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## ***ABBREVIATIONS***

2-PTS	2-propenyl thiosulfate
Br-pyr	bromo-pyruvate
CDNB	1-chloro-2,4-dinitrobenzene
DPBS	Dulbecco's Phosphate Buffered Saline
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
GSH	reduced Glutathione
GSSG	oxidized Glutathione
GST	Glutathione-S-Transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NADPH	nicotinamideadenine diphosphate reduced
p21	Cyclin-dependent Kinase Inhibitor 1A (CDKN1A)
p38	Mitogen-Activated Protein Kinase
Rhd	Rhodanese
RhdA	Rhodanese from <i>Azotobacter vinelandii</i>
TE buffer	Tris-HCl 50 mM (pH 8.0), EDTA 1 mM
Trd	Thioredoxin Reductase
Tris-HCl	tris(hydroxymethyl)aminomethane
Trx	Thioredoxin
TST	Thiosulfate:cyanide SulfurTransferase (rhodanese)

## ***INTRODUCTION***

## CHAPTER 1. *GARLIC AND CHEMOPREVENTION*

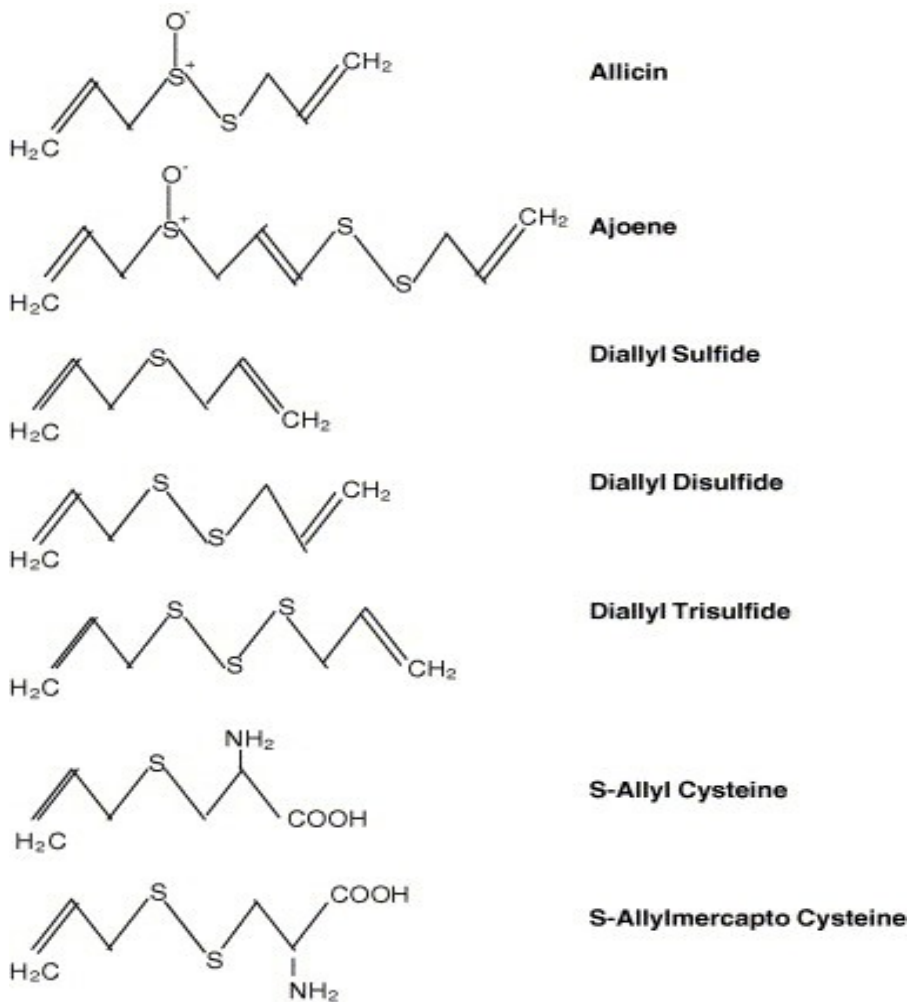
In the history of the world, food is always on the center of the chemoprevention, for the presence of active compounds [4, 65]. About the 40% of americans use alternative medicine, such as herbal medicine; in fact, the high cost, side effects and therapeutic limitation of conventional medicine are the principal reasons to the use of herbal remedies. Dietary consumption of foods and herbal medicines is a convenient method of assumption of beneficial phytochemicals. The scientific literature indicates that a diet rich of fruits and vegetables (more of 400 g/day) increase the prevention of at least 20% of all cancers, such as gastro-intestinal, mouth, pharynx, esophagus, stomach, colon and rectum cancers [4, 50, 65, 79].

The edible plants are the most versatile and play a key role on the cancer epidemiology. *Allium* species are found all over the world, except the tropics, New Zealand and Australia; includes onion, garlic, chive leek and shallot. Garlic has been used since the beginning of recorded history, and there are many ancient medical texts that prescribed medical application for garlic. Ancient people used garlic as remedy of intestinal disorder, worms, respiratory infections, skin disease, wounds, etc. Garlic has been applied since time immemorial as a culinary spice and medicinal herb and is an important constituent of chinese medicine. The garlic chemistry is quite complex and is not yet well understood, but the presence of active compounds probably is related with a self-protective mechanism against the microorganisms and other assault. [65, 80, 82].

The principal constituents in garlic are the Organ Sulfur Compounds (OSCs), that arise the typical odor in crushed garlic. When garlic is cut,

chopped or crushed, the clove's membrane disrupt and S-allyl-cysteine (SAC) sulfoxide is transformed enzymatically into allicin by allinase. The main components includes diallyl sulfide (DAS), diallyldisulfide (DADS), diallyltrisulfide (DATS), ajoene, allicin, S-allyl-cysteine (SAC), S-allylmercapto-cysteine (SAMC) (Figure 1) [50, 82]. The OSCs are branched in two subclasses, the oil-soluble and water-soluble. The oil-soluble are the most studied and active, but their use in the therapy is difficult because of their low solubility in water. On the contrary, the water-soluble compounds, instead, are less studied, and every year new active compounds are discovered and their biological effect is still object of study. For example, SAC from garlic may influence the risk of heart disease, playing an important role in the initiation of atherosclerosis. The antibacterial effect of garlic has been demonstrated against the infection of *Helicobacter pylori* [65].

Many OSCs are implicated on the enhancement of DNA repair, reduction of cell proliferation and/or apoptosis induction, and the presence of the sulfur atom and the allyl group is proportional to the antiproliferative effect of the compound. Several mechanisms have been proposed to explain the cancer-preventive effect of *Allium* vegetables and related OSCs. These include inhibition of mutagenesis by inhibiting the metabolism [24], inhibition of DNA adduct formation [20], and effect on cell proliferation and tumor growth. The antiproliferative effect of OSCs seems to be related to the induction of apoptosis. DADS and DATS treatment causes apoptosis induction in many human tumor cell lines [60, 86]. Literature of OSCs shows the propriety to induce apoptosis selectively in the tumor cells, and not in non-tumor cells; in fact, 10  $\mu$ M of DADS causes 47% of inhibition of



**Figure 1.** *Biologically active compounds from garlic (Shukla, Kaldra, 2007) [80].*



A549 lung tumor cells proliferation, whereas it did not influence the growth of non-neoplastic MRC-5 lung cells [77]. Similarly, fibroblast cells was not affected by ajoene treatment, compared to BJA-B lymphoma [78].

Recent works shows that the production of intracellular reactive species of oxygen (ROS) is one the principal causes of the antiproliferative effect of these compounds. A significant increase of intracellular ROS was induced in A549 lung cancer cells less than 30 minutes after DADS treatment, indicating that ROS may be an early event in DADS- and OSCs-modulated apoptosis [29]. Treatment of neuroblastoma SH-SY5Y cells with DADS resulted in arrest of cell cycle in G<sub>2</sub>/M phase and induced the apoptosis through the activation of the mitochondrial pathway. The earliest oxidative event observed after DADS treatment was the increase of production of ROS [28].

Hosono et al [38] have been demonstrated that DATS can induce apoptosis in human colon cancer cells through oxidative modification of  $\beta$ -tubulin. The specific oxidative modification of cysteine residues Cys-12 $\beta$  and Cys-354 $\beta$  to generate S-allyl-mercaptocysteine seems to be relevant for the DATS effect.

In the cell are present many enzymes containing redox cysteine, that are involved in many cellular process, such as thioredoxin (Trx), thioredoxin reductase (Trd), glutathione-S-transferase, rhodanese (TST), glutaredoxin (GSTRX), etc. It has been showed [39] that the redox cysteine of these enzyme must be maintained in a right redox-state, because its alteration causes enzyme inactivation. The loss of function of important enzymes involved in the maintaining of right intracellular redox-state (Trx, Trd, GSX, TST), involved in the intracellular detoxification system (GSTs), and

glutathione (GSH), can be also important for the biological effect of the OSCs.

### ***2-propenyl thiosulfate***

As described above, the water-soluble compounds are not well studied, and new compounds are discovered every year. The water-soluble are less odorous than oil-soluble compounds, but are more stable, and seems to have a higher bioavailability and seems to be able to enter in the blood reaching target organs. Moreover, their ability to be dissolved in water, allow to avoid the use of solubilization agents, such as dimethyl sulfoxide, methanol, etc.

Recently, new compounds form the aqueous phase of boiled garlic and onion was found, the 2-propenyl thiosulfate (2-PTS) and n-propyl thiosulfate (N-PTS), respectively (Figure 3-A) [100]. These compounds causes decreasing of cell proliferation, apoptosis induction in various cell lines [15] (Figure 3-B) and hemolysis in canine erythrocytes [17]. The apoptosis induction was tested in three human tumorigenic cell lines: WiDr (human colon carcinoma), 293 (human kidney embryonic cell immortalized), and HL-60 (human promyelocytic leukemia). The 2-PTS was found to be more active than N-PTS in these cell lines, and the only one to induce apoptosis. The authors describe this difference with the ability of 2-PTS to generate more superoxide. Also the intracellular content of GSH seem to be involved in this process; in fact, although GSH plays a crucial vital role in protecting cells against damage induced by ionizing radiation, oxidant, chemotherapeutic agents, previous studies show that dogs with erythrocytes

of hereditary high GSH content are more susceptible to oxidative damage produced by N-PTS than dogs with normal erythrocytes [15, 98]. This observation could lead to the hypothesis that the GSH acts as intermediate in the effect of 2-PTS, but the cytotoxicity of the N-PTS and 2-PTS is not related with the intracellular GSH content [98, 99]. Certainly, the GSH is implicated in the process, but it is not the only factor involved. The data present in literature indicate that the 2-PTS certainly participates at the anticarcinogenic properties of garlic, but its complete mechanism of action is still object of study.

We have used this OSC for our experiments because it represents a good model of study. Its chemical structure is very similar to the thiosulfate, the first identified substrate of rhodanese, and it is a water-soluble OSC, and it is still not well studied.

## **CHAPTER 2. *INTRACELLULAR REDOX AND DETOXIFICATION SYSTEM***

In the cell two essential systems are always active and tightly modulate the cell proliferation and differentiation: the redox system and the detoxification system. Several severe diseases are associated to dysfunctions or dysregulations of these systems, such as cancer development, Parkinson's disease, Alzheimer's disease.

The intracellular redox system includes all those proteins and peptides involved in redox reactions; the most important are the Thioredoxin, the Glutaredoxin, the Glutathione, the Thioredoxin reductase, the Glutathione reductase, the Catalase, the Superoxide dismutase. The intracellular redox state must be maintained in a reduced state, to neutralize the radicals generated continuously from the metabolism, to prevent redox damages to proteins and DNA. All the proteins mentioned above work to this aim, and cooperate each other, making interconnections that we have in part studied in this thesis. We suffer also from the environment many redox assaults, and during our lifetime, we are exposed to a great number of xenobiotics, including pharmaceuticals and food components. The cells have evolved a complex system of detoxification enzymes, to minimize the potential of damage from xenobiotics. There is a direct correlation between the decreasing ability to remove toxins and the etiology of chronic conditions and disease. Actually, the xenobiotics are biotransformed in two phases: functionalization, and conjugation. These two steps are named Phase I and Phase II detoxification, respectively, and to each phase includes several

enzymes and peptide. The Phase I includes the cytochrome P450 family (CypP450). The Phase II, instead, includes several enzymes that catalyze the conjugation of the xenobiotics to increment its water-solubility, such as Epoxide hydrolase, Glutathione-S-transferases, Sulfurtransferases, and various transferases. The recent discover of the antiporter activity, as a determinant factor for the develop of the Multidrug Resistance (MDR), has introduced the Phase III system, that includes two genes responsible for the antiporter activity: MDR1 and MDR2. The antiporter activity is an energy-dependent efflux activity, and it is a very important factor, because modulate the concentration of the xenobiotics, pumping it out from the cell.

These two system are essential for the correct cell vitality, and the proteins and peptide studied in this thesis are elaborate afterward.

### ***Thiosulfate:cyanide sulfurtransferase (rhodanese)***

Rhodanese (EC Number: 2.8.1.1) is a thiosulfate:cyanide sulfurtransferase (TST), widely distributed in all the organisms. In the eukaryotic cells is located in the mitochondria, and its biological function is still not completely defined [63]. Recent works highlight new implications of rhodanese in the metabolism of selenium [62], OSCs, further to the cyanide detoxification [84], implication the cluster Fe-S assembly, implication in the Friedreich's ataxia [88], colon-rectal cancer progression [8, 9] and ethylmalonic encephalopathy [91].

The presence of rhodanese in all phyla suggest an essential biological role of this enzyme, although knock-out experiment to validate this hypothesis

does not exist. Our work indicates, moreover, the importance of a right redox-state of the enzyme, that affect the activity of the enzyme. In fact, the catalytic cysteine, responsible of the transfer of the sulfur sulfane, must be in the reduced form. Oxidation of the catalytic cysteine causes a loss of activity [75].

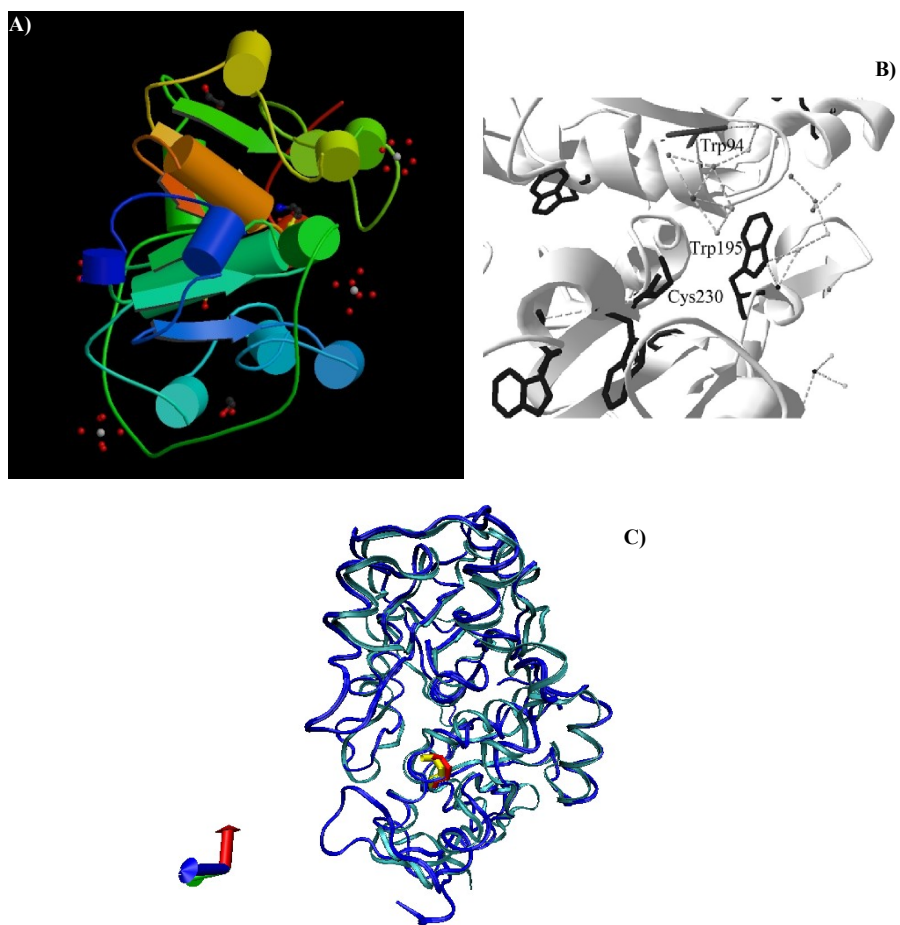
The enzyme exists in two cyclic forms, a sulfur loaded-form (ES) and a sulfur free-form (E). The E form is less stable, and the ES form is more stable, and probably in the cells is the predominant form. Rhodanese is found in mono- or bi-domain with a similar topology. Bovine liver rhodanese consist of a single polypeptide chain with molecular weight of about 32 kDa [37, 43]. The enzyme is formed by two domains with similar conformation with few structural differences. Each domains has four-stranded parallel  $\beta$ -structure, with one helix running anti parallel to the  $\beta$ -sheet (Figure 2-A).

