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DOTTORATO DI RICERCA IN BIOLOGIA CELLULARE E MOLECOLARE XXI CICLO

TESI

Melanoma cells differentiation through inhibition of p-Mek activity following treatment with 5,7-dimethoxycoumarin

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1. INTRODUCTION

1.1 The coumarins

1.1.1 Chemistry of the coumarins.

Coumarins are a group of heterocyclic compounds synthesized by numerous plant species as well as by some bacteria and fungi (O'Kennedy & Thornes, 1997), that have been widely used as a fragrance in food and cosmetic products (Lake, 1999).

They are characterized by the presence of fused benzene and α-pyrone rings as a structural nucleus and they can occur as the hydroxylated, alkoxylated or alkylated derivatives of the parent compound, the coumarin (1,2-benzopyrone), along with their glycosides (Hoult & Paya, 1996). These compounds may be located in the peel of seeds, in the roots, in the leaves and stems, but the highest concentration was found in flowers and fruits. The most representative molecule, the coumarin, has been extensively studied both in biochemical and pharmaceutical fields (Hoult & Paya, 1996); also the 7-hydroxycoumarin aroused a great interest, as the first compound derived from the metabolism of coumarin in humans. There are other

derivative molecules with important biological properties, such as furanocoumarins and pyranocoumarins; among the furanocoumarins, we should mention the 8-methoxypsoralen or xanthotoxin, a light-sensitizing agent, which can cause skin diseases in phytophage animals, if they produce abrasions and injuries to plants synthesizing these defense compounds (Timmermann *et al.*, 1999).

**Coumarin
(1,2-benzopyrone)**
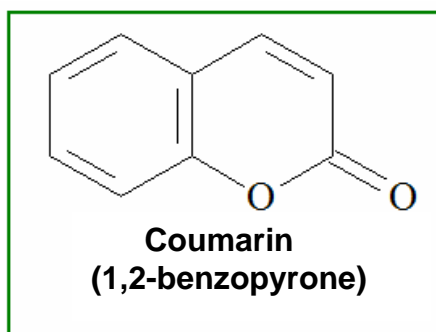
Figure 1. Molecolar structure of the coumarin.

1.1.2 The role of the coumarins in the plant.

The role of these secondary metabolites in the plants is mainly related to defense, considering their antimicrobial and deterrent properties, their shielding activity against UV and allelopathic functions (Buchanan *et al.* , 2003). The scopoletin, or 7-hydroxy-6-methoxycoumarin, is the most interesting coumarin in the study of defense mechanisms in the plants; it is the first phytoalexin to be identified in *Hevea brasiliensis* L., where it is accumulated in the leaves following infections with several fungi, such as

Mycrocyclus ulei, *Colletotrichum gloeosporioides*, and *Ophiostoma ulmi*. The accumulation of scopoletin in plant tissues is observed also when the plants are exposed to abiotic factors, such as $CuCl_2$ and UV radiation which trigger a state of physiological stress (Gutierrez *et al*., 1995). In *Corchorus olitorius* L., the synthesis of scopoletin was observed contemporaneously with that of other coumarins - the fraxinol, the isopimpinellin, the xanthotoxol - after treatment of the leaves with biotic and abiotic stress agents (Abou Zeid, 2002). Even furanocoumarins are known for their antifungal and antibacterial activities, such as 8-methoxypsoralen and 5-methoxypsoralen, identified for the first time in the leaf extract of *Ruta graveolens* L; the two phytoalexins have demonstrated a fungistatic activity against *Fusarium solani*, *Pyrenochaeta lycopersici* and *Trichoderma viride*, inhibiting the mycelial growth (Oliva *et al*., 1999). 5,8-Di-(2,3-dihydroxy-3-methylbutoxy)-psoralen and other furanocumarine (the psoralidin, the psoralin and the angelicin), extracted from the seeds of *Psoralea corylifolia* L. (Khatune *et al*., 2004), showed bacteriostatic effect, particularly against *Shigella sonnei*, *Shigella flexneri* and *Staphylococcus aureus*. The antibacterial and antifungineal activities of these plant metabolites have a great importance for human and environmental health; indeed, coumarins may be used instead of antibiotics against pathogens affecting humans, and also as fungicides in agriculture instead of the synthetic preparations (Oliva *et al*., 1999, Ojala *et al*., 2000). The coumarins are known for their allelopathic activities; the allelopathy was defined by Molisch (1937) as a chemical interaction between two plants, and may be both stimulative and inhibitory. Coumarin, for example, promotes or inhibits the growth of the plant and its allelopathic effect is species-specific and concentration-dependent (Murray *et al*., 1982). Several of the plant physiological processes

are the target of the coumarin: this compound reduces photosynthesis and it is able to inhibit respiration and electronic transport; it interferes with the absorption of nitrates in the seedlings of wheat and reduces nitrogen metabolism in cell cultures of carrot (Abenavoli *et al*., 2003). Anyway, the major effect of coumarin is the inhibition of radical growth, coupled with the reduction of diameter and branching of the roots (Abenavoli *et al*., 2004).

1.1.3 Antioxidant activity of the coumarins.

Several coumarins showed a strong antioxidant activity, due to their ability to neutralize reactive oxygen species (ROS) considered the main cause of cardiovascular disease, cancer, atherosclerosis and aging processes. If these highly reactive molecules are not neutralized by antioxidant systems, can cause oxidative damage to cellular components such as membrane lipids, proteins and DNA, resulting in the damage of cellular structures (Kaneko *et al*., 2001). The antiradical activity of the coumarins depends on the presence of hydroxyl groups (OH), linked to benzenic ring (Ng *et al*., 2000), while the α-pyrone ring contributes in small measure to antioxidant properties of these compounds (Zhang & Wang, 2004). Foti *et al*. (1996) demonstrated that the coumarins with a catechol group, which is the benzenic ring with two OH groups, had a higher antioxidant power against radical peroxide. Furthermore, it has been observed that the catechol group increases the antiradicalic activity of natural antioxidants, including coumarins; in coumarins class, antiradicalic properties decrease when the OH groups of catechol are replaced with methoxy or sugar groups (Ng *et al*., 2000; Zhang

& Wang, 2004). Among the coumarins, the 6,7-dihydroxycoumarin, or esculetin, is known to be an inhibitor of lipoxygenase enzyme (Pan *et al*., 2003); it shows neutralizing activity against the ROS, such as the superoxide and hydroxyl radicals and reduces the oxidative damage induced by butyl hydroperoxide in rat hepatocytes (Kaneko *et al*., 2003). More recently, by using human endothelial cells, it was shown the protective effect of esculetin and 4-methylesculetin against cytotoxic effects induced with linoleic hydroperoxide (LOOH), the first product resulting from the lipid peroxidation. Some coumarin derivatives, such as 7,8-dihydroxy-4-methylcoumarin and 7-hydroxy-4-methylcoumarin, inhibited significantly the peroxidation of low density lipoproteins (LDL) (Liu *et al*., 1999), which is known to contribute in atherosclerosis development (Esterbauer & Ramos, 1995; Klatt & Esterbauer, 1996).

1.1.4 Anticancer activity of the coumarins.

Several coumarins are known for their therapeutic activities and many of these have been purified from medicinal plants. In addition to their pharmacological properties, these plant metabolites showed a significant atoxicity in mammalian cells. The combination of these two characteristics makes coumarin a class of compounds of interest in pharmaceutical field (Hoult & Paya, 1996). Numerous biological activities have been attributed to simple coumarins and analogues as anti-coagulant (Suttie, 1987), hypotensive (Huang *et al*., 1992), antibacterial (Michaeli *et al*., 1970), anti-inflammatory (Paya *et al*., 1992), anticancer (Thornes *et al*., 1983; Gawron

& Glowniak, 1987; Marshall *et al*., 1987), antimutagenic (Pillai *et al*., 1999), anti-viral effects (Kostova *et al.*, 2006) and enzyme inhibition (Ghani *et al*., 2001; Gnerre *et al*., 2000), scavenging of reactive oxygen species (Vairagupta *et al*., 2000), anticarcinogenesis/chemoprevention of cancer properties (Kelly *et al*., 2000; Prince *et al*., 2006; Kleiner *et al*., 2002b; Cai *et al*., 1997; Wattenberg *et al*., 1979; Tanaka *et al*., 1998; Baba *et al*., 2002). Many of the cancer chemopreventive effects of coumarins have been attributed to their abilities to modulate xenobiotic metabolizing enzymes. For example, bergamottin was one of the most potent at suppressing mouse hepatic ethoxyresorufin O-dealkylase activity (Cai *et al*., 1993); furthermore, it inhibited the formation of the major benzo[*a*]pyrene (B[*a*]P)-DNA adduct, *anti*-benzo[*a*]pyrene-7,8-diol,9,10-epoxide-dGuo, in mouse epidermis *in vivo*, resulting in the block of B[*a*]P-induced skin tumor initiation in mice (Cai *et al*., 1997). The ability of bergamottin to suppress mouse skin tumor initiation by B[*a*]P is likely mediated by its ability to suppress cytochrome P450 1A1 dependent metabolic activation of B[*a*]P into the *anti*-benzo[*a*]pyrene-7,8-diol,9,10-epoxide. Bergamottin is a weak inhibitor of murine P450 1B1, and was not effective at suppressing P450 1B1-dependent metabolism of 7,12-dimethylbenz[a]anthracene (DMBA) into the syn-DMBA diol-epoxide derived DNA adducts (Kleiner *et al*., 2002a). In contrast, imperatorin and isopimpinellin were similarly effective at suppressing both P450 1A1 and P450 1B1 activities (Kleiner *et al*., 2003) and were effective at suppressing DNA adduct formation and skin tumor initiation from both B[a]P and DMBA (Cai *et al*., 1997; Kleiner *et al*., 2002b).

Wattenberg and colleagues (Wattenberg *et al*., 1979; Sparnins & Wattenberg, 1981) showed that coumarin enhanced forestomach glutathione

S-transferase (GST) activity and sulfhydryl levels following 2 weeks of dietary administration to mice. The compound also induced NAD(P)H quinone oxidoreductase (NQO) activity in small intestine of mice, and both coumarin and 3-hydroxycoumarin induced GST activity in the same (McMahon *et al*., 2001). Moreover, it was shown that coumarin increased the aflatoxin B1 (AFB1) aldehyde reductase, GSTA5, GSTP1, and NQO1 expression in rat liver; in the diet, it protected rats also from AFB1 hepatocarcinogenesis (Kelly *et al*., 2000). Thus, induction of GSTs and other carcinogen-detoxifying enzymes is one of the mechanisms by which the coumarins can inhibit chemical-induced carcinogenesis. Respect to anti-tumoral activity, different natural and synthetic coumarins are of great interest due to their described properties in sensitive neoplasm. Several authors reported that numerous nitrated, hydroxylated or isopentenylated coumarins and derived pyrano- and furanocoumarins show strong antiproliferative activity and induce apoptosis in various cancer cell lines (Appendino *et al*., 2004; Finn *et al*., 2002; Kawaii *et al*., 2001a; Kawaii *et al*., 2001b). Since the 1980s, some *in vivo* studies were carried out to investigate the possible use of coumarin in the treatment of kidney and renal carcinoma cells (Marshall *et al*., 1994; Kokron *et al*., 1991). Contemporaneously, *in vitro* experiments demonstrated that coumarin and 7-hydroxycoumarin were powerful cytotoxic and cytostatic agents in carcinogenic kidney cells (Marshall *et al*., 1994), following the inhibition of DNA, RNA and survival proteins synthesis. Instead, in the case of the bone cancer, coumarin and 7-hydroxycoumarin have demonstrated the properties to inhibit the growth of carcinogenic cells, inducing the cell cycle arrest in G_0/G_1 phase; induction of the apoptosis process was observed only in adenocarcinomas, and required high concentrations of the two tested

compounds (Lopez-Gonzalez *et al.*, 2004). Coumarin has also shown efficacy in treating skin cancer and was clinically defined as an agent able of preventing the recurrence of malignant melanomas; *in vitro* studies, about this, have shown that coumarin derivatives, especially the 7-hydroxycoumarin, have an antiproliferative effect both in human and murine melanoma cell lines (Marshall *et al.*, 1994, Weber *et al.* 1998). Furthermore, Lopez-Gonzalez *et al.* (2004) showed that the two compounds have cytostatic and proapoptotic actions in non-small cell lung carcinoma. On the other hand, several coumarins show synergistic effects with retinoic acid on the differentiation of human leukemic HL-60 cells (Hofmanova *et al.*, 1998); also the daphnetin behaves as a potent antiproliferative and differentiation agent in human renal cell carcinoma (Finn et al., 2004a). Moreover, it has been reported that a group of natural coumarins exhibit anti-proliferative and differentiation activity in the promonocytic U-937 cell line (Riveiro *et al.*, 2004) and more recently it has been observed that hydroxylated coumarins induce apoptosis in human leukemic cells. Moreover, nitrated and hydroxyl coumarins, the 6-nitro-7-hydroxycoumarin and 7,8-dihydroxycoumarin respectively, demonstrated a selective cytotoxic activity using two human cell lines, kidney adenocarcinoma cells and proximal tubular healthy cells (Finn *et al.*, 2002). It was also studied the selective activity of synthetic nitrate coumarins, the 6-nitro-7-hydroxycoumarin and the 3,6,8-trinitro-7-hydroxycoumarin on malignant melanocytes (SK-MEL-31) and healthy human fibroblasts from skin (Hs 613.sk). The two coumarins have showed an antiproliferative activity against SK-MEL-31 cell line, through an irreversible inhibition of cyclin A expression and a following reduction in DNA synthesis; these results allowed to propose this two nitro-coumarin derivatives as therapeutic drugs to treat melanoma, taking into account their

selective and irreversible cytotoxic properties (Finn *et al*., 2004b). These studies strongly support the potential therapeutic applications of coumarins, making these molecules attractive for their further evaluation as novel therapeutic agents for cancer treatment. However, the anti-tumor mechanisms of coumarins are not well understood. Several researchers have investigated different intracellular events in an attempt to elucidate the mechanism of action of active coumarins in cancer cells. Cellular events such as the modulation of mitogen-activated protein kinases (MAPKs) (Han *et al*., 2005; Finn *et al*., 2004; Finn *et al*., 2003a), the inhibition of protein kinases including EGF receptor tyrosine kinase, PKA and PKC (Yang *et al*., 1999) or topoisomerase II activity (Finn *et al*., 2004) have been evaluated. (proapoptotic activity of 7,8-dihydroxycoumarin). The identification of molecular targets of coumarin and its derivatives could facilitate the development of innovative and effective drug for the treatment of the cancer (Riveira et al., 2004).

