stress such as lipid peroxidation seems not to occur (Büyükavci et al.2006). The origin of melatonin-produced ROS is still ignored. A direct molecular pro-oxidant effect can be excluded on the basis of the known chemical properties of the molecule. Since the pro-radical effect was reported only as an intracellular phenomenon, we explored whether it may be the consequence of a signal transduction originated by melatonin binding to any of its cellular targets.

In this study, we show that it is not due to the interaction with MT1/MT2 receptor, providing instead evidences that it may result from binding to the lower affinity intracellular target calmodulin.

## 3. Methods

### 3.1 Cell culture

U937 are human tumor monocytes stabilized from a histiocytic lymphoma; Jurkat are a human leukemic T-cell line; E2R are an Epstein-Barr virus (EBV) positive B-cell line obtained from Burkitt's lymphoma. Cells were cultured in RPMI medium supplemented with 10% FCS. Cells were routinely checked for absence of mycoplasm by using a mycoplasm detection kit (Mycoalert TM., Cambrex Bio Science Milano, Italy). The experiments were performed on cells in the logarithmic phase of growth under condition of $\geq 98\%$ viability, as assessed by trypan blue exclusion. Peripheral blood mononuclear leukocytes (PBML) were isolated from heparinized blood samples of healthy individuals by collection with Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. Red cells were removed by hypotonic lysis. PBML were then plated in RPMI 1640 plus 10% human AB serum in culture flasks (pretreated with the same human AB serum) to promote monocytes adhesion. After 2 h, floating cells (lymphocytes) were washed out, spinned down and separately plated in RPMI 1640 plus 10% hi-FCS. Adherent monocytes were washed 3 times with RPMI to remove residual lymphocytes, detached by cell scraping, and resuspended in RPMI 1640 plus 10% hi-FCS. Treatments (see below) were performed at 20-24 h post-separation. Cell viability in both fractions was >98% as assessed by trypan blue exclusion test. Purity of the enriched fractions was controlled by labelling (20 min at room temperature) with cell-type specific antibodies: FITC-conjugated-anti-CD45 (from Becton-Dickinson) for lymphocytes; and PE-conjugated-anti-CD14 (from Becton-Dickinson) for monocytes. Cells were then washed with PBS and labelling evaluated. Both monocyte and lymphocyte fractions were >95% pure.

### 3.2 Analysis of ROS

Cells were loaded with 10 uM dichlorodihydrofluorescein diacetate (DCHFDA, Molecular Probes), by incubation at 37° for 30 min after melatonin treatments. This probe is a non-fluorescent cell-permeable compound; once inside the cell, it is de-esterified and turns fluorescent upon oxidation, fluorescence being proportional to ROS production. Analyses were performed by flow cytometry using FACScan Becton&Dickinson.

## 3.3 Evaluation of cell viability and cell number

Apoptosis and necrosis were evaluated as previously described (Colussi et al. 2000). Cells were stained with a mixture of 10 ug/ml Hoechst 33342 (for nuclear morphology) and 5ug/ml propidium iodide (for dye exclusion) in culture medium for 15 min at RT. Cells were analyzed using a fluorescence microscope; cells with nuclear apoptotic morphology, or non-excluding PI, were counted (at least 300 cells in at least 3 independent fields randomly selected to avoid biases) and the fraction of apoptotic or necrotic cells among total cells was evaluated. Hoechst 33342 and PI were purchased from Calbiochem (San Diego, CA, USA).

For cell growth, cells were incubated for 5 days with 1 mM melatonin and cell number was quantified every 24 h for 5 days. The values are related to PI-excluding cells.

## 3.4 Cell cycle analysis

U937 cells ($2x10^6$) were washed with 1% PBS and fixed in 65% ice cold ethanol for 15 min. Cells were then stained with 5ug/ml propidium iodide in RNase solution for 15 min at RT. Samples were processed for flow cytometry in a FACScan Becton&Dickinson. Cell cycle was analyzed with Cylchred software.

## 3.5 Determination of glutathione

Intracellular glutathione was assayed upon formation of *S*-carboxymethyl derivatives of free thiols with iodoacetic acid, followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives by the reaction with 1-fluoro-2,4-dinitrobenzene as previously described (Ciriolo et al. 2001). The derivatized small thiols are run on HPLC column, and the elution time of GSH and GSSG is calculated on the basis of the elution times of the standards. Data are expressed as nmoles of GSH or GSSG/mg protein.

## 3.6 Determination of protein oxidation

Carbonylated proteins were detected using the Oxyblot kit (Cayman) as previously described (Aquilano et al. 2003). Briefly, 20 µg of proteins were reacted with DNP for 15 min at 25°C. Samples were resolved on 12% SDS-polyacrylamide gels, and DNP-derivatized proteins were identified by immunoblot using an anti-DNP antibody.

## 3.7 Melatonin and analogues

Melatonin was purchased from Sigma Chemical Co (St. Louis, MO, USA), and used at the concentration of 1 mM unless otherwise specified. For the experiments, melatonin was added 1 h prior to apoptosis induction. Compounds UCM412, UCM608, UCM245, UCM92, UCM765, UCM231; UCM454, UCM353, (see table I) were synthesized according to procedures described in (Duranti et al. 1992; Spadoni et al. 1993; Spadoni et al. 1998; Tarzia et al. 1997; Rivara et al. 2007; Mor et al. 1998; Spadoni et al. 2001). The set of nine compounds was constructed by selecting known derivatives characterized by differing affinity and intrinsic activity for melatonin membrane receptors. High affinity receptor agonists (see table I, compounds # 1-5, 7,) were chosen together with derivatives endowed with lower affinity (see table I, compounds # 8-10) and different agonist potency at membrane receptors.

## 3.8 Other treatments

Trolox was used at 1 mM and added 30 before treatments. For PLC inhibition, U73122 (Sigma Chemical Co, St. Louis, M, USA) was used at 10 uM and added 30 min before treatments. Extracellular $Ca^{2+}$ influx was inhibited by incubating the cells with ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA, 650 uM) for 15 min before other treatments. G protein inhibition was achieved by pertussis toxin (PTX, 200 nM), added 24 h before the other treatments. Melatonin action on $MT_1/MT_2$ receptors was antagonized with 50 uM 2-benzyl-N-acetyltryptamine (luzindole) (Sigma), a concentration proved able to antagonize 1 mM melatonin in previous studies (Spadoni et al. 1998), which was added 30 min before the other treatments. For mitochondrial respiration inhibition a specific inhibitor of complex I (rotenone, 5 uM) was added 30 min before treatments. For calmodulin inhibition, chlorpromazine (Sigma) or calmidazolium (Sigma) respectively inhibiting or non affecting melatonin-specific binding site to calmodulin, were used at 0.5 uM. BSO was used at the concentration of 1 mM and maintained for 24 h before medium change and melatonin addition (BSO was re-added during the experiment). At this time, no GSH is detectable (see Ghibelli et al., 1998).

## 3.9 Statistical analyses

The results are presented as means +- SD. Statistical evaluation was conducted by a one-way ANOVA, followed by the Student-Newman-Keuls multiple comparisons.
Significance level was fixed at alfa=0.05.

## 4. Results

*4.1 Immediate and transient stimulation of intracellular reactive oxygen species by melatonin*

Melatonin promotes reactive oxygen species (ROS) formation on U937 tumor monocytic cells in a potent and rapid way: ROS are detected by the oxidation-sensitive probe dichlorofluorescein (DCHFDA) as early as after 1 minute of 1mM melatonin challenge, followed by a sharp increase that peaks at 2-3 h, as shown by the time course in Fig. 1A. The DCHFDA signal then decreases slowly, to reach control values at 5-6 h. The signal is efficiently scavenged by the vitamin E analogue Trolox (panel B).

A dose effect was then investigated, as shown in panel C, where the fluorescence values measured at 3 h are presented; an increase over basal levels starts at 10 uM, but is significant only at 100 uM.

To evaluate if the DCF signal was really to attribute to melatonin-produced ROS, two other oxidation-sensitive probes such as dihydrorhodamine and dihydroethidium were used; melatonin stimulates oxidation of these additional probes (albeit at different extent, see accompanying paper) thus showing that a frank oxidative stress is produced by melatonin (results not shown).

*4.2 MT1/MT2 receptor engagement is not involved in ROS production*

In order to explore the origin of melatonin-induced ROS, we evaluated whether melatonin, which is known to interact with mitochondria and their functioning, might produce ROS by disturbing the respiratory chain. However we found that rotenone, an uncoupler of mitochondrial respiratory chain, does not impair melatonin ability of producing ROS (figure 2).

Then, we evaluated the role of the intracellular melatonin binding sites, namely MT1/MT2 plasma membrane receptors (which are engaged at nM doses) and calmodulin (bound at high uM doses).

The requirement of uM melatonin doses would suggest independence of ROS production from MT1/MT2 receptors, engaged at much lower, i.e., nM concentrations. To actually investigate the role of MT1/MT2, we measured DCHFDA fluorescence in cells at 3 h of 1 mM melatonin in the presence of: luzindole, a specific MT1/MT2 antagonist; pertussis toxin, which inhibits the G protein responsible for the propagation of the signal; the PLC inhibitor U73122, and the extracellular $Ca^{2+}$ chelator EGTA. These compounds are reported to totally (luzindole) or partially (the others) inhibit melatonin-

elicited intracellular signal transductions (Radogna et al. 2007). None of these treatments impairs the ROS-promoting ability of melatonin, indicating that it occurs independently of MT1/MT2 interaction (figure 3A). To further study the involvement of MT1/MT2, we also analyzed the eventual pro-radical effect of a set of melatonin analogues interacting with MT1/MT2 with different affinities (see table I and figure 3B): none of these exerted any pro-radical activity, confirming the independence of melatonin ROS production from MT1/MT2 interaction (figure 3C).

*4.3 Possible involvement of calmodulin binding in melatonin-induced ROS production*

The doses required for melatonin pro-radical effect would be instead compatible with binding to calmodulin (63 uM). Calmodulin is a cytosolic enzyme with a Ca2+ binding site and a domain of interaction with many different protein partners. The use of inhibitors acting on the one or the other domain, namely calmidazolium and chlorpromazine, respectively, allowed establishing that melatonin interacts with the chlorpromazine-sensitive domain, being insensitive to calmidazolium (Romero et al. 1998). To explore whether the pro-radical effect of melatonin may derive from calmodulin binding, we probed the same couple of calmodulin inhibitors on melatonin-induced ROS production. As shown in fig. 4, calmidazolium was ineffective, whereas chlorpromazine efficiently prevented melatonin-pro-radical effect. This strongly suggests that the pro-radical effect of melatonin may be the consequence of calmodulin binding.

*4.4 The pro-radical effect of melatonin is general among leukocytes*

To understand if the pro-radical effect of melatonin is a general phenomenon among leukocytes, we examined a panel of cells of haematopoietic origin, i.e., lymphocytes and monocytes freshly explanted from healthy donors, and the human tumor T lymphocytic Jurkat cell line. 1 mM melatonin was able to produce a DCHFDA signal on all of these cells, independently of they being tumor or normal cells (figure 5).

*4.5 Melatonin treatment does not alter cell viability or proliferation*

Then we analyzed which consequences for the cells may derive from melatonin-induced oxidative status. First of all, we analyzed whether 1 mM melatonin may impair cell viability. Fig. 6A shows that melatonin does not elicit cell death for up to one week in the form of apoptosis/necrosis; to ascertain that scattered events of cells death may occur un-noticed, control and melatonin-treated cells were counted every 24 h; fig. 6B

shows that the proliferation rate of melatonin-treated cells perfectly overlap that of control cells. We also analyzed cell cycle in the first 24 h of melatonin treatment, but again, no substantial changes with respect to control were observed, as shown in fig. 6C. These results indicate that no toxicity can be attributed to melatonin, in spite of its pro-radical effect. Lack of toxicity/apoptosis was also found in all leukocytes examined (not shown).

*4.6 The pro-radical effect of melatonin does not imply oxidative stress*

To give a rationale to the lack of toxic effect of melatonin in spite of its pro-radical effect, we analyzed whether melatonin-produced ROS really provoke an oxidative stress. We considered three intracellular markers of oxidative stress, i.e., protein carbonylation and the level of reduced (GSH) and oxidized (GSSG) glutathione, by evaluating these parameters at different time points after 1mM melatonin treatment. Fig. 7A shows that no protein carbonylation is induced by melatonin, its level being in fact even decreased with time. GSH is substantially unaffected by melatonin (fig. 7B); interestingly, no significant oxidation of glutathione was found.

We also analyzed whether depletion of GSH by BSO would affect cell response to melatonin in terms of ROS production or toxicity/apoptosis. Fig. 7C shows that cells maintain normal viability after 1 mM BSO, independently of melatonin challenge. In terms of ROS production, BSO by itself slightly increases DCF signal (see also Cristofanon et al., 2008), but does not sensitize to melatonin-induced ROS, since the two effects appear merely additive (fig. 7D).

These results indicate that the pro-radical effect of melatonin is not translated within the cell into a proper oxidative stress.

## 5. Discussion

Promotion of free radical by melatonin within cells, though reported by several studies in the past years (Albertini et al. 2006; Büyükavci et al.2006; Medina-Navarro et al. 1999; Wolfler et al.2001), is not an openly accepted notion. We report here that the pro-radical activity is transient, peaking at 2-3 h after melatonin addition, to disappear after 6 h, and that it is elicited at melatonin concentrations ≥10 uM, 4 orders of magnitude higher than those considered as "physiological", i.e., necessary for receptor stimulation. This may explain why many studies, examining later time points and/or lower concentrations, failed to reveal it. We have shown that both normal and tumor white blood cells react to melatonin producing free radicals: thus, at least for leukocytes, the paradigm that melatonin only elicits a pro-radical effect on tumor cells is not applicable.

That a well-known anti-oxidant such as melatonin may behave as a pro-oxidant, is an (apparent) contradiction, that still needs to be explained. Anti-oxidants may often exert pro-oxidant activities according to their concentrations and/or the surrounding milieau, as shown for many endogenous or exogenous compounds, such as tocopherol or ascorbate (Maellaro et al. 1996); this is probably related with the reactivity of each redox couple. In the case of melatonin, conceivably the pro-radical effect is not a direct chemical pro-oxidant ability of melatonin (which was never demonstrated in vitro), but a cellular reaction. The effect is so rapid, that it cannot be ascribed to an exhaustion of cell anti-oxidant defenses, which in fact are unaffected in our system in the first 24 h of melatonin treatment (Albertini et al. 2006). The promptness of the response may rather suggest that it is the result of the early activation of an enzyme exerting a pro-radical activity; this may be achieved by a signal transduction pathway triggered by an intracellular target of melatonin: the search of such a target was the major goals of this study.

We show that ROS production is independent of stimulation of the plasma membrane MT1-MT2 receptors, since a), it is unaffected by the MT1-MT2 antagonist luzindole; b) requires doses 4 orders of magnitude higher than those required for MT1-MT2 binding; c) is not elicited by melatonin analogues with high affinity for the MT1-MT2 receptors. Instead, we present evidences that the pro-radical activity may derive from the binding to calmodulin, by showing that the effect is sensitive to the same calmodulin inhibitor (chlorpromazine) which was shown to prevent melatonin binding to calmodulin, being instead insensitive to calmidazolium, which did not prevent the interaction. Melatonin, as an amphipathic molecule, may freely cross the plasma membrane, thus it may rapidly accumulating within cells and react with cytosolic target.

An open, intriguing question that arises from this study is how can calmodulin binding may elicit a pro-oxidant effect. Calmodulin is an important signalling enzyme; it is activated by transient increases in cytosolic Ca2+, which binds to calmodulin inducing an allosteric change that alters the propensity of calmodulin to bind to an abundant set of target proteins, altering their activity (Benítez-King et al. 1993; Colomer et al. 2007). In this guise, calmodulin controls the immediate activation/silencing of many key cytosolic proteins, acting as a pivot of intracellular signalling (Shigeri et al. 2008). Thus, it is conceivable that calmodulin might trigger a signal that leads to the immediate (i.e., <1 min) activation of a pro-oxidant enzyme. As we describe in the accompanying paper, we delineate an intracellular signalling pathway leading from calmodulin binding to the activation of the pro-oxidant enzyme lipoxygenase, thus providing a direct evidence of the nature of melatonin-produced ROS.

The binding of calmodulin to melatonin was demonstrated from the structural point of view, but a possible physiological significance of such an interaction was not provided or hypothesized. To our knowledge, the pro-radical effect reported here is the first hypothesis of a functional role of melatonin binding to calmodulin, in spite of the relatively long time elapsed since the publication of the study (Romero et al. 1998). A possible reason for this is that the doses of melatonin necessary for this binding exceed by far the physiological blood concentration, thus possibly rendering the phenomenon a curiosity rather than a biomedical issue. However, it has been reported recently that bone marrow concentrations of melatonin are 2 orders of magnitude higher than the blood ones. Interestingly, we report the pro-radical effect of melatonin in cells of bone marrow origin; possibly, micro-environments with even higher melatonin levels might be recognized by more focused analyses, thus providing the secondary targets of melatonin, i.e., calmodulin and quinone reductase, with a more straightforward biological significance.

The involvement of an intracellular signal as possible target of melatonin for the pro-radical effect of melatonin may give a logical framework to explain how two opposite phenomena, radical scavenging and radical production, may co-exist. As an example, we report that two markers of oxidation are differently affected by melatonin: protein carbonylation is decreased (anti-oxidant effect), whereas free thiols (glutathione) are unaffected. This may be explained by the notion that the scavenging efficiency of melatonin depends on the type of radical involved (Clapp-Lilly et al. 2001); thus, the chain of oxidations primed by melatonin may be differently scavenged by melatonin

itself, thus allowing only part of radical signalling to actually take place. The potent antioxidant ability of melatonin may also explain why melatonin-produced ROS do not lead to an open oxidative stress; indeed, melatonin has often been demonstrated to protect against cellular oxidative stress (e.g., see Kimball et al. 2008).

As far as the lack of toxicity instead, it is not obvious that this can be ascribed to the anti-oxidant properties of melatonin. Melatonin is a recognized anti-apoptotic agent, preventing apoptosis induced by a variety of stimuli in many cell systems (Andrabi et al. 2004; Radogna et al. 2008); it is often given for granted that this anti-apoptotic effect is due to melatonin anti-radical activity, since very often apoptosis is triggered by, or at least implies, an oxidative stress (D'Alessio et al. 2003; Bauer et al. 1998). However, a mechanistic analysis showed that the anti-apoptotic effect of melatonin on U937 cells requires binding to MT1/MT2 receptors and the downstream signal transduction (Radogna et al. 2007); this signal leads to the translocation of the anti-apoptotic protein Bcl-2 to mitochondria, where it prevents the dimerization and activation of the cognate pro-apoptotic protein Bax (Radogna et al. 2008); down-regulation of Bcl-2 by RNA interference not only abrogates melatonin anti-apoptotic effect, but also turn melatonin into a pro-apoptotic agent (Radogna et al. 2008), thus suggesting that this Bcl-2 pathway may be required also to maintain viability in melatonin-treated cells. Other studies showed that Bcl-2 levels can be up-regulated by melatonin (Ling et al. 1999). Interestingly, Bcl-2 is especially famous for behaving as an intracellular anti-oxidant, in some instances even compensating for glutathione (D'Alessio et al. 2004): the ability of melatonin to enhance Bcl-2 levels/functions may be an alternative way of melatonin to behave as a biological (as opposed to chemical) anti-oxidant.

## 6. References

- Albertini, M.C., Radogna, F., Accorsi, A., Uguccioni, F., Paternoster, L., Cerella, C., De Nicola, M., D'Alessio, M., Bergamaschi, A., Magrini, A., Ghibelli, L. 2006. Intracellular pro-oxidant activity of melatonin deprives U937 cells of reduced glutathione without affecting glutathione peroxidase activity. Ann N Y Acad Sci. 1091; 10-16.

- Andrabi, S.A., Sayeed, I., Siemen, D., Wolf, G., Horn, T.F. 2004. Direct inhibition of the mitochondrial permeability transition pore: a possible mechanism responsible for anti-apoptotic effects of melatonin. FASEB J. 18; 869-1871.

- Aquilano, K., Rotilio, G., Ciriolo, M.R. 2003. Proteasome activation and nNOS down-regulation in neuroblastoma cells expressing a Cu,Zn superoxide dismutase mutant involved in familial ALS. J Neurochem. 85; 1324-1335.

- Bauer, M.K., Vogt, M., Los, M., Siegel, J., Wesselborg, S., Schulze-Osthoff, K. 1998. Schulze-Osthoff. Role of reactive oxygen intermediates in activation-induced CD95 (APO-1/Fas) ligand expression. J Biol Chem 273; 8048–8055.

- Baydas, G., Koz, S.T., Tuzcu, M., Etem, E., Nedzvetsky, V.S. 2007. Melatonin inhibits oxidative stress and apoptosis in fetal brains of hyperhomocysteinemic rat dams. J Pineal Res. 43; 225-231

- Benítez-King, G., Huerto-Delgadillo, L., Antón-Tay, F. 1993. Binding of 3H-melatonin to calmodulin. Life Sci. 53; 201–207.

- Bongiovanni, B., De Lorenzi, P., Ferri, A., Konjuh, C., Rassetto, M., Evangelista, de Duffard AM, Cardinali, D.P., Duffard, R. 2007. Melatonin decreases the oxidative stress produced by 2,4-dichlorophenoxyacetic acid in rat cerebellar granule cells. Neurotox Res. 11; 93-99.

- Brydon, L., Roka, F., Petit, L., De Coppet, P., Tissot, M., Barrett, P., Morgan, P.J., Nanoff, C., Strosberg, A.D., Jockers, R. 1999. Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins. Mol Endocrinol. 13; 2025-2038.

- Büyükavci, M., Ozdemir, O., Buck, S., Stout, M., Ravindranath, Y., Savaşan, S. 2006. Melatonin cytotoxicity in human leukemia cells: relation with its pro-oxidant effect. Fundam Clin Pharmacol.; 20:73-79.

- Carrillo-Vico, A., Calvo, J.R., Abreu, P., Lardone, P.J., Garcia-Morino, S., Reiter, R.J., Guerriero, J.M. 2004. Evidence of melatonin synthesis in human lymphocytes

and its physiological significance: possible role as intracrine, autocrine and/or paracrine substance. FASEB J. 18; 537–539.

- Circolo, M.R., Aquilano, K., De Martino, A., Carrì, M.T., Rotilio, G. 2001. Differential role of superoxide and glutathione in S-nitrosoglutathione-mediated apoptosis: a rationale for mild forms of familial amyotrophic lateral sclerosis associated with less active Cu,Zn superoxide Dismutase mutants. J. Neurochem. 77; 1433-1443

- Clapp-Lilly, K.L., Smith, M.A., Perry, G., Harris, P.L., Zhu, X., Duffy, L.K. 2001. Melatonin acts as antioxidant and pro-oxidant in an organotypic slice culture model of Alzheimer's disease. Neuroreport. 12;1277-1280.

- Colomer, J., Means, A.R. 2007. Physiological roles of the Ca2+/CaM-dependent protein kinase cascade in health and disease. Subcell Biochem. 45; 169-214.

- Colussi, C., Alberini, M.C., Coppola, S., Rovidati, S., Galli, F., Ghibelli, L. 2000. $H_2O_2$-induced block of glycolysis as an active ADP-ribosylation reaction protecting cells from apoptosis. FASEB J. 14; 2266-2276.

- Cristofanon S, Morceau F, Scovassi AI, Dicato M, Ghibelli L, Diederich M. 2008. Oxidative, multistep activation of the noncanonical NF-{kappa}B pathway via disulfide Bcl-3/p50 complex. FASEB J.