The catalytic site of rhodanese is located in the bottom of the cleft formed by the two domains of the enzyme [43] (Figure 2-B). It has been shown that thiol (SH) groups are essential in the rhodanese catalysis [43]. The rhodanese has an intrinsic structural flexibility and reversible conformational changes accompany catalysis. Rhodanese mechanism of action is a double displacement, where the enzyme can accept a sulfur molecule from a donor (e.s. thiosulfate) on the catalytic cysteine (247 in bovine rhodanese, 230 in *A. vinelandii*) and transfer it to an acceptor, as the cyanide. The sulfur loaded-form (ES) is characteristic for a S-S bond between the cysteine and the sulfur derived from the donor. The oxidation state of the this peculiar sulfur atom is not well defined, however is considered to be between -1 and 0, and is named “sulfur sulfane”. This characteristic makes this bond weaker than a

classical S-S bond, and facilitate the release of the sulfur to the acceptor. ES form is more stable than E form and studies indicate that the sulfur free enzyme is more susceptible to proteolysis, compared to ES form. This is imputable to the observation that the E form is more flexible than ES form, as showed in previous NMR studies [75 and citations therein].

Human rhodanese has a very conserved structure with bovine and *Azotobacter vinelandii* rhodanese (RhdA), formed by a single polypeptide chain. The homology of sequences between bovine and *A. vinelandii* rhodanese is about 22%, while the crystal of the two structures appear to be very similar [11, 21, 64], with low *rmsd* (Figure 2-C).

Microarrays analysis of colonic mucosa of normal and cancer tissues reveals that a possible cause of colorectal cancer carcinogenesis is the decreased expression of some gene, among which there is also the *rhodanese* gene, that have a significant diminution of its expression in relationship with the progression of the disease. Moreover, increase of TST expression is observed during colonocyte differentiation [72], after treatment of colon cancer cell line with sodium butyrate, that promote the differentiation of this cell line [72]. The down regulation of TST expression in some cancer cells could be explain the different sensitivity to OSCs treatment of normal and cancer cells. In fact, the minor expression of TST, as discussed in this thesis, could be related to of loss a sulfide detoxification in the cell, allowing to the toxic sulfide to damage the cell.



**Figure 2.** A) 3-D structure of *A. vinelandii* rhodanese [11]; B) active site of RhdA; C) Superimposition of bovine and *A. vinelandii* rhodanese [11, 37].



## ***Thioredoxin***

Cytosolic Thioredoxin (EC Number: 1.8.1.8), or Trx, or Trx1 is a small protein (about 12 kDa) present in all organisms, and its sequence is very conserved. Trx topology is four-stranded  $\beta$ -sheet sandwiched between two  $\alpha$ -helices, and has a characteristic and conserved sequence in the active site -C-X-Y-C-, where X and Y are often, but not necessary, hydrophobic aminoacids.

So far, three distinct variants of human Trx encoded by separate genes have been cloned and characterized in some detail. Most studied is the gene for the classical 12-kDa thioredoxin (Trx-1) [33, 36, 83]. The Trx-2 isoenzyme is located in mitochondria and includes a 60 amino acid N-terminal mitochondrial translocation signal [5]. The third, SpTrx, is a variant highly expressed in spermatozoa [52]. Trx of all organism contain a conserved sequence, that is essential for its function as a general and potent protein disulfide oxidoreductase [61]. The protein can exist in two form: reduced, with the two conserved and catalytic cysteines with a free thiol-group; and oxidized, with a disulfide bond between the two cysteines.

Trx participates to the reversible oxidation of various substrates, such as ribonucleotide reductase (RR) [5], and several transcription factors including p53, and NF- $\kappa$ B. Furthermore, reduced Trx prevents apoptosis via inhibitory binding to apoptosis signal-regulating kinase 1 (ASK-1), whereas this binding is lost when Trx is oxidized [68]. Also the TST is a substrate of Trx, that is important to maintaining in the reduced state the catalytic cysteine, so preserving its enzymatic activity [75].

The reduction of the disulfide bond can be followed by a simple

spectrophotometric technique by recording the oxidation of NADPH. Insulin is often used as Trx oxidant, in presence of thioredoxin reductase and the NADP<sup>+</sup> formation is followed at 340 nm.

### ***Thioredoxin Reductase***

Thioredoxin reductases (EC Number: 1.8.1.9), or Trd, or TrxR are enzyme belonging to the flavoprotein family of pyridine nucleotide-disulfide-reductase which includes lipoamide dehydrogenase, glutathione reductase and mercuric ion reductase. Members of this family are homodimeric proteins in which each monomer includes an FAD prosthetic group, an NADPH binding site and an active site containing a redox-active disulfide. The electron are transferred from NADPH via FAD to the active-site disulfide of Trd, which then reduces the substrate (Figure 4-A) [53].

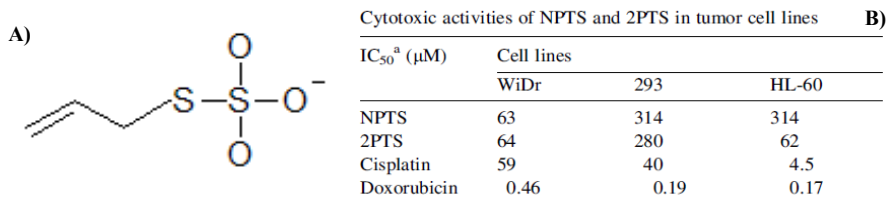
TrxRs are the only enzymes to be able to reduce the thioredoxin (Trx); is found in all know kingdoms of organism, but two types of TrxRs have evolved independently: in prokaryotes, bacteria, and some plant have one type; eukaryotes, and other plants have the other one, with a characteristic modified cysteine in the active site, a seleno-cysteine (Se-Cys). The first type containing a single domain, as showed in Fig. 4-B, containing the FAD and the NADPH domain and the conserved sequence with -C-X-Y-C- in the active site, within the FAD domain. The second type have only 31% sequence identity with prokaryotic TrxRs, but have 44% identity with eukaryotic and prokaryotic glutathione reductases [53], and have two domains with similar folding. The catalytic site of human Trd, -C-V-N-V-G-

C-. is also found in human glutathione reductase and is located in the FAD domain of the enzyme. Two TrxRs are identified in humans, TrxR1 and TrxR2. The first have a molecular mass average of 54 kDa, and works shows the presence of a particular sequence in C-terminal, -Gly-Cys-SeCys-Gly- (where the SeCys is a selenocysteine), thus the TrxR1 is a selenoprotein. The TrxR2 has a predict molecular mass of 56.2 kDa and 84% aminoacid sequence similarity with the TrxR1 protein; in addition TrxR2 has a 33-amino-acid N-terminal extension identified as a mitochondrial import sequence, in fact the TrxR2 is located in the mitochondria (Figure 4-B) [53].

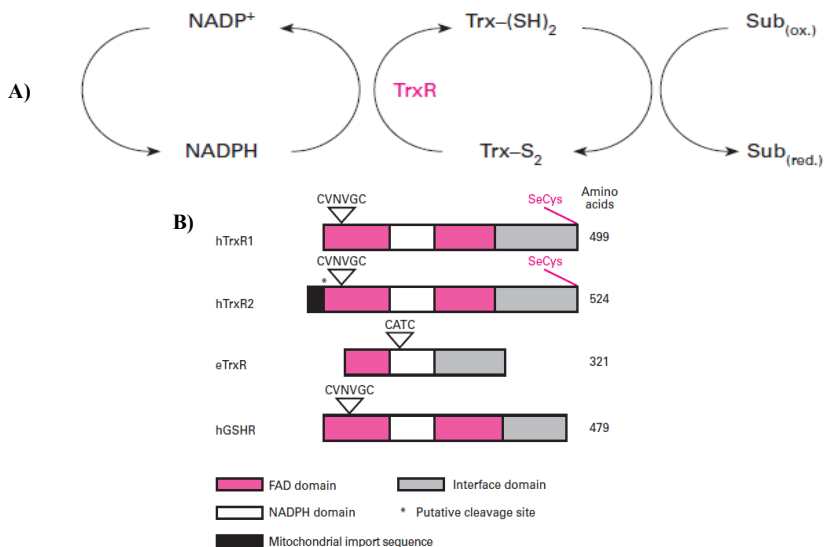
In human TrxRs the presence of SeCys is essential for its activity, but is not part of the conserved active site. It is not still well clear what are the changes that occur for the presence of the Se-Cys, but the conversion of Se-Cys in Cys causes an alteration of the activity of the Trd [53].

The activity of this enzyme is essential for the cell growth and vitality, and its activity and expression is up-regulated in several cancer. Trd influence cell proliferation, cell cycle regulation and apoptosis by regulating caspase-3 and BAX, blocking TNF- $\alpha$  [53].

So, the inhibition of its activity can be a good target for an anti-tumor therapy [7]. In fact, the use of a specific Trd inhibitor, such as motexafin gadolinium, causes the induction of the apoptosis selectively in malignant cell [7].



**Figure 3.** A) Structure of 2-propenyl thiosulfate; B) IC<sub>50</sub> values of 2-PTS and N-PTS on different cell lines. [16].



**Figure 4.** A) Representation catalytic mechanism of TrxR; B) Domain structure of human TrxR1 (hTrxR1), hTrxR2, *E. coli* hTrxR (eTrxR). The catalytic-site sequences are indicated with triangles, and the position of the C-terminal penultimate SeCys residue is shown for two human TrxR. [53].

## ***Glutathione-S-transferase***

Glutathione-S-Transferases (EC Number: 2.5.1.18), GSTs, is a family of different isoforms present ubiquitously in all the organism. They are present in the cytoplasm and in the mitochondria. At present, eight distinct classes of this enzyme is know :  $\alpha$ ,  $\kappa$ ,  $\mu$ ,  $\omega$ ,  $\sigma$ ,  $\pi$ ,  $\theta$ ,  $\zeta$ . In human and in rodents, the soluble GSTs are collectively expressed in rather large amounts, constituting as much as 4% of total soluble proteins in the liver. Although no definitive physiological role for GSTs has still been identified, several reactive endogenous molecules, including  $\alpha\beta$ -unsaturated keto-prostaglandine, and endogenous fatty acid oxidation products, such as 4-hydroxy-2-nonenal, serve as substrates for certain GSTs [27].

GSTs a phase II detoxification enzymes, and its characteristic reaction is the conjugation of reduced glutathione with a xenobiotic, to enhance their water solubility and excretion. In the last years, a large number of substrates for GSTs have been identified. Many epoxide carcinogens are detoxified by GSTs, and differences in expression of specific isoform can be an important determinant of target organ and species sensitivity. For example, mice are remarkably resistant to the hepatocarcinogenic effects of aflatoxin B<sub>1</sub> (AFB), because they constitutively express mGSTA3-3 which has high activity towards the reactive AFB-*exo*-epoxide [27]. The carcinogenic epoxide of benzo(a)pyrene (BPDE) is efficiently detoxified by hGSTP1-1 [27]. One important role of GSTs is in detoxification of endogenous products of lipids peroxidation such as 4-hydroxy-2-nonenal. Human GSTA4-4 has unusually high activity toward this substrate, and may play an important physiological role in protecting against oxidative stress induced by endogenous lipid

peroxides [27].

A number of GSTs inhibitors have been reported that are suitable for *in vitro* studies. These include ethacrynic acid, the antibiotic calvatic acid, curcumin, haloenol lactone, disulfiram and organotin compounds, to name just a few [27]. Generally, GST inhibitors relatively non-toxic, isoenzyme specific, and active *in vivo* have not yet been developed. Despite a considerable effort, so far the only *in vivo* active inhibitors of GSTs are ethacrynic acid and a number of glutathione derived structures [27].

The regulation of cytosolic GSTs is subject to a complex set of endogenous and exogenous parameters. These include developmental, sex and tissue specific factors, as well as a large number of xenobiotic inducing agents, such as polycyclic aromatic hydrocarbons, phenolic antioxidants, reactive oxygens species, isothiocyanates, trivalent arsenicals, barbiturates and synthetic glucocorticoids [27]. Most of mechanistic studies on transcriptional regulation of GSTs have utilized rodents.

Induction of GSTs by xenobiotics is mediated by several different transcriptional mechanisms. The rat *GSTA2* gene contains a glucocorticoids-response element (GRE), a xenobiotic-response element (XRE), and an antioxidant-response elements (ARE) [27]. The GRE mediates induction via glucocorticoids such as demethasone, and the XRE mediates induction by planar aromatic hydrocarbons such as 3-methyl-cholanthrene and TCDD (dioxin). The ARE is responsive to phenolic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and also mediates induction, at least in part, by natural and synthetic dithiolthione compounds such as oltipraz [27].

Because of the ability of many naturally-occurring plant products

(phytochemicals) to induce GSTs, there has been considerable interest and research in the role of dietary GST induction as a mechanistic explanation for the anticarcinogenic effect of fruits and vegetables [27]. There is substantial experimental animal evidence demonstrating that GST induction can reduce the effectiveness and potency of a variety of chemical carcinogens.

In many cancer GSTs are up-regulated, and the inhibition of their activity is a novel approach to increment the drug-sensibility of the cell; in fact, reducing the ousting of the drug, is possible to reduce the quantity to obtain the same effect. GSTs also plays an important role in the cancer ethiology and resistance to chemotherapy; in many human cells, GST $\pi$  is found to be associated with the Jun N-terminal kinase (JNK), inhibiting its activity [27]. If GSTs expression is upregulated in cancer cells, the activity of JNK can be reduced, incrementing the drug-sensitivity to induce the cell death. Thus, the discover of potent GSTs inhibitors can help to make specific strategies to cure several cancer type.

## ***Glutathione***

Glutathione (L- $\gamma$ -glutamyl-L-cysteine-glycine) is present at high concentration in most of living cells, from microorganisms to humans. Its biological significance is mainly related to the free sulfhydryl of the cysteine residue, which confers unique redox ( $E_0' = -0,24$  , for thiol-disulfide exchange) and nucleophilic properties. The GSH is the most abundant oxidant scavenger in the cell. It can exist in two interconvertible stable

states: reduced (GSH) and oxidized (GSSG). In some cells GSH can reach 5 mM concentration, and in the basal state glutathione is almost reduced. In the reduced state the thiol group of cysteine can donate a reducing equivalent ( $H^+ + e^-$ ) to other molecules, such as reactive species (ROS, NOS), becoming reactive itself, but readily reacts with another reduced glutathione, to form glutathione disulfide (GSSG) [67].