1.1.5 Metabolism and toxicity of coumarin in humans.

Coumarin is a natural product, widely used in the production of food, alcoholic drinks, soaps (Cohen, 1979); it was also applied in medicine field, considering its therapeutic properties mentioned above, at concentrations higher than 5 g/day. Although the percentage of side effects, following administration of coumarin, is very low, the compound has demonstrated toxic and carcinogenic effect in animal models (Lake, 1999). In humans, the risk of exposure to small amounts of coumarin contained in food or soap, is

nil. This amount has been calculated at around 0.06 mg/kg /day and corresponds to a lower level of 2000/3000 times respect to the dose that triggers liver cancer in rats and lung cancer in mice. Unlike the rats and the mice, where high doses of coumarin may cause toxicity and carcinogenesis, in humans there is not a significant evidence about the toxicity induced by the compound administered at therapeutic doses (Born *et al.*, 2003): infact, cases of hepatotoxicity occurred in a not significant number of patients. It has been proposed that the different effects are due to species-specific metabolism. In humans, coumarin is largely converted in its hydroxylated and not-toxic form, the 7-hydroxycoumarin, which is further metabolized into its derivatives glucuronide and sulfate before its excretion with the urine. Conversely, in mice and other rodents, the 7-hydroxylation is a process that occurs rarely in the coumarin metabolism; in these species, the coumarin is predominantly metabolized in the corresponding 3,4-epoxide (EC) and then in the hydroxyphenylacetaldehyde (o-HPA). The further oxidation of aldehyde leads to the formation of o-hydroxyphenylacetic acid (o-HPAA), the metabolite present in greater quantities in the urine of these species (Born *et al*. 2000; Lake, 1999). It was shown that the production of EC and o-HPA is the cause of coumarin-induced toxicity in rats and mice (Lake, 1999).

1.1.6 The 5,7-dimethoxycoumarin.

A coumarin derivative, the 5,7-dimethoxycoumarin, has been identified in a small group of plant species, among which *Euodia borbonica* L. var.

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borbonica (Valenciennes *et al.*, 1999), *Citrus limon* L. (Salvatore *et al.*, 2004), *Citrus bergamia* L. (Kawaii *et al.*, 1999), *Heracleum mantegazzianum* L. (Glowniak *et al.*, 2000), *Citrus medica* L. var. *sarcodactylis* (Gao *et al.*, 2002). Recently, our group has identified the compound also in *Carica papaya* L. leaves extract, at the concentration of 150 μg/g. The chemical investigation, carried out by using gas chromatography-mass spectrometry analysis, allowed to detect 5,7-dimethoxycoumarin and other phenolic compounds – such as protocatechuic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, kaempferol and quercetin (Canini *et al.*, 2007). This molecule showed a differentiating effect on HL-60 cell line (Kawaii *et al.*, 1999), a significant induction of mouse hepatic GSTs (Kleiner *et al.*, 2008) and an inhibition activity in DNA adduct formation (DMBA-DNA), induced by carcinogens, in mouse mammary gland (Prince *et al.*, 2006).
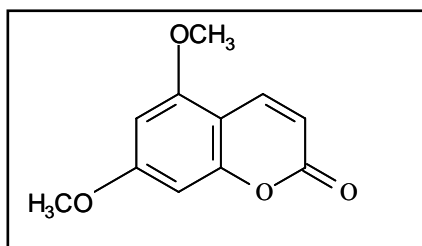

Figure 2. Molecular structure of 5,7-dimethoxycoumarin.

1.2 Melanoma

1.2.1 Biology of melanoma.

The incidence of melanoma has been increasing at an alarming rate; currently, the lifetime risk of developing melanoma is 1/58 for males in the United States, but has been reported to be as high as 1/25 for Australian males (Jemal *et al*., 2008). Although melanoma is highly curable, there is currently no effective treatment for metastatic melanoma. Despite over 20 years of trials with various therapeutic combinations, dacarbazine (DTIC) and interleukin-2 (IL-2) remain the standard treatment for advanced melanoma with response rates of only 15–25% (Tsao *et al*., 2004a). Despite this increase and an overall rise in mortality due to melanoma, the case survival rate for newly diagnosed melanoma has improved substantially; this improvement is largely attributable to earlier detection and surgery. The prognosis of patients with distant metastases not amenable to surgery remains poor and is uninfluenced by any treatment intervention (Eigentler *et al*., 2003). Thus, advances in understanding melanoma cell biology have fueled hopes for a breakthrough in the therapy of disseminated melanoma (Chudnovsky *et al*., 2005). Genetic and epigenetic alterations play an important role in the etiology and pathogenesis of this disease. Mammalian cells have numerous safeguards that protect against neoplastic transformation, and only when lesions occur in multiple genes the cancer

should become invasive (Vogelstein & Kinzeer, 2004); consequently, individual mutations are contributors to, rather than the sole causes of, cancer. Transforming mutations in oncogenes may render them constitutively active, or active under conditions in which the wildtype gene is inactive; these activations may result from chromosomal translocations, amplifications, or subtle intragenic mutations affecting crucial residues that regulate the activity of the gene product. Over the past two-and-a-half decades, application of innovative methodologies and technologies has helped to define melanoma as a highly complex, yet potentially comprehensible, disease. Early identification of melanoma oncogenes, such as NRAS, and melanoma tumor suppressor genes, such as CDKN2A, have led to a better understanding of the molecular engine (with the major genes, pathways, and networks) involved in melanoma tumorigenesis, and a more promising future in terms of therapy (sosman & Puzanov, 2006); infact, genetically informed targeted therapies have demonstrated promising results in many early clinical trials of patients with advanced melanoma.

Melanocyte and melanoblast proliferation, survival, differentiation, and migration are all critical elements of normal developmental physiology; the signaling circuitry that regulates these events is often altered at the genetic level by the aspiring melanoma cell. Two large cancer circuits—the growth-promoting Ras signaling network, the tumor-constraining CDKN2A network—and their interaction with a key downstream regulator of apoptosis, the Bcl-2/p53 network are crucial points also in therapeutical studies for melanoma treatment (Figure 3).
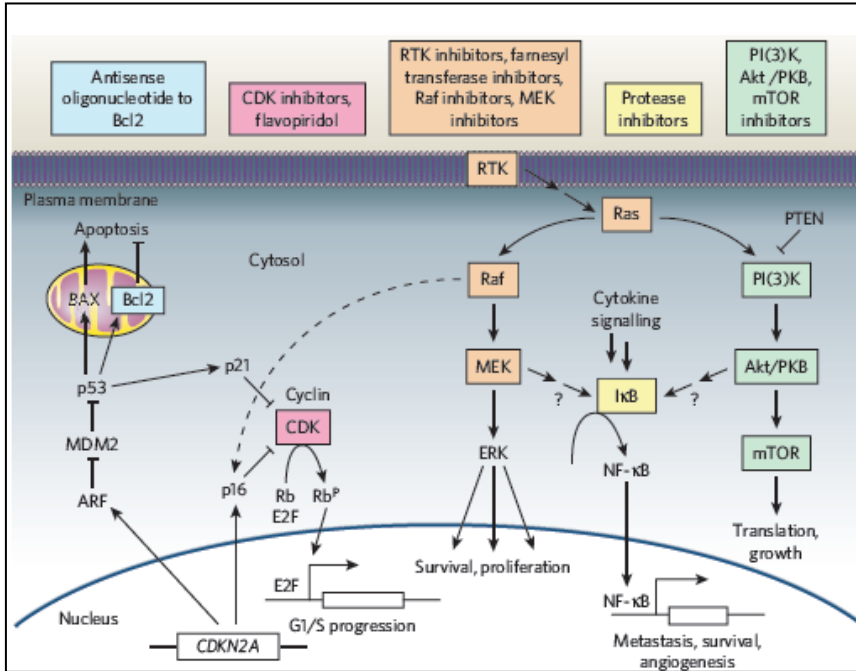
Figure 3. Melanoma drugs and their target pathways (Gray-Schopfer *et al*., 2007. Nature).

1.2.2 Genetics of melanoma: the signaling networks in melanoma proliferation and progression.

The Ras signaling pathway. The complex Ras signaling network regulates cell growth, survival, and invasion through two distinct cascades—the Ras/mitogen-activated protein kinase (MAPK) and the Ras/phosphatidylinositol-3-kinase (PI3K) signaling streams. Up to 90% of melanomas and benign melanocytic neoplasms carry activating mutations in

one of two key MAPK pathway genes, BRAF or NRAS; similarly, alterations in the PI3K pathway have been reported in up to 50–60% of melanomas (Zhou *et al*., 2000; Stahl *et al*., 2004). Functional cloning studies in the early 1980s revealed Ras GTPases as oncoproteins that were frequently mutated in a large number of human cancers. In 1984, Albino *et al*. established RAS as the first oncogene in melanoma; notably, the alterations were mainly in NRAS, a then lesser-known isoform in the RAS family, and activating NRAS mutations are relatively common (26%) (Hocker & Tsao, 2007). As Ras activates both the MAPK and PI3K pathways, mutations in NRAS have been demonstrated to occur in a reciprocal pattern with genes downstream of it in either pathway, such as PTEN and BRAF (Tsao *et al*., 2004b; Curtin *et al*., 2005). These data suggest that oncogenic NRAS promotes melanocytic proliferation, but it is not sufficient to yield true malignant transformation. Infact, when the Wellcome-Trust Sanger Institute initiated a project to resequence all relevant human protein kinases in the cancer, one of the first and perhaps most substantial findings was the discovery of a highly recurrent mutation in BRAF among different cancer types including melanoma (Davies *et al*., 2002). Although BRAF was mutated in less than 20% of most cancers, this gene was altered in 80% of short-term melanoma cell cultures and 66% of uncultured melanoma, thereby making BRAF the single most commonly mutated gene in melanoma. The discovery of a highly recurrent mutation in BRAF launched a new era in melanoma therapeutics and provided validation of the highthroughput resequencing strategy now adopted by the National Cancer Institute's Cancer Genome Project. Similar to oncogenic NRAS, the BRAF mutation is prevalent in benign nevi supporting a role for BRAF activation in melanocytic proliferation, but not full transformation (Pollock

et al., 2003; Yazdi *et al.*, 2003). This hypothesis has been experimentally validated using a zebrafish model, where it has been shown that BRAF activation leads only to development of benign nevi, whereas progression to frank melanoma requires concurrent p53 inactivation (Patton *et al.*, 2005); thus, the full oncogenic potential of BRAF appears to be dictated by the presence or absence of other genetic constraints. Although much remains to be understood regarding its exact role in melanoma biology, BRAF is a key element in melanoma tumorigenesis and crucial therapeutic target.

The kinase Ras induces also the membrane translocation and activation of PI3K, which in turn phosphorylates and activates the major signaling element of PI3K-AKT cascade, Akt. Once activated, Akt has several different enzymatic substrates including Mdm2, procaspase 9, NF-kB, mammalian target of rapamycin (mTOR), and p27, many of which contribute to tumor proliferation and survival (Sharma *et al.*, 2005). Although activating mutations in PI3K, itself, are believed to be rare, other downstream components of the PI3K pathway have been shown to be altered in up to 50–60% of melanomas (Zhou *et al.*, 2000; Stahl *et al.*, 2004); more specifically, the expression of PI3K and AKT have been shown to steadily increase during the progression from benign nevi to early melanoma, and lastly, to metastatic disease (Dhawan *et al.*, 2002; Stahl *et al.*, 2004). With regard to resistance to apoptosis, Akt is known to phosphorylate CREB, increase antiapoptotic Bcl-2, and inhibit apoptotic Bad (del Peso *et al.*, 1997). Indeed, it was recently demonstrated that a number of metastatic melanoma cell lines were resistant to independent inhibition of the MAPK or PI3K pathways, but they were susceptible to simultaneous inhibition of these pathways, with growth and invasion blocked in vitro (Smalley *et al.*, 2006).

The CDKN2A/CDK4 network. Patients that possess mutations in genes encoding for tumor suppressor, such as p16INK4A, p14ARF, RB, and TP53, have increased susceptibility to melanoma (Mooi & Peeper, 2006). The CDKN2A gene encodes two proteins involved in melanoma growth and survival (Sharpless *et al*., 2003): p16INK4A (cyclindependent kinase (CDK) inhibitor 2a) and p14ARF (alternative reading frame) p16INK4A inhibits Cdk4/6-mediated phosphorylation of the retinoblastoma protein; in the hypophosphorylated state, Rb binds and represses the E2F transcription factor and prevents G1-to- S transition. On the other hand, p14ARF directly prevents Mdm2 from accelerating the degradation of p53. Thus, loss of the CDKN2A locus negatively impacts on both the Rb and p53 pathways. CDKN2A appears to play a central role in preventing cancer formation by mediating a senescence-like state upon oncogenic stress (Serrano *et al*., 1997); indeed, oncogenic mutations in NRAS require concomitant loss of CDKN2A in order to progress to frank melanoma (Ackermann *et al*., 2005).