- D'agostino, D.P., Putnam, R.W., Dean, J.B. 2007. Superoxide (*O2- ) production in CA1 neurons of rat hippocampal slices exposed to graded levels of oxygen. J Neurophysiol. 98; 1030-1041.

- D'Alessio, M., Cerella, C., Amici, C., Pesce, C., Coppola, S., Fanelli, C., De Nicola, M., Cristofanon, S., Clavarino, G., Bergamaschi, A., Magrini, A., Gualandi, G., Ghibelli, L. 2004. Glutathione depletion up-regulates Bcl-2 in BSO-resistant cells. FASEB J. 18; 1609-1611.

- D'Alessio, M., Cerella, C., De Nicola, M., Bergamaschi, A., Magrini, A., Gualandi, G., Alfonsi, A.M., Ghibelli, L. 2003. Apoptotic GSH extrusion is associated with free radical generation. Ann N Y Acad Sci. 1010;449-452.

- Duranti, E., Stankov, B., Spadoni, G., Duranti, A., Lucini, V., Capsoni, S., Biella, G., Fraschini, F. 1992. 2-Bromomelatonin: synthesis and characterization of a potent melatonin agonist. Life Sci. 51; 479-485.

- Eskiocak, S., Tutunculer, F., Basaran, U.N., Taskiran, A., Cakir, E. 2007. The effect of melatonin on protein oxidation and nitric oxide in the brain tissue of hypoxic neonatal rats. Brain Dev. 29; 19-24

- Ghibelli L, Fanelli C, Rotilio G, Lafavia E, Coppola S, Colussi C, Civitareale P, Ciriolo MR. 1998. Rescue of cells from apoptosis by inhibition of active GSH extrusion. FASEB J. 12; 479-486.

- Kimball SR, Abbas A, Jefferson LS. 2008. Melatonin represses oxidative stress-induced activation of the MAP kinase and mTOR signaling pathways in H4IIE hepatoma cells through inhibition of Ras. J. Pineal Res. 44; 379-386

- Lai, F.P., Mody S.M., Yung, L.Y., Kam, J.Y., Pang, C.S., Pang, S.F., Wong, Y.H. 2002. Molecular determinants for the differential coupling of Galpha(16) to the melatonin MT1, MT2 and Xenopus Mel1c receptors. J Neurochem. 80; 736-45.

- Ling, X., Zhang, L.M., Lu, S.D., Li, X.J., Sun, F.Y. 1999. Protective effect of melatonin on injuried cerebral neurons is associated with bcl-2 protein over-expression. Zhongguo Yao Li Xue Bao. 20;409-414.

- Maellaro E, Bello BD, Comporti M. 1996. Protection by ascorbate against apoptosis of thymocytes: implications of ascorbate-induced nonlethal oxidative stress and poly(ADP-ribosyl)ation. Exp Cell Res. 226; 105-113.

- Medina-Navarro, R. Duran-Reyes, G., Hicks, J.J. 1999. Pro-oxidating properties of melatonin in the in vitro interaction with the singlet oxygen. Endocr Res. 25; 263-280.

- Mor, M., Rivara, S., Silva, C., Bordi, F., Plazzi, P.V., Spadoni, G., Diamantini, G., Balsamini, C., Tarzia, G., Fraschini, F., Lucini, V., Nonno, R., Stankov, B.M. 1998. Melatonin receptor ligands: Synthesis of new melatonin derivatives and comprehensive comparative molecular field analysis (CoMFA) study. J Med Chem. 41; 3831-3844.

- Mor, M., Spadoni, G., Di Giacomo, B., Diamantini, G., Bedini, A., Tarzia, G., Plazzi, P.V., Rivara, S., Nonno, R., Lucini, V., Pannacci, M., Fraschini, F., Stankov, B.M. 2001. Synthesis, pharmacological characterization and QSAR studies on 2-substituted indole melatonin receptor ligands.Bioorg Med Chem.9; 1045-1057.

- Osseni, R.A., Rat, P., Bogdan, A., Warnet, J.M., Touitou, Y. 2000. Evidence of prooxidant and antioxidant action of melatonin on human liver cell line HepG2. Life Sci 15; 68:387-399.

- Pieri, C., Recchioni, R., Moroni, F., Marcheselli, F., Marra, M., Marinoni, S., Di Primio, R. 1998. Melatonin regulates the respiratory burst of human neutrophils and their depolarization. J Pineal Res. 24; 43-49.

- Radogna, F., Cristofanon, S., Paternoster, L., D'Alessio, M., De Nicola, M., Cerella, C., Dicato, M., Diederich, M., Ghibelli, L. 2008. Melatonin antagonizes the intrinsic pathway of apoptosis via mitochondrial targeting of Bcl-2. J Pineal Res. 44; 316-325

- Radogna, F., Paternoster, L., Albertini, M.C., Cerella, C., Accorsi, A., Bucchini, A., Spadoni, G., Diamantini, G., Tarzia, G., De Nicola, M., D'Alessio, M., Ghibelli, L. 2007. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res.  43; 154-162.

- Reiter, R.J., Tan, D.X., Terron, M.P., Flores, L.J., Czarnocki, Z. 2007. Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. Acta Biochim Pol. 54; 1-9.

- Reppert, S.M., Weaver, D.R, Ebisawa, T. 1994. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron. 13, 1177-1185.

- Rivara, S., Lodola, A., Mor, M., Bedini, A., Spadoni, G., Lucini, V., Pannacci, M., Fraschini, F., Scaglione, F., Sanchez, R.O., Gobbi, G., Tarzia, G. 2007. N-(substituted-anilinoethyl)amides: design, synthesis, and pharmacological characterization of a new class of melatonin receptor ligands. J Med Chem. 50;6618-26.

- Rodriguez, C., Mayo, J.C., Sainz, R.M., Antolín, I., Herrera, F., Martín, V., Reiter R.J. 2004. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res. 36; 1-9.

- Romero, M.P., García-Pergañeda, A., Guerrero, J.M., Osuna, C. 1998. Membrane-bound in Xenopus laevis oocytes as a novel binding site for melatonin. FASEB J. 12; 1401-1408.

- Rubio, S., Estévez, F., Cabrera, J., Reiter, R.J., Loro, J., Quintana, J. 2007. Inhibition of proliferation and induction of apoptosis by melatonin in human myeloid HL-60 cells.J Pineal Res. 42; 131-138.

- Shigeri, Y., Ishida, A., Sueyoshi, N., Kameshita, I. 2008. Ca2+/calmodulin-dependent protein kinase signaling: regulatory mechanisms for switching on/off and their involvement in the pathogenesis of various diseases.  Tanpakushitsu Kakusan Koso. 53; 1360-1367.

- Spadoni G, Balsamini C, Bedini A, Diamantini G, Di Giacomo B, Tontini A, Tarzia G, Mor M, Plazzi PV, Rivara S, Nonno R, Pannacci M, Lucini V, Fraschini F, Stankov BM. 1998. 2-[N-Acylamino(C1-C3)alkyl]indoles as MT1 melatonin

receptor partial agonists, antagonists, and putative inverse agonists. J. Med. Chem. 41 ; 3624-3634.

- Spadoni, G., Balsamini, C., Diamantini, G., Tontini, A., Tarzia, G., Mor, M., Rivara, S., Plazzi, P.V., Nonno, R., Lucini, V., Pannacci, M., Fraschini, F., Stankov, B.M. 2001. 2-*N*-acylaminoalkylindoles: design and quantitative structure-activity relationship studies leading to $MT_2$-selective melatonin antagonists. J Med Chem. 44; 2900-2912.

- Spadoni, G., Stankov, B., Duranti, A., Biella, G., Lucini, V., Salvatori, A., Fraschini, F. 1993. 2-Substituted 5-methoxy-N-acyltryptamines: synthesis, binding affinity for the melatonin receptor, and evaluation of the biological activity. J. Med. Chem. 36; 4069-4074.

- Tan, D.X., Manchester, L.C., Terron, M.P., Flores, L.J., Tamura, H., Reiter, R.J. 2007. Melatonin as a naturally occurring co-substrate of quinone reductase-2, the putative MT(3) melatonin membrane receptor: hypothesis and significance. J Pineal Res. 43; 317-320.

- Tarzia, G., Diamantini, G., Di Giacomo, B., Spadoni, G., Esposti, D., Nonno, R., Lucini, V., Pannacci, M., Fraschini, F., Stankov, B.M. 1997. 1-(2-Alkanamidoethyl)-6-methoxyindole derivatives: a new class of potent indole melatonin analogs. J Med Chem. 40; 2003-2010.

- Vanecek, J. 1998. Cellular mechanisms of melatonin action. *Physiol Rev*. 78; 687–721.

- Wiesenberg, I., Missbach, M., Carlberg, C. 1998. The potential role of the transcription factor RZR/ROR as a mediator of nuclear melatonin signaling. Restor Neurol Neurosci. 12; 143-150.

- Wölfler, A., Caluba, H.C., Abuja, P.M., Dohr, G., Schauenstein, K., Liebmann, P.M. 2001. Prooxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. FEBS Lett. 502; 127-131.

- Wurtman, R.J., Axelrod, J., Fischer, J.E. 1964. Melatonin Synthesis in the Pineal Gland: Effect of Light Mediated by the Sympathetic Nervous System. Science. 143, 1328-1329.

- Yuan, L., Collins, A.R., Dai, J., Dubocovich, M.L., Hill, S.M. 2002. MT(1) melatonin receptor overexpression enhances the growth suppressive effect of melatonin in human breast cancer cells. Mol Cell Endocrinol. 192; 147-56.

**Figures Legends**

**Fig. 1. Immediate and transient stimulation of intracellular reactive oxygen species**

(A) Flow cytometric analysis of ROS measured with DCHFDA for 24 h of treatment with 1mM melatonin; the insert magnifies the first 15 min. Values indicate fold-increase with respect to control posed =1; one of 3 independent experiments with similar results is shown. Panel B shows a flow cytometric analysis of ROS measured with DCHFDA after melatonin $\pm$ 1 mM trolox C. Results indicate fold-increase with respect to control posed =1 and are the average of 3 independent experiment +/- S.D. ROS increase by melatonin over control is highly significant (p=<0.01)**; ROS scavenging by Trolox is significant (p<0.05). Panel C shows a flow cytometric analysis of ROS measured with DCHFDA after 3 h of increasing doses of melatonin. ROS production is significant starting at 10uM (p<0.05)*, and highly significant at 1 mM.

**Fig. 2. ROS production does not involve mitochondria disturbance**.

Cells were pre-treated with the mitochondria complex I inhibitor rotenone; ROS were measured by flow cytometric analysis with DCHFDA after 3 h of 1 mM melatonin. Results indicate fold-increase with respect to control posed =1 and are the average of 3 independent experiment +/- S.D. The value of Mel and Mel+Rotenone do not significantly differ.

**Fig. 3. MT1/MT2 receptor-induced signal transduction is not involved in melatonin pro-oxidant activity.**

(A) U937 cells were pre-treated with the G-protein inhibitor *pertussis toxin* (PTX), or the MT1/MT2 antagonist luzindole, or the extracellular Ca2+ chelator EGTA, or the PLC inhibitor U73122, as indicated in methods; then 1 mM melatonin (mel) was added. ROS were evaluated with DCHFDA by flow cytometry at 3 h of 1 mM melatonin treatment. Results indicate fold-increase with respect to control posed =1 and are the average of 3 independent experiment +/- S.D. For each treatment, melatonin produces a significant increase with respect to its control (p<0.001) (B) Chemical structures of the melatonin analogues listed in table I C) Flow cytometric analysis of ROS measured with DCHFDA 3 h after addition of 1 mM melatonin or its synthetic analogues (see table I). Results indicate fold-increase with respect to control posed =1 and are the average of 3 independent experiment +/- S.D.

**Fig. 4. Possible involvement of calmodulin binding in melatonin-induced ROS production**.

U937 cells were pre-treated for 30 min with two calmodulin inhibitors, which inhibit (chlorpromazine) or not inhibit (calmidazolium) melatonin binding to calmodulin; 1 mM melatonin was then added. ROS were evaluated with DCHFDA at 3 h of melatonin. Results indicate fold-increase with respect to control posed =1 and are the average of 3 independent experiment +/- S.D. Melatonin+calmidazolium does not significantly differ from melatonin alone, whereas ROS abrogation by chlorpromazine over melatonin alone is highly significant $(p<0.01)$**.

**Fig. 5. The pro-radical effect of melatonin is general among leukocytes**

(A) Flow cytometric analysis of ROS measured with DCHFDA after 3 h of 1 mM melatonin treatment in normal or tumor haematopoietic cells. Results are the average of 3 independent experiment +/- S.D. ROS generation in all cell types examined is significant $(p<0.05)$.

**Fig. 6. Melatonin does not affect cell viability or proliferation**

(A) U937 cells were incubated with melatonin 1 mM and apoptosis was quantified every 24 h for 5 days. (B) U937 cells were incubated with 1 mM melatonin and cell number was quantified every 24 h for 5 days. One of 3 independent experiments with similar results is shown. (C) U937 cells cycle was analyzed as described in Methods at different times of 1 mM melatonin treatment. One of 3 independent experiments with similar results is shown.

**Fig. 7. The pro-radical effect of melatonin does not imply oxidative stress**

U937 cells were incubated with melatonin (Mel) 1 mM. For A, at the indicated time points, cells were lysed and proteins extracts were incubated with dinitrophenylhydrazine (DNP). After derivatization, proteins (20 ug) were loaded onto 10% SDS polyacrilamide gel and carbonyls residues detected by Western blot using a rabbit anti-DNP antibody. Alpha-tubulin was used as loading control. B): at the indicated time points, cells were prepared for GSH/GSSG assay by HPLC as described under Methods; the elution time ensures the recognition of GSH or GSSG over other small thiols according to the elution times of the standards. Data are expressed as nmol of GSH or GSSG/mg total protein and reported as means +/- S.D. (n=4). C) U937 cells were pre-treated with BSO as described in Materials and Methods; cells were then analyzed for apoptosis in the presence/absence of 1 mM melatonin (4 h) as described for fig. 6A. No significant toxicity was detectable in any condition; data are mean of 3 experiments ± SD. D)

BSO pre-treated U937 were analyzed for DCH signal in the presence/absence of 1 mM melatonin (2 h); the two pro-radical effects are additive. The data are the mean of 3 experiments ± SD.

**Table I**

| Numbers | Compounds | pKi MT1 | pKi MT2 | Intrinsic activity | pKi References |
|---|---|---|---|---|---|
| 1 | Melatonin | 9.5 | 9.4 | A | |
| 2 | UCM412 | 10.5 | 9.9 | A | (Mor et al. 2001) |
| 3 | UCM 608 | 10.7 | 10.4 | A | (Mor et al. 2001) |
| 4 | UCM 245 | 8.6 | 8.6 | A | (Spadoni et al.1998) |
| 5 | UCM92 | 8.8 | 9.2 | A | (Tarzia et al. 1997) |
| 6 | Luzindole | 6.8 | 8.0 | ANT | (Dubocovich et al.1977) |
| 7 | UCM 765 | 8.4 | 10.2 | PA | (Rivara  et al.2007) |
| 8 | UCM 231 | 5.9 | 6.2 | PA | (Mor et al. 1998) |
| 9 | UCM 454 | 5.9 | 8.1 | ANT | (Spadoni et al.2001) |
| 10 | UCM 353 | 4.8 | 5.0 | ANT | (Spadoni et al.2001) |

A: Agonist; PA: Partial Agonist; ANT: Antagonist
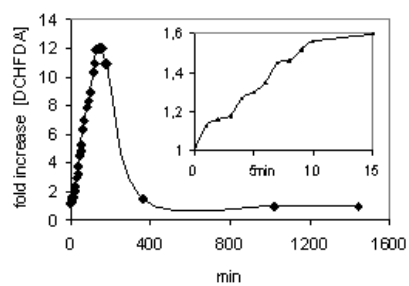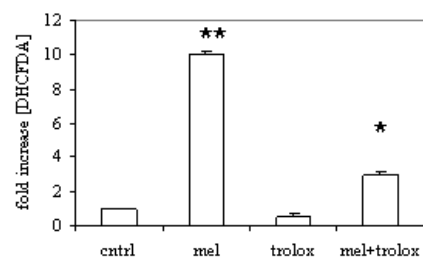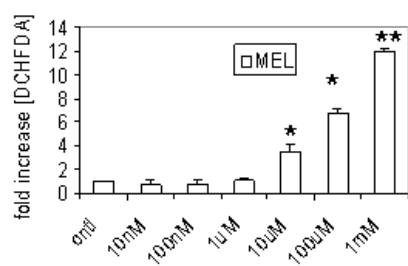
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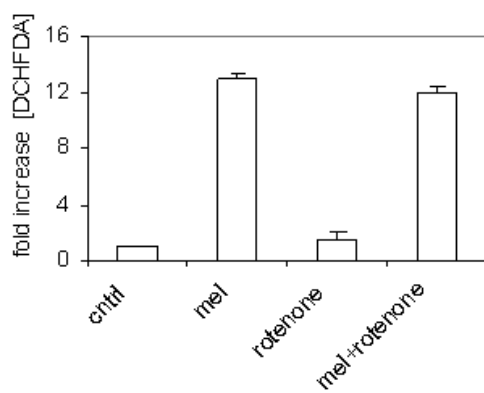
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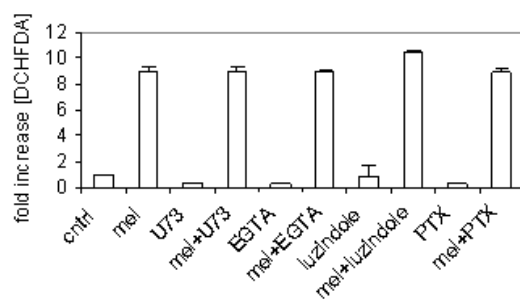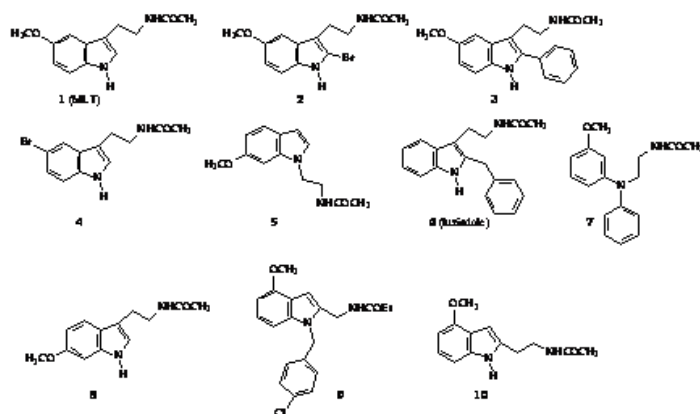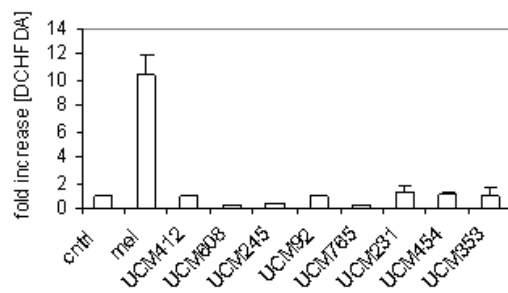
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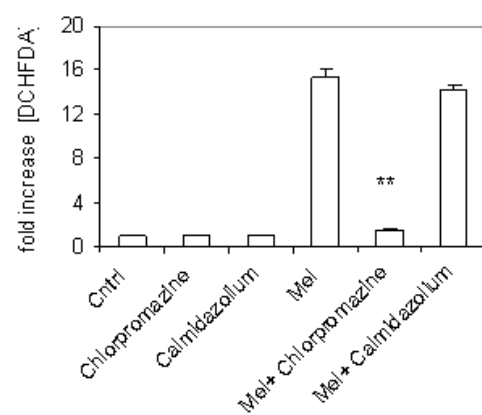
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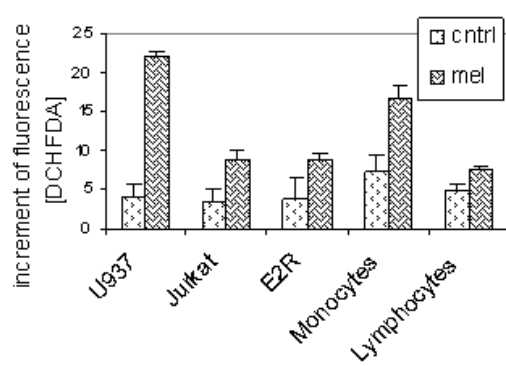
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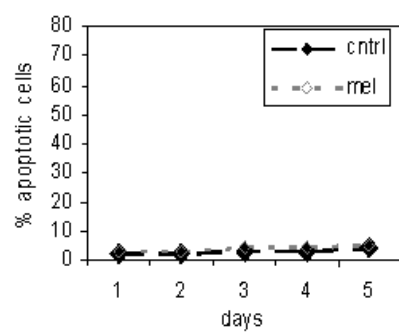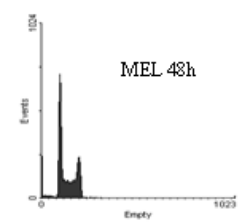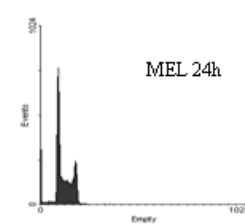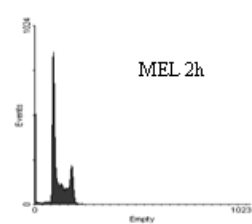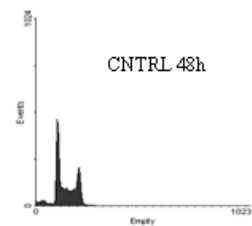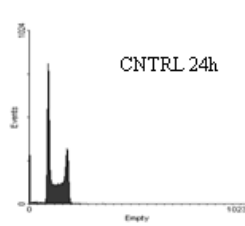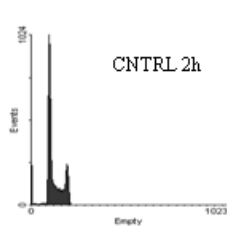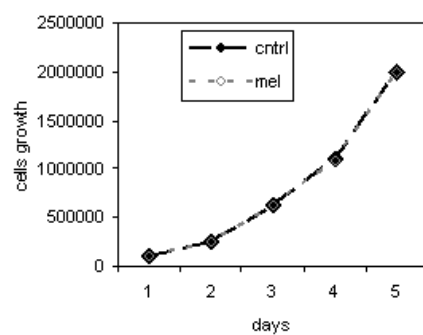
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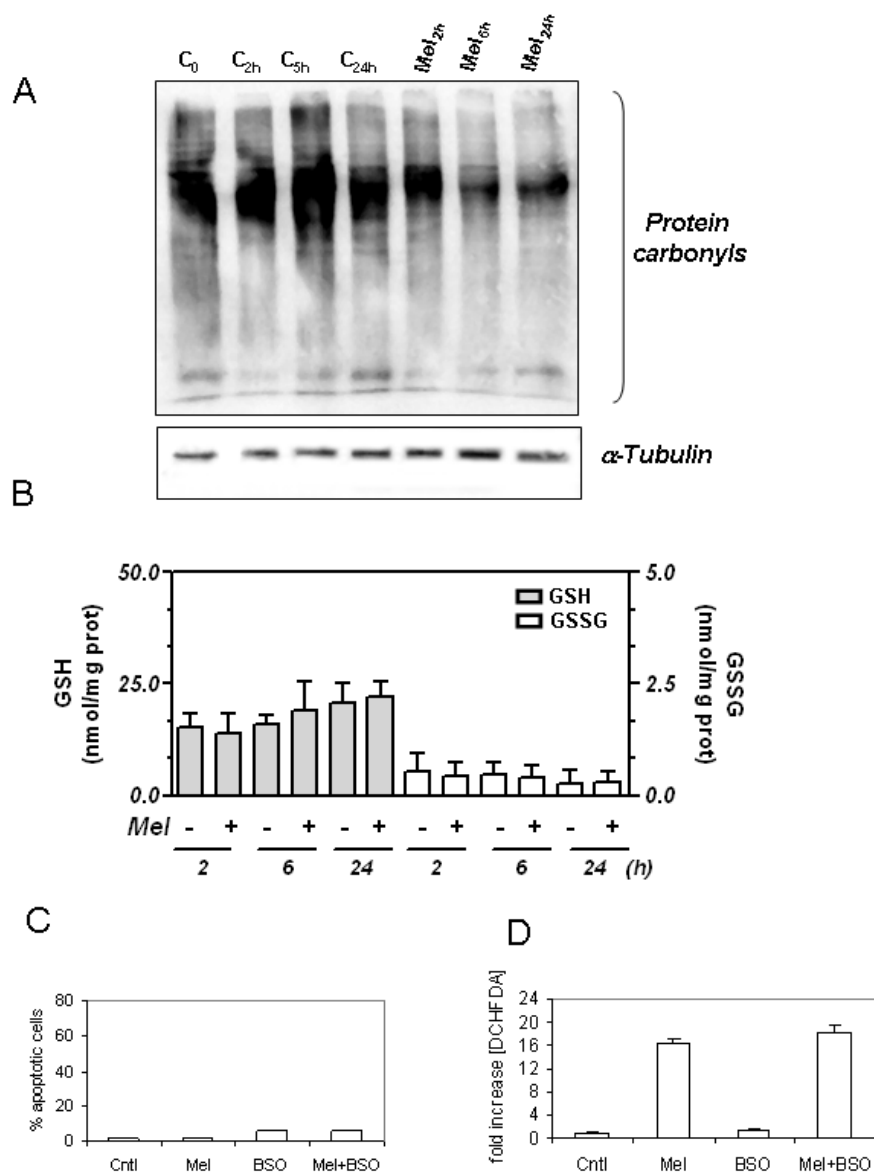
Fig.7