GSH is the main sulfur compound in eukaryotes and in living tissues, GSH assumes a pivotal role in bioreductive reactions, transport, sulfur metabolism enzyme activity, protection against harmful oxidative species, and detoxification of xenobiotics. In presence of xenobiotics, glutathione-S-transferase catalyzes the formation of GSH conjugates using the reduced glutathione. Instead, when cells are exposed to oxidative stress, GSH neutralize the radicals and the oxidant, oxidizing itself; so, the intracellular concentration of reduced glutathione decrease, while the oxidized glutathione increase. The restoration of the GSH pool was performed by the reduction of GSSG into GSH, catalyzed by glutathione reductase, consuming NADPH [67].

The ratio of reduced over oxidized glutathione is a sensitive parameter to the redox state and is associated with cell survival via redox modulation of molecular factors, such as activating protein 1 (AP-1), NF- $\kappa$ B. GSH is an essential molecule in the cell and knock-out experiments in many organisms confirms this observation [67].



## ***OBJECTIVE OF RESEARCH***

The human rhodanese has a  $K_m$  for the thiosulfate of about 3.5 mM, while for the cyanide is about 9 mM [57]. The literature indicates that bovine TST has a  $K_m$  for the reduced thioredoxin about  $10^3$  times lower than cyanide. All these observations open to important considerations to the primary role of rhodanese, in fact, primary physiological substrates of rhodanese could be others and its main biological function could be yet discovered.

Recently, some authors highlights a different functions of rhodanese, beside to cyanide detoxification. In fact, the amount of cyanide introduced by diet does not reach very toxic levels in mammals, so the conserved presence of rhodanese in all the organism reveals that this enzyme have an essential role, and our work want to investigate on the new implication of this enzyme, such as the OSCs detoxification, and the relationships with other important cellular system, such as thioredoxin-thioredoxin reductase system. Moreover we have investigated the effect of the garlic derived OSC, 2-PTS, on the GST and GSH. Our results open on new interpretations of the OSCs effect on the cell; in fact, the reactivity of the compound derived from the reaction of GSH with 2-PTS suggests that the OSCs toxicity could be related not only to a redox unbalance and a thioalkenylation of the proteic cysteines, but also to a complex network of interactions. To elucidate this network, we have studied the direct effect of 2-PTS on the enzymes mentioned above, TST, Trx, Trd, GST, and GSH. The correlation of the molecular data with the cellular data is also investigated to verify the observed effects in the cell culture. In the end, the effect of the product by reaction of 2-PTS with GSH is studied. The results obtained give us many important advices on the mechanisms of action of OSCs in the cells and on the molecular interconnections among important enzymes involved in the

cell detoxification system and in the cell redox system.

## ***RESULTS***

## ***2-PTS SYNTHESIS AND CHARACTERIZATION***

The 2-PTS is synthesized as described by Chapelet et al. [18], and as described in the experimental procedures.

The product purity is analyzed by LC-MS (Figure 5-B, C) giving about a 5% of product contamination, and a m/z of 153 relative to the 2-PTS without the Na<sup>+</sup>. Figure 5-D shows the chemical shift of H of 2-PTS: CH<sub>2</sub>= (5.07; 5.22 ppm), =CH- (6.02 ppm), -CH<sub>2</sub>- (3.71 ppm).

## ***EFFECT OF 2-PTS ON VIABILITY OF HUT 78 CELL LINE***

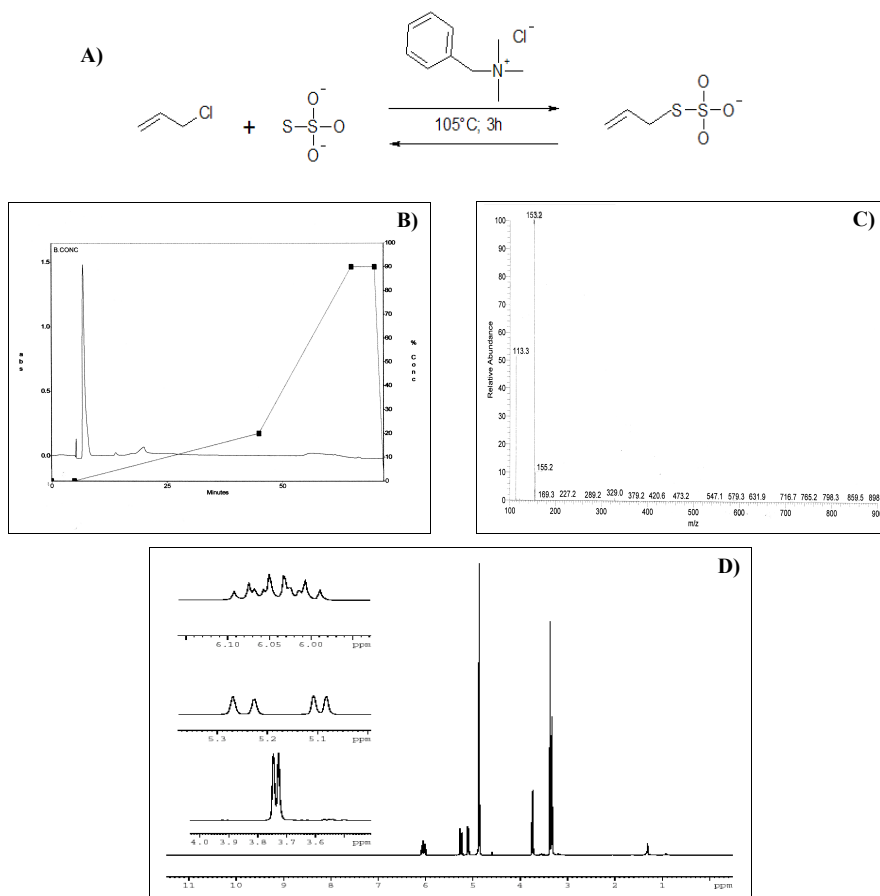
For the cellular experiments we have used a leukemic cell line, because historically, the garlic is used as a popular remedy for the leukemias, and many other works use this model system to study.

The cutaneous lymphoblastoma cell line (HuT 78) derived from the peripheral blood of a patient with Sezary's syndrome. It has the characteristic of a mature T cell with inducer/helper phenotype and release IL-2.

Literature [14, 19, 31, 34] indicates that two critic events causes the transformation of normal T-cells in malignant HuT 78: a chromosomal t(2;8) translocation, and a homozygous point mutation in codon 196 of p53. The first event causes a fusion between the proto-oncogene *c-myc* with the TCL4 gene (T-cell leukemia/lymphoma 4), resulting a fusion transcript with an half-life prolonged. The *c-myc* transcript is a transcription factor and regulate

about 15% of all the genes [31], through binding on Enhancer Box sequences and recruiting histone acyltransferases (HATs). It plays an important role in the control of cell proliferation (up-regulates cyclins, down-regulates p21), and also apoptosis (up-regulates Bcl-2). The second event, causes a conversion of CGA (arginine 196) at the end of exon 3 to TGA (stop codon), giving rise a truncated protein in the DNA-Binding Domain (DBD), with a loss of function of the gene regulator *p53* [19]. The transcription factor *p53* regulates the cell cycle, and acts as tumor suppressor. It is involved in the DNA repair, cell cycle progression, genetic stability and apoptosis. *p53* mutation is probably a common event in primary leukemic T-cell sample. In fact, the loss of normal function of the tumor suppressor gene seems to be critical in the generation of tumorigenic leukemic T cells [19].

Our results indicates that proliferation of HuT 78 cells is blocked in time-dependent manner. Trypan-blue exclusion assay shows the after 24 and 48 hours of treatment about 75% and 10% of cells are viable (Fig. 6-A, B). Flow cytometric analysis (Fig. 6-C) of HuT 78 cells treated with 0.5 mM of 2-PTS, with propidium iodide staining, reveals a dysregulation of the cell cycle, with a G<sub>2</sub>/M phase blockage after 24 hours of treatment (Fig. 6-C), with about 25% of subG<sub>1</sub> respect to the total population, reaching about 39% of value after 48 hours of treatment (Fig. 6-D, E). The G<sub>2</sub>/M phase blockage is characteristic of DNA damage in cells that have inactivating mutation in *p53*.



**Figure 5.** A) Scheme of 2-PTS synthesis [18]; B) RP-HPLC analysis of 2-PTS; C) LC-mass analysis in negative detection; D)  $^1\text{H-NMR}$  of 0.5 mg of 2-PTS dissolved in 200  $\mu\text{L}$   $\text{CD}_3\text{OH}$ , analyzed at 400 MHz spectrometer.

## ***REDOX UNBALANCE AND MONODIMENSIONAL NMR ASSAYS***

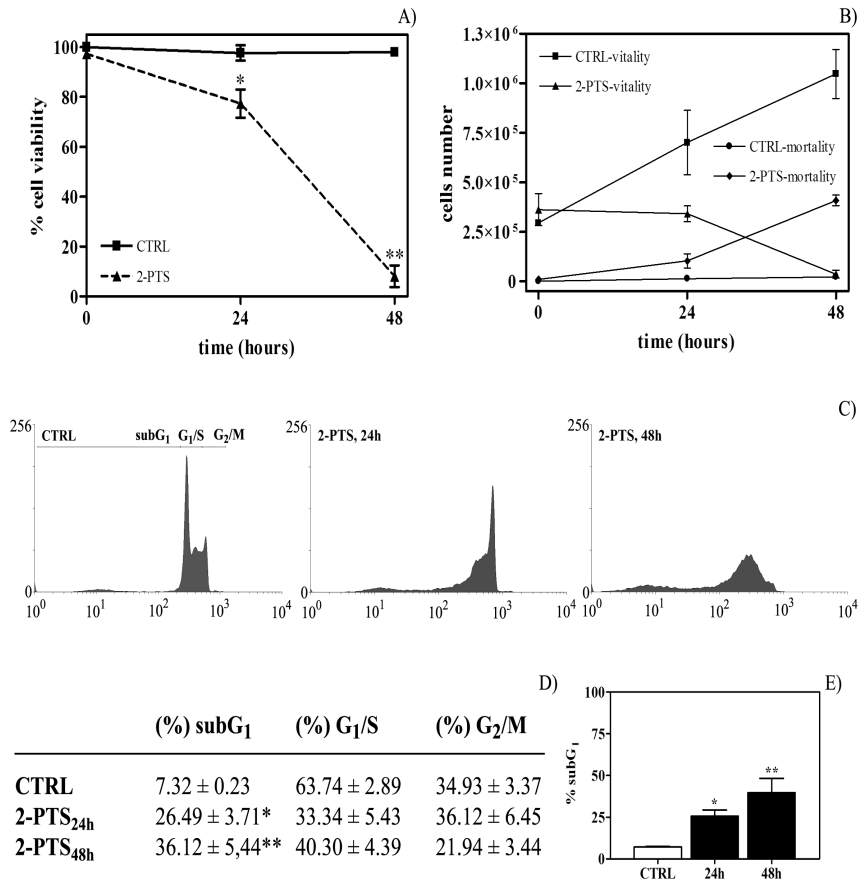
To elucidate the mechanism of action of these compounds, we have treated the cell culture with 2,7-dichlorofluorescein diacetate. FACS analysis shows the increment of fluorescence intensity of DCF due to ROS production, still after 1 hour of treatment (Figure 7-A). These results indicate that the ROS production is the early cause of the induction of cell death by 2-PTS. To verify the intracellular redox imbalance we have quantified the intracellular reduced and oxidized glutathione. Untreated cells have a concentration of about 55 nmol/mg of GSH, but 0.5 mM 2-PTS treated cells quickly increase their GSH content up to 118.5 nmol/mg (Figure 7-B). The intracellular GSSG did not alter with the same treatment (about 2 nmol/mg).

Monodimensional nuclear magnetic experiments indicate a loss of mitochondrial function. The progressive formation of mobile lipids in intact cells indicates the induction of apoptosis after treatment with 2-PTS. In fact, the time and dose dependence arising of the signals relative to the mobile lipids (-CH<sub>3</sub> at 0.89 ppm, -(CH<sub>2</sub>)<sub>n</sub>- at 1.29 ppm, -CH=CH- at 5.2 ppm), and the decreasing of the signal of the taurine (tau; 3.45 ppm), total choline containing metabolites (tCho; 3.2 ppm), and lactate (lac; 1.30 ppm) are characteristic in the HuT 78 cell line of apoptosis induction with mitochondrial dysfunction, and subsequent accumulation of long fatty acid acyl chain in triglycerid in cytoplasmic lipid bodies, induced by 2-PTS (Figure 8). Quantitative analysis of mobile lipids spectral profiles is obtained by measuring the peak area (a) ratio  $R_{chains} = a[(CH_2)_n]_{lip} / a[CH_3]_{tot}$ , as

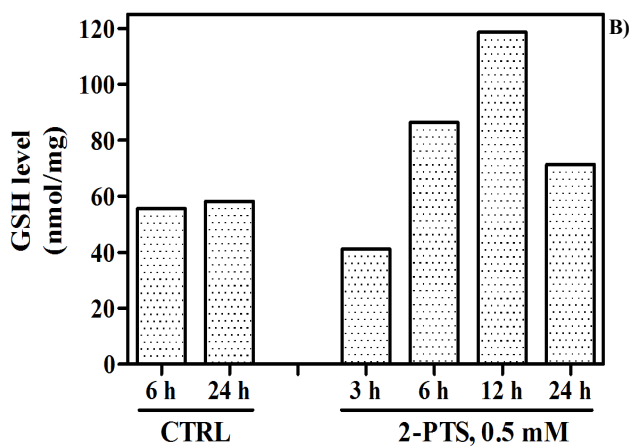
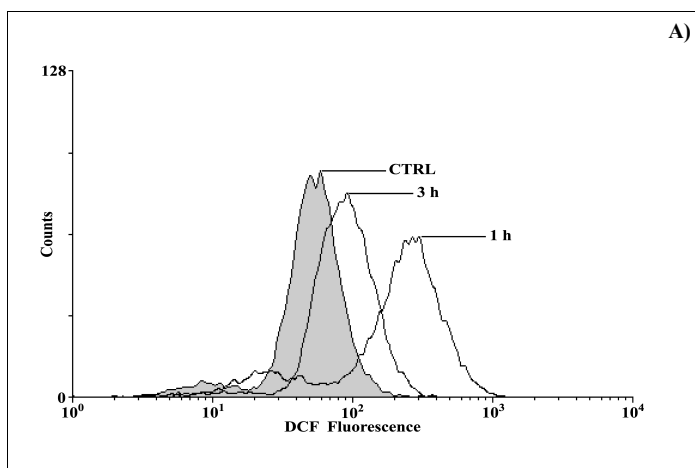


described by Iorio et al. [41], where the  $a[(CH_2)_n]_{lip}$  is the integral of the mobile lipids,  $(CH_2)_n$  is the resonance and  $a[CH_3]_{tot}$  is the integral of the total  $CH_3$  resonance at 0.9 ppm. The value  $R_{chains}$  does not change ( $0.20 \pm 0.1$ ), compared to untreated control cells and treated cells with 0.25 mM of 2-PTS at various time. When cells is treated with major amount of 2-PTS, 0.5 and 1 mM, the average of  $R_{chains}$  increase until 1.25 and 1.56, respectively, after 24 hours of treatment. After 48 hours of treatment  $R_{chains}$  still increase, reaching  $4.45 \pm 1.1$  value; the small aqueous metabolite decreased to very low levels as a result of the massive apoptosis induction probably associated with a loss of cell integrity. Quantitative analysis on  $^1H$ -NMR spectra of cell extract exposure to 0.5 mM 2-PTS for an intermediate time interval (24 h, not yet associated with late apoptosis) showed that the intracellular level of taurine (measured from the triplet centered at  $\delta = 3.45$  ppm) decreased about two-fold from a basal control of  $13.0 \pm 2.4$ , to  $6.5 \pm 1.0$ , nmol per  $10^6$  cells). Under the same experimental conditions, individual choline containing metabolites (tCho, detected under a resonance band centered at a chemical shift of 3.22 ppm) underwent differential changes; in fact, phosphocholine (Pcho) decreased by 45% (from  $15.1 \pm 1.4$ , to  $8.1 \pm 0.8$  nmol per  $10^6$  cells), while free choline and glycerophosphocholine both increased by about three-fold. The changes in the levels of water soluble choline-containing metabolites observed in cells treated with 2-PTS for 24 h probably reflect the progressive activation of phospholipases and phosphodiesterases. On the other hand, a complex network of pathways may, in principle, contribute to the measured decrease in Pcho, a metabolite particularly sensitive to conditions determining a block in cell proliferation and/or to the activation of enzyme involved in choline-phospholipid degradation. In fact, both

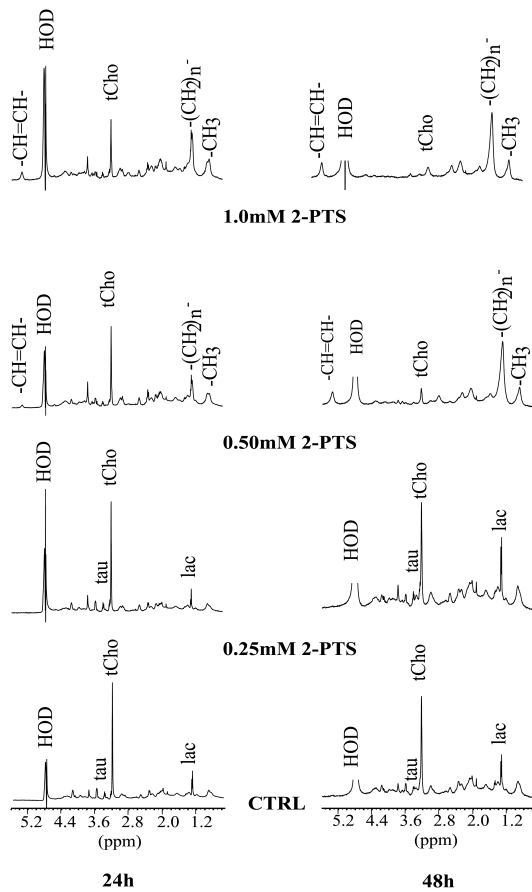
increases and decrease in Pcho have been reported to occur in different system of apoptotic induction, according to particular experimental conditions [41].