The Bcl-2 network. The Bcl-2 network is thought to be one of the most crucial regulators of melanoma cell apoptosis (Soengas & Lowe, 2003). At the core of the Bcl-2 network lives a family of anti- and proapoptotic proteins that function to regulate and execute the core mitochondrial pathway of apoptosis (reviewed in Danial & Korsmeyer, 2004). Bcl-2 has been shown to serve a physiologic role in the skin: in response to UV radiation, keratinocytes secrete NGF which binds to melanocyte receptors and leads to increased Bcl-2 expression and resistance to apoptosis (Zhai *et al*., 1996). In fact, high levels of Bcl-2 expression have been found in melanoma and melanocytes, providing a likely explanation for the resistance of melanocytes and melanoma to apoptosis induced by both physiological and therapeutic triggers (Selzer *et al*., 1998; Soengas & Lowe, 2003).

Aberrations in various signaling pathways contribute to elevated Bcl-2 levels in melanoma. Oncogenic NRAS has been shown to specifically upregulate the expression of Bcl-2 *in vitro* (Borner *et al*., 1999); the microphthalmia-associated transcription factor (Mitf) may also contribute to survival by the transactivation of Bcl-2 (McGill *et al*., 2002). Nonetheless, it has been shown that antisense suppression of Bcl-2 or mutations in Mitf, a transcriptional regulator of Bcl-2, leads to decreased melanoma cell survival and increased sensitivity to chemotherapy (Jansen *et al*., 1998; Gautschi *et al*., 2001; McGill *et al*., 2002); thus Bcl-2 has been considered an attractive therapeutic target.

Mitf gene. The Mitf gene has emerged as a master regulator of melanocyte development. This transcription factor dictates the pigment cell phenotype by regulating melanocyte-specific proteins such as MART-1, enhances and controls melanoblast survival, and melanocyte proliferation and survival (Hodgkinson *et al*., 1993; Widlund & Fisher, 2003; Nishimura *et al*., 2005). Mitf has multiple downstream effects, which together define numerous aspects of normal pigment cell physiology. Mitf directly promotes melanocyte and possibly melanoma cell survival by inducing the expression of the protooncogene, Bcl-2, as one of its transcriptional targets (McGill *et al*., 2002). Despite the emerging view of Mitf in melanoma biology, critical questions about its biology remain unanswered. In contrast to its oncogenic behavior, Mitf has been shown to mediate G1 cell-cycle arrest by upregulating p21Cip1 (Carreira *et al*., 2005). One model posits that cell-cycle arrest and differentiation takes place at high levels of Mitf expression whereas apoptosis occurs at very low, or null, expression levels (Gray-Schopfer *et al*., 2007); thus, a tumor-favorable Mitf level may be required. Increases in MAPK signaling leads to ubiquitin-dependent proteolysis of

Mitf (Wu *et al*., 2000). As complete loss of Mitf is incompatible with cell survival, melanoma specimens with constitutively active BRAF require mechanisms that maintain Mitf levels in the range compatible with tumorigenesis. Mitf amplification occurs in up to 20% of melanomas and thus may account for some of this Mitf recovery; however, the fact that only a small percentage of melanomas with oncogenic BRAF and NRAS possess Mitf amplifications suggests that other mechanisms are likely involved. Mitf is a downstream target of β-catenin, a critical regulator of melanoma cell growth (Widlund *et al*., 2002); thus, an alternative mechanism of Mitf recovery could involve stabilizing mutations in β-catenin leading to induction of Mitf. Given that Mitf plays a central role in melanocyte and tumor survival, further delineation of the genetic network that regulates, and is regulated by, Mitf is warranted.

1.2.3 Therapy of melanoma: Ras/Raf/Mek/Erk MAPKs as targeted growth signaling pathway.

Dacarbazine (DTIC)—which possesses response rates as low as 10–13% and complete responses in only 5%—has remained the mainstay of single-agent chemotherapy treatment for advanced melanoma for over 20 years (Eigentler *et al*., 2003). Nitrosoureas, platinum analogs, vinca alkaloids, and the taxanes all have been shown to have only modest activities against metastatic melanoma; temozolomide (TMZ) is a newer oral alkylating agent that functions like DTIC and possesses excellent bioavailability. The generally poor efficacy of all current single-agent chemotherapeutic

approaches has prompted myriad studies involving combination chemotherapy, biochemotherapy, and tumorspecific immunologic therapies. Unfortunately, over the past 20 years, there have been no substantial benefits with regard to median survival, and many of the combination regimens have the drawback of increased toxicity (Eigentler *et al*., 2003; Gogas *et al*., 2007). Much of melanoma's resistance to traditional chemotherapy is believed to arise intrinsically, by virtue of potent growth and cell survival-promoting genetic alterations. Therefore, significant attention has recently been focused on developing previously unknown targeted therapies that aim to selectively shut down the genetic aberrations that fuel this tumor's oncogenic engine, as well as agents that are capable of decreasing this tumor's inherent resistance to chemotherapy. Given the importance of the RAS signaling network in promoting melanoma proliferation and survival, therapies specifically aimed at this pathway are ideal for many reasons. First, this network is the most frequently altered in melanomas: up to 90% of melanomas possess oncogenic alterations in NRAS or one of its downstream pathways. Second, activation of the MAPK and PI3K pathways has been shown to lead to increased proliferation, cell survival, and contributes to melanoma's inherent chemoresistance by disrupting the death receptor-, mitochondrial-, and endoplasmic reticulum stress-induced apoptotic pathways (Eisenmann *et al*., 2003; Zhang *et al*., 2003; Hersey *et al*., 2006; Jiang *et al*., 2007). For example, lonafarnib blocks Ras activation and preliminary data suggest that lonafarnib with cisplatin may enhance melanoma apoptosis *in vitro*. (Smalley & Eisen, 2003; Morgillo & Lee, 2006). Instead, sorafenib is a Raf inhibitor that has been shown to abrogate MAPK signaling biochemically and to harbor antimelanoma effects *in vitro* (Karasarides *et al*., 2004); early clinical trials have failed to show significant

efficacy with sorafenib as monotherapy in advanced melanoma (Eisen *et al.*, 2006), but recent results showed that sorafenib in combination with carboplatin, paclitaxel, TMZ and DITC have been more promising. Finally, in preclinical studies CI-1040, a small molecular weight MEK inhibitor, was found to completely suppress growth of BRAF mutant melanoma xenografts with regression of pulmonary metastases and prevention of new pulmonary metastases (Collisson *et al.*, 2003). PD0325901, a second generation Mek inhibitor, is 100 times more potent and is 490-fold more potent in suppressing pERK relative to CI-1040. A recent phase I/II trial of PD0325901 enrolled 27 patients and demonstrated partial responses in 2/27 and stable disease in 5/27 (Lorusso *et al.*, 2005a). Significantly, tumors from partial responders showed substantial ERK inhibition, a critical component of the targeted mechanism.

2. PURPOSE

Since research in melanoma cancer therapy is focused on discovery of novel drugs able to reduce its proliferative capacity and induce terminal differentiation and/or apoptosis (Jiang *et al*., 1994), the aim of this work is:

1)   to investigate the antigrowth activity of a coumarin derivative, the 5,7-dimethoxycoumarin, on melanoma cell lines, such as murine B16 F1 and human A375;

2)   to analyse modulation of Ras/Raf/Mek/Erk MAPKs signaling pathway that is the most frequently altered network in melanomas (Wellbrock *et al*., 2005);

3)   to examine cellular processes triggered by 5,7-dimethoxycoumarin following reduction of cell proliferation, which can be related to differentiation and/or apoptosis programmes;

4)   to define the molecular mechanisms involved in 5,7-dimethoxycoumarin effects, such as downstream effectors of Ras/Raf/Mek/Erk signaling pathway and /or other molecular networks cross-talking with MAPKs.

3. MATERIALS AND METHODS

3.1 Test compound.

Synthetic 5,7-dimethoxycoumarin was purchased from Sigma-Aldrich (116238). The compound was dissolved in methanol and a solution with a concentration 20 mM was obtained. In the treatment of cells, a volume of this solution was added to culture medium and its percentage respect to medium was 0.5, 1.25 and 2.5 % for 100, 250 and 500 μM, respectively.

3.2 Cell lines and culture conditions.

Cell lines used in this work are: murine melanoma B16 F1, human melanoma A375, human breast adenocarcinoma MCF7, human prostate adenocarcinoma PC3, human colorectal carcinoma SW620. Cancer cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). As control, non-neoplastic murine cardiofibroblasts were used. Cells were cultured in RPMI 1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS), 1% L-glutamine (v/v), 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

3.3 MTT assay.

5,7-dimethoxycoumarin activity on cells growth was estimated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, that is based on the cleavage of the yellow tetrazolium salt to purple formazan crystals by intracellular dehydrogenases (Mosmann *et al*., 1983). Briefly, 1-$2x10^3$ cells/well were seeded into sterile 96-well plates. After 24 h, 5,7-dimethoxycoumarin was added to cell culture medium over a final concentration range of 0-500 μM and the cells were incubated at 37°C and 5% CO_2 for a period of 24-72 h. After incubation period, MTT (Sigma-Aldrich) was added to each well and incubated for further 4 h. Then, medium was removed, blue crystals of MTT reduced by cells were dissolved with DMSO and cellular metabolism was determined by measuring absorbance of samples at 570 nm in a microelisa reader. IC_{50} values were estimated following 72 h incubation.

3.4 Trypan blue exclusion test.

B16 cells, A375 cells and cardiofibroblasts were seeded at a density of 1-$2x10^4$ cells/well in 24 well plates. After 24 h cells were treated with 100, 250 and 500 μM 5,7-dimethoxycoumarin and the plates were incubated at 37°C and 5% CO_2 for a period of 24-72 h. After treatment time, floating cells in the medium of each well were transferred to centrifuge tubes; adherent cells were washed, collected by trypsinization and mixed with the corresponding

floating cells before centrifugation. Cells were stained with 0.4% Trypan blue and counted in triplicate with optic microscope with the aim to estimate the number of live and dead cells. Cell viability was expressed as a percentage of treated cells respect to appropriate vehicle-treated controls, and toxicity as a percentage of dead cells respect to total number of cells. Moreover, B16 cells treated for 72 h were washed several times with medium to remove the excess of 5,7-dimethoxycoumarin, counted and replaced in fresh medium at 1×10^4 cells/well without the compound. After 48, 72 and 96 h incubation at 37°C and 5% CO_2, the irreversible or reversible growth inhibitory effect of compound was observed by trypan blue test.

The B16 treated cells were observed with an inverted microscope and photographed at 10X and 20X with a digital camera Nikon Coolpix 995 to detect any drug-induced morphological changes.

3.5 Treatment of B16 cells with 5,7-dimethoxycoumarin and U0126 and Trypan blue assay.

B16 cells were seeded at a density of 1×10^4 cells/well in 24-well plates. After 24 h, cells were treated with 100, 250, 500 μM 5,7-dimethoxycoumarin or with concentration 1, 5, 10 μM of U0126 (Sigma-Aldrich), a specific inhibitor of Mek 1/2 (Favata *et al.*, 1998); moreover, 5,7-dimethoxycoumarin was used in combination with U0126. After 72 h treatment, floating cells in the medium of each well were transferred to centrifuge tubes; adherent cells were washed, collected by trypsinization and mixed with the corresponding

floating cells before centrifugation. Cells were stained with 0.4% trypan blue and counted in triplicate with an optic microscope with the aim to estimate the number of live and dead cells. Cell viability was expressed as a percentage of treated cells with respect to appropriate vehicle-treated controls.

3.6 Cell cycle analysis.

B16 and A375 cells were seeded and treated as described in section of Trypan blue exclusion test. After 24, 48 and 72 h incubation at 37°C and 5% CO_2, cells were washed, harvested, pelleted, fixed with PFA (paraformaldehyde) 1% in PBS for at least 30 min at 4°C and stained with propidium iodide (PI) staining solution containing 50 μg/ml PI, 0,5 % RNase A and 0.1% Triton X-100. After incubation for 30 min at 4°C in the dark, cell cycle distributions were analyzed by flow cytometry on a FACScalibur (Becton Dickinson, Mountain View, CA). Using Cellquest Pro software, the percentage of cells at different phases of the cell cycle was determined. PI was excited at 488 nm, and fluorescence analyzed at 620 nm. A total of 10,000 events in each sample were acquired.

3.7 Protoporphyrine IX (PpIX) analysis and phenotypic characterization with flow cytometry.

B16, A375 and SW620 cells were seeded at a density of $1\text{-}1.5\text{x}10^4$ cells/ml in 24-well plates. After 24 h were treated with methanol control or 100, 250 and 500 μM of 5,7-dimethoxycoumarin. After 24, 48 and 72 h incubation at 37°C and 5% CO_2, cells were washed with PBS, harvested bytrypsinization, pelleted and resuspended in PFA 1%. After at least 30 min incubation at 4°C, the samples were analyzed on a FACScalibur flow cytometer using the 488 nm line from an argon laser (Becton Dickinson, Mountain View, CA). In all, 10,000 cells, for each sample, were measured: scattering properties and fluorescence emitted from the cells at 670 nm were collected and analysed.