# PAPER VII

*Lipoxygenase-mediated pro-radical effect of melatonin via stimulation of arachidonic acid metabolism*

# LIPOXYGENASE-MEDIATED PRO-RADICAL EFFECT OF MELATONIN VIA STIMULATION OF ARACHIDONIC ACID METABOLISM

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**Key words:** U937, lipoxygenase, phospholipase A2, arachidonic acid, 5-HETE, calmodulin

**Running title**: Transient pro-inflammatory effect of melatonin

## 1. Abstract

We have shown that melatonin immediately and transiently stimulates intracellular free radical production on a set of leukocytes, possibly as a consequence of calmodulin binding. We show here that melatonin-induced ROS are produced by lipoxygenase (LOX), since they are prevented by a set of LOX inhibitors, and are accompanied by increase of the 5-LOX product 5-HETE. LOX activation is accompanied by strong liberation of AA; inhibition of Ca2+-independent, but not Ca2+-dependent, phospholipase A2 (PLA2), prevents both melatonin-induced arachidonic acid and ROS production, whereas LOX inhibition only prevents ROS, indicating that PLA2 is upstream with respect to LOX, as occurs in many signaling pathways. Chlorpromazine, an inhibitor of melatonin-calmodulin interaction, inhibits both ROS and arachidonic acid production, thus possibly placing calmodulin at the origin of a melatonin-induced pro-radical pathway. Interestingly, it is known that Ca2+-independent PLA2 binds to calmodulin: our results are compatible with PLA2 being liberated by melatonin from a steady-state calmodulin sequestration, thus initiating an arachidonate signal transduction. These results delineate a novel molecular pathway through which melatonin may participate to the inflammatory response.

## 2. Introduction

Melatonin, a neuro-hormone principally produced by the pineal gland (Wurtman et al. 1964), is being receiving much attention as a regulator of organism homeostasis; its potential therapeutic use in the treatment of many pathological conditions is a main goal of pharmacological research, since its natural origin would ensure lack of important side effects. From this point of view, very important was the discovery of its strong antioxidant ability (Rodriguez et al. 2004), since many critical pathologies are originated, or progress, in instances of insufficient radical scavenging. Melatonin biological activity results also from its binding to high (plasma membrane MT1/MT2 receptors) (Reppert et al. 1994) and low (quinone reductase, calmodulin) (Tan et al. 2007; Benítez-King et al. 1993) affinity targets within cells, in the nano- or micro-molar range, respectively. Thus, on the one hand, MT1/MT2 engagement triggers a canonical intracellular signal transduction (Radogna et al. 2007), whose results depend on the signaling and enzymatic asset of each target cell; on the other hand, binding to the cytosolic enzymatic targets alters the activity of the bound enzyme. It is not clear whether the high micromolar levels of melatonin required for quinone reductase (Tan et al. 2007) or calmodulin binding (Benítez-King et al. 1993) may be actually reached in the organism, thus questioning whether these interactions may have a physiological meaning.

Recently, increasing evidences showed that many non-neuroendocrine tissues produce, and respond to, melatonin; this is especially true for the white blood cells compartment (Carrillo-Vico et al. 2006), in which melatonin seems to play a role in controlling their number and functioning. This proposes melatonin as a possible modulator of the inflammatory/immune response. Indeed leucocytes possess all the enzymatic machinery necessary to synthesize melatonin from tryptophan (Cubero et al. 2006) as well as the proper receptors (Carrillo-Vico et al. 2004), thus being an autonomous compartment as far as melatonin responses are concerned. Many evidences suggest that melatonin may exert an anti-inflammatory effect; it has been proposed that melatonin antioxidant ability may contrast the onset and progression of inflammation, phenomenon that implies production of reactive oxygen species and activation of pro-oxidant enzymes (Cuzzocrea et al. 2002). Since the antioxidant effect of melatonin improves at supra-physiological doses, this opens an important avenue for the pharmacological use of melatonin as an anti-

inflammatory adjuvant.

The inflammatory response begins with an insult that activates and recruits leukocytes to the inflamed tissue through the fine tuning of a huge set of molecular mediators; an important role in the early events is played by phospholipase A2 (PLA2), an enzyme that cleaves membrane phospholipids liberating membrane bound arachidonic acid (AA), which is processed by cyclooxygenases (COX) and lipoxygenases (LOX) to produce important inflammatory mediators such as prostaglandins and leukotrienes (González-Périz et al. 2007), respectively. In examples of experimental inflammation induction in vivo and ex vivo, melatonin was shown to prevent/contrast the up-regulation or activation of all the enzymes involved in the arachidonate response, PLA2 (Li et al. 2000), LOX (Zhang et al.1999) and COX (Deng et al. 2006). In some instances, i.e., reduction of PLA2 up-regulation, the mechanism involved may depend on non-oxidative means, such as the engagement of MT1/MT2 receptors (Li et al. 2000); thus, many different actions of melatonin (i.e., receptor stimulation, radical scavenging) seem to cooperate to achieve a common final goal (i.e., contrast the inflammatory cascade).

In spite of the clear chemical anti-oxidant action of melatonin, many reports indicate a surprising pro-oxidant activity that may occur within cells (see accompanying paper; Albertini et al. 2006; Buyukavic et al.2006; Clappy-Lilly et al. 2001). The issue is still unclear, many perplexities arising from the paradox that an anti-oxidant molecule may, in a biological environment, behave as a pro-oxidant. With the goal of clarifying the issue, we analyzed the possible mechanisms involved in the pro-radical effect of melatonin; in the accompanying paper, we show that a set of normal and tumor leukocytes immediately (<1min) and transiently (up to 4-5 h) produce reactive oxygen species in response to melatonin treatment, and that this requires high micromolar doses; a mechanistic analysis on U937 tumor monocytes revealed that the pro-radical effect is a cell reaction rather than a direct chemical effect, thus possibly solving the apparent contradiction of the opposite effects of melatonin on oxidations. This cell reaction does not involve stimulation of the MT1/MT2 receptor, possibly requiring instead binding to calmodulin. In this study, we investigate on the origin of melatonin-produced ROS, showing that they result from the sequential activation of PLA2 and LOX; the notion that PLA2 can be sequestered by melatonin gives a rationale for the role of calmodulin binding as an event that liberates

PLA2, thus triggering the arachidonate metabolism; this delineates a novel intracellular signaling pathway triggered by melatonin that may contribute to clarify the possible role played by melatonin in the onset of inflammation.

## 3. Methods

### 3.1 Cell culture

U937 are human tumor monocytes stabilized from a histiocytic lymphoma. Jurkat are a human leukemic T-cell line. Cells were cultured in RPMI medium supplemented with 10% FCS. Cells were routinely checked for absence of mycoplasm by using a mycoplasm detection kit (Mycoalert TM., Cambrex Bio Science Milano, Italy). The experiments were performed on cells in the logarithmic phase of growth under condition of ≥ 98% viability, as assessed by trypan blue exclusion. Peripheral blood mononuclear leukocytes (PBML) were isolated from heparinized blood samples of healthy individuals by collection with Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. Red cells were removed by hypotonic lysis. PBML were then plated in RPMI 1640 plus 10% human AB serum in culture flasks (pre-treated with the same human AB serum) to promote monocytes adhesion. After 2 h, floating cells (lymphocytes) were washed out, spinned down and separately plated in RPMI 1640 plus 10% human serum. Adherent monocytes were washed 3 times with RPMI to remove residual lymphocytes, detached by cell scraping, and resuspended in RPMI 1640 plus 10% FCS. Treatments (see below) were performed at 20-24 h post-separation. Cell viability in both fractions was >98% as assessed by trypan blue exclusion test. Purity of the enriched fractions was controlled by labelling (20min at room temperature) with cell-type specific antibodies: FITC-conjugated-anti-CD45 (from Becton-Dickinson) for lymphocytes; and PE-conjugated-anti-CD14 (from Becton-Dickinson) for monocytes. Cells were then washed with PBS and labelling evaluated. Both monocyte and lymphocyte fractions were >95% pure.

### 3.2 Analysis of ROS

Cells were loaded with 10 uM dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes), 2 uM dihydrorhodamine (DHR, Molecular Probes) or 5 uM dihydroethidium (DHE, Molecular Probes) by incubation at 37° for 30 min after melatonin treatments. These probes are non-fluorescent cell-permeable compounds; once inside the cell, they are de-esterified and turn fluorescent upon oxidation, fluorescence being proportional to ROS

production. Analyses were performed by flow cytometry using FACScan Becton&Dickinson.

*3.3 Measurement of extracellular release of [3H]-arachidonic acid (AA)*

Cells were labeled with $[^3H]$-AA (0.5 µCi/ml, Amersham) and grown for 18 h. Before treatments, the cells ($2x10^5$) were washed twice with saline A, supplemented with 0.3% fatty acid-free bovine serum albumin and resuspended in a final volume of 1 ml of complete culture medium. The cell suspension was then separated and centrifuged at 5000 x g for 3 min; 900 µl of the resulting supernatant were removed and radioactivity was determined in a Packard liquid scintillation counter. The data are given as extent of 3H-arachidonic acid derived radiolabel released; for brevity, in the legend of each figure this is named "arachidonic acid release" even though no molecular determination of the tritiated materials released is presented.

The calcium ionophore ionomycin was included in each set of experiments as a positive control to promote maximal AA release (Hoffman et al. 1990); it was used at 2 uM and AA release measured after 30 min.

*3.4 Reverse Phase-HPLC analysis of 5-HETE*

Reversed-phase HPLC (RP-HPLC) is a useful technique for analyzing LOX products and an isocratic elution separation has been performed (Jacobsonn et al. 1991). We used a C18, 5-um nucleosil column (0.46 x 25 cm) to elute the extracts at a flow rate of 1.2 ml/min with methanol/water/TFA (70:30:0.007 by volume) and by UV detection at 270 nm. The samples were also co-chromatographed with the 5-HETE standard (furnished by Biomol International) to identify the elution peak of interest and quantified by the peak area. 5-HETE concentration was calculated by using the peak area of the standard.

*3.5 Melatonin and other treatments*

Melatonin was purchased from Sigma Chemical Co (St. Louis, MO, USA), and used at the concentration of 1 mM unless otherwise specified. Melatonin action on $MT_1/MT_2$ receptors was antagonized with 50 uM 2-benzyl-N-acetyltryptamine (luzindole) (Sigma)**,** a

concentration proved able to antagonize 1mM melatonin in previous studies (Durati et al. 1992), which was added 30 min before the other treatments. For lipoxygenase inhibition a general LOX inhibitor (CAPE) was used at 17 uM, a specific inhibitor of 5-LOX (AA861) acting with a redox mechanism was used at 20 uM and an inhibitor of the adaptor protein FLAP, (MK886), was used at 30 uM. Any of these compounds was added 30 min before treatments. For PLA2 inhibition was used a general PLA2  4-bromophenacil bromide (BPB, 50 uM) and the Ca2+-independent PLA2 inhibitor bromoenol lactone (bel, 50 uM) purchased from Sigma and a cytosolic PLA2 (cPLA2) inhibitor arachidonyl trifluoromethyl ketone (AACOCF3, 5 uM) added 30 min before other treatments. Possible necrogenic effect of the inhibitors were monitored with the trypan blue exclusion test; no toxicity was detected for the doses and times for all the inhibitors. For calmodulin inhibition calmidazolium (Sigma) and chlorpromazine (which inhibits melatonin-specific binding site to calmodulin, Sigma) were used at 0.5 uM.

*3.6 Statistical analysis*

The results are presented as means +- SD. Statistical evaluation was conducted by a one-way ANOVA, followed by the Student-Newman-Keuls multiple comparisons. Significance level was fixed at alfa=0.05.

## 4. Results

*4.1. Melatonin produces reactive oxygen species by stimulating lipoxygenase*

We have described an immediate and transient intracellular pro-radical effect of melatonin, evaluated as the increment of dichlorofluorescine (DCF) signal (see accompany paper). Re-addition of 1mM melatonin after 5 h from the initial challenge, i.e., after the peak of DCF signal is over, produces a new burst of radicals with similar duration (~2 h) as the first one, but much lower intensity (fig. 1A). This indicates that a desensitization of the target has occurred, supporting the notion that the effect is due to the stimulation of an intracellular signal. This prompted us to investigate the nature of such a signal, exploring the possible involvement of pro-oxidant enzymes.

It was recently shown that, in addition to ROS detection, DCFH (the reduced form of DCF) is a direct substrate of the pro-oxidant enzyme lipoxygenases (LOX) (Pufahl et al. 2007), and that DCF fluorescence may be a function of LOX activity (Pufahl et al. 2007). Thus, we analyzed the effect of two additional, LOX-independent oxidation-sensitive probes, namely dihydrorhodamine (DHR) and dihydroethidium (DHE); the 3 probes recognize a wide spectrum of ROS, DCFDA and DHR being more sensitive to $H_2O_2$ and OH•, DHE to anion superoxide. Melatonin increases the fluorescence of these additional probes (fig. 1B), confirming the notion of melatonin as an intracellular pro-oxidant agent. However, the extent of DCF fluorescence is much higher than DHR and DHE. Thus, the different sensitivity to DCF vs. DHR and DHE might be diagnostic for LOX activity; indeed, DCF signal may be the sum of a frank oxidation (the extent shared by DHR and DHE) plus a direct interaction with LOX.

To explore the issue, we measured melatonin-produced ROS in the presence of a set of LOX inhibitors, i.e., a general LOX inhibitor (CAPE), a specific inhibitor of 5-LOX (AA861), and the inhibitor of the adaptor protein FLAP, (MK886), which prevents 5-LOX feeding of available arachidonic acid. All these inhibitors abolished the pro-radical effect of melatonin (fig. 1C). Inhibition of other pro-radical enzymes such as cycooxygenase, does not abrogate melatonin-produced ROS (see below) indicating that LOX activation may be the mechanism through which melatonin produces intracellular ROS.

In support to this, we measured the intracellular concentration of 5-HETE, one of the main products of 5-LOX. As shown in Fig. 1D, melatonin produces a substantial

increase of 5-HETE at 2 and 6 h, that decreases at 24 h below control levels. This shows that melatonin activates 5-LOX, and that the effect is transient in the same guise as the DCF signal.

In order to ascertain whether 5-HETE production was the result of LOX activity, we analyzed whether CAPE or AA861 may prevent their accumulation following melatonin treatment. We observed that in the presence of CAPE or AA861 melatonin is no longer able to produce 5-HETE: both inhibitors slightly reduced the basal 5-HETE levels (by about 15%), and strongly reduced melatonin-induced increase (from 1.7 to 1.1 folds, results not shown). This shows that the increase of 5-HETE by melatonin is the result of increased LOX activity.

*4.2. Melatonin promotes lipoxygenase-mediated reactive oxygen species formation in other leukocytes.*

To understand if the pro-radical effect of melatonin is a general phenomenon among leukocytes, we examined a panel of cells of haematopoietic origin, i.e., lymphocytes and monocytes freshly explanted from healthy donors, and the human tumor T lymphocytic Jurkat. 1mM melatonin was able to produce a DCF signal on all of these cells, which was prevented by CAPE (or AA861, which completely prevents melatonin-dependent ROS in Jurkat cells), showing that it occurs with a similar mechanism, i.e., stimulation of LOX, on all the tested leukocytes (figure 2).

*4.3. Melatonin stimulates the release of arachidonic acid (AA)*

To understand the mechanism of LOX activation by melatonin, we explored whether it could derive from an increased availability of its substrate AA.

We measured the extent of AA release by quantifying the radio-labeled material released after incubating cells for 24 h with tritiated arachidonic acid (see materials and methods); this value is in fact the sum of arachidonic acid as such plus its molecular derivatives elaborated by AA-metabolizing enzymes (COX and LOX), thus providing the total measure of the extent of AA liberation. We compared the extracellular accumulation of radiolabel derived from membrane-bound AA released by untreated vs. melatonin-treated U937. Fig. 3A shows that 1mM melatonin determines a strong release of AA; this is

a very early event, which peaks at 60min. A dose-effect experiment (fig. 3B) reveals that stimulation requires at least 10 uM melatonin, being significant at 1 mM. When comparing ROS production and AA release, it becomes evident that the two phenomena are correlated, occurring with the same kinetics and requiring the same doses of melatonin. Ionomycin was used as a positive control; curiously, the addition of an excess of cold AA in the assay did not impair the ability of ionomycin to promote liberation of AA, whereas it completely prevented the effect of melatonin. This suggests that the enzymes involved in the two types of stimulation may be different, with different sensitivity to end-product inhibition.

*4.4. Ca2+-independent PLA2 mediates melatonin-induced AA release and ROS production*
To understand whether AA is released by PLA2, and in case by which isoform, we analyzed the effect exerted by different inhibitors of PLA2 on ROS production and AA release.

We found that melatonin-produced ROS are abrogated by the general PLA2 inhibitor brophenacil bromide (BPB). In the search for the PLA2 isoform actually involved, we used bromoenol lactone (BEL), an inhibitor of the Ca2+-independent form (iPLA2); BEL prevented ROS production by melatonin, indicating that this isoform of PLA2 is involved (see fig. 4A).

BEL also abrogated melatonin-induced AA release (see fig. 4B), supporting the involvement of the Ca2+-independent form of PLA2. For AA analysis (which unlike DCF signal does not imply fluorescence detection), we could also make use of the fluorescent compound arachidonyl-trifluoromethyl ketone (AACOCF3), which specifically inhibits Ca2+-dependent PLA2 (cPLA2) at the concentration used (5 uM) (Kim et al., 1997). Interestingly, AACOCF3 does not prevent melatonin-induced AA release (fig. 4B), indicating that the latter isoform is not implicated. In order to verify the ability of AACOCF3 to actually inhibit cPLA2 in our system, we tested the compound on ionomycin, a well established activator of cPLA2: in this instance, 5 uM AACOCF3 inhibits AA release by about 65% (not shown), reinforcing the notion that melatonin and ionomycin stimulate AA release by activating different forms of PLA2.

Overall, these results indicate that AA is liberated by melatonin through PLA2, and specifically by iPLA2.

Since AA is a substrate of LOX, its release should be an upstream event with respect to ROS production; thus, we expect that inhibitors of LOX prevent ROS but not melatonin-induced AA. Indeed, as shown in fig. 4C, AA861 not only allows, but even increases the extent of AA release; this piece of data shows that AA release is upstream to ROS production, as expected. The increase of AA release by AA861 is in line with the notion that if AA is freely released, its LOX-peroxidized derivatives are substantially retained.

AA is also a substrate for cyclo-oxygenase (COX); thus, we explored the effects of COX inhibition with indomethacine in melatonin-induced AA release and ROS production. Indomethacine increases the levels of released AA, thus behaving as the LOX inhibitor (fig. 4C); this is conceivable and in line with the notion that also COX-elaborated AA products are retained. Intriguingly, indomethacin did not abrogate, but even increased, the DCF signal produced by melatonin (not shown). This indicates that COX inhibition possibly leaves more AA available to LOX, implying that COX activity does not substantially contribute to melatonin-induced increase in DCF signal, which is essentially due to increase LOX activity.

*4.5. Possible involvement of calmodulin in the melatonin-induced AA/ROS pathway*

As the next step, we explored how melatonin could activate iPLA2. It is known that this isoform may bind to calmodulin, thus being sequestered in the cytosol; its liberation leads to membrane translocation and activation (Jenkins et al. 2001). But also melatonin binds to calmodulin (Benítez-King et al. 1993), and we have shown that this interaction may initiate the pro-radical activity of melatonin (see accompanying paper). All this evidence strongly pushed us examining whether also AA release may depend on calmodulin-melatonin interaction.

To this purpose, we used two inhibitors of calmodulin, chosen according to their ability of preventing or not melatonin binding, namely chlorpromazine or calmidazolium, respectively, as already described (Romero et al. 1998; and accompanying paper). The two inhibitors were used to challenge AA release (fig. 5A), and compared to their effect on ROS production (fig. 5B). Both parameters are affected by chlorpromazine, being instead unaffected by calmidazolium. This result is compatible with an involvement of calmodulin

as the initial target of the pathway leading to PLA2 and LOX activation and ROS formation.

As a control, we probed also luzindole, the specific MT1/MT2 antagonist; luzindole was shown not to affect ROS production; we show here that luzindole is also ineffective in preventing melatonin induced AA release (fig. 5A and B), confirming that the MT1/MT2 plasma membrane receptors are not involved in the pro-radical signaling pathway.

## 5. Discussion

The apparent contradiction that a well known anti-oxidant such as melatonin may behave as a pro-oxidant, may be more easily accepted by the finding that the pro-radical effect is not a direct chemical pro-oxidant ability of melatonin, but the result of the stimulation of a signaling pathway (via calmodulin-PLA2-5-lipoxygenase), thus implying that the biological pro-radical effect and the chemical radical scavenging activity of melatonin may actually co-exist.

We show here that melatonin-produced ROS derive from the activation of LOX, by many orders of evidences: first, it is prevented by all of the LOX inhibitors tested (CAPE, AA861, MK866); second, is marked by a transient increase in the intracellular levels of 5-HETE; third, the differential stimulation that melatonin exerts on the probe DCF over the DHR or DHE (12 fold vs. 2-3 fold): DCFH is a direct substrate for the enzyme, and on this basis, a fluorescence-based enzyme assay of human 5-lipoxygenase has been developed in *in vitro* systems (Pufahl et al. 2007); also in *in vivo* systems, a similar correlation with LOX expression and DCF over DHE fluorescence was recently reported (Belfiore et al. 2007). Thus, our results suggest that DCF fluorescence increase by melatonin is partly due to oxidation by ROS, and partly to be ascribed with direct interaction with LOX. Our data point to the activation of 5-LOX; however, the involvement of 5-LOX over other subtypes (i.e., 15 or 12) is not unequivocal from our results. FLAP is as a specific protein link between AA and 5-LOX, and MK866 is considered a specific FLAP inhibitor; however, many studies report that MK866 prevents LOX activity also in cells lacking FLAP (Uz et al. 2008; Hatzelmann et al.1994). Moreover, AA861, considered a specific inhibitor of 5-LOX (Hassan and Carraway, 2005), is reported to inhibit also other isoforms (Nagasawa et al. 2007). Inter-conversion of HETE after the double bond is formed is beginning to be described (Petrich et al. 1996), thus possibly rendering the measurement of 5-HETE less a stringent proof for 5-LOX activation than previously thought. It is important to mention that some clones of U937 cells seem not to express 5-LOX at the protein or mRNA level (Maccarrone et al. 2003). Thus, if our findings do show LOX activation by melatonin, further analysis will be necessary to demonstrate which isoform is actually involved.