**Figure 6.** Effect of 0.5 mM on the HuT 78 cells. A) cell viability; B) cell vitality and mortality; C) FACS analysis; D) statistic analysis of FACS experiments; E) increment of subG<sub>1</sub> population after treatment.



**Figure 7.** A) DCF fluorescence of HuT 78 treated cells after 1 and 3 hours; B) intracellular GSH levels of treated HuT 78 cells at various time.



**Figure 8.**  $^1\text{H-NMR}$  of intact Hut 78 cells exposed at various concentrations of 2-PTS analyzed at 24 and 48 hours.

## ***2-PTS AND RHODANESE***

To investigate on the involvement of the mitochondria in this process, we have monitored the expression and the activity of some enzymes and peptide involved in the maintaining of the redox state and in the detoxification system, such as Trx, Trd, TST, GST, and glutathione.

A decrease of the TST activity is observed in the cell lysates treated with 2-PTS (Fig. 9-B). After 24 hours there is a significant decrease (about 25%); after 48 hours the residue TST activity is about 30%, while the expression of the TST does not reveal alteration (Fig. 9-A), thus we can think that the alteration of the activity could be due to a direct inhibition of the rhodanese activity by 2-PTS.

To verify the effective direct interaction of 2-PTS with rhodanese, we have conducted fluorescence experiments. Figure 10-A, shows the changing of the intrinsic fluorescence intensity of RhdA when interchange between the sulfur-free form (E) and the sulfur-loaded form (ES) by addition of cyanide or thiosulfate, respectively. The changing in the fluorescence intensity is a fast analysis to monitoring both the presence or the absence of the sulfur linked to the catalytic cysteine [30].

As we see in the Figure 10-A, when the enzyme is in the ES form have a characteristic fluorescence profile, that undergo to an increasing of its intensity by addition of the cyanide; the further addition of 2-PTS does not lead changes of fluorescence intensity. By contrast, we can observe a quenching effect of 2-PTS in dose-dependent manner, starting to the E form of RhdA (Figure 10-B). This effect could be related to a specific interaction of 2-PTS in the active site of the rhodanese. Figure 10-B, show also the

major quenching effect of 2-PTS respect to the thiosulfate ( $\Delta F\%_{\text{thiosulfate}} = 15,7\%$ ;  $\Delta F\%_{2\text{-PTS}} = 27,2\%$ ), probably due to the presence of the allyl group that could interact with some Trp residues close to the active site of the enzyme (Fig. 2-B). Moreover, the addition of the cyanide after the 2-PTS does not restore the E form of the enzyme, differently to the case of the thiosulfate (Fig. 10-C).

The preliminary data obtained with the fluorescence have been implemented with enzymatic studies, that indicate the capacity of 2-PTS to inhibits the TST activity of RhdA in dose- and time-dependence manner (Figure 11-A, B). The figure 10-A shows the complete inhibition of TST activity after 100 minutes of direct incubation of RhdA with 2-PTS. The following experiments (Figure 11-B) show a typical behavior of a competitive inhibition of 2-PTS with the classic substrate of rhodanese, thiosulfate. The Lineweaver-Burk linearization consents to extrapolate an apparent  $K_i$  of 2-PTS of  $3.31 \pm 0.71$  mM, in the thiosulfate:cyanide sulfurtransferase activity.

To deeply understand the inhibition we have purified by dialysis the RhdA after 1,30 hours of incubation with 2-PTS and KCN at 37°C, and the dialysis does not restore the active form of the RhdA. By contrast, the incubation of RhdA with 2-PTS without the KCN (ES-form) does not leads to an inhibition of TS activity.

TST activity of 2-PTS inhibited form (RhdA-PS) can be restored by presence of DTT (Figure 12-A) for 30 minutes at room temperature. In fact, the presence of a strong reducing reactive agent restore the 53,6% of the TST activity, compared with the ES-form before the treatment.

The dialyzed RhdA (E-form) and RhdA-PS has been analyzed by LC-

MS, and the data obtained shows a  $m/z$  of 31063.8 for the E-form of RhdA, and a  $m/z$  of 31138.9 for the RhdA inhibited with the 2-PTS. These results point out that the inhibition on the RhdA is due to an thioalkenylation of the unique catalytic cysteine (230) by propenyl sulfide ( $m/z$  73).

The TST activity can be restore also by presence of Trx by direct dependence of its concentration (Fig.12-B). The ratio between RhdA-PS and Trx 1 : 2 leads to about 66% of TST activity after 2 hours at room temperature of direct incubation.

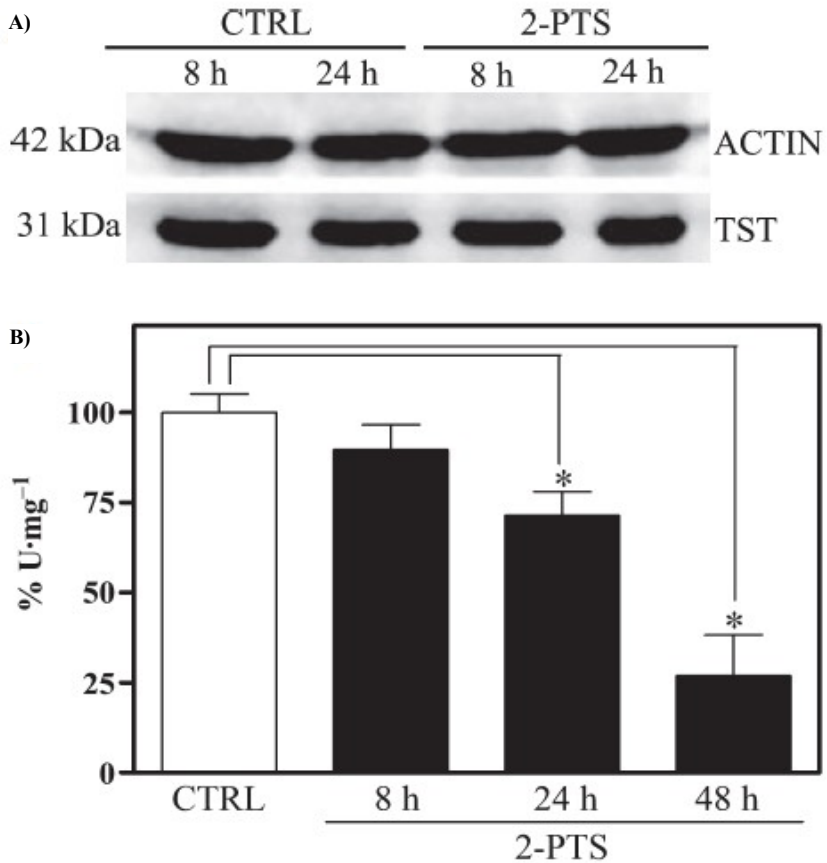
The recovery of the thiosulfate:cyanide sulfurtransferase activity of RhdA-PS is faster and complete if in the solution is present Trd and NADPH. In fact, 100% recovery of TST activity has been obtained within 70 minutes and with a minor concentration of Trx was required (0.5  $\mu$ M), as showed in Fig. 12-C. All these results indicates the ability of 2-PTS to oxidase the cysteines.

Further experiments are performed to characterize the RhdA-PS form. Limited proteolysis of globular proteins generally occurs at sites that contain the most flexible regions of the polypeptide chain within a domain or at the flexible hinges between two domains. Therefore, a limited trypsin digestion of RhdA-PS was performed to investigate the flexibility of this modified enzyme. As previously observed, the RhdA(ES) [75, and citations within] appeared to be quite resistant to limited proteolysis. In fact, RhdA(ES), which was treated in the same manner as for RhdA-PS, but in the absence of cyanide and 2-PTS, was not proteolyzed by trypsin and remained intact, even after incubation overnight (Fig. 13-A). By contrast, RhdA-PS showed a higher sensitivity to proteolysis than RhdA(ES), as shown in Fig. 13-B, and *a* band rapid digestion was observed. A stable daughter band (*b* band), of

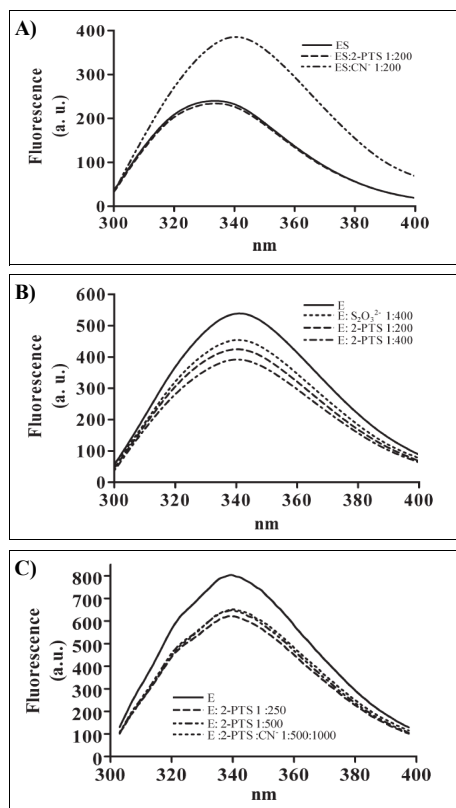


about 17.3 kDa, appeared after a few minutes of proteolysis and remains also after many hours of digestion. These data suggest that RhdA-PS is more flexible than RhdA(ES), probably as a result of local conformational differences.

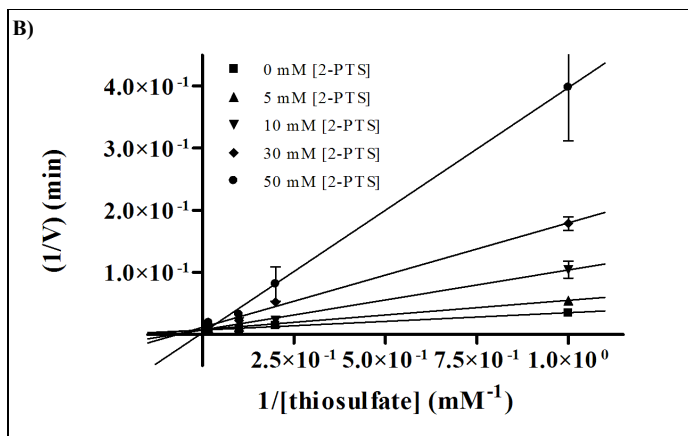
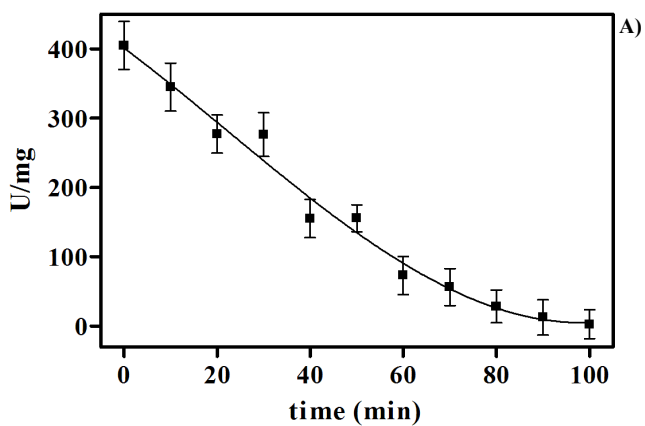
RhdA-PS showed behavior very similar to that previously observed for the alkylated and oxidated forms of the bovine rhodanese [75, and citations within]. In fact, limited proteolysis of the alkylated and oxidized forms yielded fragments that were about half of the apparent molecular mass of the protein, as a result of cleavage at the interdomain tether that connects the two domains into which the single polypeptide chain protein is folded [75, and citations within]. Thus, the inactivation of the rhodanese by 2-PTS induces local conformational changes that make the enzyme much more sensitive to proteolytic degradation.



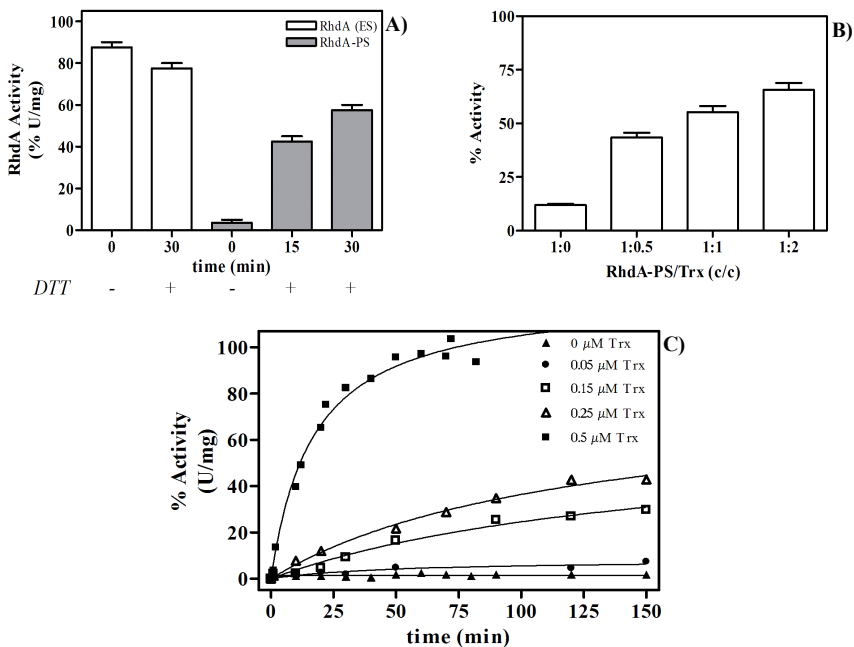
**Figure 9.** TST activity (A) and expression (B) in HuT 78 cell lysates treated with 0.5 mM of 2-PTS, at various times. A) The WB analysis shows that TST expression does not change; B) TST activity in cell lysates indicates a decreasing after treatment with 0.5 mM 2-PTS. CTRL (control) was taken as 100% of TST activity. TST activity was detected using the Sörbo assay.