3.8 Melanin synthesis in B16 and A375 cells.

B16 and A375 cells were seeded and treated as reported in section of Trypan blue exclusion test, in order to measure extracellular and intracellular melanin according to the method of Hill et al. (1989). After 72 h incubation at 37°C and 5% CO_2, the culture medium was removed, centrifuged (700xg, 10 min) and the supernatant was collected for extracellular melanin quantification; 1 ml of 0.4M HEPES buffer (pH 6.8) and EtOH (9:1, v/v) was added to 1 ml of the medium and the OD at 475 nm was measured to quantify extracellular melanin by using a calibration curve obtained with synthetic melanin solutions. Cells were collected by trypsinization, pelleted,

washed twice with PBS and digested in 1 ml 1N NaOH for 16 h at room temperature; intracellular melanin was measured as described above. The stimulation in melanogenesis following the treatment was estimated as percentage of total melanin (μg/ml), calculated by the sum of intracellular and extracellular melanin, synthesized in treated cells respect to vehicle-treated controls.

3.9 Tyrosinase assay.

B16 cells were seeded and treated as described above in order to determine tyrosinase activity according to the method of Lin et al. (2002). After 24, 48 and 72 h treatment time, cells were washed, collected by trypsinization, stained with 0.4% trypan blue and counted in triplicate with an optic microscope to estimate the number of live cells. For each sample, 7 x 10^4 cells were then lyzed in 1% Triton-X 100 in 0.1 M Na phosphate buffer (pH 6.8) for 20 minutes; lysates were incubate with an equal volume of 3,4-dihydroxy-L-phenylalanine
(L-DOPA), 3 mg/ml in 0.1 M Na phosphate buffer for 3 h at 37°C. Tyrosinase activity was determined by measuring sample absorbance at 490 nm. The stimulation of tyrosinase activity following the treatment was estimated as fold increase in treated cells with respect to vehicle-treated controls.

3.10 Western blot analyses.

B16 and A375 cells were seeded at a density of $1x10^4$ cells/well in 6-wells plates and treated as described above. After 24, 48 and 72 h treatment time, cells were washed, collected by trypsinization and suspended in RIPA lysis buffer [1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 NaCl, 0.01 M sodium phosphate (pH 7.2), 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate and 100 units/ml aprotinin]. After 1 h on ice, the cell lysates were cleared by centrifugation at 13000 x g for 15 min and the resultant supernatants collected and used for Bradford assay (1976) to estimate protein concentration. Then, 40 μg of total protein lysates were resolved on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and the separated proteins were transferred to PVDF membrane. The level of protein expression in each sample was detected using specific mouse monoclonal primary antibody diluted in TTBS solution containing 1% (w/v) of BSA: antibodies against phospho-Mek 1/2 (Ser 218/Ser 222), phospho-Erk 1/2 (Tyr-204), Mitf (N-15), PBG-D (A-16), p-CREB-1 (Ser 133) and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After, the membranes were incubated with a specific HRP-conjugated secondary antibody and developed using the enhanced chemiluminescent substrate from Pierce; then, they were stripped and reprobed with α-tubulin (4G1) primary antibody as a protein loading control.

3.11 Mek 1/2 immunoprecipitation and kinase assay.

B16 and A375 cells were seeded and treated as described above; after 72 h treatment time, cells were washed, collected by trypsinization and lysed by using the same experimental procedure of the previous section. Equal protein aliquots of precleared cell lysates (300 µg) were incubated overnight with Anti-Mek antibody and Ezview Red Protein A Affinity Gel beads (Sigma-Aldrich) at 4 °C with gentle rotation. The immune complexes were collected by centrifugation, washed three times and incubated for 90 minutes at 30 °C with non activated Erk substrate solution (Sigma-Aldrich) for kinase assay. Then, the samples were boiled in SDS sample buffer and loaded on 12% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE); the separated proteins were transferred to PVDF membrane. The level of diphosphorylated Erk 1/2 was detected using a monoclonal antibody against the activated MAP kinase (Sigma-Aldrich); the membrane was then incubated with a specific HRP-conjugated secondary antibody (Sigma-Aldrich) and developed using the enhanced chemiluminescent substrate from Pierce.

3.12 Statistical analysis.

Student's t test was employed to determine the significance of any reduction in cellular viability and the increase of melanin synthesis following the treatment. A probability of 0.05 or less was deemed statistically significant.

4. RESULTS

4.1 Reduction of metabolic activity of murine and human cell lines after treatment with 5,7-dimethoxycoumarin.

In the first set of experiments, the antineoplastic activity of 5,7-dimethoxycoumarin in various human melanoma and adenocarcinoma cell lines was measured using MTT assay. The compound significantly reduced metabolic activity, in dose-dependent manner up to 70% after 72 h treatment respect to the control. The concentration 100 μM did not show a significant growth inhibition activity, except for B16, PC3 and MCF7 lines. The concentrations 250 μM and 500 μM inhibited significatively cellular proliferation at each incubation time for every cell line (Figure 1). In Table 1, IC_{50} values, estimated after 72 h treatment, are reported and evidenced that 5,7-dimethoxycoumarin exhibited a clear effect not only on melanoma but also on adenocarcinoma cell lines.
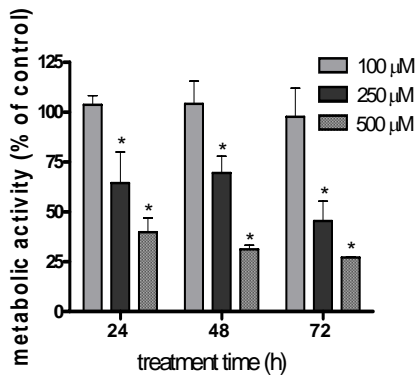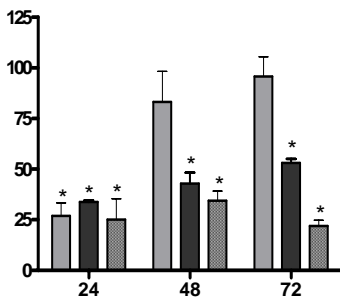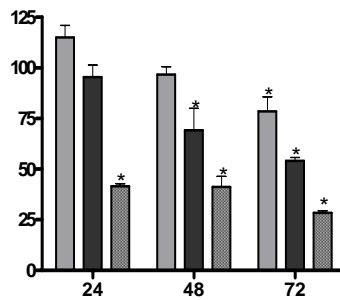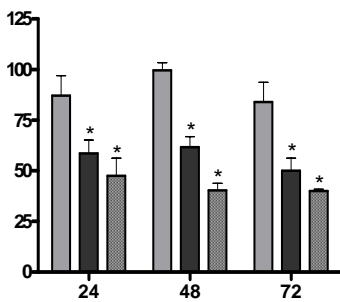
A)

B)

C)

D)

E)

4.2 Effect of 5,7-dimethoxycoumarin on growth and death of melanoma cell lines.

   MTT assay did not reveal if the reduction of proliferation was related to growth arrest or cell death since both mechanisms could result in reduced cell numbers and an apparent loss of viability. To establish that, a Trypan blue exclusion test was carried out on melanoma cell lines. The results reported in Figure 2A show that 5,7-dimethoxycoumarin possessed an antiproliferative activity on B16 cells growth at all the concentrations tested. The compound inhibited the growth of B16 cells significatively, as compared with the control group, at 48 h and 72 h, accounting for 35 % to 47 %, 48 % to 68 % and 61 % to 72 %, at the doses of 100, 250 and 500 μM, respectively. The inhibitory effect was time-dose dependent.

In the same way, the treatment with 5,7-dimethoxycoumarin reduced significatively proliferation of A375 cells as well (Figure 2B), accounting for 0 % to 45 %, 17 % to 68 % and 41 % to 80 % of reduction at the doses of 100, 250 and 500 μM, respectively. The inhibition of cell growth was significant respect to the control in each treatment time, except for the concentration 100 μM at 24 h. The inhibitory effect was time-dose dependent.

A)

B)
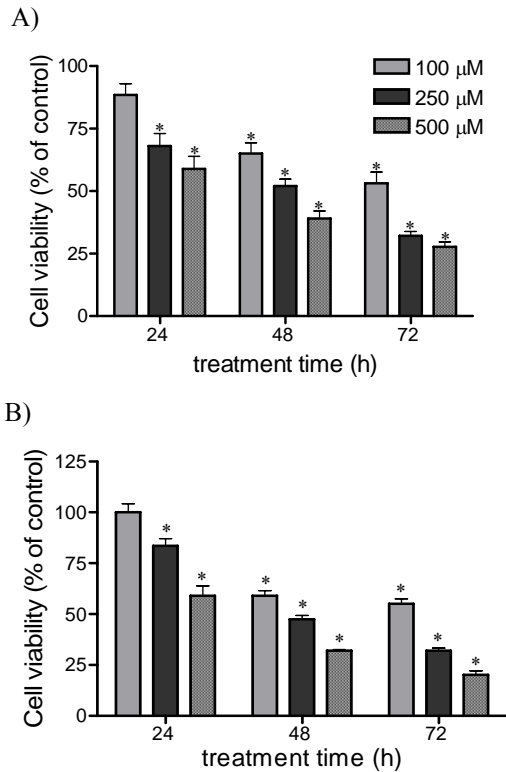
Figure 2. (**A**) B16 and (**B**) A375 cell viability following 24, 48 and 72 h treatment with 5,7-dimethoxycoumarin. Cells were incubated with or without the compound using 100, 250 and 500 µM doses and Trypan blue exclusion test was carried out. Data are expressed as % of cell viability respect to vehicle control as described in the section of Materials and methods. Results are the mean ± SD from three separate experiments. *$P<0.05$ versus vehicle control treated cells.
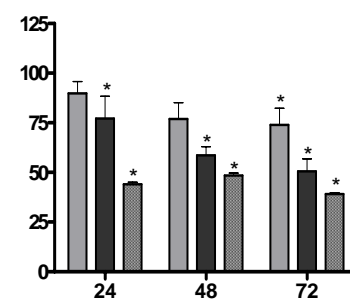
IC_{50} values at 72 h were obtained also through Trypan blue test and they accounted for 109 µM and 142 µM in B16 and A375, respectively (Table 1). Interestingly, they were lower than those derived from MTT assay at the same time of treatment. That probably is a consequence of the minor

reliability of an indirect assay, such as MTT assay, respect to a direct one as Trypan blue test (Jabbar *et al*., 1989).

Table 1. IC_{50} of 5,7-dimethoxycoumarin in different cell lines following 72 h treatment. Cells were incubated with or without the compound using 100, 250 and 500 μM doses; then, MTT assay and Trypan blue exclusion test were carried out, as described in the section of Materials and methods. A grapf of viability versus drug concentration was used to calculate IC_{50} values for each cell line.

Histological type	Cell line	[1] IC_{50} (μM)	[2] IC_{50} (μM)
Human Melanoma	A375	300	142
Murine Melanoma	B16	260	109
Human Adenocarcinoma	MCF7	250	-
	PC3	285	-
	SW620	325	-

[1] IC_{50} values as obtained by MTT assay.
[2] IC_{50} values as obtained by Trypan blue exclusion test.

Moreover, the effect of the compound on murine melanoma cells was long lasting, since the same cells previously treated at doses of 100, 250, and 500 μM, washed, reseeded and cultured without treatment, showed a significant reduced performance of proliferation respect to untreated cells. After 96 h incubation at 37°C and 5% CO_2, the growth reduction produced by previous treatment was 42 %, 61 % and 57 % at the doses of 100, 250 and 500 μM, respectively. Statistical differences were observed between the values obtained with 100 μM and 250 μM doses, and with 100 and 500 μM doses (Figure 3). These results evidenced that the antiproliferative effect of the

compound was irreversible since proliferation did not return to control levels even at the end of 96 h recovery period.

The percentage of dead cells calculated as explained in Materials and Methods sections, after treatments for both cell lines was also evaluated; no significant cytotoxicity was observed at any experimental time and dose respect to the control (data not shown).

The 5,7-dimethoxycoumarin did not change significatively the growth of non-neoplastic cardiofibroblasts for none of the used concentrations (Figure 4).
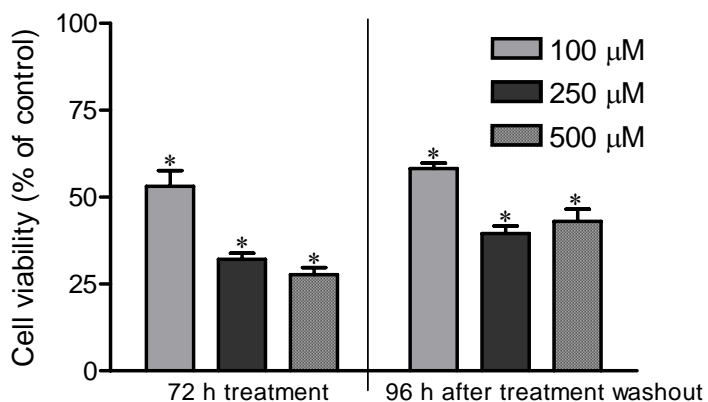
Figure 3. The irreversible effect of 5,7-dimethoxycoumarin on B16 cell growth. Cells were incubated with or without the compound using 100, 250 and 500 µM doses for 72 h; then, they were reseeded without treatment for 96 h. Data are expressed as % of cell viability respect to vehicle control as described in the section of Materials and methods. Results are the mean ± SD from three separate experiments. *P<0.05 versus vehicle control treated cells.
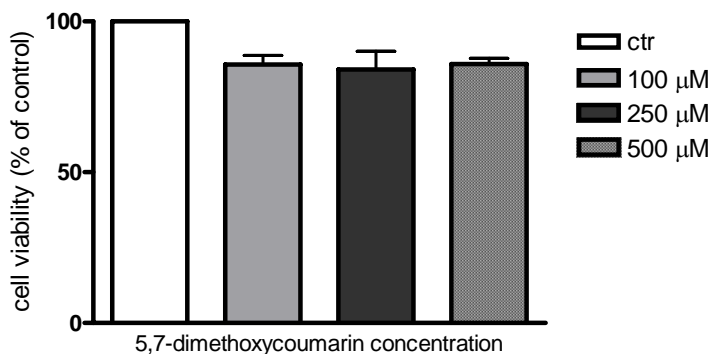
Figure 4. Cell viability of fibroblasts following 72 h treatment with 5,7-dimethoxycoumarin. Cells were incubated with or without the compound using 100, 250 and 500 µM doses and Trypan blue exclusion test was carried out. Data are expressed as % of cell viability respect to vehicle control as described in the section of Materials and methods.