The ability of melatonin to elicit the activation of LOX provides a possible mechanism to explain why the pro-radical effect of melatonin does not create any toxicity

to U937 cells (see accompanying paper). Indeed 5-LOX has been shown to supply pro-survival signals to many cells (Belfiore et al. 2007; Zhou et al. 2007; Wu et al. 2003); in tumor cells, LOX has been implicated in chemo-resistance (Zhou et al. 2007; Wu et al. 2003); we have reported that 5-LOX expression is required to maintain viability of EBV-converted B lymphocytes, its inhibition being a rapid and efficient mean of killing EBV+ cells (Belfiore et al. 2007); all these findings have suggested the notion that LOX may play a role in facilitating tumor progression (Pidgeon et al. 2007). We have recently shown that freshly explanted blood monocytes require LOX-derived signals and ROS to maximize in vitro maturation and viability by triggering over-expression of Bcl-2 (Cristofanon et al. 2008), suggesting that LOX may control survival also in normal cells.

The molecular mechanism of LOX activation is a still obscure issue; the current opinion is that it can be activated by pro-inflammatory agents via increased Ca2+ influx (Ford-Hutchinson et al. 1994). As far as the mechanism of LOX activation by melatonin, we have shown that melatonin does stimulate Ca2+ influx (Radogna et al. 2007), but its inhibition with EGTA does not affect melatonin pro-radical effect (accompanying paper), implying that this is not the mechanism for LOX activation by melatonin. We found that a large amount of AA is released upon melatonin treatment thus possibly being made accessible to LOX; it remains to be explored whether the plain increased availability of AA is sufficient for LOX activation, or if an additional undiscovered effect of melatonin is required. We did not explore whether the other major users of AA, i.e., cyclooxygenase, may also be activated by such a large availability of substrate; however, the finding that indomethacine increases melatonin-produced DCF signal may be interpreted in the sense of competition between LOX and COX for the same substrate, implying that COX might be activated by melatonin in the same guise as LOX, thus recommending further analysis. In particular, it will be interesting to analyze the AA-derived molecular species liberated by melatonin, both within and outside the cell, to understand which molecular pathways undergoes the tritium signal derived from incorporation of 3H-arachidonic acid. This may provide information about the involvement of each AA-modifying enzymes acting downstream of AA liberation, thus allowing to get insight about the possible role of melatonin in modulating the inflammatory response.

The major responsible for the liberation of AA from the phospholipids bi-layer is

PLA2, which exists in various isoforms that are differently activated and are sensitive to different inhibitors (Yedgar et al. 2006; Balsinde et al., 1999). Functional criteria for identifying the isoforms are involved in the phenomena under study are based on the sensitivity to the couple of inhibitors BEL vs. AACOCF3: if the former is reported to be specific for iPLA2, the latter is specific for the cPLA2 only at low concentrations ($\leq$5 uM, Kim et al., 1997), whereas at higher concentrations also iPLA2 results inhibited; thus, sensitivity to BEL and lack of sensitivity to AACOCF3 is diagnostic for iPLA2 involvement (Alzola et al., 1998). In our system, the inhibitors pattern that modulates melatonin-induced AA release and ROS production indicates that the form of PLA2 involved is the Ca2+-independent. This was a very important point in this study, because it is known that this isoform can be bound and sequestered by calmodulin, and that its release is an activator event (Jenkins et al. 2001). This provides a possible interpretation of the mechanism through which melatonin may activate PLA2 and LOX, since calmodulin is one of the low affinity targets of melatonin. The melatonin/calmodulin binding was found sensitive to the calmodulin inhibitor chlorpromazine but not calmidazolium (Romero et al. 1998); the specific action of these inhibitors led the authors to suggest that melatonin binds to the calmodulin domain interacting with protein partners (Romero et al. 1998). We explored the possibility that melatonin pro-radical effect may be actually triggered upon binding to calmodulin, and presented two evidences in support. First, melatonin binds to calmodulin at concentrations (Kd=63uM (Romero et al. 1998)) compatible with the melatonin concentrations required for the pro-radical effect and the AA release (see fig. 1 and fig. 3). Second, our analysis showed that also AA release and ROS production are sensitive to chlorpromazine but insensitive to calmidazolium. These evidences suggest for the first time a possible function for the high-dose calmodulin binding. We hypothesize the existence of a pathway where melatonin binds to calmodulin, thus inducing the release of sequestered Ca2+-independent PLA2; this is thus free to move to membranes and release high doses of AA; in turn, liberated AA feeds 5-LOX to produce free radicals and 5-HETE (see fig. 6).

The finding that leukocytes possess the machineries necessary both for synthesizing (Carrillo-Vico et al. 2004) and responding to (Carrillo-Vico et al. 2004) melatonin, provides a rationale for its potential physiological role as a modulator of the immune and

inflammatory responses (Carrillo-Vico et al. 2006; Liebmann et al. 1997) recommending for possible pharmacological applications to reach higher, more effective doses.

In particular, it has been shown that melatonin down-regulates, or inactivates, the pro-inflammatory enzymes PLA2 (Li et al. 2000) and LOX (Zhang et al. 1999, Steinhilber et al. 1995). In this context, the activation of the same enzymes, LOX and PLA2 that we report here, may seem a paradox. However, we have shown that the melatonin-induced increases of ROS, 5-HETE and AA are all transient phenomena. This is consistent with single-hit LOX stimulation: indeed, LOX participates to the early phases of the inflammatory response, since leukotrienes promote diapedesis by acting as vessel permeabilizing agents and chemo-attractants. In the absence of further signals, LOX effects are of short duration, and extinguish within 2-3 h: this coincides with the time required for the decrease of DCF signal and AA after melatonin treatment. We have also shown that re-addition of new melatonin 5 h after the first treatment produces a weaker stimulation of DCF fluorescence, indicating that a de-sensitization of the signal has occurred. It is worth mentioning that melatonin was shown to rapidly down-regulate 5-LOX via interaction with ROR/RZR (melatonin high affinity nuclear receptors) (Steinhilber et al. 1995), and PLA2 via MT1/MT2 receptors (Li et al. 2000). Thus, it is conceivable that the extinguishment of the DCFDA signal that we report here may be due to down-regulation of either 5-LOX or PLA2. In line with this is the finding that the levels of 5-HETE at 24 h of melatonin are lower than untreated cells (see fig. 1D).

A conciliation between our results with those available in the literature suggests that melatonin may transiently activate LOX via calmodulin binding, while working for its silencing via other targets/mechanisms; an important implication of this reasoning would be that melatonin might facilitate promoting the inflammatory response, at the same time limiting it by providing to its resolution via down-regulation of LOX (or PLA2), thus helping avoiding complications of chronic inflammation. Indeed, if LOX is required for a correct inflammatory response, its altered/prolonged activation may favor chronic inflammation and the onset of extremely serious diseases such as atherosclerosis, tumor progression, infarction (Pidgeon et al. 2007; Danielsson et al. 2008; Sexton et al. 2007).

A molecular pathway such as those here described, if provides a physiological meaning for melatonin binding to calmodulin, leaves unsolved the problem that it requires

supra-physiological doses; however, it was recently discovered that bone marrow concentrations of melatonin are much higher that those found in the blood stream (Tan et al.1999). It would be interesting to explore if even higher micro-environmental doses might be reached in specific areas, thus possibly providing a physiological/physiopathological role for the very high melatonin concentrations required for the pro-radical effect presented here and in other studies (Albertini et al. 2006; Buyukavic et al.2006; Osseni et al. 2000).

The overlapping of the many effects exerted by melatonin, i.e., radical scavenging, ROS promotion, stimulation of one or another of the intracellular targets, dose and persistence of melatonin around target cells, combined with different cell sensitivities, produce a huge variety of effects in different compartments of the organism, suggesting a highly sophisticated fine tuning. This deserves much attention for focusing on possible therapeutic effects of melatonin as such, and for the design of specific derivates that may maintain only selected actions of melatonin, according to the requirement of the specific pathologies/disturbances to be treated.

**References**

- Aggarwal, B.B., Shishodia, S. 2006. Molecular targets of dietary agents for prevention and therapy of cancer.Biochem Pharmacol. 71;1397-13421.

- Albertini, M.C., Radogna, F., Accorsi, A., Uguccioni, F., Paternoster, L., Cerella, C., De Nicola, M., D'Alessio, M., Bergamaschi, A., Magrini, A., Ghibelli, L. 2006. Intracellular pro-oxidant activity of melatonin deprives U937 cells of reduced glutathione without affecting glutathione peroxidase activity. Ann N Y Acad Sci. 1091; 10-16.

- Alzola, E., Pèrez-Etxebarria, A., Kabrè, E., Fogarty, D.J., Mètioui, M., Chaib, N. , Macarulla, J.M., Matute, C.,. Dehaye J.P,  Marino, A. 1998. Activation by P2X7 agonists of two phospholipases A2 (PLA2) in ductal cells of rat submandibular gland. J.B.C. 273; 30208–30217.

- Balsinde, J., Balboa, M.A., Insel, P.A., Dennis, E.A. 1999. Regulation and inhibition of phospholipase A2.  Annu Rev Pharmacol Toxicol. 39; 175-189.

- Belfiore, M.C., Natoni, A., Barzellotti, R., Merendino, N., Pessina, G., Ghibelli, L., Gualandi, G. 2007. Involvement of 5-lipoxygenase in survival of Epstein-Barr virus (EBV)-converted B lymphoma cells. Cancer Lett. 254; 236-243.

- Benítez-King, G., Huerto-Delgadillo, L., Antón-Tay, F. 1993. Binding of 3H-melatonin to calmodulin. Life Sci. 53; 201–207.

- Büyükavci, M., Ozdemir, O., Buck, S., Stout, M., Ravindranath, Y., Savaşan, S. 2006. Melatonin cytotoxicity in human leukemia cells: relation with its pro-oxidant effect. Fundam Clin Pharmacol.; 20:73-79.

- Carrillo-Vico, A., Calvo, J.R., Abreu, P., Lardone, P.J., Garcia-Morino, S., Reiter, R.J., Guerrero, J.M. 2004. Evidence of melatonin synthesis in human lymphocytes and its physiological significance: possible role as intracrine, autocrine and/or paracrine substance. FASEB J. 18;537–539,

- Carrillo-Vico, A., Reiter, R.J., Lardone, P.J., Herrera, J.L., Fernández-Montesinos, R., Guerrero, J.M., Pozo, D. 2006. The modulatory role of melatonin on immune responsiveness. Curr Opin Investig Drugs. 7; 423-431.

- Clapp-Lilly, K.L., Smith, M.A., Perry, G., Harris, P.L., Zhu, X., Duffy, L.K. 2001. Melatonin acts as antioxidant and pro-oxidant in an organotypic slice culture model of Alzheimer's disease. Neuroreport. 12;1277-1280.

- Cristofanon, S., Nuccitelli, S., D'Alessio, M., Dicato, M., Diederich, M., Ghibelli, L. 2008.Oxidation-dependent maturation and survival of explanted blood monocytes via Bcl-2 up-regulation. Biochem Pharmacol.

- Cubero, J., Valero, V., Narciso, D., Rivero, M., Marchena, J.M., Rodríguez, A.B., Barriga, C. 2006. L-tryptophan administered orally at night modifies the melatonin plasma levels, phagocytosis and oxidative metabolism of ringdove (Streptopelia roseogrisea) heterophils. Mol Cell Biochem. 293;79-85.

- Cuzzocrea, S., Reiter, R.J. 2002. Pharmacological actions of melatonin in acute and chronic inflammation. Curr Top Med Chem. 2; 153-65.

- Danielsson, K.N., Rydberg, E.K., Ingelsten, M., Akyürek, L.M., Jirholt, P., Ullström, C., Forsberg, G.B., Borén, J., Wiklund, O., Hultén, L.M. 2008. 15-Lipoxygenase-2 expression in human macrophages induces chemokine secretion and T cell migration. Atherosclerosis. 199; 34-40.

- Deng, W.G., Tang, S.T., Tseng, H.P., Wu, K.K. 2006. Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding. Blood. 108; 518-24.

- Duranti, E., Stankov, B., Spadoni, G., Duranti, A., Lucini, V., Capsoni, S., Biella, G., Fraschini, F. 1992. 2-Bromomelatonin: synthesis and characterization of a potent melatonin agonist. Life Sci. 51; 479-485.

- Ford-Hutchinson, A.W., Gresser, M., and Young, R.N. 1994. 5-Lipoxygenase. Annu. Rev. Biochem. 63; 383-3417.

- González-Périz, A., Clària, J. 2007. New approaches to the modulation of the cyclooxygenase-2 and 5-lipoxygenase pathways. Curr Top Med Chem. 7; 297-309.

- Hassan, S., Carraway, R.E. 2005. Involvement of arachidonic acid metabolism and EGF receptor in neurotensin-induced prostate cancer PC3 cell growth.Regul Pept. 133;105-114.

- Hatzelmann, A., Fruchtmann, R., Mo h, K.H., Raddatz, S., Matzke, M., Pleiss, U., Keldenich, J., Müller-Peddinghaus, R. 1994. Mode of action of the leukotriene

synthesis (FLAP) inhibitor BAY X 1005: implications for biological regulation of 5-lipoxygenase. Agents Actions. 43; 64-68. Review.

- Hoffman ,T., Brando, C., Lizzio, E.F., Lee, C., Hanson, M., Ting, K., Kim, Y.J., Abrahamsen, T., Puri, J., Bonvini, E. 1990. Functional consequences of phospholipase A2 activation in human monocytes. Adv Exp Med Biol. 279;125-136.

- Jakobsson, P., Odlander, B., Steinhilber, D.; Rosén, A. Claesson, H.E. 1991. Human B Lymphocytes possess 5-lypoxygenase activity and convert arachidonic acid to leukotriene B4. Biochemical and Biophysical Research Communications 178; 302-308

- Jenkins, C.M., Wolf, M.J., Mancuso, D.J., Gross, R.W. 2001. Identification of the calmodulin-binding domain of recombinant calcium-independent phospholipase A2beta. implications for structure and function. J Biol Chem. 276; 7129-35.

- Kim, S.S., Kim, D.K., Suh, Y.H. 1997. Cerebral cortical phospholipase A2 activity of senescence-accelerated mouse is increased in an agedependent manner, Neurosci. Res. 29; 269–272.

- Li, B., Zhang, H., Akbar, M., Kim, H.Y. 2000. Negative regulation of cytosolic phospholipase A(2) by melatonin in the rat pineal gland. Biochem J. 351;709-716.

- Liebmann, P.M., Wölfler, A., Felsner, P., Hofer, D., Schauenstein, K. 1997. Melatonin and the immune system. Int Arch Allergy Immunol. 112; 203-211.

- Maccarrone, M., Battista, N., Meloni, M., Bari, M., Galleri, G., Pippia, P., Cogoli, A., Finazzi-Agrò, A. 2003. Creating conditions similar to those that occur during exposure of cells to microgravity induces apoptosis in human lymphocytes by 5-lipoxygenase-mediated mitochondrial uncoupling and cytochrome c release. J Leukoc Biol. 73; 472-481.

- Nagasawa, K., Kakuda, T., Higashi, Y., Fujimoto, S. 2007. Possible involvement of 12-lipoxygenase activation in glucose-deprivation/reload-treated neurons. Neurosci Lett. 429;120-125.

- Osseni, R.A., Rat, P., Bogdan, A., Warnet, J.M., Touitou, Y. 2000. Evidence of prooxidant and antioxidant action of melatonin on human liver cell line HepG2. Life Sci. 68; 387-399.

- Petrich, K., Ludwig, P., Kuhn, H., Schewe, T. 1996. The suppression of 5-lipoxygenation of arachidonic acid in human polymorphonuclear leucocytes by the 15-lipoxygenase product (15S)-hydroxy-(5Z,8Z,11Z,13E)eicosatetranoic acid: structure-activity relationship and mechanism of action, Biochem. J. 314 ; 911-916.
- Pidgeon, G.P., Lysaght, J., Krishnamoorthy, S., Reynolds, J.V., O'Byrne, K., Nie, D., Honn, K.V. 2007. Lipoxygenase metabolism: roles in tumor progression and survival. Cancer Metastasis Rev. 26; 503-524. Review.
- Pufahl, R.A., Kasten, T.P., Hills, R., Gierse, J.K., Reitz, B.A., Weinberg, R.A., Masferrer, J.L. 2007. Development of a fluorescence-based enzyme assay of human 5-lipoxygenase. Anal Biochem. 364; 204-212.
- Radogna, F., Paternoster, L., Albertini, M.C., Cerella, C., Accorsi, A., Bucchini, A., Spadoni, G., Diamantini, G., Tarzia, G., De Nicola, M., D'Alessio, M., Ghibelli, L. 2007. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res. 43;154-162.
- Reppert, S.M., Weaver D.R., Ebisawa, T. 1994Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron. 13; 1177-1185.
- Rodriguez, C., Mayo, J.C., Sainz, R.M., Antolín, I., Herrera, F., Martín, V., Reiter, R.J. 2004. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res.  36; 1-9.
- Romero, M.P., García-Pergañeda, A., Guerrero, J.M., Osuna, C. 1998. Membrane-bound in Xenopus laevis oocytes as a novel binding site for melatonin. FASEB J. 12; 1401-1408.
- Sexton, A., McDonald , M., Cayla, C., Thiemermann, C., Ahluwalia, A. 2007. 12-Lipoxygenase-derived eicosanoids protect against myocardial ischemia/reperfusion injury via activation of neuronal TRPV1.FASEB J. 21; 2695-2703.
- Steinhilber, D., Brungs, M., Werz, O., Wiesenberg, I., Danielsson, C., Kahlen, J.P. 1995. The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes. J Biol Chem. 270; 7037-7040.

- Tan, D.X., Manchester, L.C., Reiter, R.J., Qi, W.B., Zhang, M., Weintraub, S.T., Cabrera, J., Sainz, R.M., Mayo, J.C.1999. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochim Biophys Acta. 1472; 206–214.

- Tan, D.X., Manchester, L.C., Terron, M.P., Flores, L.J., Tamura, H., Reiter, R.J. 2007. Melatonin as a naturally occurring co-substrate of quinone reductase-2, the putative MT(3) melatonin membrane receptor: hypothesis and significance.J Pineal Res. 43; 317-320.

- Uz, T., Dimitrijevic, N., Imbesi, M., Manev, H., Manev, R. 2008. Effects of MK-886, a 5-lipoxygenase activating protein (FLAP) inhibitor, and 5-lipoxygenase deficiency on the forced swimming behavior of mice. Neurosci Lett. 436; 269-272.

- Wu, X., Biswal, S.S., Kehrer, J.P. 2003. Roles of 5-lipoxygenase activating protein in cell proliferation and apoptosis. Cell Biol Toxicol. 19; 135-43.

- Wurtman, R.J., Axelrod, J., Fischer, J.E. 1964. Melatonin Synthesis in the Pineal Gland: Effect of Light Mediated by the Sympathetic Nervous System. Science. 143;1328-1329.

- Yedgar, S., Cohen, Y., Shoseyov, D. 2006. Control of phospholipase A2 activities for the treatment of inflammatory conditions. Biochim Biophys Acta. 1761; 1373-1382.

- Zhang, H., Akbar, M., Kim, H.Y.. 1999. Melatonin: an endogenous negative modulator of 12-lipoxygenation in the rat pineal gland. Biochem J. 2; 487-93.

- Zhou, G.X., Ding, X.L., Huang, J.F., Zhang, H., Wu, S.B. 2007. Suppression of 5-lipoxygenase gene is involved in triptolide-induced apoptosis in pancreatic tumor cell lines. Biochim Biophys Acta. 1770; 1021-1027.

**Figures Legend**

**Fig. 1. Melatonin produces reactive oxygen species by stimulating lipoxygenase.**

(A) Flow cytometric analysis of ROS measured with DCFDA for 24 h of treatment with 1mM melatonin; re-addition of a second dose of 1mM melatonin 5 h after the first addition is indicated by the arrow. Values indicate differences with respect to control posed =1; 3 experiments have been performed with similar results. In (B) it is shown the flow cytometric analysis of ROS measured with three different redox-sensitive probes, DHR, DCFDA or DHE after 3 h of 1 mM melatonin treatments. Results indicate differences with respect to control posed =1 and are the average of 3 independent experiment +/- SD. ROS increase measured with DCFDA is highly significant ($p<0.01$)**; by DHR and DHE is significant ($p< 0.05$)*. (C) U937 cells were pre-treated for 30 min with either the general LOX inhibitor CAPE, or the specific 5-LOX inhibitor AA861, or the FLAP inhibitor MK866; 1 mM melatonin was then added. ROS were evaluated with DCFDA at 3 h of melatonin. Results indicate differences with respect to control posed =1 and are the average of 3 independent experiment +/- SD. ROS prevention by CAPE, AA861 and MK66 is significant with respect to melatonin alone ($p<0.05$)*. (D) shows the time course of 5-HETE levels after melatonin addition, as results from HPLC separations of cell extracts at the different time points; the HPLC peaks were graphically quantified considering the time of elution of the standard 5-HETE. Results are the average of 3 independent experiment +/- SD. The increase of 5-HETE at 2 h melatonin is significant.

**Fig. 2. Melatonin promotes lipoxygenase-mediated reactive oxygen species formation in other leukocytes.**

(A) Flow cytometric analysis of ROS measured with DCFDA after 3 h of 1 mM melatonin treatment +/- CAPE in normal or tumor haematopoietic cells. Results indicate differences with respect to fluorescence values of the control of each type of cells, posed =1; actual fluorescence values of untreated cells expressed in arbitrary units were as follows: U937=3.93, Jurkat=3.47, monocytes=7.29, lymphocytes=4.76. Results are the average of 3 independent experiment +/- SD. ROS generation in all cell types examined

is significant, as well as its prevention by CAPE (p<0.05).

**Fig. 3. Melatonin stimulates the release of AA.**
(A) shows the time course of extracellular release of [3H]-AA (see Methods) for 90 minutes of treatment with 1 mM melatonin; 3 experiments have been performed with similar results; 2 uM ionomicin was used as a positive control (11,514±312). (B) shows the measurement of extracellular release of [3H]-AA (see Methods) after 1 hr of treatment with different doses of melatonin. Results are the average of 3 independent experiment +/- SD.  The increase of AA release is significant starting from 100 uM melatonin (p<0,05)*.