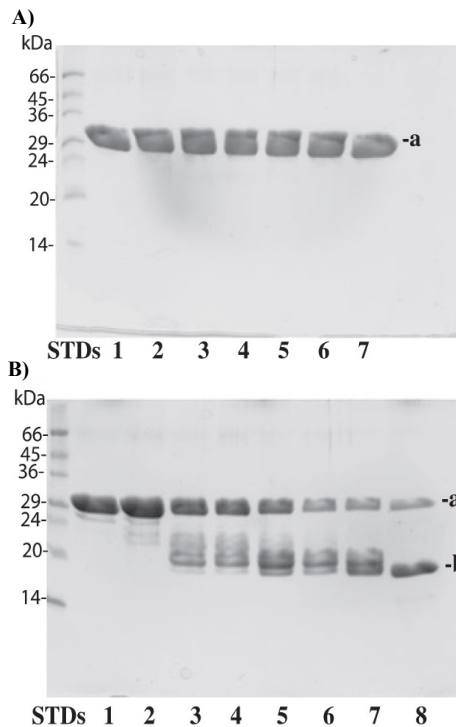
**Figure 10.** Monitoring of fluorescence intensity changing of *RhdA*. A) *RhdA*(ES) 2.3  $\mu\text{M}$  (continuous line), after the addition of 460  $\mu\text{M}$  2-PTS (dashed line), and 460  $\mu\text{M}$  CN<sup>-</sup> (dotted line); B) *RhdA* (E) 4.6  $\mu\text{M}$  (continuous line), after the addition of thiosulfate (E : thiosulfate; 1 : 200 c/c) (dashed line), 2-PTS (E : 2-PTS; 1 : 200 c/c) (dotted line) and 2-PTS (E : 2-PTS; 1 : 400 c/c) (dash-dotted line); C) *RhdA* (E) 5  $\mu\text{M}$  (continuous line) in presence of 2-PTS (E : 2-PTS; 1 : 250 c/c) (dotted line), 2-PTS (E : 2-PTS; 1 : 500 c/c) (dash-dotted line) and after the addition of CN<sup>-</sup> (E : 2-PTS : CN<sup>-</sup>; 1 : 500 : 1000c/c)(dotted line). a.u.: arbitrary units.



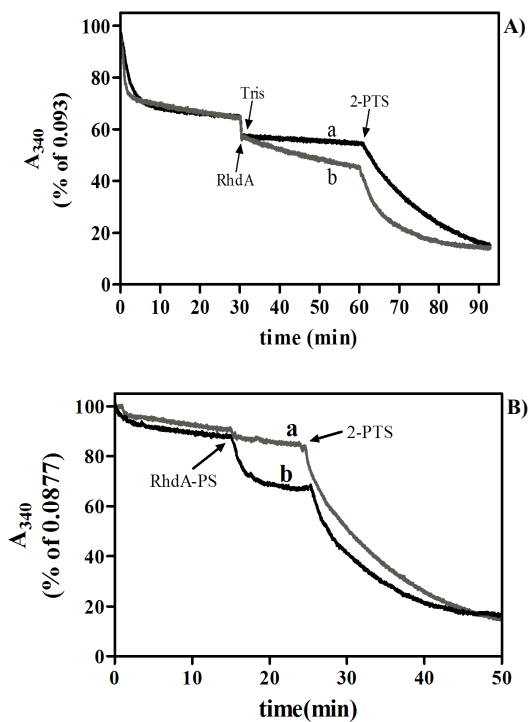
**Figure 11.** A) Time dependence inhibition of TST activity of RhdA (8.9  $\mu\text{M}$ ) by incubation with 2-PTS 4 mM, in Tris-HCl 50 mM (pH 8.0), NaCl 0,3 M, and KCN 15 mM. B) Lineweaver-Burk linearization of RhdA inhibition by 2-PTS.



**Figure 12.** A) Effects of DTT on the TST activity of RhdA(ES) and RhdA-PS. The proteins were incubated with 4 mM DTT at room temperature and the TST activity was assayed after 0, 15 and 30 min. RhdA(ES) and RhdA-PS were treated identically, except that KCN and 2-PTS were absent during the treatment of RhdA(ES). The TST activity of the enzyme before treatment was taken to represent 100% activity; B) Recovery of the TST activity of 17  $\mu$ M RhdA-PS detected using the Sörbo assay. Recovery of TST activity after 2 h of incubation at 25°C in the absence and in the presence of thioredoxin at molar ratios 1 : 0.5, 1 : 1 and 1 : 2 c/c RhdA-PS/Trx. All values are expressed as a percentage of the TST activity value of RhdA(ES); C) Recovery of the TST activity of 8.1  $\mu$ M RhdA-PS after incubation in the presence of 0.1 U Trd, 50  $\mu$ M NADPH and different concentrations of Trx (0, 0.05, 0.15, 0.25 and 0.5  $\mu$ M).



**Figure 13.** Time course of trypsin digestion of *Rhda-PS* and *Rhda(ES)*. Three-hundred micrograms of enzyme was subjected to limited digestion with 1% (w/w) trypsin in 1 mL of 50 mM Tris-HCl buffer, pH 8, at room temperature. After the reaction the samples were subjected to SDS-PAGE. A) Lanes 2-7, proteolysis products of *Rhda(ES)* (lane 1) at 0, 5, 10, 15, 20 and 30 min and overnight incubation; B) Lanes 2-8, proteolysis products of *Rhda-PS* (lane 1) at 0, 5, 10, 15, 20, 30, 60 min and overnight incubation, respectively. 'a' and 'b' bands are the parent and daughter bands, at about 29.7 and 17.3 kDa, respectively. Molecular markers are on the left. STDs, molecular mass standards.



**Figure 14.** Trx oxidation by 2-PTS, in the presence and in the absence of rhodanese, by measurement of NADPH (50  $\mu$ M) consumption (absorbance at 340 nm), in 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, with 4  $\mu$ M Trx, 0.1 U Trd and in the presence of 0.5 mM 2-PTS. (A) Tris-HCl buffer (a) or 4  $\mu$ M RhdA(ES) (b) was added to the solution after 30 min at 37°C, and, after stabilization, 2-PTS was added. The data were normalized against an A<sub>340</sub> of 0.093 (which represents 100%). (B) Tris-HCl buffer (a) or 4  $\mu$ M RhdA-PS (b) was added to the solution after 15 min of incubation at 37°C and, after stabilization (about 10 min), 2-PTS was added. The data were normalized against an A<sub>340</sub> of 0.085 (which represents 100%).

## ***2-PTS AND THIOREDOXIN, AND THIOREDOXIN REDUCTASE***

As showed before and in other articles [56, 57], the Trx can interact strongly with rhodanese, in fact, the  $K_M$  of Trx is about 1000 times lower than the  $K_M$  of the cyanide for the bovine rhodanese. To clarify the role of the Trx in this mechanism of oxidation, we have performed experiments where the Trx oxidation was monitored, by the absorbance decrease of NADPH at 340 nm.

As observed in other articles [56, 57] RhdA can act like Trx-oxidase. RhdA can oxidase the Trx and the addition of the 2-PTS causes a further oxidation of the Trx, leading a decrease of the NADPH absorbance (Fig. 14-A). Moreover the presence of RhdA make the Trx oxidation more rapid, than only in presence of 2-PTS (Fig. 14-A, line *a* and *b*). If RhdA-PS is used, the Trx oxidation was more faster and also the ability of the 2-PTS to oxidize the Trx is marked.

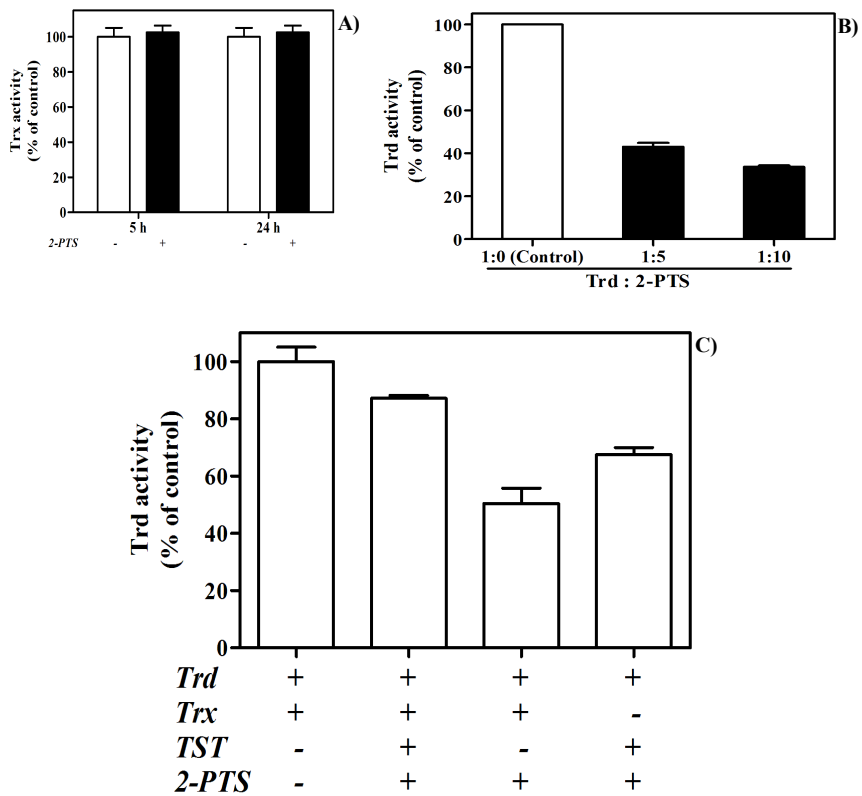
To complete the global picture, the direct incubation of 2-PTS with Trx and Trd is conducted. The Trx is not inhibited by 2-PTS after 5 and 24 hours of incubation at room temperature (Fig. 15-A). Instead, a very marked inhibition is observed in the case of Trd and after 15 minutes of direct incubation of 2-PTS with Trd at very low concentration, 100 and 200 nM (Trd : 2-PTS = 1 : 5, and 1 : 10, respectively) induces a dramatic decrease of Trd activity, with a residual activity of 45% and 35%, respectively (Fig. 15-B). Major concentration of 2-PTS cannot be evaluated because the oxidizing effect of 2-PTS on the Trx is more evident. The inhibition of the Trd activity can be due to a similar thioalkenylation of their catalytic cysteines; in fact,



Trd from *E.coli* used for these experiments contains two redox cysteines in its active site, that could be irreversibly oxidized by 2-PTS.

A closing experiment is conducted, where the ability of the global Trx-Trd-TST system to detoxify from 2-PTS is evaluated. Figure 15-C shows that 2-PTS induces a light inhibition of Trd activity about of 10%, if TST is present; in absence of TST or Trx, the percentage of Trd inhibition is major of about 50% and 25%, respectively.

All these results indicates that the Trx-Trd-TST system is not inhibited by 2-PTS, but can act as a detoxification system, neutralizing the oxidant effect of 2-PTS, and probably also for other OSCs.



**Figure 15.** A) Incubation of Trx 133  $\mu$ M with 2-PTS 0.67 mM (1:5 c/c), in 38 mM Hepes (pH 7.4), 17  $\mu$ M EDTA, for 5 h and 24 h at r.t.; B) Incubation of 2-PTS 100 nM and 200 nM with Trd 20 nM for 15 min in TE buffer at r.t.; C) Different incubations. 2-PTS 50  $\mu$ M, Trd 5  $\mu$ M, RhdA 5.8  $\mu$ M, Trx 6.3  $\mu$ M, NADPH 0.3 mM, in TE buffer. All the incubations was effectuated at r.t., and assayed after 30 minutes. NADPH and buffers only does not interfere with the assays.

## ***2-PTS AND GLUTATHIONE-S-TRANSFERASE***

GST role in the detoxification from the OSCs has been investigated. In fact, the GSTs are ubiquitously enzymes that catalyzes the nucleophilic attack of glutathione on the electrophilic center of a number of toxic compounds and xenobiotics. The cellular experiment seem suggest that GSTs expression and activity are not altered by 2-PTS. However, the molecular studies indicates that GSTs could be involved in the process of the detoxification of 2-PTS. The human GSTP1-1 is extensively studied for of the clinical interest in it as a potential marker during chemical carcinogenesis and the potential role in the mechanism of multidrug resistance.

First, we have monitored the expression and the activity of GSTP1-1 in the HuT 78 cell lysates, after 8 and 24 hours of treatment with 0.5 mM 2-PTS. As showed in Figure 16-A, GSTP1-1 expression does not have relevant changes. Also the activity of the GSTs in the cell lysates does not undergo to a evident variationsm and only a very little increment is observed after 24 hours of treatment(Fig. 16-B).

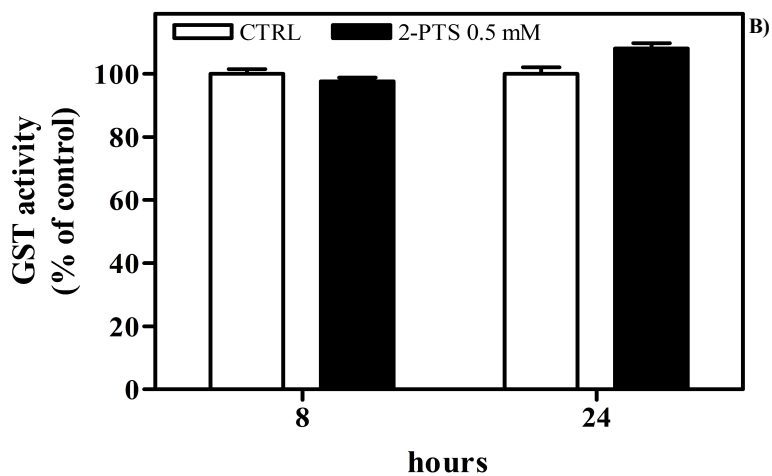
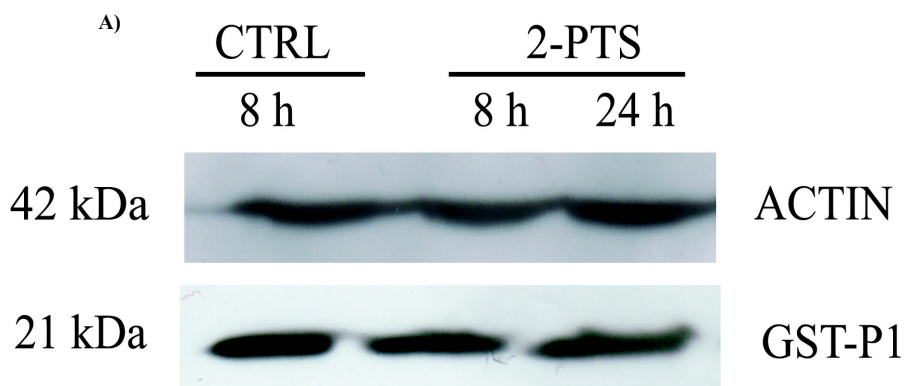
As described above, 2-PTS can interacts with the TST, Trd inducing an inhibition of their activity. Thus, we have incubated, for about 2 hours at room temperature, 0.15 mM GSTP1-1 with 30 mM 2-PTS (1 : 200 c/c). After, the protein has loaded on NAP-25 column in 100 mM phosphate buffer, to remove all the residue 2-PTS. After, their activity has evaluated, compared to the control protein treated in the same manner, but using ddH<sub>2</sub>O instead of 2-PTS, which was defined as 100% of activity. As showed in Fig. 17-A, the GSTP1-1 have a residual activity of about 10% after incubation with 2-PTS, probably due to a similar thioalkenylation of their catalytic

cysteines, similarly observed for TST. To confirm this hypothesis, we have incubated the inhibited 7.8  $\mu\text{M}$  GSTP1-1 (GST-PS) with increase of Trx concentrations (1 : 0.025, 1 : 0.05, 1: 0.1 c/c), in presence of 0.1 U of TrxR and 50  $\mu\text{M}$  NADPH. As we can see in Fig. 17-B, the restoring of GST-activity is time- and dose-dependent of Trx concentration. After 40 min of incubation at r.t. of GST-PS with 0.4  $\mu\text{M}$  of Trx, the activity of GST is restored until the 80%; this result lead us to investigate more deeply on the cysteines involved in the inhibition of GST-activity.