4.3 Block of cell cycle in G0/G1 phase after treatment of melanoma cells with 5,7-dimethoxycoumarin.

Since the strong inhibitory effect induced by 5,7-dimethoxycoumarin could be related to a decrease of DNA synthesis, we then investigated its effect on cell cycle events. After 24 h treatment, we demonstrated, by FACS analysis, that 5,7-dimethoxycoumarin induced a strong and dose dependent arrest of cell cycle progression in G0/G1 phase in B16 and A375 cell lines, when compared to control sample, with reduction of S and G2/M phases

population. In regard to B16 cells (Figure 5A), the accumulation in G0/G1 phase was significant at high dose and consistent with growth arrest when compared to the control. An accumulation of melanoma cells in G0/G1 phase was detected also after 48 and 72 h treatment, although the differences respect to the control were less significative than 24 h of incubation (data not shown). Similarly, in A375 cells, the 500 μM dose induced a G0/G1 arrest after 24 h (data not shown), which did not change at later treatment times; also 100 and 250 μM doses of 5,7-dimethoxycoumarin showed a significant increase in G0/G1 cell population after 48 h treatment (Figure 5B).
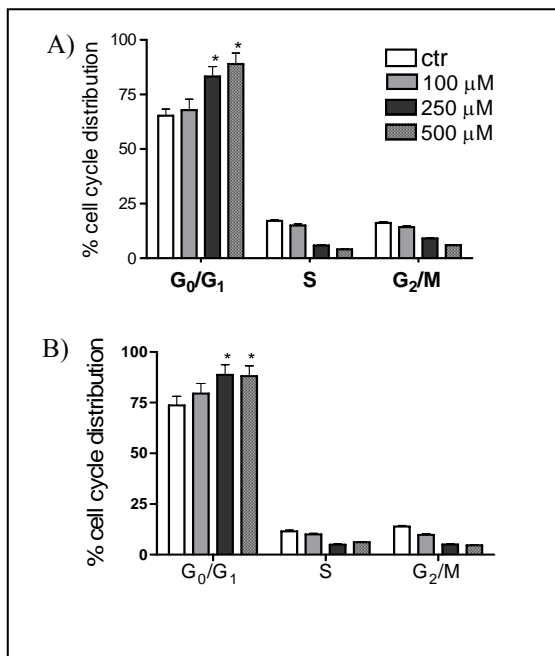
Figure 5. Effect of 5,7-dimethoxycoumarin on cell cycle progression in B16 cells after 24 h (**A**) and A375 cells after 48 h treatment (**B**). Cells were incubated with or without the compound using 100, 250 and 500 μM doses. After treatment, cells were stained with propidium iodide and flow cytometric analysis was performed as described in Materials and methods. The percentage of cell cycle distribution data for each treatment group are shown. Results are the mean ± SD from three separate experiments. *$P<0.05$ versus vehicle control treated cells.

**4.4 Effect of Mek 1/2 inhibitor on 5,7-dimethoxycoumarin antigrowth activity in B16 cell line.**

   In the previous paper, we detected that 5,7-dimethoxycoumarin inhibited the growth of melanoma cells. Now, to further investigate the role of MAPKs signalling pathway Ras/Raf/Mek/Erk in antineoplastic activity of 5,7-dimethoxycoumarin, we used the compound in combination with the Mek 1/2 inhibitor U0126. After 72 h treatment, U0126 reduced cell growth with a precentage of 8%, 29% and 43% for 1, 5 and 10 µM doses, respectively (Figure 6A). In the combination treatment, the inhibitor augmented growth inhibition induced by 5,7-dimethoxycoumarin, as shown in Figure 6 (B-D). When 100 µM dose was combined with increasing amounts of U0126, cell viability was reducEd respect to control from 52.1% to 45.4-22.3%; for 250 µM, from 16.2% to 10.8-4.1% and for higher dose, from 11% to 5.1-2.7%. These data suggest that Mek 1/2 inhibition can sensitize B16 cells to tested compound treatment and then, the combination therapy increased antiproliferative activity of 5,7-dimethoxycoumarin in synergic or additive mode.
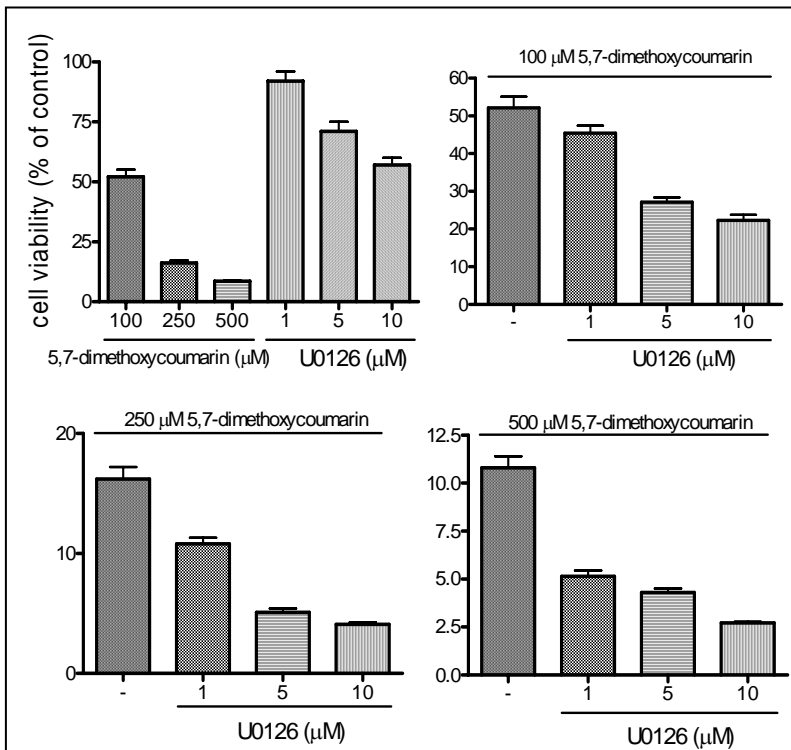
Figure 6. The effect of Mek 1/2 inhibitor U0126 on 5,7-dimethoxycoumarin activity in murine melanoma cell line. B16 cells were incubated with the compound using 100, 250 and 500 µM doses or with U0126 using 1, 5 and 10 µM doses **(A)**; and in combination treatment with 5,7-dimethoxycoumarin 100 **(B)**, 250 **(C)** and 500 µM **(D)** and U0126 (1, 5 and 10 µM). After 72 h incubation, Trypan blue exclusion test was carried out. Data are expressed as % of cell viability respect to vehicle control as described in the section of Materials and methods. Results are the mean ± SD from three separate experiments. *$P<0.05$ versus vehicle control treated cells.

4.5 Morphological changes in melanoma cells after treatment with 5,7-dimethoxycoumarin.

Since the treatment with 5,7-dimethoxycoumarin showed low cytotoxicity, we hypothesised that growth inhibition was due to the induction of differentiation, a process that is generally coupled to a block in cellular proliferation (Umek *et al*., 1981; Zermati *et al*., 2000). To verify this hipothesis first we evaluated the morphology of treated melanoma cell lines under an inverted microscope. B16 cells analysis by digital images (Figure 7) showed a dose dependent decrease of cellular density in the culture treated with different doses of 5,7-dimethoxycoumarin. This result is in accordance with those obtained with Trypan blue test. Moreover, a clear morphological change was detected in cells. Infact the treatment induced the formation of dendrite-like projections which give a star-like shape to the cells respect to rounded untreated cells. This effect was more evident with the increase of treatment time and concentration and after 5,7-dimethoxycoumarin washout it did not recover the control morphology (data not shown). Similar results were obtained for A375 cells regarding cellular density; anyway, morphological changes were less evident respect to those obtained in B16 cells (data not shown).

A)

B)

C)

D)
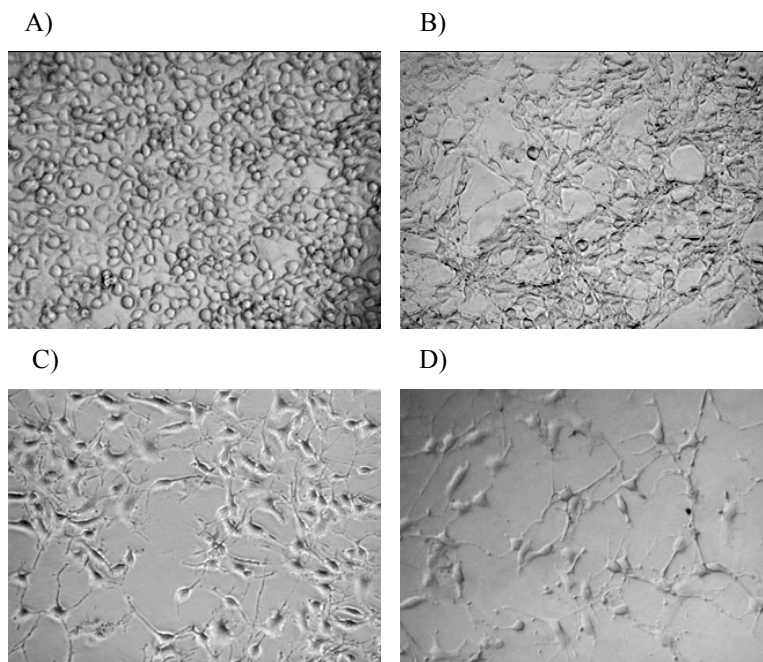
Figure 7. Effect of 5,7-dimethoxycoumarin 100 µM (**B**), 250 µM (**C**) and 500 µM (**D**) respect to control (**A**) on B16 cells growth and morphology after 72 h incubation. Cells were seeded and treated as described in the section of Materials and Methods; then digital images were obtained by observation with an inverted microscope at 10X.

4.6   Phosphorylation   of   ERK1/2   after   treatment   with 5,7-dimethoxycoumarin.

To unravel the potential mechanism underlying 5,7-dimethoxycoumarin-induced antigrowth effect, we examined the alterations in activation of a MAPK signalling pathway following treatment. The effects of 5,7-

dimethoxycoumarin on Erk 1/2 phosphorylation in B16 cell line after 72 h treatment are shown in Figure 8: the treatment inhibited the activation of the protein in a dose-dependent manner resulting in a moderate to complete inhibition. Densitometric analysis allowed to calculate the effect of treatment, as compared to control untreated cells, in ERK phosphorylation: the compound reduced p-Erk 1/2 of 60%, 85% and 96% for 100, 250 and 500 μM, respectively. The compound was likewise effective on A375 cells bringing an inhibition, after 72 h treatment, of the Erk phosphorylation with a percentage of 70% in the case of higher dose (Figure 9).
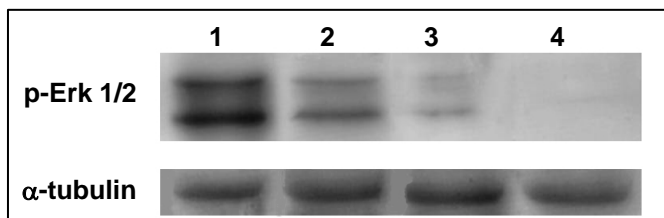
Figure 8. The effect of 5,7-dimethoxycoumarin on Erk 1/2 activity in B16 cells following treatment 72 h. Cells were incubated with the compound using 100, 250 and 500 μM doses: lanes **2** (100 μM), **3** (250 μM), **4** (500 μM); or with the vehicle solvent: lane **1**. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in the section of Materials and methods. Activation level of Erk 1/2 was analysed with its corresponding phospho-specific antibody.
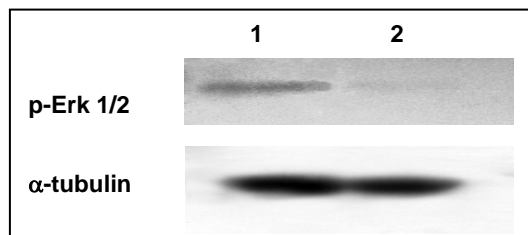
Figure 9. The effect of 5,7-dimethoxycoumarin on Erk 1/2 phosphorylation in A375 melanoma cells following treatment 72 h. Cells were incubated with (lane **2**) or without (lane **1**) the compound using 500 μM doses. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in the section of Materials and methods. Activation level of Erk 1/2 was analysed with its corresponding phospho-specific antibody.

4.7 Phosphorylation of MEK 1/2 and inhibition of its kinase activity in melanoma cells after treatment with 5,7-dimethoxycoumarin.