**Fig. 4. Ca2+-independent PLA2 mediates melatonin-induced AA release and ROS production.**
 (A) U937 cells were pre-treated for 30 min with either the general PLA2  4-Brophenacil Bromide (BPB) and a specific Ca2+-independent inhibitor Bromoenol Lactone (bel), 1mM melatonin was then added. ROS were evaluated with DCFHDA at 3 h of melatonin. Results indicate differences with respect to control posed =1 and are the average of 3 independent experiment +/- SD. ROS inhibition by 4-BB+Mel and Bel+Mel   is significant with respect to melatonin alone (p<0.05)* (B) shows the measurement of extracellular release of [3H]-AA (see Methods) after a pre-treatment for 30 min with bel or the cytosolic (Ca2+-dependent) PLA2 (cPLA2) inhibitor Arachidonyltrifluoromethyl ketone (AACOCF3), and then 1 hr of 1mM melatonin treatment. Ionomicin was used as a positive control. Results are the average of 3 independent experiment +/- SD. ROS inhibition by Bel+Mel is significant with respect to melatonin alone (p<0.05)*, whereas the value of AACOCF3+Mel does not significantly differ with respect melatonin alone. C) shows the extent of AA release in the presence of melatonin ± the LOX or COX inhibitors AA861 or indomethacine, respectively. Ionomicin was used as a positive control. Results are the average of 3 independent experiment +/- SD. The increase of AA release of Mel+AA861 and Mel+Indometacin over melatonin alone is significant (p<0.05)*.

**Fig. 5. Possible involvement of calmodulin in melatonin-induced AA/ROS pathway.**

U937 cells were pre-treated for 30 min with calmidazolium or chlorpromazine and then 1 mM melatonin was added. ROS were evaluated with DCFDA at 3 h of 1 mM melatonin (A), whereas extracellular release of [3H]-AA was estimated after 1hr of 1 mM melatonin treatment (B). Results indicate differences with respect to control posed =1 and are the average of 3 independent experiment +/- SD. 1 hr pre-treatment with 50 uM Luzindole does not alter melatonon-produced ROS (A) or AA release (B). Results are the average of 3 independent experiment +/- SD. ROS inhibition by Cloropromazine+Mel is highly significant with respect to melatonin alone (p<0.01)**. The AA release inhibition by Cloropromazine+Mel is significant with respect to melatonin alone (p<0,05)*. Calmidazolium does not significantly alter melatonin-induced ROS production or AA release.


**Fig. 6. Melatonin-induced pro-radical pathway: a hypothesis.**

Melatonin might bind to calmodulin, thus inducing the release of sequestered Ca2+-independent PLA2; this is thus free to move to membranes and release high doses of AA; in turn, liberated AA feeds 5-LOX to produce free radicals and 5-HETE. The inhibitors used, as well as the products enhanced by melatonin (HETE, ROS, AA), which allowed proposing this model, are indicated.

Fig.1

A
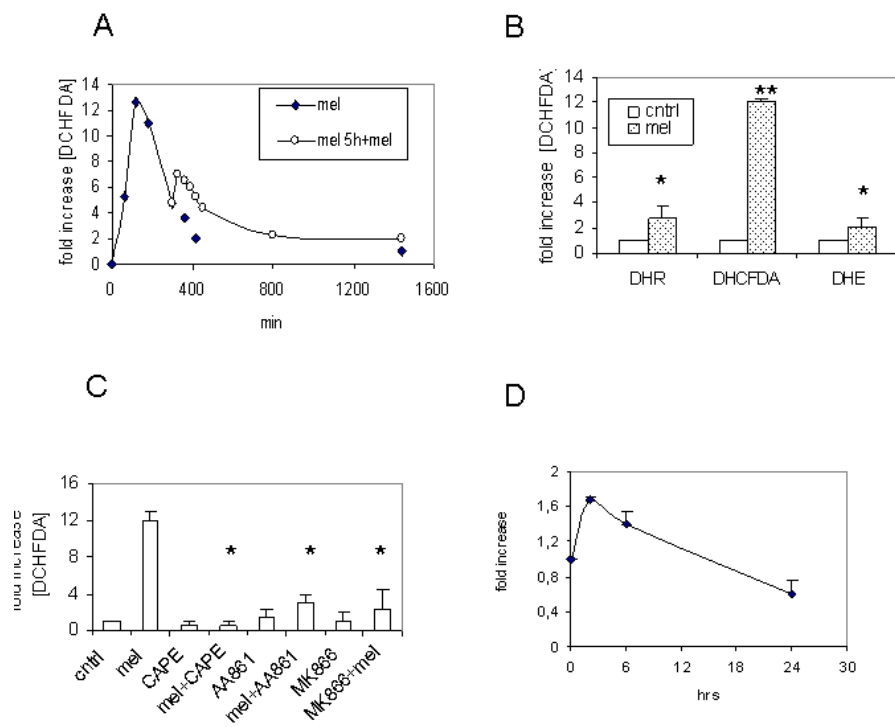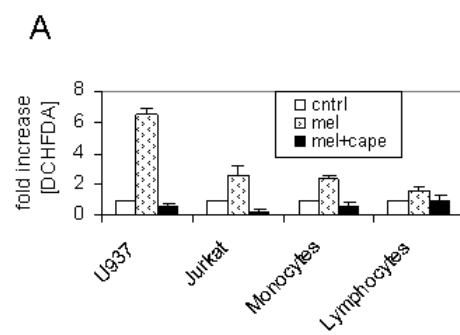


Fig.2

A
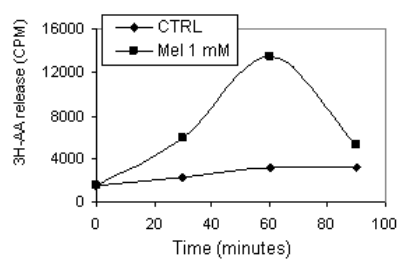


B
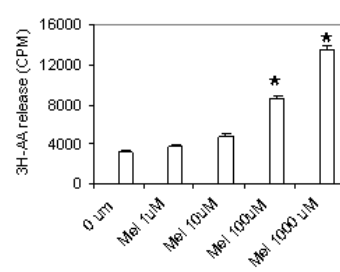


Fig.3

A
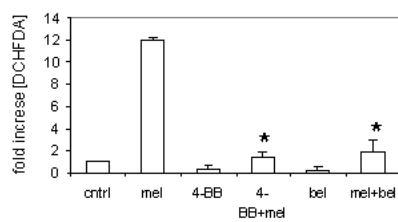


B
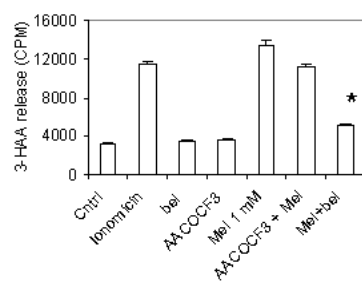


C
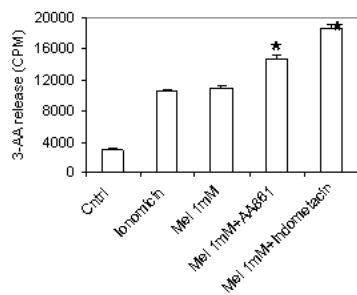


Fig.4

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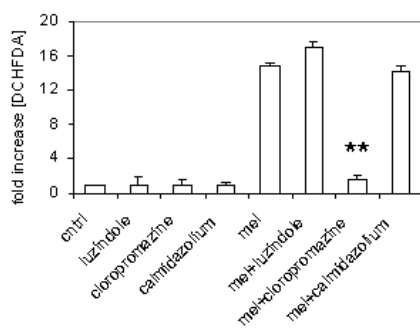


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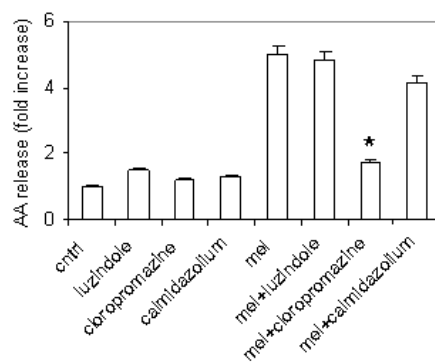


Fig.5
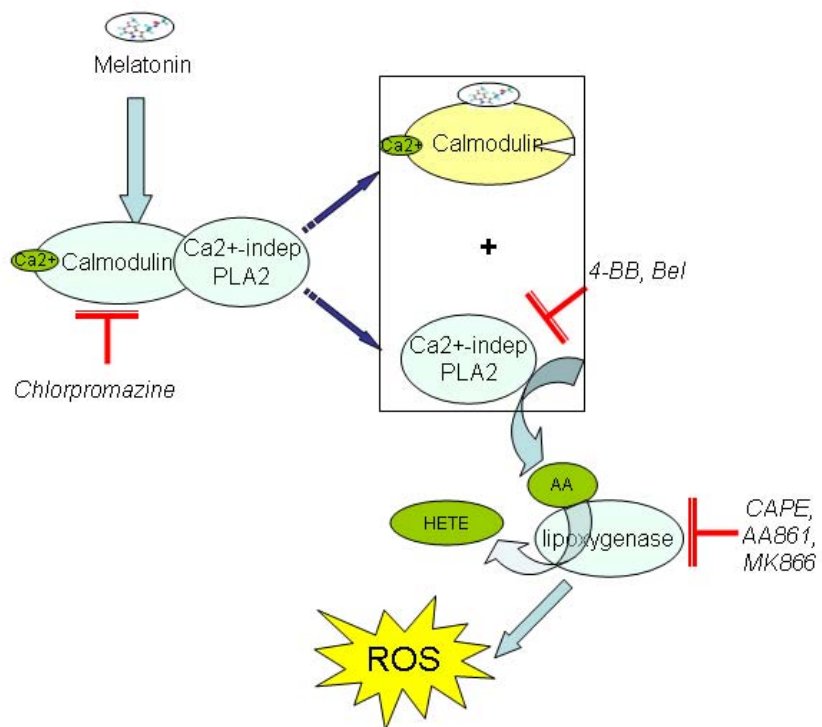
Fig.6

# PAPER VIII

*Melatonin antagonizes apoptosis by concurrent stimulations of high and low affinity targets*

# MELATONIN ANTAGONIZES APOPTOSIS BY CONCURRENT STIMULATIONS OF HIGH AND LOW AFFINITY TARGETS

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**Running title**: convergence of multiple signals for melatonin apoptosis antagonism

**1. Abstract (200)**

Melatonin exerts its functions by stimulating high (nanomolar) affinity plasma membrane receptors (MT1/MT2) and additional lower (micromolar) affinity targets such as calmodulin. Melatonin contrasts apoptosis with mechanisms that need clarification. We reported that melatonin contrasts Bax activation and the intrinsic pathway of apoptosis promoting re-localization of Bcl-2 to mitochondria via interaction with MT1/MT2 plasma membrane receptors. Surprisingly, the doses necessary for apoptosis antagonism are >10,000 higher than those required for MT1/MT2 stimulation; thus, we investigated the requirement of an additional, lower affinity target. We showed that melatonin activates 5-lipoxygenase as a consequence of a signal pathway triggered by calmodulin binding, at the same doses than required for apoptosis inhibition. Here we show that melatonin anti-apoptotic effect is abolished by the inhibition of 5-lipoxygenase, and restored by the addition of its product 5-HETE; this allows sequestration of Bax in mitochondria by interaction with Bcl-2, and impairment of the intrinsic apoptotic pathway. In the presence of exogenous 5-HETE, i.e., bypassing the bottleneck of lipoxygenase requirement, melatonin antagonizes apoptosis at nanomolar doses, demonstrating that the independent and simultaneous interaction with high (MT1/MT2) and low (lipoxygenase) affinity targets are required for, and converge into, the anti-apoptotic effect.

**Introduction**

Increasing evidence for melatonin non-neurological functions is supported by the discovery that many non-neuronal tissues possess all the necessary machineries to synthesize [1], and properly respond to [2], melatonin. Much attention is receiving the role melatonin plays in regulating immune/inflammatory responses [3, 4]; intriguingly, melatonin was found in higher concentrations in bone marrow than in other tissues [5].

Large part of the immuno-modulatory functions of melatonin deals with the control of leucocyte apoptosis; during the differentiation/activation processes, leukocytes go through apoptosis-prone and apoptosis-resistant phases [6], mostly controlled by the pro- or anti-apoptpotic members of the Bcl-2 family, such as the up-regulation of Bax in uncommitted neutrophils [7] or the up- and down-regulation of Bcl-2 as part of the B lymphocytes differentiation pathways [8]. Interestingly, melatonin's control of apoptosis is exerted, at least in part, at the level of the Bcl-2 family members [7, 9], whose concentration/localization are controlled by melatonin through different mechanisms [9, 10], thus modulating the proneness of cells to apoptosis.

Melatonin has many cellular targets. MT1/MT2 are G-protein linked plasma membrane receptors engaged at high affinity (kD in the nanomolar range) [11], which transducer an intracellular signal involving phospholipase C [12] and/or adenylate cyclase [13] pathways, and is prevented/blocked by the melatonin antagonist luzindole [14]. Additional lower affinity targets are quinone reductase 2 [15] and calmodulin [16], two cytosolic enzymes that are inhibited by melatonin binding; whether interaction with these enzymes is able to elicit a physiologically meaningful melatonin signal is not known. In addition, melatonin well known radical scavenging ability, also elicited at micromolar doses, is responsible of many biological actions of melatonin, buffering oxidative stress in organic fluids and within cells [17].

Apoptosis is a process of cell suicide activated by stress/cell damage (intrinsic pathway) or ligand/receptor interaction (extrinsic pathway) [18]. At least for the intrinsic pathway,

intracellular oxidations play a role in the finalization of apoptosis [19]. Interestingly, the doses required for experimental antagonism of apoptosis are similar to those required for radical scavenging [20]. For these reasons, it is generally believed that the anti-apoptotic effect of melatonin may deal with its radical scavenging ability [20].

We have recently shown that melatonin antagonizes the intrinsic pathway of apoptosis on a set of tumor and normal cells of haematopoietic origin [21]. This requires interaction with MT1/MT2 receptors, even though the doses necessary are 10,000 fold higher [22]. This poses an apparent concentration paradox. Here, we address this problem by hypothesising that MT1/MT2 interaction is necessary but not sufficient for apoptosis antagonism, and that the independent interaction with a second, lower affinity target is required. We have recently reported that at the same doses of the anti-apoptotic effect, melatonin transiently activates 5-lipoxygenase (5-LOX). Intriguingly, 5-LOX is receiving much attention as a promoter of pro-survival pathways [23], antagonizing apoptosis with a still obscure mechanism. Thus, we wanted to check whether 5-LOX may be the co-effectors of MT1/MT2 signalling for melatonin anti-apoptotic effect. Here, we report that the exogenous addition of the LOX metabolite 5-HETE lowers the melatonin dose necessary for apoptosis antagonism to a level that is consistent with MT1/MT2 stimulation, thus providing an explanation for the concentration paradox.

**Materials and Methods**

**Cell culture**

U937 are human tumor monocytes stabilized from a histiocytic lymphoma [34]; cells were cultured in RPMI medium supplemented with 10% FCS. Cells are routinely checked for absence of mycoplasm by using a mycoplasm detection kit (Mycoalert TM., Cambrex Bio Science Milano, Italy). The experiments were performed on cells in the logarithmic phase of growth under condition of ≥ 98% viability, as assessed by trypan blue exclusion. Peripheral blood mononuclear leukocytes (PBML) were isolated from heparinized blood samples of healthy individuals by collection with Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. Red cells were removed by hypotonic lysis. PBML were then plated in RPMI 1640 plus 10% human AB serum in culture flasks (pre-treated with the same human AB serum) to promote monocytes adhesion. After 2hrs, floating cells (lymphocytes) were washed out, spinned down and separately plated in RPMI 1640 plus 10% hi-FCS. Adherent monocytes were washed 3 times with RPMI to remove residual lymphocytes, detached by cell scraping, and resuspended in RPMI 1640 plus 10% hi-FCS. Treatments (see below) were performed at 20-24hrs post-separation. Cell viability in both fractions was >98% as assessed by trypan blue exclusion test. Purity of the enriched fractions was controlled by labelling (20min at room temperature) with cell-type specific antibodies: FITC-conjugated-anti-CD45 (from Becton-Dickinson) for lymphocytes; and PE-conjugated-anti-CD14 (from Becton-Dickinson) for monocytes. Cells were then washed with PBS and labelling evaluated. Both monocyte and lymphocyte fractions were >95% pure.

**Induction and evaluation of apoptosis**

Apoptosis was induced with the protein synthesis inhibitor puromycin (PMC, 10 μg/ml). Apoptosis was evaluated after 4 h of PMC continuous treatment. Apoptosis was quantified as previously described [35]. Briefly, cells were stained with the Hoechst 33342; cells with nuclear apoptotic

morphology, detected using a fluorescence microscope, were counted (at least 300 cells in at least 3 independent fields) and the fraction of apoptotic cells among total cells was evaluated. PMC was purchased from Sigma Chemical Co (St Louis, MO, USA), Hoechst 33342 from Calbiochem (San Diego, CA, USA).

**Melatonin**

Melatonin was purchased from Sigma Chemical Co (St. Louis, MO, USA), and used at the concentration of 1 mM. For the experiments, melatonin was added 1 h prior to apoptosis induction.

**Other treatments**

For lipoxygenase inhibition a general LOX inhibitor (CAPE) was used at 17uM, a specific inhibitor of 5-LOX (AA861) was used at 20uM. and added 30 min before treatments. PLC inhibition: U73122, (from Sigma Chemical Co, St. Louis, M, USA) was used at 10 uM, and added 30 min before treatments. 5-HETE 30nM, 50nM and 15-.HETE 50nM (furnished by Biomol International) was added 2 hrs before melatonin treatments.

*$IP_3$ assay*

After the treatments, U937 cells were collected, washed and resuspended at $6 \times 10^6$ cells/ml in RPMI medium. 200 μl of 60% ice-cold perchloric acid were added to the cell suspension. The cell suspension was then centrifuged and aliquots of the supernatant were neutralized with 10 M KOH for $IP_3$ (Inositol 1,4,5-trisphosphate) assay. $IP_3$ was assayed using a commercially available kit (D-myo-Inositol 1,4,5-trisphosphate ($IP_3$) [3H] Biotrak Assay System, Amersham Biosciences UK Limited). The assay is based on competition between unlabelled and tritium-labelled $IP_3$ (the tracer) in the standard or samples, for binding to a binding protein prepared from bovine adrenal cortex. The bound $IP_3$ is separated from free $IP_3$ by centrifugation leaving the bound fraction adherent to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled $IP_3$ in the

sample to be determined by interpolation from a standard curve. The data represent the mean of 5 experiments as indicated in figure legends.

**Immunofluorescence analysis**

U937 cells were fixed with 4% paraformaldehyde. Fixation was stopped by adding the same volume of 50 mM NH$_4$Cl in PBS for 20min. cells were washed twice in PBS and incubated with 2 ug/ml anti Bax mouse monoclonal antibody 6A7 (BD Bioscieciences  Pharmingen, San Diego, CA, USA,) or anti Bax mouse polyclonal antibody  D21: sc6236 (Santa Cruz Biotechnology, Santa Cruz, CA, USA,) or anti Bcl-2 mouse monoclonal (Oncogene, San Diego, CA, USA;  Ab #0P60,) in a solution of PBS, 1% BSA, 0.05% saponin for 45 min followed for 45 minutes at room temperature, as described in [24]. Then cells were washed with PBS and incubated with 10 ug/ml of of TRIC- or FITC-conjugated secondary antibodies (Dako, Glostrup, Denmark) in PBS, 0.05 % saponin for 30 min. Nuclei were visualized by staining with Hoechst 33342. In order to study the mitochondrial localization of Bcl-2 protein, U937 were stained, before the paraformaldehyde fixation, with MitoTrackerRed (Molecular Probes, Eugene, OR, USA) for 20 minutes at 37°C. Images were captured with a Nikon Eclipse TE 200 microscope. The merge and the analysis were performed with Adobe Photoshop software.

**Subcellular fractionation and immunoblot assay**

Western blot analysis of cytochrome c: cytosolic fraction from U937 cells were isolated by a quick cell lysis method with digitonin. Under vortexing, lysis buffer (9.4 ug digitonin/10$^6$ cells, 500mm sucrose in PBS: NaH$_2$PO$_4$ 2mM, Na$_2$HPO$_4$ 16 mM, NaCl 150 mM , pH 7.6) was added to a cell suspension of 5x10$^6$ cells in PBS (1mM DTT and  protease inhibitor cocktail, SIGMA P 8340, were added just prior to use). Heavy organelles and cell debris were pelletted for 60 seconds at 14000g at 4°C. The 30ug of protein of cytosolic fraction (supernatant) was analysed for cytochrome C by 12%SD-Page and immunoblotting (PharMingen, San Diego, CA, USA). The specific protein was

identified using the ECL system by Amersham (Piscataway, NJ, USA) with HRP-conjugated secondary antibody (Dako).

Western blot analysis of Bax: to obtain mitochondrial and cytosolic extracts for the determination of Bax localisation, the subcellular fractionation procedure was performed as described [26], with minor modification. Briefly, sample of $20x10^6$ cells were washed with ice cold PBS (all the following procedures were performed at +4°C) and resuspended in 5x volume of hypotonic buffer (10mM NaCl, 1.5mM MgCl2, 10mM Tris-HCl, pH 7.5, 1mM DTT, 1mM PMSF and protease inhibitor cocktail, see above). The cells were incubated on ice until they completed swelling, and then dounced with the B pestle (Kontes Glass Company, Vineland, NY, USA) until 90% of cells were Trypan Blue positive. A volume of 2.5x MS buffer (525mM mannitol, 175mM sucrose, 12.5mM Tris-HCL pH 7.5, 2.5mM EDTA pH7.5, 1mM DTT, 1mM PMSF and protease inhibitor cocktail) was quickly added to the lysate to achieve 1 x MS. The mixture was subjected to centrifugation at 1300g. The resulting supernatant was centrifuged two more times to completely remove the nuclei and unlysed whole cells. The supernatant was then subjected to centrifugation at 10000 g to isolate mitochondria. The pellet (mitochondrial fraction) was re-suspended in hypotonic buffer containing 0.5% Triton x-100 (Calbiochem) and rotated for 30 min at +4°C.

The resulting fractions were placed in loading buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 5 % beta-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue) for Western blot analysis.

For samples separated in non-reducing gels, the same procedure as above was followed; except that cells were lysed in non-reducing hypotonic buffer (DTT was omitted), and the sample buffer was modified by omission of beta-mercaptoethanol. For western blot analysis, 50ug of cytosolic or mitochondrial protein extracts were loaded into a 12% standard SDS-PAGE and blotted to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The filter was incubated for the immunoreactions with the Bax polyclonal antibody (Santa Cruz, D21).

Western Blot analysis of Bcl-2 protein levels: to obtain mitochondrial and cytosolic extract, for the detection of Bcl-2 cellular localization, we perform the same procedure as above was followed for

Bax. Whole cell extracts were prepared with M-PER Mammalian Protein Extract Reagent (Pierce, Rockford, IL, USA); briefly, $10x10^6$ U937 were washed in PBS and the pellet was resuspended in 400ul of the solution containing protease inhibitor, vortexed horizontally for 15 minutes at 4°C and then centrifugated at 10000g for 15 minutes. 5ug of the supernatant were loaded into a 12% standard SDS-PAGE and blotted to nitrocellulose membrane. The filter was incubated for the immunoreactions with the Bcl-2 monoclonal antibody (Calbiochem).

Western Blot bands were quantified by image analysis with NIH Image 1.4 software. Following values were normalised against Ponceau red staining of the nitrocellulose filter.

**Immunoprecipitation**

For immunoprecipitation aliquots of 107 U937 cells with or without melatonin treatment were lysed and prepared as previously described [25]. The fraction of each sample were incubated 1h at room temperature and then over night at 4°C with 2 ug of Bcl-2 policlonal antibody (Santa Cruz). The day after, sample were incubated for 1 h at room temperature with 2 mg/ml protein A-Sepharose (Amersham Bioscienceds) and then, after centrifugation and washing, analyzed by SDS-PAGE and Western Blot. Immunoblot analysis was performed with anti-protein Bax monoclonal antibody (Santa Cruz).