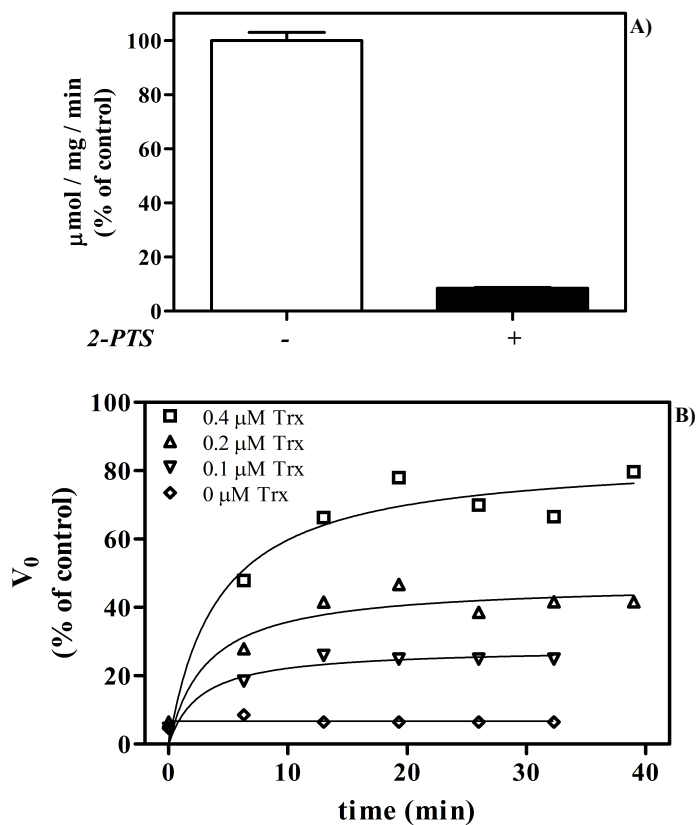
The GSTP1-1 has four cysteines in its primary structure, but only three are available to DTNB titration, Cys47, 101 and 169. The Cys169 is slowly titrated by DTNB, and Cys14 is deeply buried in the protein structure, and it is not titrated by DTNB [49]. Using UV spectroscopy and bromo-pyruvate (Br-pyr) it is possible to determine the oxidation state of the thiolic Cys47 and 101, as described by Lo Bello et al. [49]. The conversion of Cys47 and 101 into Ala, in fact, changes the UV spectra in presence of Br-pyr, which bond the cysteine in a thiolic state. The interaction of GSTP1-1 with 2-PTS seems to modify covalently the Cys (Fig. 17-A, B), giving rise to a similar form of Cys47-converted to Ala.

The mass spectroscopy analysis indicates that the GST-PS undergo to three different types of thioalkenylation. The LC-MS analysis indicates one  $m/z$  for the GSTP-1, of 23375.6 (MW theoretical = 23355.8), corresponding to the GSTP1-1 with a  $\text{Na}^+$  ion; however, the LC-MS analysis of GSTP1-1 treated with 2-PTS (GST-PS) shows, instead, three different  $m/z$ , 23440.5, 23505.4, 23579.6, corresponding to GSTP1-1 with one, two, and three thioalkenylation, respectively (MW theoretical = 23428.8, 23501.8, 23574.8, respectively), on three different cysteines of the protein.

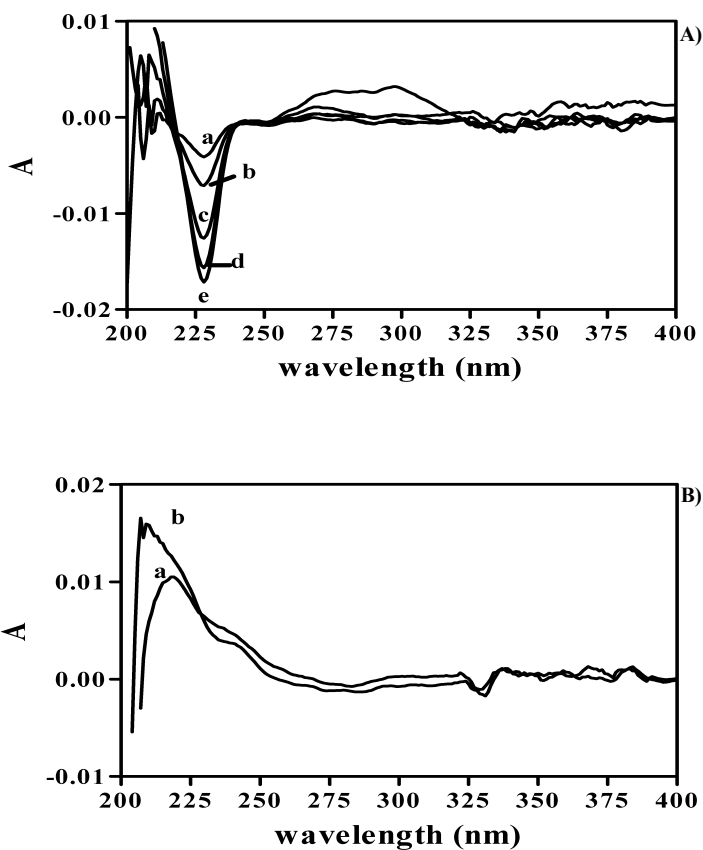
All these results indicates that the inhibition of GST is due to a thioalkenylation of the thiolic cysteines, like the TST. In fact, in the case of the RhdA, only a cysteine is present in the primary structure, the catalytic Cys230; in the case of GSTP1-1, there are available three thiolic cysteines, as described above, the Cys47, Cys101 and Cys169. The chemical modification of the three cysteines causing the fast inactivation process, even though these residues are not involved in the catalytic mechanism. The reason for this finding may rely on the particular position of these residues, in fact, Cys47 is situated at the end of the flexible helix  $\alpha$  2. Crystallographic analysis of the GSTP1-1, in complex with S-hexilglutathione (a GSTs inhibitor), reveals that Cys47 is located on the surface with its thiol group pointing into a small hydrophobic pocket, where the residue that compose it are involved, on the opposite site in the binding of GSH. So this could explain why chemical modification of Cys47 inhibits the binding of GSH.



**Figure 16.** A) Western blotting of GSTP1-1; B) GSTs activity in cell lysates, treated or not (CTRL) with 2-PTS 0.5 mM. All the assays has been performed in DPBS, GSH 2 mM and CDNB 1 mM, at 37°C, reading the absorbance increment at 340 nm.



**Figure 17.** A) GSTP1-1 0.15 mM incubated with 30 mM 2-PTS or ddH<sub>2</sub>O (control) for 2 hours at r.t.; after NAP-25 purification, the GST activity is valuated, and the control has taken as 100% of activity; B) Recovery of GST activity by direct incubation of GST-PS and Trx (at various ratio), in presence of 0.1 U TrxR and 50  $\mu\text{M}$  NADPH. Incubation with GSH alone at 2 mM does not restore GST activity.



**Figure 18.** UV difference spectra of GSTP1-1 and GST-PS after reaction with Br-pyr: GSTs (3  $\mu$ M) are solubilized in 0.1 M potassium phosphate buffer (pH 7.2), and incubated with 16  $\mu$ M Br-pyr; A) GSTP1-1, curves *a, b, c, d, e*: time 0, 5, 10, 15 and 30 minutes of incubation; B) GST-PS, curves *a, b*: time 0 and 30 minutes of incubation.



## ***2-PTS AND GLUTATHIONE***

The 2-PTS can interact with GSH *in vitro*, at physiological conditions. The incubation for 2 hours at r.t. in 50 mM Tris-HCl buffer (pH 8.2) of 2-PTS with GSH leads to the production of another compound, characterized by LC-MS and <sup>1</sup>H-NMR, as showed in Fig. 19.

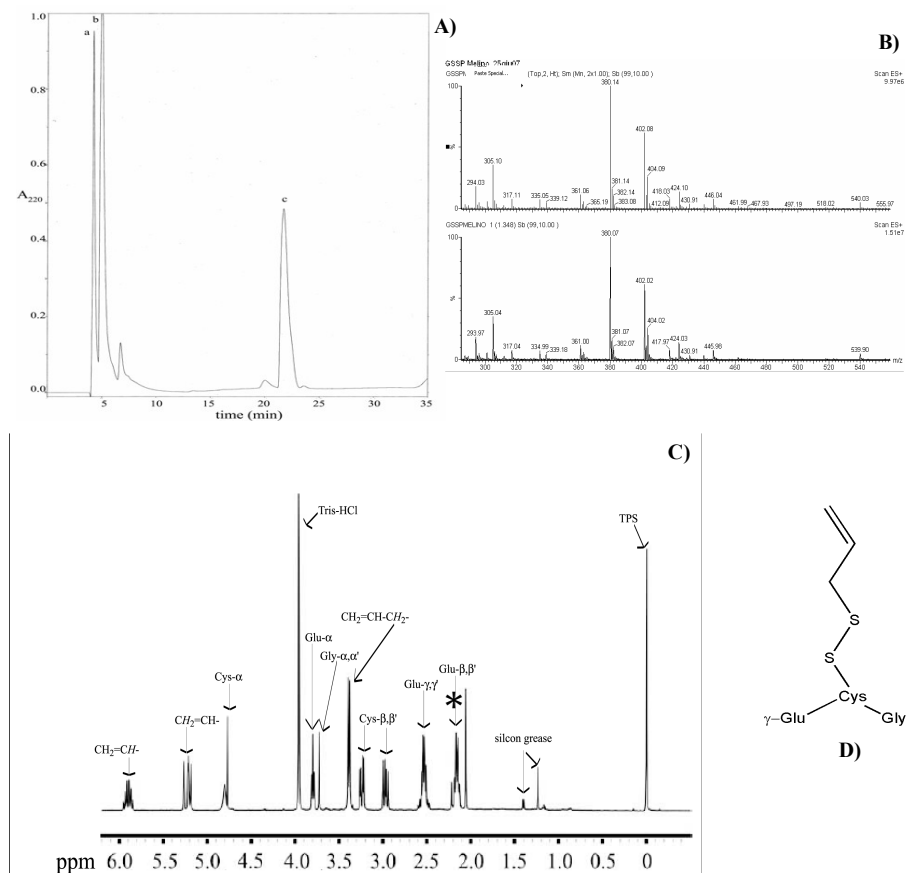
The LC-MS analysis reveals the presence of 3 predominant peaks *a*, *b*, and *c*, with *m/z* of 153, 308, and 380, respectively (Fig. 19-A, B). The first two peaks are imputable to unreacted 2-PTS and GSH, while the peak *c* is a diverse molecule. The <sup>1</sup>H-NMR (Fig. 19-C), in fact, reveals that the structure of this molecule correspond to the S-allyl-mercaptogluthathione, GSSP (Fig. 19-D).

## ***S-ALLYL-MERCAPTOGLUTATHIONE AND TST, AND GST***

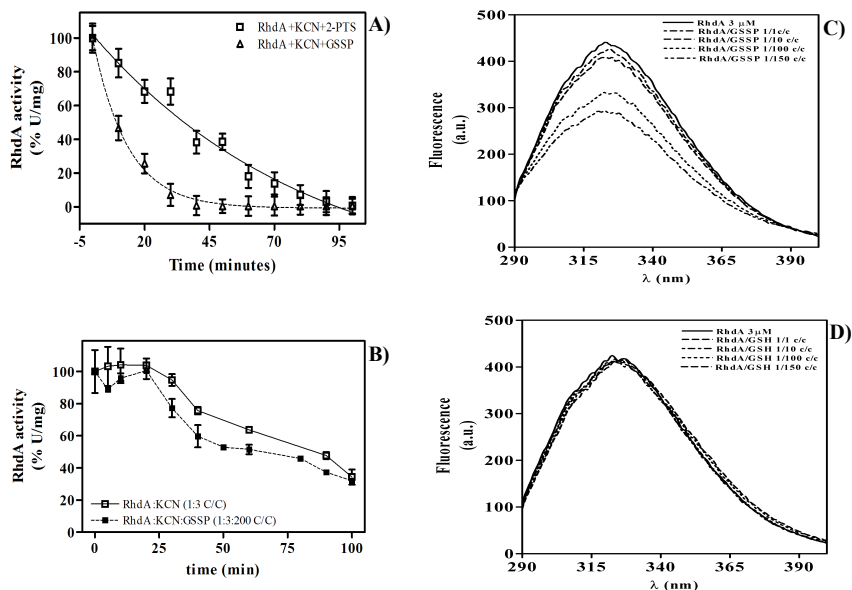
Relatively to the experiments effectuated with 2-PTS on TST, first of all the mixture of reaction of 2-PTS and GSH (GSSP<sub>mix</sub>) (after 2 hours of reaction and before purification) is tested on the TST activity. Figure 20-A shows that the GSSP<sub>mix</sub> is able to inhibits the TST activity faster than only 2-PTS. This could be due to the marked production of highly reactive allyl sulfide. By contrast, using purified GSSP by HPLC, the TST activity seems to be not changed (Fig. 20-B). The Fig. 20-A shows that GSSP purified by RP-HPLC causes a quenching of the intrinsic fluorecence of rhodanese. The

increase of the GSSP amount leads to a dose-dependence decreasing of the fluorescence of RhdA until about 55%, for the higher ratio (1 : 150). The control, using GSH instead of GSSP, shows that GSH does not causes any variation of intrinsic fluorescence of RhdA. On the contrary, the activity assays, in Fig. 20-B, indicates that purified GSSP does not causes variations of enzymatic activity of RhdA. These results suggests that GSSP can interact with RhdA, but does not causes an inhibition of its activity, like the case of 2-PTS.

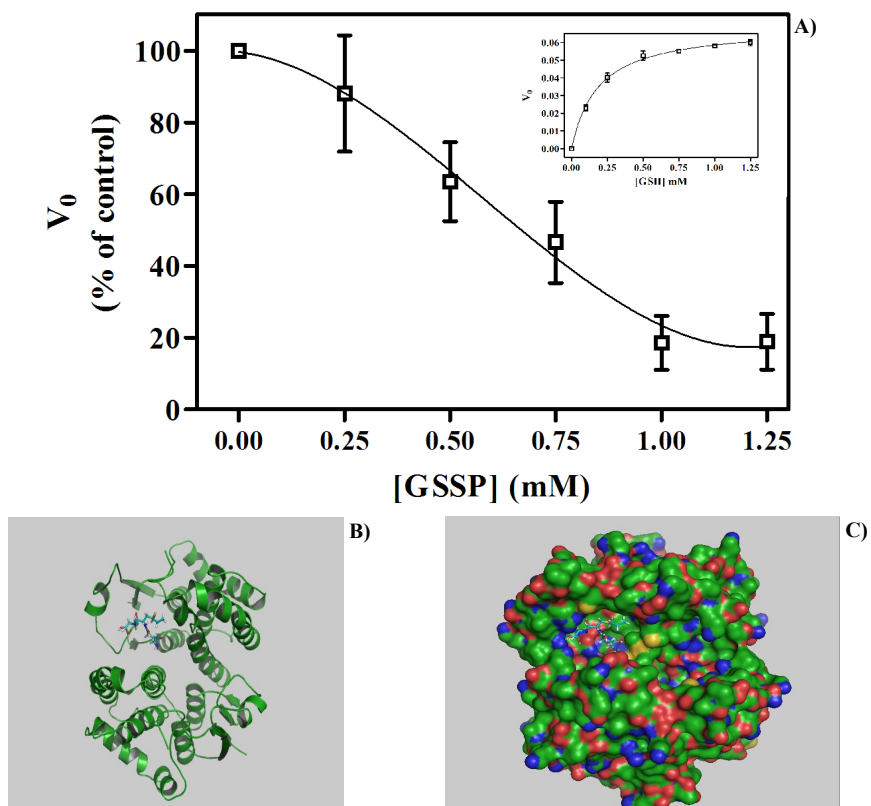
Literature shows the existence of many inhibitors of GSTs, but the principal problem is the low affinity of these inhibitors, due to the capacity to recognize wide range of substrates. Thus, we have tested the effect of GSSP on the recombinant GSTM1-1. Figure 21 shows the curve fitted on the initial rates to determine the  $IC_{50}$ , and the  $K_i$  values of GSSP on the GSTM1-1; the extrapolated  $IC_{50}$  value is about 0.6 mM. By Chang-Prusoff equation is possible also to obtain the  $K_i$  value, about 0.1 mM. The calculated  $K_i$  value of GSSP for GSTM1-1 is comparable to the  $K_i$  value of another competitive inhibitor of GSTM1-1 the S-methyl-glutathione. The Figure 21-B and -C shows the predicted 3D-structure of GSTM1-1 complexed with the GSSP, based on the structure of GSTM1-1 with the S-methyl-glutathione.