In the next step, we sought to examine, following a 72 h treatment, the alterations in phosphorylation level of Mek 1/2, the kinase which phosphorylates Erk 1/2. The effects of 5,7-dimethoxycoumarin on the MAP kinase kinase activation, in B16 and A375 cells, are shown in Figure 10A and 10B, respectively: the treatment increased level of phospho-Mek 1/2 (p-Mek 1/2) in a moderate manner in both cell lines as compared with control samples. The augmentation of protein phosphorylation, estimated by densitometric analysis, was 16%, 32% and 85% for 100, 250 and 500 μM, respectively in B16 cells; it was 42%, 67% and 72% in A375 cell line. Considering promoting activity of 5,7-dimethoxycoumarin on Mek phosphorylation in melanoma cells, we were interested to understand the key

point in the phosphorylation cascade of this signalling pathway where the compound exerted, directly or indirectly, its antiproliferative activity. Up to now we described that treatment reduced Erk 1/2 phosphorylation, but it triggered a moderate augmentation in Mek 1/2 phosphorylation in both used cell lines.
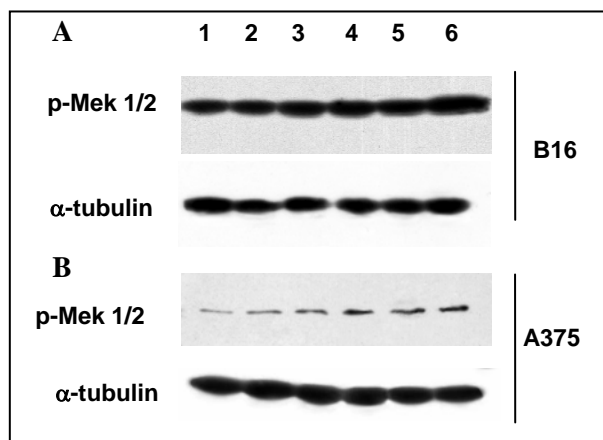
Figure 10. The effect of 5,7-dimethoxycoumarin on Mek 1/2 phosphorylation in B16 (**A**) and A375 (**B**) melanoma cells following treatment 72 h. Cells were incubated with the compound using 100, 250 and 500 µM doses: lanes **2** (100 µM), **4** (250 µM), **6** (500 µM); or with respective vehicle solvent: lanes **1**, **3**, **5**. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in the section of Materials and methods. Activation level of Mek 1/2 was analysed with its corresponding phospho-specific antibody.

Then, the first question was regarding the effect of treatment on enzimatic activity of p-Mek, which was tested using Erk, which is activated upon dual phosphorylation by Mek, as its substrate in a kinase assay following immunoprecipitation of Mek 1/2. Immunoblot analyses results (Figure 11),

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after incubation with a p-Erk 1/2 specific antibody, allowed to observe a significant decrease in p-Erk, demonstrating that 5,7-dimethoxycoumarin exerts its antiproliferative activity through inhibition of Mek 1/2 kinase activity; then, next reduction in Erk 1/2 triggered a terminal differentiation in treated melanoma cells, with melanin synthesis and dendricity.
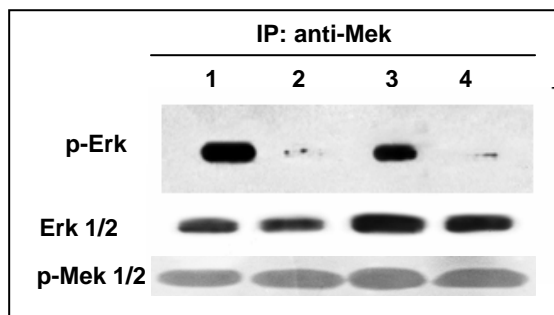
Figure 11. The effect of 5,7-dimethoxycoumarin on *in vitro* kinase activity of p-Mek 1/2 in B16 and A375 melanoma cells. B16 (**1,2**)and A375 cells (**3,4**) were incubated for 72 h with the vehicle solvent (**1,3**) or with the compound using 500 μM dose (**2,4**). Mek 1/2 was immunoprecipitated with anti-Mek 1/2 antibody from equal protein aliquots of cell lysates and immune complexes were incubated with non activated ERK substrate solution for *in vitro* kinase assay. After Western blotting, phosphorylation level of Erk 1/2 was analysed with its corresponding phospho-specific antibody.

4.8 Alteration of phenotypic characteristics in melanoma cells after treatment with 5,7-dimethoxycoumarin.

Phenotypic alterations were observed through cytofluorimetric analysis of both treated melanoma cell lines. A change in light scatter properties of B16

and A375 cells treated with 5,7-dimethoxycoumarin was detected. In particular, a decrease of FSC (forward scatter) mean values respect to the solvent control was observed; at the same time, SSC (side scatter) mean values increased strongly following the treatment. This effect was more pronounced in B16 than A375 cells. In Figure 12 results relative to the treatment with 500 µM dose for 72 h are shown. Also phenotypic characterization of SW620 cells was carried out, but changes in FSC and SSC were not detected after treatment, using the same experimental conditions (data not shown).
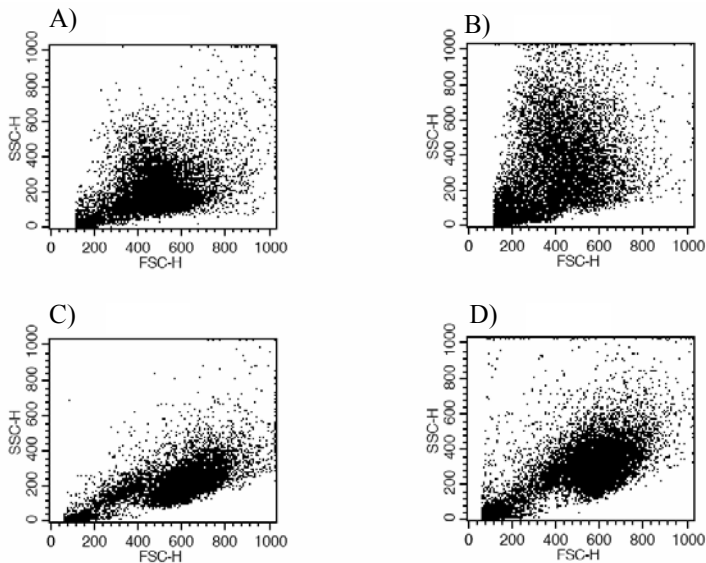
Figure 12. Light scatter of B16 (**A-B**) and A375 cells (**C-D**) incubated or not with 5,7-dimethoxycoumarin 500 µM for 72 h. In the scattergrams 5,7-dimethoxycoumarin-treated cancer cells (**B-D**) exhibited less forward scatter and much more side scatter than solvent-treated cells (**A-C**).

4.9 Melanogenesis process induced in B16 and A375 cells after treatment with 5,7-dimethoxycoumarin.

Since the changes in light scatter properties of B16 and A375 cells could be related to an increase of cellular complexity, we further investigated the melanogenesis process in these cell lines following treatment with tested compound. After 72h incubation, 5,7-dimethoxycoumarin demonstrated to induce melanin synthesis in B16 and A375 treated cells as shown in Figure 13 (A and B). In B16 cells, both the extracellular and intracellular melanin quantity significatively increased respect to the control for all tested doses. Total melanin calculation revealed that the melanogenesis was significantly increased in dose-dependent manner around 2.12, 3.0, 3.55-folds respect to the control for 5,7-dimethoxycoumarin 100, 250 and 500 μM, respectively (Figure 13A). In A375 cell line, the extracellular melanin quantity significantly increased respect to the control for all tested doses, while intracellular increase was signigicant only for the higher dose. Total melanin calculation, for A375 cells, allowed to detect that the melanogenesis increased in dose-dependent manner around 1.7, 2.9, 5.2-fold respect to the control for the concentration 100, 250 and 500 μM, respectively (Figure 13B). Then, these results also demonstrated that 5,7-dimethoxycoumarin induced a differentiation program in B16 and A375 cells since stimulation of melanogenesis is considered a well known marker of differentiation for melanoma cells (Edward *et al*., 1988).

A)   B)
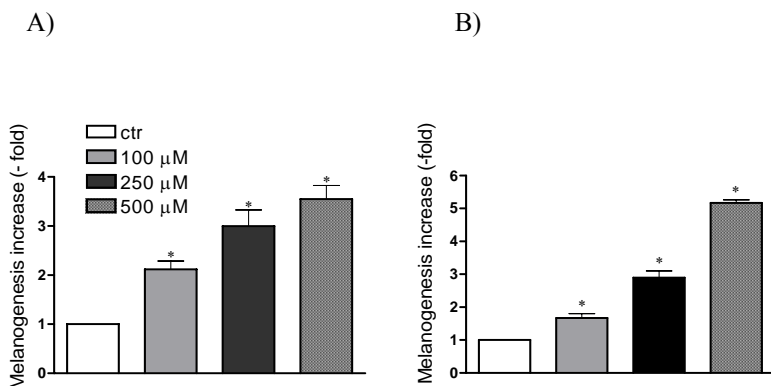
Figure 13. The effect of 5,7-dimethoxycoumarin on melanin synthesis in B16 (**A**) and A375 (**B**) cells following treatment 72 h *in vitro*. Cells were incubated with or without the compound using 100, 250 and 500 μM doses. The melanin concentrations (μg/ml) and the increase in melanin production were estimated as described in the section of Materials and Methods. Results are the mean ± SD from three separate experiments. *$P<0.05$ versus vehicle control treated cells.

Then, activity of enzyme tyrosinase, which catalyses the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) followed by the oxidation of L-DOPA to dopaquinone in the synthesis of melanin (Lin *et al*., 2002), was analyzed after treatment with the aim to delucidate the melanogenesis pathway activated by the compound. In B16 cells (Figure 14), the treatment increased significatively enzymatic activity after 72 h incubation around 2.59, 2.64, 3.3-fold respect to the control for the concentration 100, 250 and 500 μM, respectively; the increase was dose-dependent, although significative differences were not detected between the effects of concentration 100 and 250 μM.
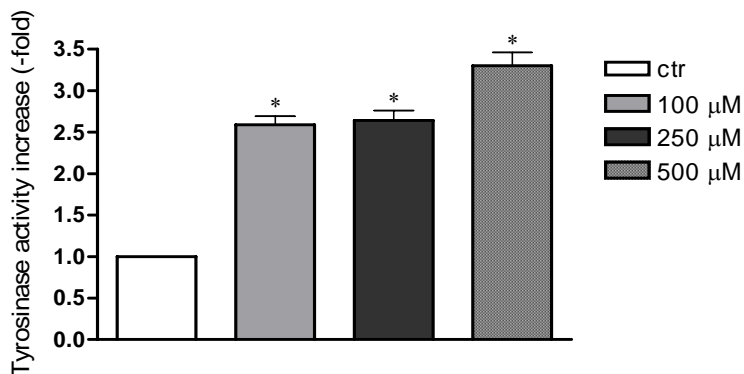
Figure 14. The effect of 5,7-dimethoxycoumarin on tyrosinase activity in B16 melanoma cells following treatment 72 h *in vitro*. Cells were incubated with or without the compound using 100, 250 and 500 μM doses. The tyrosinase activity was estimated as described in the section of Materials and Methods. Results are the mean ± SD from three separate experiments. *P<0.05 versus vehicle control treated cells.

4.10 CREB phosphorylation and expression of Mitf in melanoma cells after treatment with 5,7-dimethoxycoumarin.

In literature, it is known that the transcription of gene encoding for tyrosinase is activated from microphthalmia-associated transcription factor (Mitf) (Busca & Ballotti, 2000), another substrate of kinase p-Erk 1/2 involved in differentiation, proliferation and survival of melanocytes (Wellbrock & Marais, 2005). During melanogenesis, the Mitf activity is regulated through the c-AMP mediated pathway with the activation of protein kinase A (PKA) that upregulates the Mitf gene transcripion by

phosphorylation of the transcriptional factor CREB (cAMP-response element-binding protein) (Busca & Ballotti, 2000). Based on these knowledges, we investigated if alterations in CREB phosphorylation and Mitf expression occurred in melanoma cells following treatment with 5,7-dimethoxycoumarin.

Western blot of B16 cells incubated with 5,7-dimethoxycoumarin for a period of 24 h allowed to detect the increase of CREB phosphorylation, as shown in Figure 15A: infact, by using densitometric analysis, we calculated that augmentation in expression of activated transcriptional factor was 1.4- and 2.3-fold with respect to the control for 250 and 500 μM, respectively; no alteration was detected for lower tested dose at this time and for every tested dose following a treatment of 72 h (data not shown). Moreover, we detected an increase of Mitf expression in B16 cells after 72 h incubation (Figure 15A): augmentation in protein expression was evaluated 3.0- and 2.1-fold with respect to the control for 250 and 500 μM doses, respectively; instead, the expression level of this protein remained unchanged after lower dose treatment. These results are consinstent with melanogenesis process caused in B16 cells by 5,7-dimethoxycoumarin, probably through induction of thyrosinase activity, as elucidated above. Interestingly, in A375 cell line Mitf expression remained almost unchanged during an incubation of 24-72 h (Figure 15B); instead, it was possible to detect that CREB phosphorylation increased in moderate manner also in A375 treated cells respect to methanol control after 24 h treatment, as shown in Figure 15B.

Figure 15. The effect of 5,7-dimethoxycoumarin on CREB phosphorylation and Mitf expression in B16 **(A)** and A375 **(B)** melanoma cells following treatment 24 (for p-CREB) and 72 h (for Mitf). Cells were incubated with the compound using 100, 250 and 500 μM doses: lanes **2** (100 μM), **4** (250 μM), **6** (500 μM); or with respective vehicle solvent: lanes **1**, **3**, **5**. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in the section of Materials and methods. Expression levels of p-CREB and Mitf were analysed with corresponding specific antibodies.

4.11 PpIX accumulation and expression of porphobilinogen deaminase (PBG-D) in melanoma cells after treatment with 5,7-dimethoxycoumarin.