**siRNA transfection**

2ug of siRNA Bcl-2 (Dharmacon, Lafayette, CO, USA) were transfected into $10^6$ cells using kit V from AMAXA (Cologne, Germany) according to the manufacturer's protocol and program V-001 an AMAXA's nucleofactor device. Treatments were performed 48hours after transfection. 1ug of enhanced green fluorescent protein expression vector (pmaxGFPTM) was co-transfected with the siRNA; percent of transfected cells based on the GFP-green signal were measured by flow cytometer and selected by microscope for the in situ apoptosis analysis.

**Statistical analysis**

Statistical analyses were performed using Student's *t*-test for unpaired data and *P* values < 0.05 were considered significant. Data are presented as mean ± SEM.

**Results and Discussion**

We have demonstrated that melatonin reduces the extent on apoptosis on U937 cells by activating a signal transduction triggered by interaction with MT1/MT2 plasma membrane receptor. The dose-response effect of melatonin on apoptosis induced by puromycin (PMC) on U937 cells indicates a significant effect at high micro molar doses (fig. 1, see also [22]), which is 4 orders of magnitude higher than what required for standard MT1/MT2 stimulation. Thus we investigated whether stimulation of MT1/MT2 signal transduction would occur at the standard doses also in U937. To this purpose, we measured the levels of inositol-3-phosphate (IP3), which is produced as part of MT1/MT2 signalling. Fig. 1 shows that the standard doses are sufficient to produce IP3, thus confirming that receptors are stimulated in a standard way

Thus, the paradox that melatonin protects from apoptosis by stimulating the MT1/MT2 receptor at low doses, though requiring high doses, must be explained otherwise.

We investigated the possible requirement of an additional, cooperating pathway. We recently showed that at doses similar to those required for apoptosis reduction, melatonin activates an independent pathway, possibly by interaction with the low affinity target calmodulin. This binding activates a signal transduction culminating with activation of 5-lipoxygenase and production of 5-HETE. Thus, we evaluated if this pathway is required for the anti-apoptotic effect by probing whether inhibition of 5 LOX may revert melatonin's anti-apoptotic effect.

Two different LOX inhibitor, the generic CAPE and the specific AA861 revert melatonin anti-apoptotic effect on U937, Jurkat and also normal lymphocytes and monocytes from peripheral blood of healthy individuals (Fig. 2). This indicates that 5-LOX is necessary for melatonin anti-apoptotic effect.

To prove this assumption, and also to investigate the mechanism through which 5-LOX may affect apoptosis, we performed the same set of experiments in U937 cells pre-treated with 5-HETE, one of the main products of 5-LOX, which was chosen because it is implicated in survival pathways of tumours cells [23]. Fig .3A shows that 5-HETE restores melatonin

protection when it was reversed by LOX inhibition; interestingly, 5-HETE do not exert any effect in the absence of melatonin, indicating that they need an additional pathway (possibly the one triggered by MT1/MT2) to work as anti-apoptotic agents. The presence of exogenous HETE ameliorates melatonin anti-apoptotic effect, possibly increasing HETE total (endogenous plus exogenous) concentration. Indeed, increasing exogenous 5-HETE strengthen melatonin anti-apoptotic effect. Interestingly, also 15 HETE is effective, indicating that the biochemical effect exerted are similar, though not necessarily implying activation of other lipoxygenases (Fig. 3B).

This indicates that the calmodulin-LOX pathway triggered by melatonin at high doses is necessary (though not sufficient) for melatonin anti-apoptotic effect. If this hypothesis is correct, then in the presence of exogenously added HETE, i.e., bypassing the bottleneck of requirement of high doses of melatonin, the anti-apoptotic effect would come to depend only by MT1/MT2 stimulation, that is would occur at the same nanomolar doses.

Indeed, this is what actually occurs, as shown in fig. 4.

This demonstrates that two independent pathways are independently induced by melatonin at different doses, and cooperate to the anti-apoptotic effect being both necessary but not sufficient.

Next, we investigated the role that HETE play in melatonin anti-apoptotic effect.

We observed that treatment with melatonin induces the substantial translocation of Bax to mitochondria (Fig. 5A). This was an unexpected finding, since mitochondrial localization of Bax is synonym of its activation, whereas we have shown that melatonin contrasts apoptosis by Bax inactivation. To investigate the issue, we analyzed whether this translocation might be in fact a sequestration rather than an activation process. Since Bax is known to be sequestered by Bcl-2, and we had shown that impairment of Bax function by melatonin requires Bcl-2, we analyzed whether melatonin might promote the physical interaction between the two proteins. Fig. 5 B shows the immuno-precipitation assay where Bax is revealed after IP with anti-Bcl-2

antibodies; the amount of precipitated Bax is strongly increased by melatonin, indicating that an interaction, conceivably a sequestration, is occurring. Mitochondrial localization of Bax is prevented by LOX inhibition, indicating that LOX is necessary for its re-localization. (fig. 5 C).

Interestingly, Bax re-localization requires Bcl-2, since it is prevented by Bcl-2 silencing with siRNA (see fig. 6A). Thus, Bax requires both LOX activation (HETE) and Bcl-2 to translocate. Instead, the reversal is not true, since in the presence of LOX inhibitors Bcl-2 is still able to be localized to mitochondria by melatonin (Fig. 6B). This suggests that it is Bcl-2 that drives Bax to mitochondria, implying that their interaction occurs in the cytosol.

In conclusion, melatonin independently activates two pathways that cooperate for the anti-apoptotic effect. Amazingly, they end up interfering with the behaviour of the Bcl-2 family of proteins (Fig.7). It remains to be understood how Bcl-2 is induced to translocate by the signal transduction elicited by MT1/MT2 stimulation, and how HETE stimulate Bax to interact with Bcl-2 in the cytosol. Bcl-2 phosphorylation state is known to be responsible for transloaction to mitochondria [26]; many kinases may modify Bcl-2, including protein kinase C (PKC), which is conceivably activated by the signal transduction activated by MT1/MT2 interaction; interestingly, protection requires Ca2+ influx [22], an event that is required for PKC activation [26]. On the Bax side, it will be interesting to analyze the modality through which HETE affect apoptosis on tumor cells, possibly analyzing whether this may be related to the staus of Bcl-2 (level and basal localization) in the different tumor cells.

# References

1. Carrillo-Vico, A., Calvo, J.R., Abreu, P., Lardone, P.J., Garcia-Morino, S., Reiter, R.J., Guerrero, J.M. 2004. Evidence of melatonin synthesis in human lymphocytes and its physiological significance: possible role as intracrine, autocrine and/or paracrine substance. FASEB J. 18;537–539,

2. Carrillo-Vico, A., Reiter, R.J., Lardone, P.J., Herrera, J.L., Fernández-Montesinos, R., Guerrero, J.M., Pozo, D. 2006. The modulatory role of melatonin on immune responsiveness. Curr Opin Investig Drugs. 7; 423-431.

3. Li, B., Zhang, H., Akbar, M., Kim, H.Y. 2000. Negative regulation of cytosolic phospholipase A(2) by melatonin in the rat pineal gland. Biochem J. 351;709-716.

4. Zhang, H., Akbar, M., Kim, H.Y.. 1999. Melatonin: an endogenous negative modulator of 12-lipoxygenation in the rat pineal gland. Biochem J. 2; 487-93.

5. Tan, D.X., Manchester, L.C., Reiter, R.J., Qi, W.B., Zhang, M., Weintraub, S.T., Cabrera, J., Sainz, R.M., Mayo, J.C.1999. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochim Biophys Acta. 1472; 206–214.

6. Wesche-Soldato De, Swan Rz, Chung Cs, Ayala A. The apoptotic pathway as a therapeutic target in sepsis. Curr Drug Targets. 2007; **8**:493-500. Review.

7. Hoijman E, Rocha Viegas L, Keller Sarmiento Mi, et al. Involvement of Bax protein in the prevention of glucocorticoid-induced thymocytes apoptosis by melatonin. Endocrinology. 2004; **145**:418-25.

8. Lu L, Osmond Dg. Apoptosis and its modulation during B lymphopoiesis in mouse bone marrow. Immunol Rev. 2000;**175**:158-174. Review.

9. Eck-Enriquez K, Kiefer Tl, Spriggs Ll, Hill Sm. Pathways through which a regimen of melatonin and retinoic acid induces apoptosis in MCF-7 human breast cancer cells.Breast Cancer Res Treat. 2000; **61**:229-39.

10. Luchetti F, Canonico B, Curci R, et al. Melatonin prevents apoptosis induced by UV-B treatment in U937 cell line.J Pineal Res. 2006 ; **40**:158-67.

11. Reppert, S.M., Weaver D.R., Ebisawa, T. 1994Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron. 13; 1177-1185.

12. Bach Ag, Wolgast S, Muhlbauer E, Peschke E. Melatonin stimulates inositol-1,4,5-triphospathate and $Ca^{2+}$ release from INS1 insulinoma cells. J Pineal Res 2005;39:316-323.

13. Vanecek, J. 1998. Cellular mechanisms of melatonin action. *Physiol Rev*. 78; 687–721.

14. Dubocovich Ml. Luzindole (N-0774): a novel melatonin receptor antagonist. J Pharmacol Exp Ther 1998;246:902-910.

15. Tan, D.X., Manchester, L.C., Terron, M.P., Flores, L.J., Tamura, H., Reiter, R.J. 2007. Melatonin as a naturally occurring co-substrate of quinone reductase-2, the putative MT(3) melatonin membrane receptor: hypothesis and significance.J Pineal Res. 43; 317-320.

16. Benítez-King, G., Huerto-Delgadillo, L., Antón-Tay, F. 1993. Binding of 3H-melatonin to calmodulin. Life Sci. 53; 201–207.

17. Rodriguez, C., Mayo, J.C., Sainz, R.M., Antolín, I., Herrera, F., Martín, V., Reiter, R.J. 2004. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res. 36; 1-9.

18. Coppola S And Ghibelli L. GSH extrusion and the mitochondrial pathway of apoptotic signaling. Biochem. Soc. Trans. 2000; **28**: 56-61.

19. D'alessio M, De Nicola M, Coppola S, et al. Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis. FASEB J. 2005; 19:1504-1506.

20. Reiter, R.J., Tan, D.X., Terron, M.P., Flores, L.J., Czarnocki, Z. 2007. Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. Acta Biochim Pol. 54; 1-9.

21. Radogna, F., Cristofanon, S., Paternoster, L., D'Alessio, M., De Nicola, M., Cerella, C., Dicato, M., Diederich, M., Ghibelli, L. 2008. Melatonin antagonizes the intrinsic pathway of apoptosis via mitochondrial targeting of Bcl-2. J Pineal Res. 44; 316-325

22. Radogna, F., Paternoster, L., Albertini, M.C., Cerella, C., Accorsi, A., Bucchini, A., Spadoni, G., Diamantini, G., Tarzia, G., De Nicola, M., D'Alessio, M., Ghibelli, L. 2007. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res. 43;154-162.

23. Wu, X., Biswal, S.S., Kehrer, J.P. 2003. Roles of 5-lipoxygenase activating protein in cell proliferation and apoptosis. Cell Biol Toxicol. 19; 135-43.

24. Jungas T., Motta I., Duffieux F., et al. Glutathione levels and bax activation during apoptosis due to oxidative stress in cells expressing wild-type and mutant cystic fibrois transmembrane conductance regulator The Journal of Biological Chemistry, 2003; **227** :27912-27918.

25. Frouin I., Maga G., Denegri M., Riva F., Savio M., Spadari F., Prosperi E., Scovassi A.I. 2003. Human proliferating cell nuclear antigen, poly(ADP-ribose) polymerase-1, and p21 waf/cip1. A dynamics exchanges of partners. J. Bio Chem. 278, 39265-39268.

26. Deng X, Kornblau Sm, Ruvolo Pp And May Ws Jr. Regulation of Bcl2 phosphorylation and potential significance for leukemic cell chemoresistance. J Natl Cancer Inst Monogr. 2001; **28**:30-7. Review.

*Figures Legends*

Fig. 1. Melatonin reduces apoptosis according to $MT_1/MT_2$ receptor affinity A) U937 cells, jurkat cells and were treated with. Apoptosis was evaluated at 3 hrs of puromycin (PMC, 10 ug/ml) treatment before 1 hrs of 1 mM melatonin pre-treatment in normal or tumor haematopoietic cells. Results are the average of at least 3 independent experiments ±SEM. B) U937 cells were incubated with different doses of melatonin and $IP_3$ intracellular concentration was measured (see Material and Methods) Values are the average of four measurement in at least 3 independent experiments ±SEM.

Fig.2. Lipoxygenase is required for melatonin anti-apoptotic effects. A) U937 cells and Jurkat cells were incubated with a generic LOX inhibitor CAPE 17uM for 30 min and followed by 1 mM melatonin treatment and then apoptosis was induced with PMC 10 ug/ml. Results are the average of at least 3 independent experiments ±SEM. B) U937 cells and Jurkat cells were incubated with a specific LOX inhibitor AA861 20 uM, for 30 min and followed by 1 mM melatonin treatment and then apoptosis was induced with PMC 10 ug/ml. Results are the average of at least 3 independent experiments ±SEM. C) PBMC cells were incubated with a generic LOX inhibitor CAPE 17 uM, for 30 min and followed by 1 mM melatonin treatment and then apoptosis was induced with PMC, 10 ug/ml. Results are the average of at least 3 independent experiments ±SEM.

Fig.3. Lipoxygenase participates to melatonin anti-apoptotic effect through its product 5-Hete. A) U937 cells were incubated with 5-Hete 50nM for 2 hrs then treated with AA861 for 30 minutes before melatonin 1mM treatment. Apoptosis was evaluated at 3 hrs of PMC 10 ug/ml treatment. Results are the average of at least 3 independent experiments ±SEM.B) U937 cells were incubated with 5-Hete 30nM, 50nM and 15-Hete 50nM for 2 hrs then treated with AA861 for 30 minutes before melatonin 1mM treatment. Apoptosis was evaluated at 3 hrs of PMC 10 ug/ml treatment. Results are the average of at least 3 independent experiments ±SEM.

Fig.4 The calmodulin-LOX pathway triggered by melatonin at high doses is necessary (though not sufficient) for melatonin anti-apoptotic effect. U937 cells were pre-treated with 5-Hete 50nM for 2 hrs before 1 mM melatonin treatment. Apoptosis was evaluated at 3 hrs of PMC 10 ug/ml treatment.
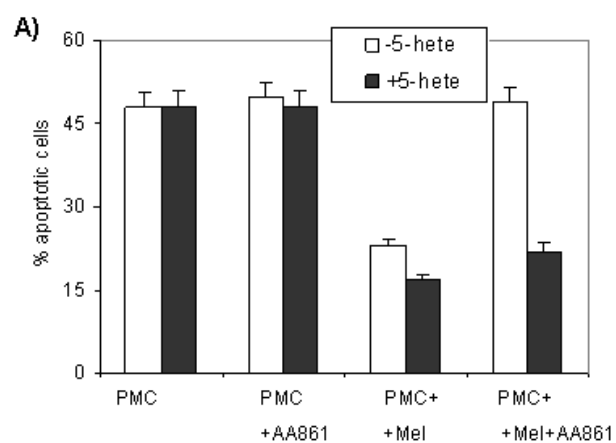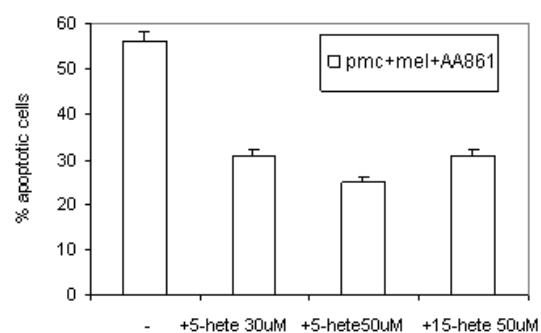
Fig.5. Melatonin induced the translocation of Bax to mitochondria. via LOX pathway and its sequestration by interaction with Bcl-2. A) Western blot analysis of Bax with polyclonal antibody delta21 on the mitochondrial fraction (purity was > 70% as assessed by anti-actin and cytochrome c oxidase antibody labeling, not shown) on U937 cells, untreated, or treated with with 1mM melatonin. B) Bcl-2 and Bax immuno-precipitation analysis; the revealing antibody is Bax, whereas the precipitating antibody is Bcl-2; red arrow shows a dimer which perhaps it's not cleaved on SDS page. C) The pattern of Bax localization as assessed by bi-parametric analysis performed by double-labelling cells with polyclonal antibody delta21 on U937 (treatments as above) versus mitotracker red to label active mitochondria. The merging between the mitochondrial and Bax pattern is shown in the right column of panel C. >3 experiments have been performed with similar results.

Fig.6. Bax requires Bcl-2 to translocate and not the reversal. A) U937 cells were nucleofected with specific Bcl-2 siRNA and Green Fluorescence Protein (GFP) then immuno-stained with TRIC-conjugated anti-Bax antibodies (transfection efficiency = 50%, not shown). Results show the fraction of cells separately scored according to the presence/absence of green fluorescence, and are the average of three independent experiments ± SEM.

Fig.7. Propose a mechanism for melatonin anti-apoptotic effect.
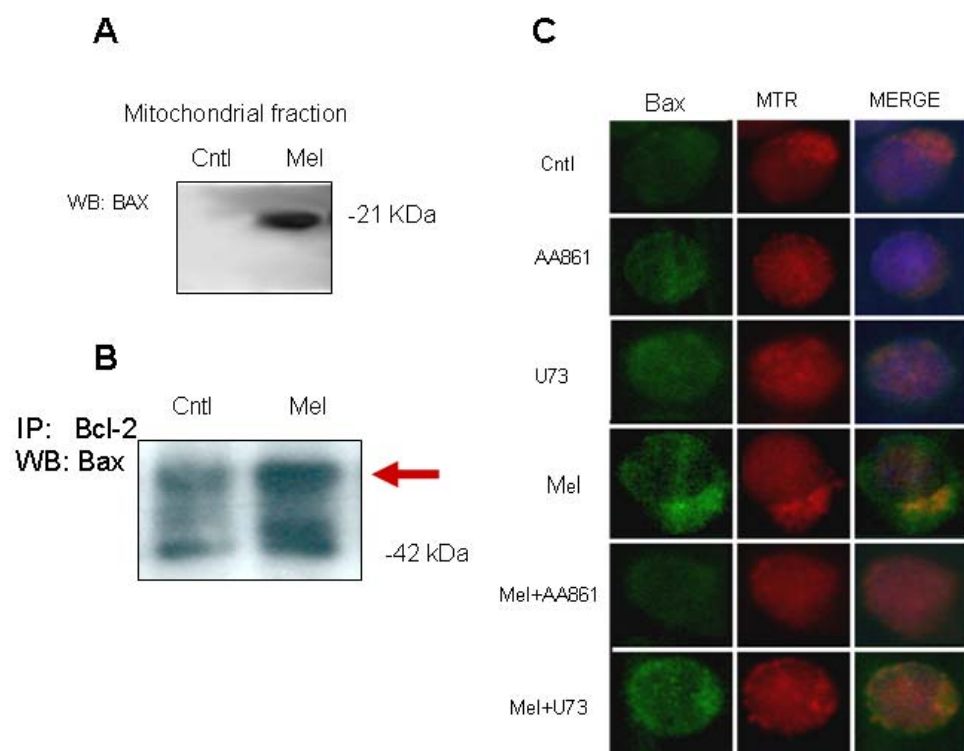
**A**