**Figure 19.** A) RP-HPLC of reaction of 40 mM 2-PTS and 20 mM GSH after 2 hours of incubation at r.t. in 50 mM Tris-HCl buffer (pH 8.2), give rise at three predominant peaks *a*, *b*, *c*; B) ESI(+)-MS spectroscopy analysis reveals that peak (*a*) is the 2-PTS (153 *m/z*), (*b*) GSH (302 *m/z*) and peak (*c*) has a 380 *m/z*; C) <sup>1</sup>H-NMR analysis of peak (*c*), solubilized in CD<sub>3</sub>OH reveals that structure is S-allyl-mercaptogluthathione (GSSP) (D).



**Figure 20.** A) Rhda ( $8.8 \mu\text{M}$ ) is inhibited more efficiently by  $\text{GSSP}_{\text{mix}}$  ( $2 \text{ mM GSH} + 4 \text{ mM 2-PTS}$ ) than 2-PTS ( $4 \text{ mM}$ ). The time-course inhibitions are effectuated in presence of KCN ( $1 : 3$ ; Rhda : CN) at r.t., to generate the E-form of Rhda, necessary for interact with the 2-PTS. The GSH alone does not alter the activity; B) Incubation of Rhda ( $8.7 \mu\text{M}$ ) in presence of KCN ( $26 \mu\text{M}$ ) or GSSP purified ( $3.45 \mu\text{M}$ ), in  $50 \text{ mM Tris-HCl}$  buffer ( $\text{pH } 8.0$ ),  $0.3 \text{ M NaCl}$ ; C) Fluorescence of Rhda and GSH or GSSP (D) purified by RP-HPLC. All the fluorescence assays has been performed in  $50 \text{ mM Tris-HCl}$  buffer ( $\text{pH } 8.0$ ),  $0.3 \text{ M NaCl}$  with  $3 \mu\text{M Rhda}$ .



**Figure 21.** A) Dose-response curve of GSSP on the GSTM1-1 activity. All the assays have been conducted in DPBS at 37°C with 0.51  $\mu$ M GSTM1-1, and the substrates has been maintained constant: GSH 1 mM, CDNB 1 mM; B,C) 3-D modeling of GSTP1-1 complexed with S-allyl-mercaptogluthathione, based on the structure of GSTP1-1 complexed with S-methyl-glutathione.

## ***EFFECTS OF S-ALLYL-MERCAPTOGLUTATHIONE ON THE CELL VIABILITY***

We have valuated the cytotoxic effect of the obtained GSSP on HuT 78 cells. In Figure 22-A and -B, it possible to see that GSSP causes a decreasing of cell viability in time- and dose-dependence manner. From the graph is possible to determine an  $IC_{50}$  of about 0.9 mM, at 48 hours of treatment. The GSSP seems to inhibits the cell proliferation, but does not lead to a marked cell death. In fact the higher tested concentration (0.5 mM) causes about a 20% of cell death, respect to the control. The cytofluorimetric analysis indicates a  $G_1/S$  phase blockage, and an increment of the sub $G_1$  population, as showed in the Figure 22-C.

To investigate the effect of the GSSP on the protein expression, we have performed also Western Blotting experiments (Fig. 23). After 9 hours of 0.5 mM GSSP treatment a marked activation of p38 is observed, and the phosphorylation is consistent still after 24 hours. The MAPK p38 is very important in the regulation of cell survival, it is linked with the regulation of production of IL-2 and IL-10, and can regulate directly the activity of p53, and indirectly of p21 [46]. p38 MAPK activated can translocate in the nucleous and regulate the expression of many important transcription factors, such as c-myc, stat1, creb [13, 44, 74].

The inhibitions of GST by GSSP, observed in the molecular experiments, was studied also HuT 78 cells treated with GSSP. As showed in figure 24-A, the treatment at 0.5 mM GSSP does not alter the expression of GSTP1-1,

and also the activity seems do not change; a little decrement is observed after 4 hours of treatment.

These preliminary results and the few other works about the GSH-derived compounds suggest that this compounds maybe does not be able to enter in the cells, and probably can interact with receptor and/or membrane proteins, for example TGF $\beta$ , FASL, and ABC-transporters. Particularly, the ABC-transporter, such as the Permeability Glycoprotein (P-gp) could have an active role in this process. In fact, the membrane transporters play a crucial role in the resistance to many anticancer drugs, exporting the conjugates between G $\Omega$ I and the xenobiotics [81]. Specific GST inhibitor has been used to enhance the cytotoxicity of a well-known chemotherapeutic drug, the Doxorubicin (Doxo). To deeply understand the mechanism of action of the GSSP we have conducted some experiments in co-treatment with the doxorubicin.

## ***CO-TREATMENT OF S-ALLYL-MERCAPTOGLUTATHIONE AND DOXORUBICIN ON HUT 78 CELL LINE***

First of all, we have tested some concentrations of Doxo on HuT 78 cell line, to define the cytotoxic concentrations. Using a concentration minor of 200 ng/mL, no cytotoxic effect was observed (Fig. 25-A). We have choose a fixed concentration of Doxo (50 ng/mL) and of GSSP (0.5 mM) for the co-treatment. As showed in Figure 25, the Doxo at the concentration of 50

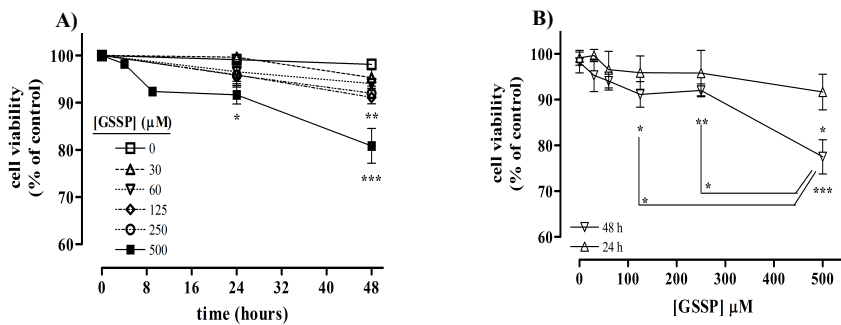
ng/mL have no effect, but in presence of 0.5 mM GSSP, an decrease of cell viability is observed (Fig. 25-B, -C). The cytofluorimetric analysis confirm the cytotoxicity of 50 ng/mL Doxorubicin in presence of 0.5 mM GSSP. The GSSP after 48 h of treatment causes a G<sub>1</sub>/S phase blockage (Fig. 25-D) with a low percentage of subG<sub>1</sub> population (about 14%), but in presence of Doxo the percentage increase of about 20%.

The western blotting analysis of the cellular lysates indicates that in the case of the co-treatment there is not the phosphorylation of p38, but by contrast, there is an increase of p21 expression. In the case of the treatment with the only GSSP, p21 is not very expressed, but p38 is strongly activated. In the co-treatment the result is reversed, as showed in Figure 26-A. The densitometric analysis (Fig. 26-B) indicate an increment of p21 expression of about twice in the case of the co-treatment respect to the control.

On the base of these observations, we have investigated on the causes of the major cytotoxic effect of the Doxo in presence of GSSP. One possible cause is that, in presence of GSSP, the Doxo remains in the cell for much more time, or by an inhibition of its detoxification or by blockage of its export out of the cell. We have hypothesized that the blockage of the export could be the hypothesis more probable, because before we don't have seen any variations on the GST activity and expression (Fig. 24-A, B).

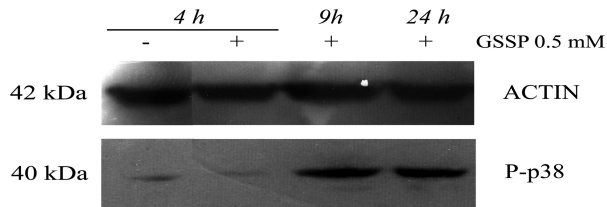
Using the intrinsic fluorescence of Doxo, is possible to quantify the incorporation of Doxo by a confocal microscope. After 2 hours of treatment, there a marked increase of intracellular Doxo when GSSP is present (Fig. 27). The quantitative fluorescence measurements indicates that there is an increment of about 3 time of intracellular Doxo.



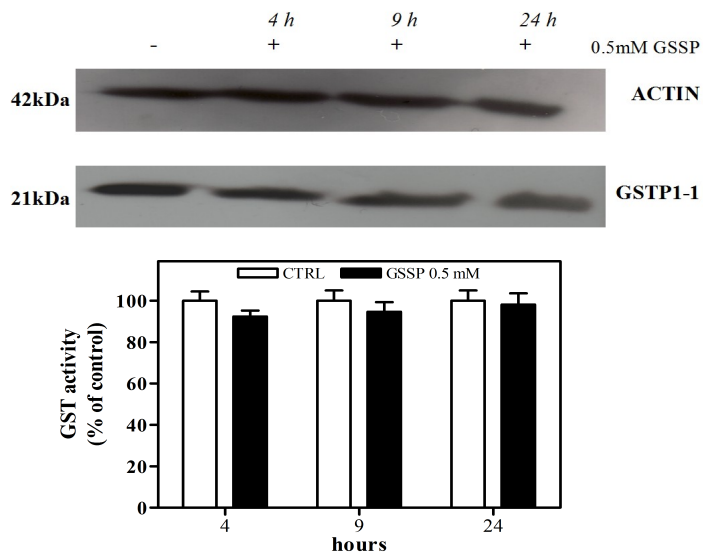


C)	% subG <sub>1</sub>	% G <sub>1</sub>	% S	% G <sub>2</sub> /M
CTRL <sub>24h</sub>	1,43 ± 0,33	50,64 ± 0,83	17,86 ± 0,44	30,21 ± 1,51
CTRL <sub>48h</sub>	2,23 ± 0,61	66,29 ± 2,23	10,84 ± 0,57	21,26 ± 2,47
<b>30<sub>24h</sub></b>	2,08 ± 0,50	51,84 ± 1,22	17,35 ± 0,50	29,32 ± 1,54
<b>30<sub>48h</sub></b>	2,20 ± 0,20	69,66 ± 0,92	10,38 ± 0,13 *	18,67 ± 1,27
<b>60<sub>24h</sub></b>	2,08 ± 0,41	51,01 ± 0,53	17,52 ± 0,97	29,79 ± 0,07
<b>60<sub>48h</sub></b>	2,52 ± 0,01	68,89 ± 0,18	11,05 ± 0,37	18,32 ± 0,31
<b>125<sub>24h</sub></b>	1,98 ± 0,18	50,12 ± 0,98	18,73 ± 0,52	29,52 ± 0,36
<b>125<sub>48h</sub></b>	3,64 ± 0,18	73,79 ± 0,71	9,54 ± 0,22 *	14,18 ± 0,21
<b>250<sub>24h</sub></b>	3,12 ± 0,52	50,26 ± 0,17	19,68 ± 0,28 *	27,15 ± 1,19
<b>250<sub>48h</sub></b>	3,12 ± 0,17	70,60 ± 2,94	10,68 ± 0,21	16,28 ± 3,19
<b>500<sub>24h</sub></b>	10,14 ± 0,40 **	36,15 ± 0,44 **	20,80 ± 0,04 *	30,81 ± 0,11 *
<b>500<sub>48h</sub></b>	14,84 ± 0,91 **	43,33 ± 1,60 *	24,14 ± 0,79	12,10 ± 0,09

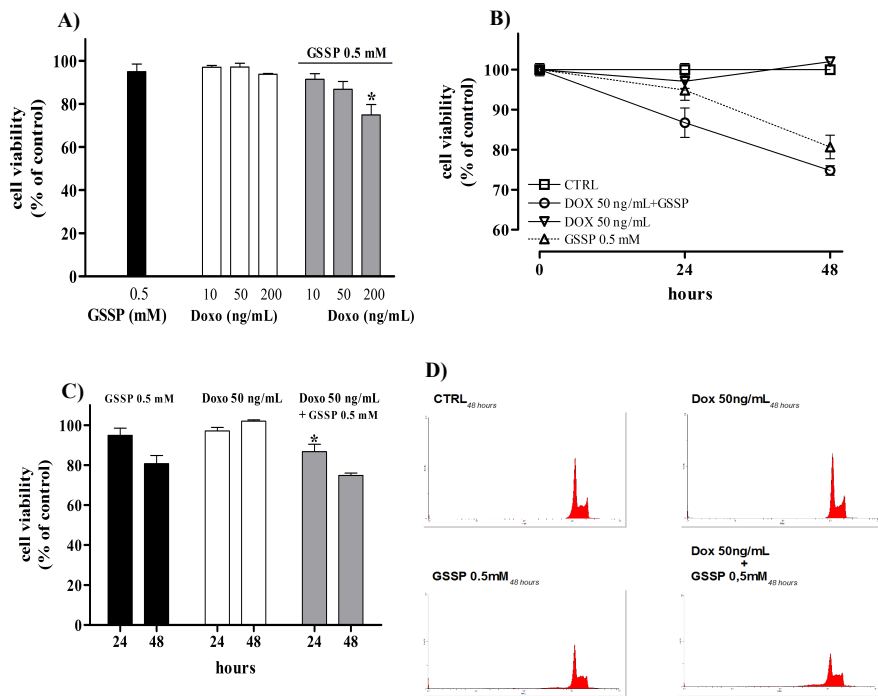
**Figure 22.** A) Cell viability graph. The GSSP causes a decreasing of cell viability after 24 and 48 hours of treatment, in dose- and time-dependent manner; B) FACS analysis at various concentrations of GSSP, at 24 and 48 hours of treatment, indicates a G<sub>1</sub>/S phase blockage and a subG<sub>1</sub> population increment.



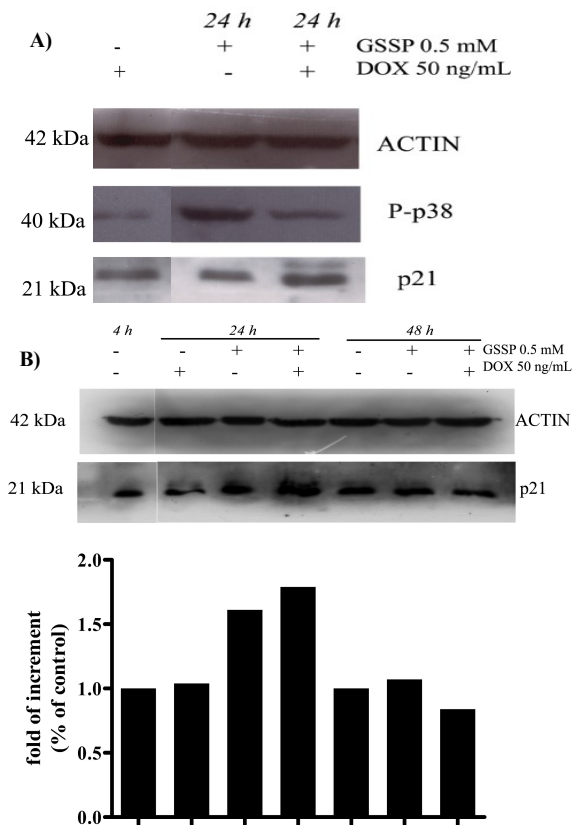
**Figure 23.** Western Blotting analysis of HuT 78 cells treated with 0.5 mM of GSSP. A consistent activation of MAPK p38 is observed after 9 hours of treatment. The phosphorylation remains still after 24 h.