Recently, cellular differentiation has been related to an increase of intracellular PpIX, the compound synthesized during heme biosynthesis and considered a differentiation marker for some cancer cell lines including B16 cells (Ickowicz Schwartz *et al.*, 2004). To investigate if the treatment with

5,7-dimethoxycoumarin induced a significative augmentation in PpIX production, melanoma cell lines were treated with doses of 100, 250 and 500 µM for 72 h. As shown in Figure 16 (A and B), PpIX accumulation enhanced following increase of the dose of tested compound for both B16 (Figure 16A) and A375 (Figure 16B) cell lines. After 72 h incubation at higher concentration of 5,7-dimethoxycoumarin used, the increase of PpIX was 3.3 (B16) and 1.6 (A375) folds respect to the control. Instead, in SW620 cells, used as control, accumulation of PpIX was unchanged after treatment in the same experimental conditions (data not shown).
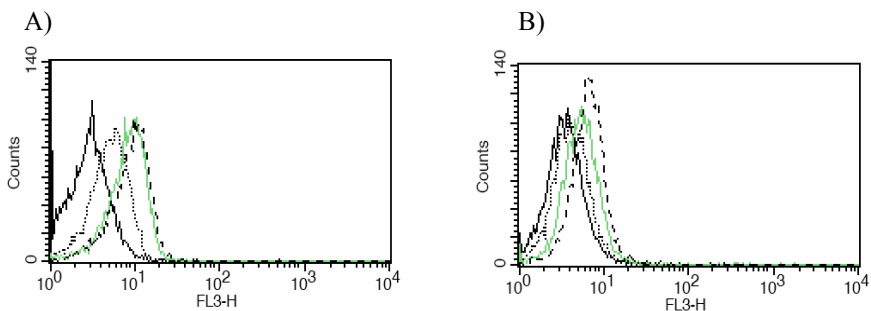
Figure 16. PpIX production in B16 (**A**) and A375 (**B**) cells treated with solvent (solid black line) or with 5,7-dimethoxycoumarin 100 µM (dotted line), 250 µM (green line) and 500 µM (dashed line) for 72 h, observed through cytofluorimetric analysis. The data shown are representative of 4 indipendent experiments.

Treated melanoma cells emitted a PpIX fluorescence higher than untreated cells, during analysis with FACScalibur, owing to a synthesis increase and intracellular accumulation of PpIX during treatment. Then, porphobilinogen

deaminase (PBG-D) expression was investigated with the aim to support previous fluorescence results; infact, this is a key enzyme in heme synthesis and catalyse the combination of four molecules of PBG-D into hydroxymethylbilane, which is turned into uroporphyrinogen III. As shown in Figure 17, Western blot analysis revealed a dose-dependent increase in total PBG-D protein both in B16 and A375 cell lines after a 72 h treatment with 5,7-dimethoxycoumarin. In B16 cells (Figure 17A), the augmentation of enzyme expression was estimated 8.0- and 2.8-fold with respect to the control for 100 and 500 µM doses, whereas no alteration in band intensity was observed between 250 µM dose and the solvent; in the case of A375 cells (Figure 17B), it was 1.9-, 1.5- and 2.1-fold for 100, 250 and 500 µM doses, respectively.
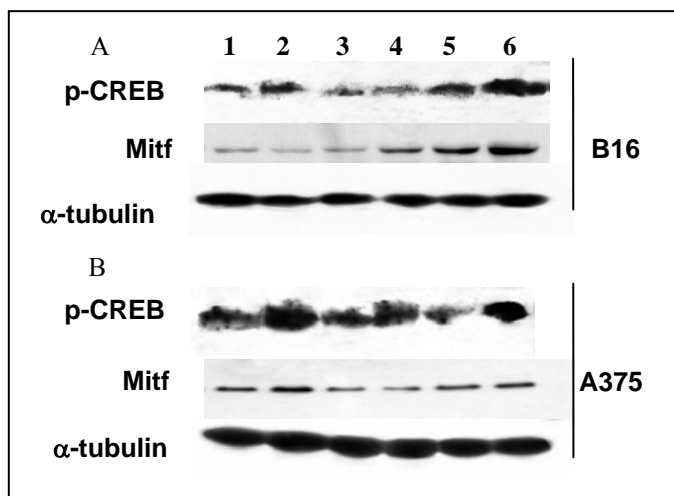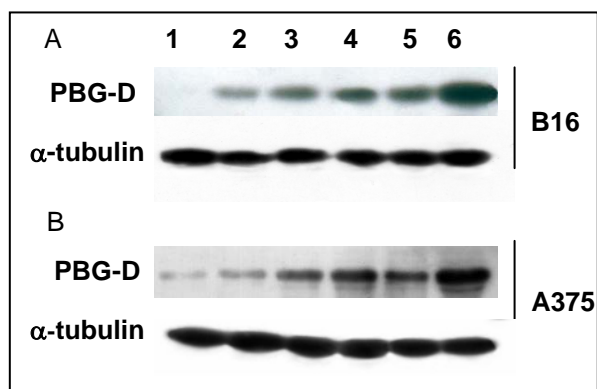

Figure 17. The effect of 5,7-dimethoxycoumarin on PBG-D expression in B16 (**A**) and A375 (**B**) melanoma cells following treatment 72 h. Cells were incubated with the compound using 100, 250 and 500 µM doses: lanes **2** (100 µM), **4** (250 µM), **6** (500 µM); or with respective vehicle solvent: lanes **1**, **3**, **5**. After treatment, total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting as described in the section of Materials and methods.

5. DISCUSSION

Among the biological properties possessed by coumarins, one that makes these compounds attractive is the antitumour activity showed by some members of this chemical group. In particular, 5,7-dimethoxycoumarin, a coumarin derivative identified in few vegetable species and recently also in *Carica papaya* L. (Canini *et al*., 2007), was the object of our investigation since it has been scarcely studied up to now. We decided to test the antitumour efficacy of this compound on different cancer cell lines *in vitro* by using first MTT assay, widely used for high through-put screening of new drugs. Our results indicated that 5,7-dimethoxycoumarin inhibited growth of several cell lines, of melanoma and carcinoma origin, in a comparable manner. Since MTT is an indirect colourimetric assay, Trypan blue test was then used to directly assess the cell survival rate in melanoma lines following treatment. Antineoplastic activity of the molecule was confirmed, but IC_{50} values obtained after a 72 h treatment were considerably lower than those from MTT test. This discrepancy in test sensitivity could be explained considering that MTT assay is based on intracellular dehydrogenases activity whose activity could be altered by the tested compound (Pagliacci *et al*., 1993); anyway, not shown experiments allowed us to exclude the feasibility of a direct MTT reduction by 5,7-dimethoxycoumarin. By direct cell counting it is possible to distinguish between cytostatic and cytotoxic effect; in this case, our results indicated a cytostatic activity exerted by this molecule, at least in used experimental conditions. Anyway, we can not ruled out that higher doses and longer incubation times could exert cytotoxic

activity, since our experimental observations were extended up to 72 h of culture and at maximum dosage of 500 µM in presence of 5,7-dimethoxycoumarin.

FACS analysis demonstrated that 5,7-dimethoxycoumarin caused an arrest of cell cycle in G0/G1 phase with a decrease of cell population in synthesis and mitosis phases in melanoma cells. Other coumarins with anticancer properties blocked cell cycle either in G0/G1 phase (Lopez-Gonzales *et al*., 2004) or in S phase (Finn *et al*., 2004a). Moreover, cytostatic activity proved irreversible by culturing the B16 cells, previously treated, without the compound; after some days of culture in fresh medium, the treated cells did not recover control growth. Generally, an irreversible growth inhibition is determined by either apoptotic cell death or differentiation process (Agarwal *et al*., 2000). In B16 and A375 cells, following treatment with 5,7-dimethoxycoumarin, we observed significant morphological changes and characteristic markers specific of melanoma cell differentiation: infact, after incubation, melanoma cells took a star-like shape with dendritic projections. Morphological alterations were also revealed through flow cytometry analysis; infact, treated melanoma cells exhibited a subtle reduction of FSC respect to the control, indicating that the incident light was absorbed rather than transmitted through the cells presumably due to an augmentation of light absorbing melanin. But we can not rule out the possibility that such changes could be due to cell size reduction or to the presence of other cellular components that absorb incident light. On the contrary, we observed a high increase of SSC, that generally indicates the granularity of a cell, in melanoma treated cells, especially in B16 cell line, after treatment with 5,7-dimethoxycoumarin. We hypothesised that this reflects an increase of intracellular number of melanosomes, supported by the evidence that

SW620, a human adenocarcinoma cell line, did not change light scatter properties after treatment with the compound. Based on these data, we investigated the melanin content in murine B16 and human A375 melanoma cells: an increase in melanin synthesis was observed in treated cells, as hypothesised above. Morphological changes with dendrite outgrowth and melanogenesis are considered specific markers of differentiation for melanocytes and melanoma cells (Umek *et al.*, 1991). It is well known from literature that some melanoma differentiation agents, such as UV-B (Medrano *et al.*, 1995) and cyclic adenosine monophosphate (cAMP) (Kato *et al.*, 1994), stimulate dendricity and melanogenesis after blocking cells in G0/G1 phase as well as our tested compound. We suggested that 5,7-dimethoxycoumarin induced a terminal differentiation in B16 cells that in the end causes cell death, even if the ultimate fate of the growth arrested cells was not analysed. This hypothesis is supported by the observation that exposure to the highest dose of compound for 72 h led to a small accumulation of cells in sub-G1 phase (data not shown); moreover, the compound induced irreversibly both cell growth inhibition and morphological changes. In literature, it is reported that novobiocin, an other coumarin derivative with antibiotic properties, inhibited B16 cell proliferation and induced cellular differentiation (Nordenberg *et al.*, 1992). In the last years, novel synthetic nitrated coumarins, namely 6-nitro-7-hydroxycoumarin and 3,6,8-trinitro-7-hydroxycoumarin have been shown to be anti-proliferative and differentiation agents in human melanoma cells (Finn *et al.*, 2005).

With the aim to elucidate the mechanism by which 5,7-dimethoxycoumarin reduced proliferation and induced differentiation in treated B16 cells, we focused our attention on phosphorylation status of Erk 1/2 following

treatment. Erk 1/2 (extracellular signal-regulated kinase) is a member of the Ras/Raf/Mek/Erk mitogen-activated protein kinase (MAPK) signalling pathway that regulates cell survival, proliferation and differentiation processes following transmission of a wide variety of extracellular stimuli from the cell surface to the nucleus (Torii *et al.*, 2006). Its phosphorylation by Mek 1/2 in serine and threonyne residues activates a series of transcription factors in the nucleus, such as c-Myc, NF-IL6 and Elk-1, initiating the transcription of genes encoding for proteins that promote cell cycle progression through G0/G1 phase and from G0/G1 to S phase, such as the cyclins D and E, and then cell proliferation (Torii *et al.*, 2006, Henson & Gibson, 2006). Using Western blot analysis, we detected a reduction in the phosphorylation of Erk 1/2, following incubation of B16 and A375 cells with 5,7-dimethoxycoumarin. In murine cell line, the inhibition of the MAPK phosphorylation was more evident with increase of tested compound concentration and this data was comparable with cell cycle analysis and trypan blue assay results, since reduction of Erk phosphorylation caused the block in G0/G1 phase and next growth reduction and differentiation. Moreover, our results indicated that Erk phosphorylation is inhibited in the human melanoma cell line with a lesser extent than the murine; and this different behaviour could partially explain the differences in values of IC_{50}, i.e. 109 μM for B16 and 142 μM for A375 cells.

In literature, it is reported that Ras/Raf/Mek/Erk pathway is constitutively activated, following mutation in the catalytic domain of these protein kinases, in a high percentage of human melanoma cells; particularly, an activating mutation of B-Raf, as a consequence of a substitution of a glutamate for valine at position 600 (V600E), triggers a constitutive activation of Mek and Erk with a subsequent induction of cell proliferation

and a strong reduction of dendricity and pigmentation (Wellbrock *et al.*, 2005). The treatment of melanoma cells with either U0126 (Mek 1 inhibitor) or PD098059 (Mek 2 inhibitor) causes an inhibition of cell growth and a reduction in levels of matrix metalloproteinases MMP-1 and MMP-2, involved in tumor cell invasion, demonstrating that activation of Erk is an important event in melanoma development (Namkoong *et al.*, 2006). Based on this assumption, the MAPKs of Ras/Raf/MEK/ERK signalling pathway are considered molecular targets of novel therapy in melanoma cancer research and several agents that target this pathway are already undergoing clinical testing (Panka *et al.*, 2006). Since Mek 1/2 is a key regulator in the MAPKs pathway (Sharma *et al.*, 2002) and phosphorylates Erk 1/2, its phosphorylated form (p-Mek) was examined: surprisingly, the treatment with 5,7-dimethoxycoumarin was shown to increase p-Mek, although in a moderate manner, both in B16 and A375 cells. The increase in Mek phosphorylation, followed by Erk disactivation, promped us to analyze the kinase activity of p-Mek following treatment. Our results of immunoprecipitation following kinase assay of Mek 1/2, by using Erk as substrate, demonstrated for the first time that the 5,7-dimethoxycoumarin inhibited enzymatic activity of the kinase derived from melanoma treated cells respect to not treated cells, triggering a significant reduction of Erk phosphorylation. Up to now, it has been reported that some compounds, in the class of coumarins, inhibit the activation of unphosphorylated Mek 1 by binding to an allosteric site in the inactive conformation of this kinase; but they can't inhibit the activity of the activated Mek 1 (Han *et al.*, 2005). According to the literature, equol, another natural compound of flavonoids class, greatly inhibited Mek 1 kinase activity in mouse epidermal cells, binding directly to the kinase; moreover, also this compound suppressed

phosphorylation of Erk, but did not show effect on phosphorylation of Mek (Kang *et al*., 2007). Besides, it is known that cyclin-dependent protein kinase 5 (cdk5), involved in neuronal development and neurite outgrowth, can down regulate the Map kinase pathway by inhibiting Mek 1 activity: cdk5 targeted specifically the Raf-phosphorylated Mek 1 or a constitutively active form of Mek 1 through phosphorylation of a threonine residue (Thr286), thereby inhibiting its enzymatic activity (Sharma *et al*., 2002). Then, we can presume either a direct or an indirect modulation of Mek 1/2 by 5,7-dimethoxycoumarin: the compound could bind to one site of the activated kinase Mek 1 and inhibit its enzymatic activity; alternatively, it could up-regulate other kinases, such as cdk5, that in turn will phosphorylate and inactivate Mek 1/2 in irreversible manner. Our results suggested for the first time that 5,7-dimethoxycoumarin, by targeting activated Mek 1/2 in melanoma cells and reducing its kinase activity, lead to developing of dendrite-like projections and induced cellular differentiation with activation of melanogenesis, following cell cycle block.