**B**



**Fig.1**

Fig.2

**A**



**B**



**Fig.3**

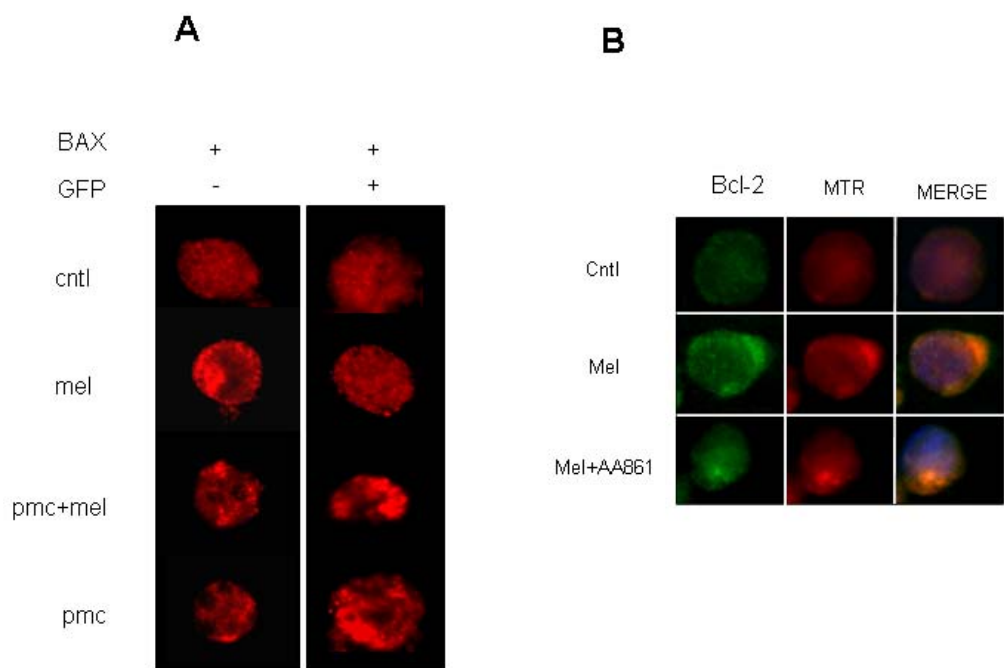Fig.4

Fig.5

**A**

BAX   +   +
GFP   −   +

cntl

mel

pmc+mel

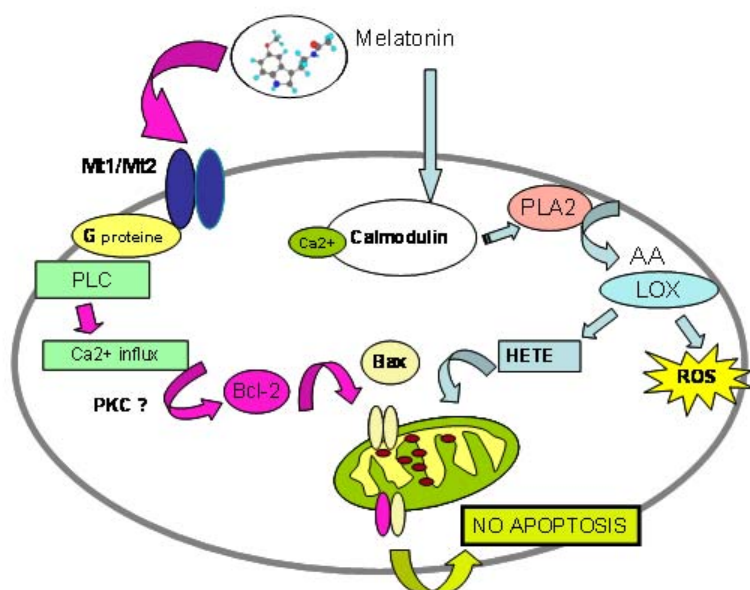pmc

**B**

Bcl-2   MTR   MERGE

Cntl

Mel

Mel+AA861
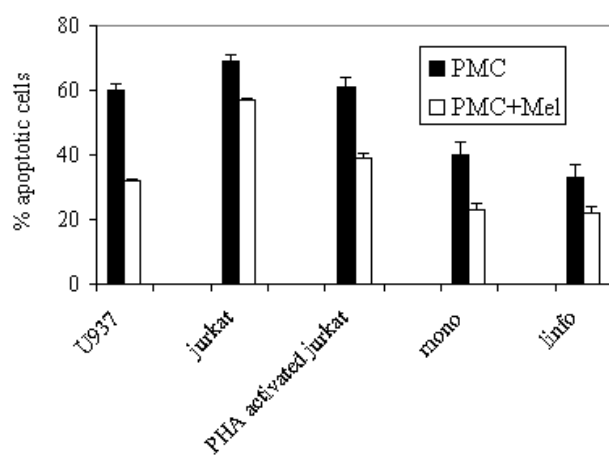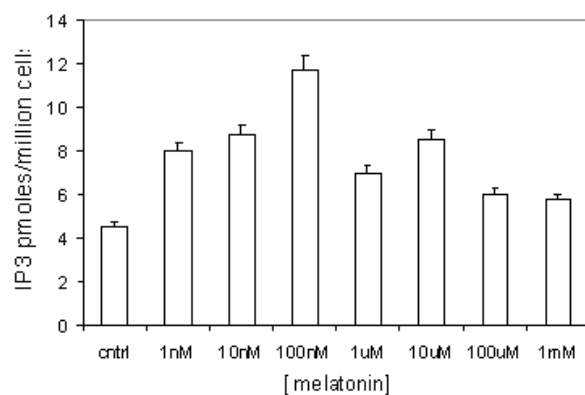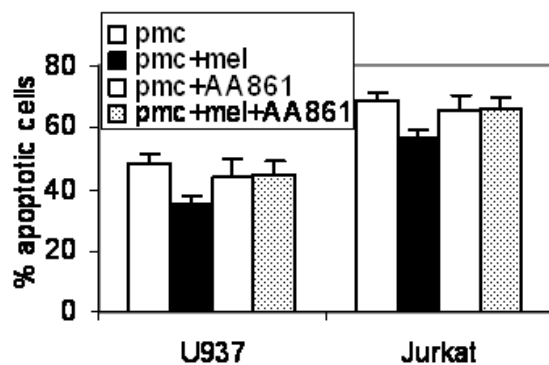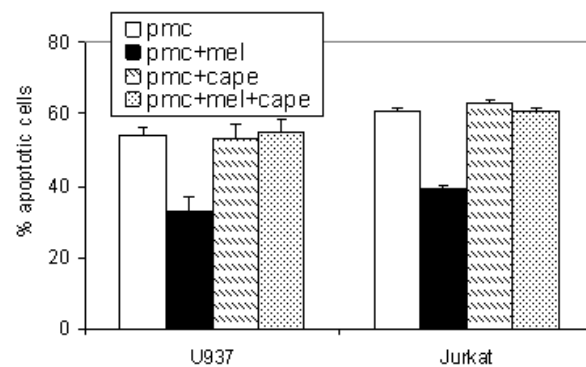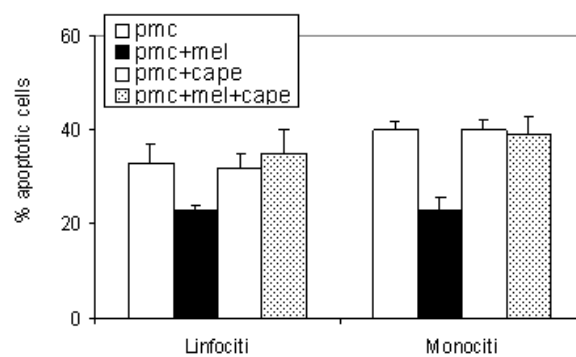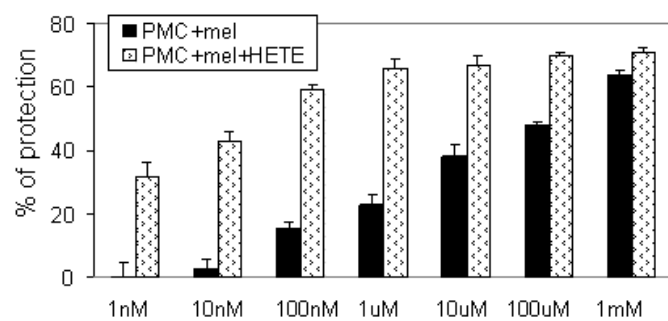
**Fig.6**

**Fig.7**

# PAPER IX

*Intracellular pro-oxidant activity of melatonin induces a survival pathway involving NF-kB activation*

# INTRACELLULAR PRO-OXIDANT ACTIVITY OF MELATONIN INDUCES A SURVIVAL PATHWAY INVOLVING NF-κB ACTIVATION

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**Running title:** Melatonin induces canonical NF-κB activation

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**Key words:** melatonin, NF-κB, ROS, survival

## Abstract

We have shown that melatonin exerts a pro-oxidant activity in U937 cells, a tumor human promonocytic cell line[1]. Here we show that melatonin induces a strong canonical activation of NF-κB, inducing IκBα degradation and the consequential nuclear translocation of p50/p65 subunits. The timing of NFkB activation overlaps with the timing of reactive oxygen species (ROS) production due to melatonin. Overexpression of dominant negative IκB, which prevents a possible NF-κB activation, transformed melatonin in a pro-apoptotic molecule. These data indicate for the first time that melatonin is able to trigger NFkB activation and might suggest a possible role for ROS induced by melatonin. Results indicate a possible involvement in survival pathway of melatonin generated ROS as secondary messenger.

### Introduction

Melatonin is a pineal hormone derived from tryptophan by a multi-step enzymatic reaction chain[2]. Considered for years to be exclusively produced in the pineal glands, more recently, melatonin production was detected in many other tissues[3]. Melatonin regulates the circadian rhythm of several biological functions and plays an important role in immuno-regulation[4] and inflammatory responses[5]. Numerous papers are based on powerful antioxidant functions of melatonin[6-9] that is believed to scavenge hydroxyl free radicals and many other related reactants[10]. In various experimental models of tissue damage, the protective role of melatonin was reported by reducing oxidative stress and lipid per-oxidation[11, 12]. The potential ability of melatonin to reduce radical production, increasing the activity of many antioxidant enzymes[13, 14], comes from *in vivo* evidences in oxidant-related pathological situations, where radical species are measured in extra-cellular fluids[15, 16]. However, studies based on the activity of melatonin in cell culture conditions give a completely different picture of its effect, describing an intense pro-oxidant activity of melatonin[1, 17]. Recently, in human leukemia Jurkat cells, was described a strong radical production due to a range of concentrations (from micro- to millimolar) of melatonin correlated to a decrease of glutathione level[17]. Moreover, we have also observed that in U937, human tumour promonocytes, melatonin acts as a pro-oxidant treatment, inducing a time related increase of intracellular ROS, between seven- and threefold more than in untreated cells, and promoting glutathione (GSH) depletion[1].

Even if ROS are predominantly implicated in causing cell damage[18], they also play a major physiological role in several aspects of intracellular signaling and regulation[18]. Cells are able to generate endogenous ROS which are utilized in the induction and maintenance of signal transduction pathways[19]. The initiation of several signal transduction pathways depend on the action of ROS as signaling molecules, which may act on different levels in the signal transduction cascade[19]. Thus, ROS can play a very important role as secondary messengers[20, 21] and, probably, their most significant effect on signaling pathways has been observed in the mitogen-activated protein kinase pathways[22]. These involve activation of nuclear transcription factors that control the expression of protective genes that are related with the life and the death of a cell.

A number of reports published during recent years indicate that some ROS are able to affect the activation or activity of NFκB transcription factors[23].

NFκB is a transcription factor family that responds to cytokine receptor stimulation to bind a common DNA sequence motif, the κB box[24], thus promoting transcription of genes involved in many cellular dynamics (activation, proliferation, apoptosis) that control the inflammatory and immune response[25]. The NFκB family of proteins is constituted by five different subunits (RelA or p65, RelB, cRel, p50 and p52); they are differently assembled to produce different active homo- or hetero-dimers[26, 27] displaying selective promoter targeting, thus accounting for the differential set of genes trans-activated by each dimer[28]. In untreated cells, NF-κB is sequestered in the cytoplasm by the interaction with a member of the inhibitory (IκB) family. Activation of NF-κB occurs in response to a wide variety of extracellular stimuli that promote the degradation of IκB, thereby allows entry of NF-κB into the nucleus and binds κB regulatory elements. NF-κB regulates several genes involved in cell transformation, proliferation and survival[29]. Reactive oxygen species have been implicated as second messengers involved in the activation of NF-κB *via* tumour necrosis factor (TNF) and interleukin-1[23].

The effect of melatonin on NFκB activation is not well described in the literature. Indeed, a majority of the studies focus on the melatonin inhibitory effect on injury/inflammatory induced NF-κB activation [30-32], rather than on the possible effect of melatonin as inducer of NF-κB activation. So far no paper reports the effect of the melatonin alone on the constitutive NFκB activity. Because little is known about the link between melatonin and the NF-κB, we examined the nature of this relation in U937 cells, a cell line where we have already showed the pro-oxidant nature of melatonin[1].

### Materials and Methods

*Cell culture and treatments*

U937 cells were cultured as described[33]. All the experiments were performed in complete medium at cell density of $7 \times 10^5$ cells/ml. Apoptosis was quantified as the fraction of apoptotic nuclei upon staining with the DNA specific dye Hoechst 33342 (Sigma Chemical Co., St.Louis, MO, USA) as described[34]. Melatonin (Mel) (Fluka, Switzerland) was added to the cell culture at the final concentration of 1mM. Vitamin E analogue Trolox C (Fluka, Switzerland) was used as radical scavenger[35]; it was added to the cell culture at the final concentration of 500uM (1hour prior to Melatonin when used together). To induce NFκB activation, 20ng/ml TNFα (Sigma) was added in the culture medium for 2 or 4 hours.

*Western Blot Analysis*

Nuclear and cytoplasmic proteins are extracted from $10^7$ cells in different experimental conditions according to Schreiber et al.[36]. During this procedure, the cells were lysed in a hypertonic detergent medium containing protease inhibitors. The extraction was performed on ice to avoid denaturation of the proteins. Whole cell extracts were prepared with M-PER Mammalian Protein Extraction Reagent, according to manufacture's instruction (Pierce, Rockford, IL, USA); briefly, $10^7$ U937 cells were washed in PBS and the pellet was resuspended in 400ul of the solution containing protease inhibitor, vortexed horizontally for 15min at 4°C and then centrifuged at 13000 rpm for 15min. Equivalent amount of proteins were separated through 10% SDS-PAGE gel and electrotransferred to nitrocellulose membranes. Immunoblotting was performed by blocking over-night aspecific binding with 5% non-fat milk in PBS-Tween 0.1%. Blots were then incubated 1h with 0.5-1ug/ml of following antibodies diluted in a solution of PBS-Tween 0.1% and Milk 5%: anti-NF-κB -p50 rabbit polyclonal (NLS), anti-NF-κB -p65 rabbit polyclonal (A), anti-IκBα rabbit polyclonal (C-21) (all obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Tubulin mouse monoclonal (Calbiochem), anti-Actin mouse monoclonal (Sigma) and anti-Lamin A/C mouse monoclonal (VisionbioSystems Novocastra, Newcastel, UK). The membranes were washed and incubated for 1h with a secondary antibody-HRP conjugated goat anti-rabbit or goat anti-mouse (all obtained from Santa Cruz). Then, the specific immunoreactive protein bands were identified using the ECL system by Amersham, Little Chalfont, UK.

*Electrophoretic Mobility Shift Assay*

Nuclear extracts were prepared after lysis in high salt extraction buffer as described [37] and stored at -80 °C. Aliquots of nuclear extracts (20μg of protein) were incubated with $^{32}$P-labelled κB DNA probe, consensus NFkB site 5'-AGTTGAGGGGACTTTCCCAGGC-3' (Eurogentec, Liege, Belgium) followed by analysis of DNA binding activities by EMSA. Binding reaction was performed as described[37]. Complexes were analyzed by electrophoresis on a non-denaturing 5% poly-acrylamide gel. DNA-protein complexes migrate more slowly than the unbound radioactive probe and consequently they are visualized as distinct bands of radioactivity. The acrylamide gel image was detected by autoradiography.

*Transient transfection*

Transient transfections of U937 cells were performed as described previously[38]. A 5μg of DN-IκBα plasmid (kindly provided by Dr. Bharat B. Aggarwal) and 5μg of pDsRed-Monomer-N1 (BD Biosciences) were used for each pulse. Following electroporation, the cells were resuspended in growth medium (RPMI/FCS 10%) and incubated at 37 °C and 5% of CO2. 48 h after transfection, the cells were harvested and re-suspended in growth medium (RPMI/FCS 10%) to a final concentration of 106 cells/mL and then treated for 3 h with melatonin 1mM. All treatments were performed with a 40% of transfected cells.

*Statistical analysis*

Statistical differences were determined using Student's t-test for unpaired data, and p values <0,05 were considered significant.

### Results

We have previously shown that melatonin leads to a significant ROS production which corresponds to a significant depletion of glutathione[1], without affecting cell viability. Here, we investigated whether melatonin-induced ROS production might be important for survival. To this purpose, we prevented any ROS formation in melatonin-treated cells by using the well-known radical scavenger Trolox C, an analogue of Vitamin E; then, cell viability was analyzed. As shown in Fig.1, Trolox C, that *per se* is unable to affect viability, as melatonin alone [39], sensitizes U937 cells to apoptosis. This indicates that ROS are required to trigger a survival response to melatonin treatment in our cells.

It is reported that ROS production may trigger an inflammatory/survival response, *via* the activation of NF-κB and AP-1[23, 40]. Next, we analyzed the possible involvement of NF-κB in melatonin-induced cell response. Fig.2A reports the time-course of NF-κB activation, performed by Electrophoretic mobility shift assay. Melatonin induces a time-dependent increase (up to 5 hrs of melatonin exposure) in NFkB activation, with timing similar to TNFα. Thus, melatonin is able to activate NFkB.

NFkB activation may follow a canonical *vs.* non-canonical pathway of activation, the first one only requiring the formation and translocation from the cytosol to the nucleus of the heterodimer p50/p65[25]. To investigate which pathway was involved in melatonin-dependent NFkB activation, we analyzed the localization of p65 and p50 in both nuclear and cytosolic fractions of melatonin-treated cells. Fig.2B shows the western blot analysis of nuclear and cytosolic fractions for NFκB subunits p50 and p65. Melatonin induces a nuclear translocation of both NFkB subunits p50 and p65, as TNFα that activates NFkB via the canonical pathway, (Fig.2B), thus indicating the ability of melatonin to induce NFkB in a canonical way. The canonical pathway of NFκB activation is dependent to IkBα degradation[25]. We expect to detect also IkBα degradation following melatonin treatment. As shown in Fig.2C, melatonin effectively leads to a very early IκBα degradation (1hour), thus confirming that melatonin activates NFkB via a canonical IκBα-dependent mechanism.

If such activation is responsible for their survival, the inhibition of the NFκB canonical pathway should affect cell viability. To explore this possibility, we study the effect on survival of melatonin, by preventing NFκB pathway activation via over-expression of dominant negative form of IκBα (IκBα-DN). Transient transfection of IκBα-DN

expression plasmide induces in the cells an overexpression of IκBα lacking the phosphorylation site (lacking residues 1–36) responsible for its degradation. Moreover, we co-transfected U937 with a pDsRed-Monomer-N1 plasmide that enables us to detect transfected cells by microscopy. It was possible to perform an *in situ* analysis of the transfected cells. By applying this approach, 48 hours after the IκBα-DN plasmid transfection, U937 cells were treated with melatonin for further 3 hours and then analysed for apoptosis (nuclear vesciculation) by image analysis, evaluated only on the fraction of succesfully transfected cells. Fig.3 shows that the over-expression of IκBα dominant negative *per se* induces a basal level of apoptosis, and that melatonin treatment becomes an apoptotic treatment in NFκB inhibited cells. This indicates an involvement of NFκB activation in the survival to melatonin treatment.

### Discussion

In this study, we have shown that melatonin is able to activate NF-κB via the canonical pathway. This activation is crucial for cells to survive to melatonin. To our knowledge, this is the first time that the ability of melatonin to activate NF-κB is demonstrated. Our results are in strong contrast with the data reported in literature, showing instead an inhibitory effect of melatonin on the canonical NF-κB activation. Published data show that the effect of melatonin on NF-κB activation is always analyzed in cellular systems where NF-κB is constitutively activated or previously activated by injuring condition, without showing an effect of melatonin alone on NF-κB[30-32, 41]. Thus, we cannot exclude that in pathological systems in which several cell parameters are altered, melatonin might have different levels of action or a different target also leading to the inhibition of NF-κB, as previously described.

Melatonin is considered to have an oncostatic action in cancer cells, as revealed by its ability to control the tumor growth. Here, we show that melatonin is able to induce a pro-survival pathway in which the activation of NF-κB is paradoxically necessary to survive to the same melatonin treatment. This might imply that melatonin could *per se* be a potent stress agent affecting cell viability. We have previously shown that this neurohormone desensitizes tumor cells of haematopoietic origin to apoptosis induced by several cell-damaging agents[39, 42]. We have ascertained that this antiapoptotic effect is due to the ability of melatonin to promote the mitochondrial targeting of Bcl-2 in order to antagonize the intrinsic pathway of apoptosis (Radogna et.al, *in press*). In addition, we have proved that this effect depends on the melatonin-specific receptor stimulation. The dose of melatonin used in our experiments is much higher than the dose normally required for receptor stimulation. Thus, at this level of our study, we might hypothesize that the effect of melatonin on Bcl-2 re-localization and on NFkB, might belong to two different pro-survival pathways, whose activation depends on a receptorial *vs.* non-receptorial melatonin-dependent pathways. The only correlation described in theliterature between Bcl-2 protein and NF-κB activation is the NF-κB mediated trans-activation of the Bcl-2 protein after melatonin exposure. However, we can already exclude this option because we have never observed any Bcl-2 upregulation after 3-5 hours of melatonin exposure in U937. Therefore, Bcl-2 re-localization is induced by low concentrations of melatonin involving the activation of its receptors. NF-κB activation might be involved in the pro-oxidant activity of melatonin.

**References**

1.      Albertini, M. C.*, et al.* 2006. Intracellular pro-oxidant activity of melatonin deprives U937 cells of reduced glutathione without affecting glutathione peroxidase activity. Ann N Y Acad Sci. **1091**: 10-6.
2.      Reiter, R. J. 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr Rev. **12**: 151-80.
3.      Brzezinski, A. 1997. Melatonin in humans. N Engl J Med. **336**: 186-95.
4.      Zhang, Z.*, et al.* 1997. Melatonin, immune modulation and aging. Autoimmunity. **26**: 43-53.
5.      Reiter, R. J.*, et al.* 2000. Melatonin and its relation to the immune system and inflammation. Ann N Y Acad Sci. **917**: 376-86.
6.      Hung, M. W.*, et al.* 2008. Protective effect of melatonin against hippocampal injury of rats with intermittent hypoxia. J Pineal Res. **44**: 214-21.
7.      Reiter, R. J.*, et al.* 2003. Melatonin as an antioxidant: biochemical mechanisms and pathophysiological implications in humans. Acta Biochim Pol. **50**: 1129-46.
8.      Reiter, R. J. & D. X. Tan. 2003. Melatonin: a novel protective agent against oxidative injury of the ischemic/reperfused heart. Cardiovasc Res. **58**: 10-9.
9.      Omurtag, G. Z.*, et al.* 2008. Melatonin protects against endosulfan-induced oxidative tissue damage in rats. J Pineal Res.
10.     Poeggeler, B.*, et al.* 1993. Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. J Pineal Res. **14**: 151-68.
11.     Pieri, C.*, et al.* 1995. Melatonin is an efficient antioxidant. Arch Gerontol Geriatr. **20**: 159-65.
12.     Reiter, R. J.*, et al.* 1998. Melatonin as a pharmacological agent against oxidative damage to lipids and DNA. Proc West Pharmacol Soc. **41**: 229-36.
13.     Barlow-Walden, L. R.*, et al.* 1995. Melatonin stimulates brain glutathione peroxidase activity. Neurochem Int. **26**: 497-502.
14.     Rodriguez, C.*, et al.* 2004. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res. **36**: 1-9.
15.     Princ, F. G.*, et al.* 1998. In vivo protection by melatonin against delta-aminolevulinic acid-induced oxidative damage and its antioxidant effect on the activity of haem enzymes. J Pineal Res. **24**: 1-8.
16.     Allegra, M.*, et al.* 2003. The chemistry of melatonin's interaction with reactive species. J Pineal Res. **34**: 1-10.
17.     Wolfler, A.*, et al.* 2001. Prooxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. FEBS Lett. **502**: 127-31.
18.     Droge, W. 2002. Free radicals in the physiological control of cell function. Physiol Rev. **82**: 47-95.
19.     Valko, M.*, et al.* 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. **39**: 44-84.
20.     Lowenstein, C. J., J. L. Dinerman & S. H. Snyder. 1994. Nitric oxide: a physiologic messenger. Ann Intern Med. **120**: 227-37.
21.     Storz, P. 2005. Reactive oxygen species in tumor progression. Front Biosci. **10**: 1881-96.
22.     Sun, Y. & L. W. Oberley. 1996. Redox regulation of transcriptional activators. Free Radic Biol Med. **21**: 335-48.
23.     Gloire, G., S. Legrand-Poels & J. Piette. 2006. NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol. **72**: 1493-505.

24.    Sen, R. & D. Baltimore. 1986. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell. **47**: 921-8.

25.    Gilmore, T. D. 2006. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. **25**: 6680-4.

26.    Thanos, D. & T. Maniatis. 1995. NF-kappa B: a lesson in family values. Cell. **80**: 529-32.

27.    Saccani, S., S. Pantano & G. Natoli. 2003. Modulation of NF-kappaB activity by exchange of dimers. Mol Cell. **11**: 1563-74.

28.    Natoli, G*., et al.* 2005. Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. Nat Immunol. **6**: 439-45.

29.    Amiri, K. I. & A. Richmond. 2005. Role of nuclear factor-kappa B in melanoma. Cancer Metastasis Rev. **24**: 301-13.

30.    Chetsawang, B*., et al.* 2006. Melatonin protects against hydrogen peroxide-induced cell death signaling in SH-SY5Y cultured cells: involvement of nuclear factor kappa B, Bax and Bcl-2. J Pineal Res. **41**: 116-23.

31.    Li, J. H*., et al.* 2005. Melatonin reduces inflammatory injury through inhibiting NF-kappaB activation in rats with colitis. Mediators Inflamm. **2005**: 185-93.

32.    Alonso, M., P. S. Collado & J. Gonzalez-Gallego. 2006. Melatonin inhibits the expression of the inducible isoform of nitric oxide synthase and nuclear factor kappa B activation in rat skeletal muscle. J Pineal Res. **41**: 8-14.

33.    Ghibelli, L*., et al.* 1995. Non-oxidative loss of glutathione in apoptosis via GSH extrusion. Biochem Biophys Res Commun. **216**: 313-20.

34.    Ghibelli, L*., et al.* 1998. Rescue of cells from apoptosis by inhibition of active GSH extrusion. Faseb J. **12**: 479-86.

35.    Casini, A. F., A. Pompella & M. Comporti. 1984. Glutathione depletion, lipid peroxidation, and liver necrosis following bromobenzene and iodobenzene intoxication. Toxicol Pathol. **12**: 295-9.

36.    Schreiber, S. S. 1989. Ethanol, acetaldehyde and cardiac protein synthesis: the relation to cardiomyopathy. Br J Addict. **84**: 133-9.

37.    Duvoix, A*., et al.* 2004. Expression of glutathione S-transferase P1-1 in leukemic cells is regulated by inducible AP-1 binding. Cancer Lett. **216**: 207-19.

38.    Folmer, F*., et al.* 2006. Inhibition of TNFalpha-induced activation of nuclear factor kappaB by kava (Piper methysticum) derivatives. Biochem Pharmacol. **71**: 1206-18.

39.    Radogna, F*., et al.* 2006. Melatonin as an apoptosis antagonist. Ann N Y Acad Sci. **1090**: 226-33.

40.    Tonks, N. K. 2005. Redox redux: revisiting PTPs and the control of cell signaling. Cell. **121**: 667-70.

41.    Chuang, J. I*., et al.* 1996. Effect of melatonin on NF-kappa-B DNA-binding activity in the rat spleen. Cell Biol Int. **20**: 687-92.

42.    Radogna, F*., et al.* 2007. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res. **43**: 154-62.

**Figures legends**

**Fig.1 Melatonin induces NF-κB activation in U937.**

A): Image analysis of nuclei morphology with Hoechst 33342. Apoptosis was evaluated by quantification of nuclear fragmentation as described in materials and methods

B): Time course of NFκB activation upon melatonin as detected by gel mobility shift analysis (EMSA); equal (15ug) amount of protein was loaded for each sample as described in Materials and Methods.

**Fig.2 NF-κB activation by melatonin occurs via IkB-dependent mechanisms.**

A): Cellular localization of p50 and p65 on nuclear and cytosolic extracts of U937 treated as indicated; tubulin and lamin A/C are shown as loading and fraction purity control. TNFα treatment (20ng/ml) for 2 hours was used as positive control for the nuclear translocation of p50 and p65 subunits.

B): Time course of IκB levels in U937 treated with Melatonin (1mM) at the indicated times, as detected by western blot. 15μg total protein extracts were used.

**Fig.3 Involvement of NF-κB activation in the survival to melatonin treatment.**

Apoptosis was evaluated in cells co-transfected with 5μg of IκBα-DN plasmid and 5μg of pDsRed-Monomer-N1 in the absence or presence of melatonin 1mM. The viability after 3hr is significantly reduced upon IκBα dominant negative overexpression. Results are the average of three independent experiments ± SD.
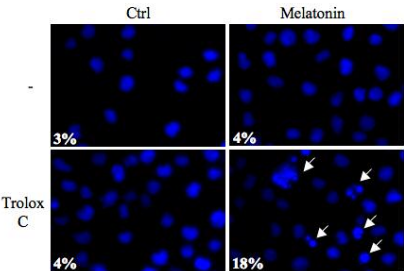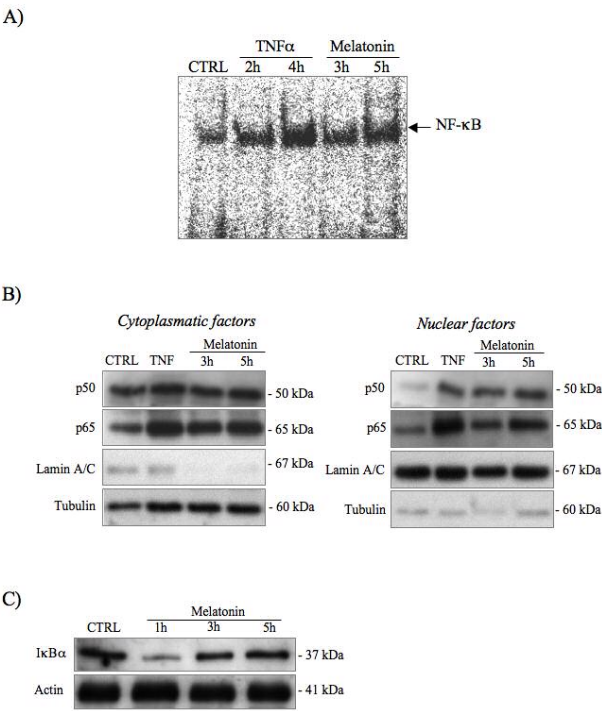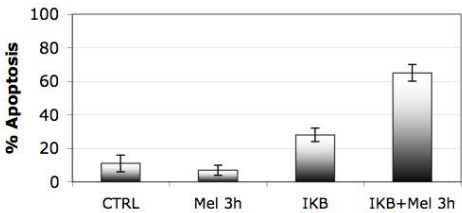
Fig.1

Fig.2

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C)

Fig.3

# PAPER X

*Neuroprotection by melatonin on astrocytoma cell death*

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Radogna *et al*.: Melatonin's protective effect on astrocytoma.

# NEUROPROTECTION BY MELATONIN ON ASTROCYTOMA CELL DEATH

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**ABSTRACT**

Glial cells play an active role in the homeostatic regulation of central nervous system (CNS). Astrocytes, the most abundant glial cell types in the brain, provide mechanical and metabolic support for neurons. The regulation of astrocyte apoptosis, therefore, is important for physiological and pathological processes in CNS. Melatonin is a neuro-hormone that regulates target cells via binding to specific high affinity plasma membrane receptors, MT1/MT2. In addition to regulating circadian rythms, melatonin is recently attracting much interest for its potential regulation of cell apoptosis. We recently showed that melatonin antagonizes apoptosis on U937 cells via intersecting signal transduction events involving binding to MT1/MT2 and activation of lipoxygenase. In this study, we describe the neuro-protective potential of melatonin, showing that melatonin significantly reduces damage-induced apoptosis in astrocytoma cells. The mechanism of protection is different with respect to that shown on U937, since it does not involve MT1/MT2 or lipoxyegenase; likewise, Ca2+ influx is not involved. Intriguingly, inhibition of PLC with neomycin reverses melatonin protection, suggesting that a PLC-dependent signal transduction, different from that triggered by MT1/MT2, is involved in the anti-apoptotic pathway of melatonin.

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## INTRODUCTION

Glial cells, including astrocytes and microglial cells, are well known protectors of neurons[1,2]. Astrocytes contribute to the neuroprotection and preserve neuron survival; thus any astrocytic dysfunction seriously affects neuronal viability[3]. Apoptosis is an essential physiological process for maintenance of brain homeostasis and plays a critical role in ischemia, neurodegenerative disease and brain tumors[3,4].

Melatonin plays a central role in circadian rhythms and regulation of seasonal reproductive cycles in vertebrate physiology[5]. It is biosynthesized from L-tryptophane by a multi-step enzymatic reaction in pinealocytes[5] and leucocytes[6]. This neuro-hormone, is involved in many regulatory functions of the cells, possibly through receptor engagement, i.e., it modulates the immune response[6] regulates the apoptotic response on a number of cell types[7], regulates signal transduction reactions[8]. On the basis of the molecular structure, three subtypes of the melatonin receptors have been described: $Mel_{1A}$ or MT1 (expressed in mammalian and bird brain), $Mel_{1B}$ or MT2 (expressed mainly in mammalian retina), $Mel_{1C}$ or mt3 (found in amphibian melanophores, brain, and retina, and also in bird and fish brain)[9]. Melatonin receptors regulates several second messengers: cAMP, cGMP, diacylglycerol, inositol trisphosphate, arachidonic acid, and intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$)[8].

Melatonin is known to be an endogenous free-radicals scavenger and an antioxidant in many systems, especially effective in reducing/preventing oxidative damage in the brain[10-12]. It has been demonstrated that melatonin protects neurons from cell death induced by several neurotoxins at pharmacological and physiological doses[13-15].

However, the mechanism by which melatonin prevents apoptosis on CNS is not clarified. Thus, it is important to understand whether the neuro-protective effect of melatonin derives from its ability to engage specific receptors[5], or from its antioxidant ability to directly pass the blood-brain barrier and scavenge free radicals[5,16,17].

In this study, we analyse the protective effect of melatonin in cultured astrocytoma cells induced to apoptosis by the cell-damaging agent puromycin, focusing on the the role of

receptor interaction and the subsequent signal transduction pathway as possible mechanisms involved, in view of the important therapeutic potential that melatonin may exert in the treatment of neurodegenerative disease.

**MATERIALS AND METHODS**

*Cell culture*

Human astrocytoma cell line 132 1N1 was maintained in 25-cm$^2$ flasks in DMEM with 10% inactivated fetal calf serum (FCS), 2mM L-glutamine, 100 IU/ml penicillin and streptomycin, and kept in a controlled atmosphere (5%CO2) incubator at 37°C. Cells were trypsinized weekly and reseeded for experiments. Medium was renewed every 2–3 days and after 7-8 days the cells became sub-confluent and ready for use.

*Treatments*

Melatonin (Mel) was used at the final concentration of 1mM, unless otherwise specified, and added to the culture medium 1 hour before apoptosis induction. Melatonin action on MT1/MT2 receptors was antagonized by 2-benzyl-N-acetyltryptamine (luzindole). Luzindole was added at the concentration of 50 µM, 30 minutes before other treatments.   For phospholipase C (PLC) inhibition, neomycin was used at 100µM, 30 minutes before other treatments. 10 µM nifedipine, a Ca2+ fluxes inhibitor, was added 30 minutes before other treatments.   For  lipoxygenase inhibition, the specific 5-LOX inhibitors AA861 was used at 20 µM and added 30 minutes before other treatments.

*Induction of apoptosis*

Apoptosis was induced by the protein synthesis inhibitor puromycin (PMC, 10  µg/ml). Apoptosis was evaluated after 15 hours of PMC incubation.

*Identification and quantification of apoptotic cells*

Nuclear morphology of control and treated cells was analysed by fluorescence microscopy after staining with Hoechst 33342; apoptotic cells were characterized by nuclear condensation of chromatin and/or nuclear fragmentation. Apoptosis was evaluated among the Hoechst-stained cells by counting at least 300 cells in at least three randomly selected fields.

**RESULTS**

*Melatonin reduces apoptosis in astrocytoma cells*

We investigated melatonin effects on stress-induced apoptosis. Fig.1A shows that melatonin reduces PMC-induced apoptosis in a dose-dependent fashion, protection being highly significant for the 1 mM dose. The doses required for melatonin anti-apoptotic effect ($\geq$10uM) are not compatible with MT1/MT2 receptor stimulation since melatonin receptor affinity is 1nM. To definitely exclude that melatonin protective effect may depend on receptor interaction, we used luzindole, a specific melatonin membrane receptor antagonist. Luzindole is an important tool in melatonin research, as it specifically inhibits G protein activation by a melatonin-engaged MT1 or MT2 receptor[18]. Fig.1B shows that luzindole does not inhibit melatonin anti-apoptotic effects. Thus, this result confirms that melatonin is able to protect astrocytoma cells from apoptosis in a receptor-independent way.

*Melatonin protective effect is sensitive to neomycin.*

In U937 monocyte cells, melatonin exerts an anti-apoptotic effect by the interaction of two independent pathways: one involving MT1/MT2 receptor-interaction[21] and the other involving lipoxygenase (LOX) (Radogna et al., in preparation). In order to understand whether also in astrocytoma cells LOX was involved in melatonin protection, we investigated if AA861, a specific 5-lipoxygenase inhibitor, could abrogate melatonin protection. Fig.2A reported that, unlike U937, AA861 have not effect on melatonin protection on astrocytoma..

Instead, we found that neomycin, which inhibits phospolipase C (PLC) by protecting its substrate PIP2, partially abrogates melatonin protection (Fig.2B), suggesting a role of PLC in the anti-apoptotic pathway of melatonin. Activation of PLC stimulates the production of diacylglycerol (DAG) and IP3[19], which in turn stimulates Ca2+ influx from the extracellular environment. Since melatonin was shown to mobilize Ca2+ in other cell systems[20]. and that this plays a role in melatonin protection from apoptosis in U937 cell[21], we investigated the involvement of Ca2+ influx in melatonin protection on astrocytoma cells with the L-type

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Ca2+ channel inhibitor nifedipine. Fig.2C shows that nifedipine did not abrogate melatonin

protection. Thus, Ca2+ influx is not involved in the anti-apoptotic effects of melatonin.

**DISCUSSION**

Glial cells play an active role in CNS development and are essential for neuronal differentiation and neuronal survival[2]. Thus, the study of the modulation of apoptosis in astrocytes is particularly important, considering that any changes in astrocyte shape and function seriously affects neuronal viability[3,4]. Apoptosis has been associated with a variety of acute disease and chronic pathologies in which neuronal cell death correlates with an increase of oxidative stress[22-24]. With the goal to clarify the therapeutic potential of melatonin in neurodegenerative disease and neurological disorder, we investigated the role of melatonin on apoptosis of astrocytoma.

In a recent study we have demonstrated that melatonin exerts a similar protective effect on U937 monocytes, at the same doses[21]. In that cell system, the signal transduction elicited by melatonin MT1/MT2 receptor stimulation is the mechanism through which melatonin exerts its anti-apoptotic effects [21]. Here, we show that melatonin prevents apoptosis induced by the cell-damanging agents puromycin also in astrocytoma cells. Thus, we investigated whether the anti-apoptotic effect of melatonin in astrocytoma cells could follow the same mechanisms. Unexpectedly, we found that on astrocytoma cells the mechanism is very different. The doses required for the anti-apoptotic effect ($\geq$10uM) are not compatible with receptor stimulation, since melatonin receptor affinity is 1nM. Moreover, the MT1/MT2 antagonist luzindole does not revert the effect. Thus, on astrocytoma melatonin anti-apoptotic effect occurs in a receptor-independent way. However, it is likely that other signaling pathway may be mobilised by melatonin since the inhibition of PLC, a key actor in intracellular receptor-induced signaling, is sufficient to reverse melatonin anti-apoptotic effect in astrocytoma cells. However, this PLC protective pathway does not involve Ca2+ flux, as was instead demonstrated in U937 cells[21].

In U937 monocytic cells, we have found that melatonin anti-apoptotic effect requires the cooperation of an additional mechanisms involving lipoxygenase (LOX) (Radogna et al., in

preparation). However, unlike U937, the 5-LOX inhibitors AA861does not reverse melatonin anti-apoptotic effect, showing that 5-LOX is not involved in astrocytoma protection.

These findings demonstrate a clear melatonin protective effect in astrocytoma cell death that is different with respect to what we reported on leukocytes. Thus it is very important to consider that melatonin may exert very different effects depending on the cell type under study, and that even if the end result is similar, it may take place according to different mechanisms. In astrocytoma, antagonism of apoptosis occurs in a lipoxygenase-independent, MT1/MT2 receptor-independent way, but with the possible involvement of the PLC pathway. We cannot rule out other possible explanations; indeed, neomycin may have other cellular targets, and other means of PLC inhibition will help to identify more clearly the role of PLC. Moreover, we have to consider the possible role of melatonin as an antioxidant in contrasting apoptosis. Some groups indeed reported protection against programmed cell death in neuronal cells by antioxidant activity of melatonin[13-15]. Further investigations are under study to elucidate the mechanism of this effect, since the identification of the mechanism of melatonin protective effect may contribute to generate novel strategies for treating CNS injuries.

**REFERENCES**

1. Blanc, E.M., A.J. Bruce-Keller, & M.P. Mattson. 1998. Astrocytic gap junctional communication decreases neuronal vulnerability to oxidative stress-induced disruption of $Ca^{2+}$ homeostasis and cell death. J. Neurochem. **70**: 958–970.

2. Nedergaard, M., B. Ransom & SA. Goldman. 2003. New role for astrocytes: Redefining the functional architecture of the brain. Trends Neurosci. **26**:523–530.

3. Takuma, K., A. Baba, & T. Matsuda. 2004. Astrocyte apoptosis: implications for neuroprotection. Prog. Neurobiol. **72**:111–127.

4. Kobayashi, K., M. Hayashi, H. Nakano, et al. 2002. Apoptosis of astrocytes with enhanced lysosomal activity and oligodendrocytes in white matter lesions in Alzheimer's disease. Neuropathol. Appl. Neurobiol. **28**: 238–251.

5. Reiter, R.J. 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr. Rev. **12:** 151-180.

6. Carrillo-Vico, A., J.R. Calvo, P. Abreu, et al. 2004. Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possibile role as intracrine, autocrine, and/or paracrine substance. FASEB J. **18**: 537-539.

7. Sainz, R.M., J.C. Mayo, C. Rodriguez, et al. 2003. Melatonin and cell death: differential actions on apoptosis in normal and cancer cells. Cell Mol Life Sci. **60**: 1407-26.

8. Vanek, J. 1998. Cellular mechanism of melatonin action. Physiol.Rev. **78**:687-721.

9. Dubocovich, M.L. & M. Markowska  2005. Functional MT1and MT2 melatonin receptor in mammals. Endocrine **27** :101-10.

10. Tan D-X, L-D. Chen, B. Poeggeler et al. 1993. Melatonin: a potent, endogenous hydroxyl radical scavenger. Endocr J. **1**: 57– 60.

11. Reiter, R.J., D. Acuña-Castroviejo, D-X. Tan et al. 2001. Free radical-mediated molecular damage. Mechanisms for the protective actions of melatonin in the central nervous system. Ann N Y Acad Sci. **939**: 200– 215.

12. Reiter RJ. 1998. Oxidative damage in the central nervous system: protection by melatonin. Prog Neurobiol.**56**: 359– 384.

13. Pappolla, M, M. Sos, RA Omar *et al.* 1997. Melatonin prevents death of neuroblastoma cells exposed to Alzheimer's amyloid peptide. J Neurosci. **17**: 1683– 1690.

14. Post, A, F. Holsboer, C. Behl. 1998. Induction of NF-kappaB activity during haloperidol-induced oxidative toxicity in clonal hippocampal cells, suppression of NF-kappaB and neuroprotection by antioxidants. J Neurosci. **18**: 8236– 8246.

15. Mayo, JC, RM. Sainz, H. Uria *et al.* 1998. Melatonin prevents apoptosis induced by 6-hydroxydopamine in neuronal cells, implications for Parkinson's disease. J Pineal Res.**24**: 179– 192.

16. Martín, V., Sainz, RM., Antolín, I. et al. 2002. Several antioxidant pathways are involved in astrocyte protection by melatonin. J Pineal Res. **33**: 204-12.

17. Reiter, RJ., J. Cabrera, RM. Sainz, et al. 1999. Melatonin as a pharmacological agent against neuronal loss in experimental models of Huntington's disease, Alzheimer's disease and parkinsonism. Ann N Y Acad Sci. **890**:471-85. Review.

18. DUBOCOVICH ML. 1998. Luzindole (N-0774): a novel melatonin receptor antagonist. J Pharmacol Exp Ther. **246**:902-910.

19. BACH, AG., S. WOLGAST, E. MUHLBAUER, E. PESCHKE. 2005. Melatonin stimulates inositol-1,4,5-triphospathate and $Ca^{2+}$ release from INS1 insulinoma cells. J Pineal Res. **39**:316-323.

20. MARKOWSKA, M., A. MROZKOWIAK, J. PAWLAK, K. SKWARL-SONTA 2004. Intracellular second messengers in melatonin signal transduction in chicken splenocytes in vitro. J Pineal Res. **37**:207-212.

21. Radogna, F., L. Paternoster, MC. Albertini, et al. 2007. Melatonin antagonizes apoptosis via receptor interaction in U937 monocytic cells. J Pineal Res. **43**:154-62.

22. Olanow CW. A radical hypothesis of neurodegeneration. 1993. Trends Neurosci. **16**: 439– 444.

23. Wellington CL, & MR. Hayden. 2000. Caspases and neurodegeneration: on the cutting edge of new therapeutic approaches. Clin Genet. **57**: 1– 10.

24. Halliwell B. Reactive oxygen species and the central nervous system. 1992. J Neurochem. **59**: 1609– 1623.

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**FIGURES LEGENDS**

**FIGURE 1.** Melatonin protect astrocytoma cells from apoptosis in a receptor-indipendent way. a) Astrocytoma cells were incubated in complete medium (see material and methods) with different doses of melatonin for for 1 hr, followed by puromycin (PMC; 10ug/ml). Apoptosis was evaluated at 15 hours. Results are the average of at least 5 independent experiments +/-SD. Apoptosis reduction is significant starting from melatonin 10uM ($p = <0.05$)*. b) Melatonin anti-apoptotic effect is sensitive to luzindole. Luzindole 50 uM was added 30 min before incubation with melatonin 1 mM and then apoptosis was induced with puromycin (PMC; 10ug/ml). Apoptosis was estimated at 15 hours. Results are the average of at least 3 independent experiments +/-SD.

**FIGURE 2.** Melatonin protective effect  require phospholipase C but not Ca2+fluxes and lipoxygenase. a) Lipoxygenase is not required for melatonin anti-apoptotic effect. AA861 20uM, a 5-LOX inhibitor, was added 30 min before  melatonin 1mM treatment, and then apoptosis was induced with with puromycin (PMC; 10ug/ml). Results are the average of at 3 independent experiments +/-SD. b) Astrocytoma cells were incubated with neomycin 100uM, a PLC inhibitor, added 30 min before melatonin 1mM treatment, and then apoptosis was induced with with puromycin (PMC; 10ug/ml). Results are the average of at 3 independent experiments +/-SD. c) Astrocytoma cells were incubated with nifedipine 10uM, inhibitor of Ca2+ fluxes, added 30 min before melatonin 1mM treatment, and then apoptosis was induced with with puromycin (PMC; 10ug/ml). Results are the average of at 3 independent experiments +/-SD.
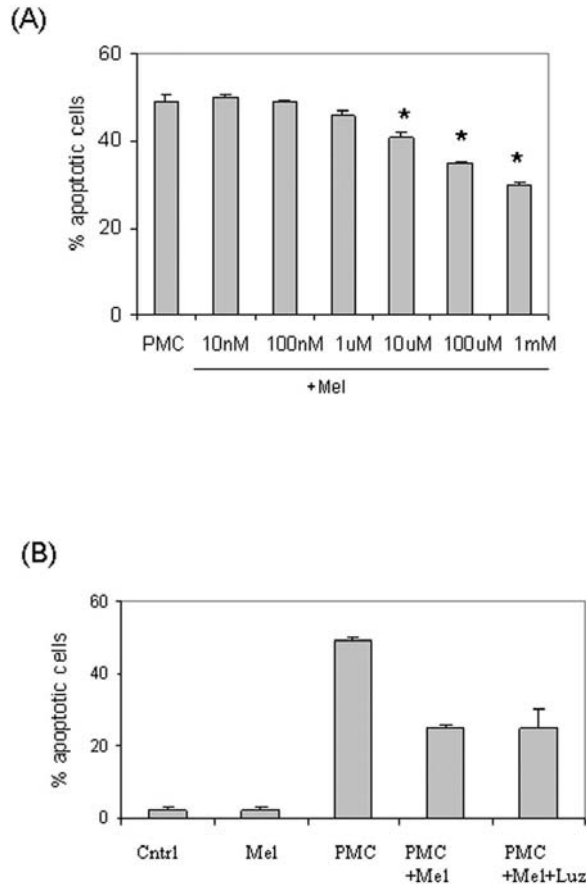
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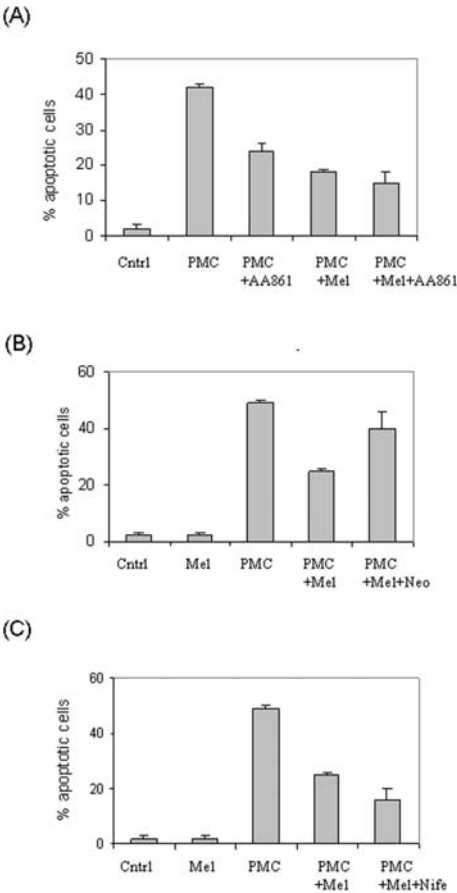88x121mm (300 x 300 DPI)

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