Next, we tried to determine whether 5,7-dimethoxycoumarin induced melanin synthesis in melanoma cells, through modulation of cAMP pathway, which plays a key role in the regulation of melanogenesis stimulated by some agents, including α-melanocyte (α-MSH) stimulating hormone and forskolin (Costin *et al*., 2007). Infact, cAMP activates protein kinase A (PKA), that in turn translocates to the nucleus and phosphorylates cAMP-response element-binding proteins (CREB) that finally upregulates transcription of specific genes (Kadekaro *et al*., 2003). Among the others, there is the gene encoding for microphthalmia-associated transcription factor (Mitf), which is involved in differentiation, proliferation and survival of melanocyte (Mansky *et al*., 2002; Vance & Goding, 2004). It promotes the

transcription of the gene encoding for tyrosinase, which catalyses two key steps in the pathway of melanin synthesis: the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), followed by the oxidation of L-DOPA to dopaquinone (Ito, 2003). For this reason, it is known that Mitf is required for the cAMP-induced melanogenesis (Busca & Ballotti, 2000).
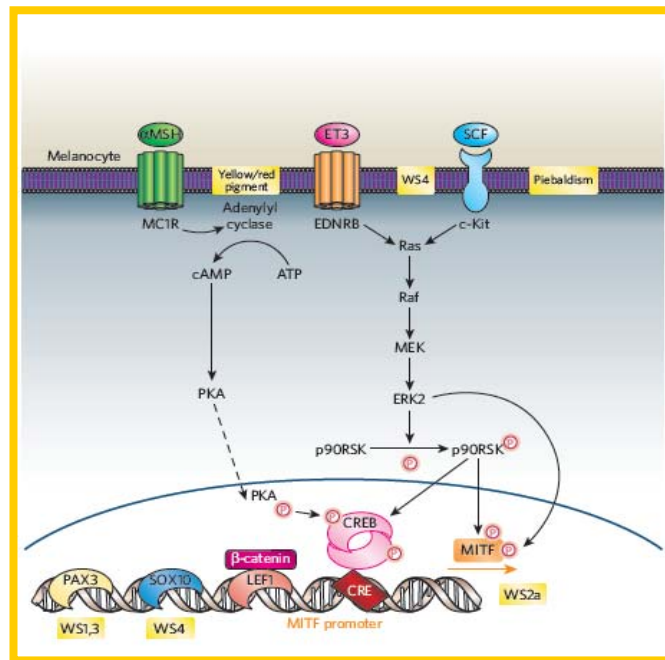

Figure 1. Regulation of Mitf expression and activity in melanogenesis process (Lin & Fisher, 2007. Nature)

We observed, by using Western blotting analyses, that in B16 cells the treatment enhanced CREB phosphorylation associated to an augmentation of Mitf expression; furthermore, a subsequent stimulation of tyrosinase activity,

which finally led to increase of melanin synthesis, was detected. Since p-Erk 1/2 downregulates Mitf by phosphorylation at serine 73 and its subsequent degradation (Hemesath *et al.*, 1998), it can be hypothesized that in tretaed B16 cells, Mitf expression is up-regulated, in addition to transcriptional factor CREB, also through inhibition of Erk1/2 phosphorylation induced by the tested compound. In literature, it is known that the MAPKs play a key role in tyrosinase expression and melanogenesis process; particularly, p-Erk 1/2 downregulates Mitf by phosphorylation at serine 73 and subsequent degradation. Englaro *et al.* (1998) observed that inhibtion of Ras/Raf/Mek/Erk MAPK pathway led to the B16 cell differentiation and melanogenesis, increasing tyrosinase expression and activity. Furthermore, Mitf can control cell cycle progression in melanoblasts and melanoma cells, since it induces a G0/G1 cell-cycle arrest through upregulation of p21Cip1 (cyclin-dependent kinase inhibitor gene) expression and this will contribute to activation of the melanogenesis and the differentiation programme (Carreira *et al.*, 2005). Previous studies showed that V600EB-Raf triggers Mitf degradation in mouse and human melanocytes and that its reexpression suppresses melanoma cells proliferation (Wellbrock *et al.*, 2005). Considering these evidences, we demonstrated, as shown in Figure 2, that the treatment of B16 with 5,7-dimethoxycoumarin inhibited Mek 1/2 kinase activity and consequent Erk phosphorylation, which is fundamental for transcription of the genes encoding for proteins involved in cellular proliferation; at the same time, 5,7-dimethoxycoumarin increased expression of Mitf, reducing its phosphorylation raised by p-Erk and promoting CREB phosphorylation (perhaps, CREB phosphorylation is following c-AMP signaling pathway activation). Then, it might be possible that also Mitf promoted cell cycle arrest in G0/G1 and differentiation, probably through

p21 expression, and melanin synthesis following to the increase of tyrosinase gene transcription. Finally, we suggest that the moderate augmentation in Mek 1 phosphorylation, observed both in B16 and A375 cells, could be explained with a parallel increase in cAMP pathway activation; infact, the cAMP and Ras-Raf-Mek-Erk signalling pathways interact through several cross-talks such as the PKA-induced activation of Erk 1/2 interceded through B-Raf (Dumaz & Marais, 2005; Gerits *et al.*, 2008).
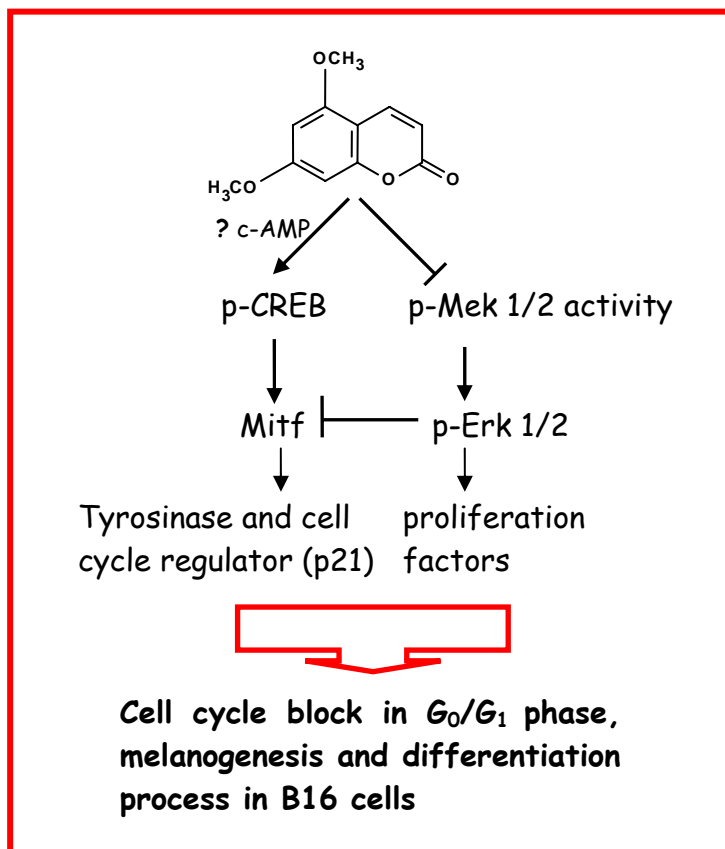
Figure 2. Signalling pathways modulated in B16 murine melanoma during treatment with 5,7-dimethoxycoumarin.

Also in human A375 cells, an inhibition of Mek 1/2 kinase activity and p-Erk 1/2 expression were detected following treatment with 5,7-dimethoxycoumarin as described above. However, although the compound stimulated CREB phosphorylation and the melanogenesis process, considerable increases in Mitf expression was not observed in A375 cells. We could partially justify this discrepance with the assumption that the regulation of Mitf expression and activity is controlled by several agents, both at the transcriptional and post-translational level. For example, GSK3β can phosphorylate MITF on serine 298, enhancing the binding activity of MITF to the tyrosinase promoter, whereas MAPK and Rsk-1 phosphorylates it at serine 73 and 409, respectively, and promote its activation followed by its degradation (Levy *et al*., 2003). Furthermore, MITF may be a direct target of the p38 MAPK signaling pathway that is necessary, in example, for osteoclast differentiation and lipopolysaccharide (LPS)-induced melanogenesis (Mansky *et al*., 2002; Ahn *et al*., 2008). Then, 5,7-dimethoxycoumarin could stimulate melanogenesis process in A375 cells through regulation of Mitf like in B16 cells, although significant alterations in its expression have not been detected. We could investigate the several phosphorylation states of the transcriptional factor, since probably in this cell line the treatment controls it in post-translational level.

As a further marker of differentiation, we monitored the production of PpIX, a photosensitiser compound synthesized and localized in the mithocondria as intermediate molecule in the heme biosynthesis pathway, of which the 5-aminolevulinic acid (ALA) is the first intermediate. The treated melanoma cells emitted a PpIX fluorescence higher than untreated cells owing to a

synthesis increase and intracellular accumulation of PpIX during treatment; furthermore, the compound increased expression of total PBG-D protein, the key enzyme involved in heme synthesis. In literature, it is reported that the induction of differentiation enhanced PpIX accumulation in some cell lines, such as prostate cancer cells (Ortel *et al.*, 1998), fibroblasts, B16 cells (Schoenfeld *et al.*, 1994) and keratinocytes (Sato *et al.*, 2007); however, the relationship between tumour cell differentiation and photosensitizer accumulation is poorly defined (Siboni *et al.*, 2003) since some differentiation agents, as butyrate, did not enhance PpIX accumulation whereas others, as hexamethylene bisacetamide (HMBA) and methotrexate, did it (Ickowicz Schwartz *et al.*, 2004; Sinha *et al.*, 2006). To the best of our knowledge, this is the first evidence that a member of coumarin group enhanced PpIX accumulation at least in melanoma cells; this observation was not reproduced in SW620 cells suggesting a cell-type specific effect. PpIX production is exploited clinically in photodynamic therapy of cancer (PDT), accepted for the care of superficial epithelial neoplasias. This therapy uses 5-aminolevulinic acid (ALA), an inert pharmacological precursor that remains inactive until it is converted into a photosensitising agent (PpIX) that is activated by light to generate reactive oxygen species (ROS): this process leads to cancer cell death via necrosis or apoptosis (Iinuma *et al.*, 1994). A combination therapy with use of a differentiation agent -that enhances specifically PpIX production in cancer cells- and the PpIX precursor could be very useful, because tumour eradication with ALA-PDT is not always obtained. Based on this premise, our data result particularly interesting, although more studies are needed to establish the interaction between 5,7-dimethoxycoumarin and ALA-PDT.

Furthermore, it is known that PpIX induces melanogenesis through the cAMP-mediated pathway; infact, it activates the soluble guanylate cyclase and elevates intracellular cGMP (cyclic guanine monophosphate) content which was demonstrated to increase cAMP content by inhibition of cAMP phosphodiesterase (Ignarro *et al.*, 1984; Brechler *et al.*, 1992). We suggest that 5,7-dimethoxycoumarin treatment of melanoma cells stabilizes cAMP signalling pathway also following production of PpIX; then, CREB phosphorylation and Mitf expression increased, thus triggering to differentiation and melanogenesis process, in harness with Ras/Raf/Mek/Erk modulation.

In combination treatment of B16 melanoma cells, the Mek 1/2 inhibitor U0126 significantly potentiated growth inhibition induced by 5,7-dimethoxycoumarin, in synergic or additive mode; moreover, the development of dendrite-like projections was more evident respect to the treatment with the 5,7-dimethoxycoumarin alone (data not shown). It is known that Mek 1/2 inhibitors, such as U0126 or PD098059, cause inhibition of the cell growth, induce a differentiation process in melanoma cells and reduce tumor cell invasion. Our results indicates that down-regulation of the Ras/Raf/Mek/Erk pathway, through Mek 1/2 inhibitor, sensitizes melanoma cells to 5,7-dimethoxycoumarin treatment, suggesting a possible regimen for preclinical and clinical therapy. Recently, the use of combinations of anticancer agents is in clinical trials and appears an effective strategy to strengthen their anticancer effect, since this could reduce the doses of each agent and then their toxicity (Pagliacci *et al.*, 1993).

Overall, in this study, we attempt to delucidate some molecular mechanism involved in antineoplastic and differentiation programme activated by 5,7-dimethoxycoumarin in melanoma cells (Alesiani *et al.*, 2008; Alesiani *et al.*,

2009). It will be of note to carry out *in vitro* analyses to investigate the antimetastatic activity of the tested compound. Furthermore, we are going to effect *in vivo* studies with animal models, using also the combination treatment with 5,7-dimethoxycoumarin and Mek inhibitors; this could allow to demonstrate cancer antigrowth and antimetastatic properties of compound also in preclinical trials.

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