**Figure 24.** Expression and activity at various time of GST in lysates of HuT 78 cells, treated with 0.5 mM of GSSP. GSTP1-1 expression remains unchanged, and also the activity of GST seems do not change with the treatment. A very little decrement is observed after 4 h, but it seems be not significant.

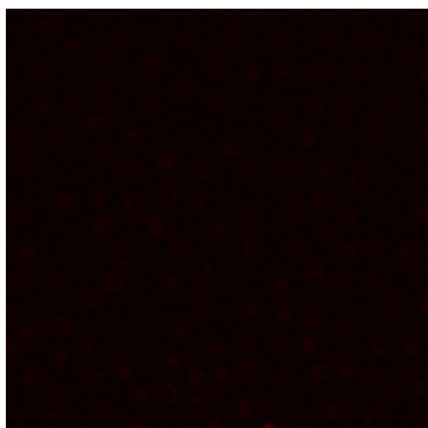


**Figure 25.** Co-treatment with GSSP and Doxorubicin on HuT 78 cells. The co-treatment indicates that the presence of GSSP enhance the cytotoxic effect of Doxorubicin. A) After 24 h of treatments, GSSP has a light effect on cell viability, but in presence of Doxo, an increment of its cytotoxicity is observed; B) Using a fixed concentration of GSSP and Doxo there is an increment of cytotoxicity of Doxo in time dependence; C) Bars representation of cell viability after co-treatment with GSSP and Doxo; D) FACS analysis after 48 h of treatment confirm the increment of the mortality in the case of co-treatment associated with a G1/S phase blockage.

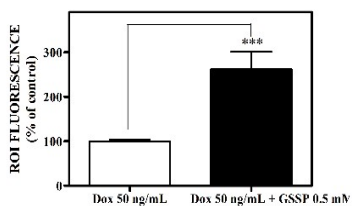
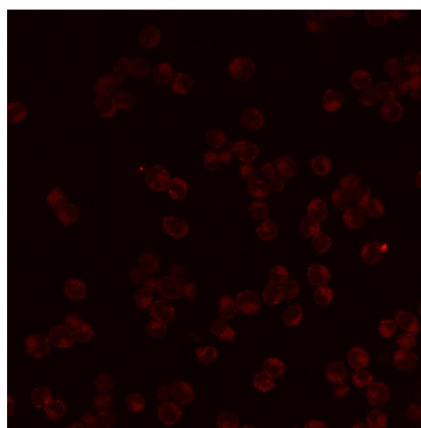


**Figure 26.** Western Blotting analysis of HuT 78 cell lysates. A) Different response to the treatment with the only GSSP or Doxo, and the co-treatment. In the case of the only GSSP treatment, p38 is activated, but in the co-treatment, p38 phosphorylation return to the basal state, and p21 expression is augmented; B) Densitometric analysis indicates that after 24 h of co-treatment there is an expression of p21 about two-times more than control.

Doxorubicin 50 ng/mL 2h



Doxorubicin 50 ng/mL + GSSP 0.5 mM 2h



About 3 times of increment of Doxorubicin incorporation in presence of GSSP.

**Figure 27.** *Quantification of intracellular Doxorubicin in HuT 78 treated cells by confocal microscopy.* The presence of GSSP causes an intracellular accumulation of Doxo, about 3 times more than using only Doxo. This could be related to an inhibition of the drug efflux (for example, P-gp inhibition), and it could explain the major cytotoxic effect observed in the case of the co-treatment.

## ***DISCUSSION***

Studies in experimental animals indicates that the benefits of *Allium* vegetables are not limited to one species, tissue, or carcinogen. Organosulfur compounds can hinder activation of active carcinogen from precursor increasing its metabolic detoxification, or preventing its reaction with vulnerable target cells. The ability of allylsulfides (present in all the *allium* vegetables) to suppress carcinogen-activating P450 (CYP2E1), induce phase II GST and scavenge ultimate carcinogenic species may contribute, singly or in combination to the reduction of tumorigenesis [35]. Additional mechanisms include a delay or a reversion of the expression of malignancy by antiproliferative activity in tumor cells and modification of signal transduction mechanism ultimately leading to inhibition of carcinogenic insult. Overall, evidence shows that *allium* vegetables have strong cancer-preventive activity but there are many horizon that still need to be explored.

The literature [38, 39, 75] indicates that the thioalkenylation of cysteines of many proteins ( $\beta$ -tubulin, GSTs, TST, TrxR, etc.) is an important event that causes the induction of cell death. On the other hand, the ability to prevent the tumor development by *allium* compounds is confined to the events described above, but none authors handle the question of the different response to the treatment with garlic extract of normal and cancer cells. The different expression of important proteins involved in the detoxification of these compounds, such as TST, could be related to the different sensibility. In fact, as described before, in colon cancer cells the TST expression is lower compared to normal cells, and we have demonstrated that the presence of TST can prevent the inhibition of the essential Trx-TrxR system. When TST is absent, or in minor concentration, the prevention of this inhibition is lacking, and the cell could be more vulnerable to the action these

compounds. The TST-Trx-Trd system can be seen as a new detoxification system, and the proposed scheme of action of 2-PTS in figure 28, can be expanded to other OSCs.

In addition, the oxidative inhibition of Trd and GSTs by OSCs can be very important in the induction of cell death. The Trx can be seen as a switch of the apoptosis induction, in fact its oxidation leads to a dissociation from ASK-1 that can initiate the apoptotic program, activating JNK and p38. Screening for ASK-1 associated proteins, using the yeast two hybrid system, leads to the identification of thioredoxin [76]. Thioredoxin association with ASK-1 was found in non-stressed cells and required the amino-terminal domain of ASK-1. Thioredoxin association with ASK-1 inhibits its activity as a kinase. Thioredoxin inhibition of ASK-1 is subject to attenuation by ROS, which induce dimerization of thioredoxin. If Trd is inhibited, the Trx cannot be reduced and it remains in the oxidized state leaving ASK-1 free.

ASK-1 has been implicated in the activation of MKK3/6, MKK4/7, and subsequently, p38 or JNK [76], resulting in the phosphorylation of respective p38 and JNK substrates, including ATF2, c-Jun [28] and p53 [1]. The key role of these substrates in the cellular protection from stress and damage suggests that their kinases should be tightly regulated. One indicator for such regulation is the low basal activity of JNK in cells, even if maintained under high concentration of growth factors. This suggests that JNK activity is inhibited in non-stressed. Indeed, extract prepared from normal growing cells contains an inhibitory component which was purified and identified through microsequencing as GSTp [2]. GSTp inhibition of JNK activity requires its association, which, in turn, limits the degree of Jun phosphorylation under non-stressed growth conditions. GSTp-JNK



association was primarily found in non-stressed cells. Upon treatment with H<sub>2</sub>O<sub>2</sub> or UV, GSTp dissociated from JNK and formed dimers/multimers. Under physiological conditions GSTp inhibition was limited to JNK and did not involve the upstream JNK kinase MKK4. Furthermore, in the cells over-expressing GSTp, constitutively active MEKK1 efficiently phosphorylated both MKK4 and JNK, but did not elicit Jun phosphorylation due to GSTp-mediated inhibition. GSTp inhibitors as well as a GSH-derived peptides were able to alter the degree of JNK activity prior to and after UV-irradiation, further defining the nature of this inhibition. Cells derived from GSTp null cells were found to have high basal JNK activity, which was reduced upon forced expression of GSTp cDNA [2]. Being a key regulator of glutathione and, in turn, of redox potential, the identification of GSTp as a JNK regulator provides an important link between cellular redox potential and the regulation of stress kinase activities.

The prevention of the inhibition of important enzyme, such Trd and GST by TST, could reduce the OSCs toxicity, but, when TST concentration is reduced, as a consequence of disease for example, the cell are more susceptible to oxidant action of these compounds.

In mammalian cell, the most abundant non-protein thiol is the reduced glutathione, GSH. The intracellular GSH concentration is about 100-fold higher than cysteine, 2 - 5 mM, and is plausible that the OSCs, after passing the cell membrane reacting with GSH producing S-allyl-mercaptogluthathione (GSSP). Many studies indicate the production of S-allyl-mercaptocysteine and its effect on different cell lines, but only two works shows the production of GSSP by reaction between allicin and GSH, and the direct effect of GSSP on enzyme or on the cell viability were not studied.

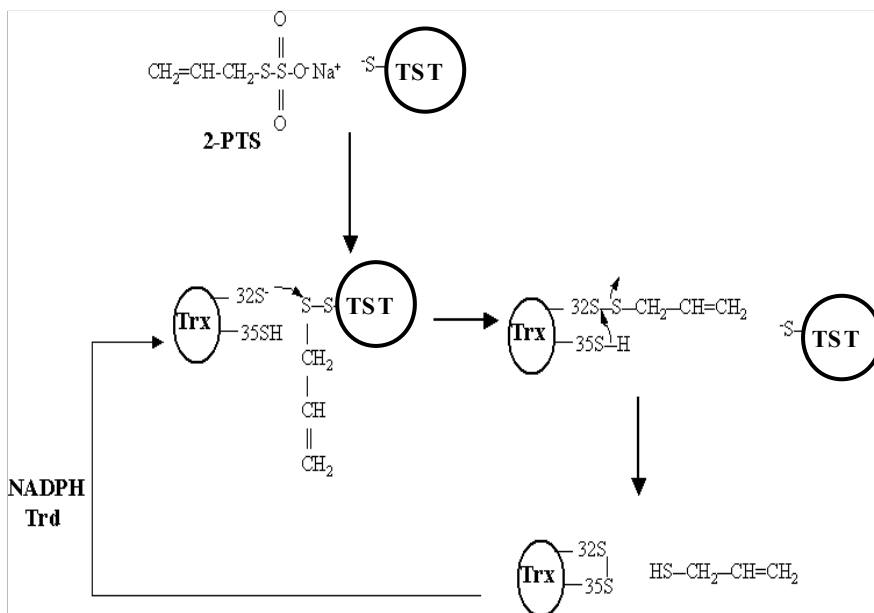
The p38 MAPK is an important regulator in the T-cells [93]. In fact, its degree of phosphorylation is a cellular switch between the proliferation and not-proliferation, because regulates two cytokines with opposite function, IL-2 and IL-10. The interleukine 2 (IL-2) is a cytokine that promotes the cell proliferation and in HuT 78 cells its production is major respect to normal cells. The interleukine 10 (IL-10), instead plays an opposite role respect to IL-2, promoting the inhibition of IL-2 production. The increase of p38 phosphorylation causes an increment of IL-10 production and an inhibition of IL-2 production, leading a decrease of cell proliferation. Although, the role of p38 on the IL-2 gene expression is not still clear, appears evident that p38 plays a central role in the regulation of the balance between IL-2 and IL-10. Thus, the discover of the ability of GSSP to increase the phosphorylation of p38 is very important to the comprehension of the effect of this class of compounds that it is not still well studied.

Moreover, the discovery of the effect of the GSSP on the cell viability and its effect on the Doxorubicin efflux, open new questions and opportunity on the use of the glutathione-derived compounds in co-treatment with other chemotherapeutic drugs. In fact, many frequently used drugs for the cancer treatment have relevant side effects, and one is the multidrug resistance (MDR). The MDR is a condition originating to the over-activity of some membrane transporters, such as P-gp or MDR-associated proteins, that can be over-expressed, increasing the efflux of many xenobiotic, among there is the Doxorubicin. The incremented efflux of the drugs, causes the use of major doses of the drugs, increasing the other side effects of the drug used on the organism. So, the new approach using combined treatments allow to use minor amount of both drugs. In fact, Doxorubicin has many side effects,

among cardiotoxicity and ROS production. The reduction of the drug dose, using a natural compound derived from garlic, such as S-allyl-mercaptogluthathione, confirm the important role of garlic compounds introduced by diet, and open new perspectives on the study of derived-gluthathione compounds for therapeutic use in the cancer.

The progression of the cell cycle is tightly controlled in the normal cells; when a cell collects dysregulations of its components of control, the cell could be replicate without regulation, resulting the development of tumor. The cyclin-dependent kinase inhibitors (CKIs) plays an essential role of control of the cell cycle, and p21<sup>WAF/CIP1</sup> is one of the most important CKIs, its gene is regulated by another important protein, the tumor suppressor protein p53. Activated p53, after various stress stimuli, can promote p21 expression, that promote the G<sub>1</sub> phase cell cycle arrest, through the binding and inhibition of cyclin-CDK2 and -CDK4 complex, resulting in a blockage of the cell proliferation. When p53 have mutations that inactive its activity, p21 expression can be reduced and the one of the check-point of control of cell cycle is abolished. In HuT 78 cells, the *p53* gene, as mentioned in the introduction, have a mutation that give rise to a truncated form of p53, incapable to bind the DNA and regulate its target gene expression, as well as *p21/WAF1*. Recent works try to restore the normal p53 target gene expression; Wikhanskaya et al. [94] have been demonstrated that inactive mutant of p53, with a repression of p21 expression, if treated with trichostatin (TSA) regains the normal p21 expression. It seems possible because some drugs can induce a p53-independent p21 expression, acting directly on the promoter of *p21/WAF1* gene. The discover of drug or mixed treatment that enhance the expression of p21 is widely used as cancer

treatments, and the p21 expression in the case of the co-treatment with GSSP and doxorubicin open on interesting advance, that should be further on investigated.



**Figure 28.** Proposed scheme of action of 2-PTS. The 2-PTS inhibits the TST activity by a thioalkenylation of Cys230, after, the Trx restore the active form of TST, oxidizing itself, and it can be reduced by Trd using NADPH.

## ***EXPERIMENTAL PROCEDURES***

## ***2-PTS SYNTHESIS***

Briefly, 230 mmol of thiosulfate and 190 mmol of allyl chloride are dissolved in 330 mL of ddH<sub>2</sub>O and 420 mL of toluene, respectively; 8 mmol of benzyl trimethyl ammonium chloride is added and warmed at 105°C for 3 h in agitation (Figure 4-A). After evaporation of the precipitate, it is dissolved in the minimum volume of methanol, and incubated at 4°C overnight. The supernatant is separated from the precipitated and loaded into a silica column, using as elution solvent a mix of methanol and chloroform (45:55 v/v).

## ***FLUORESCENCE MEASUREMENTS***

All fluorescence measurements were made using an LS50 Perkin Elmer spectrofluorimeter equipped with a thermostated stirrer cell holder. The temperature was always maintained at 23°C. The excitation and emission bandwidths were 5 and 3 nm respectively. The excitation wavelength was set at 286 nm and the spectra were recorded from 300 to 400 nm. The changes in fluorescence intensity at 340 nm ( $F_{\text{obs}}$ ) were given as  $\Delta F\%$  :

$$\Delta F\% = [(F_{\text{obs}} - F_0) / F_0] \times 100$$

, where  $F_0$  is the original fluorescence intensity of RhdA. The fluorescence measurements were performed in the presence of different concentrations of allyl compounds with an enzyme concentration of 3-5  $\mu\text{M}$  in 50mM Tris-HCl buffer, pH 7.2.

## ***ENZYMATIC ACTIVITY ASSAYS***

The thioredoxin oxidase activity of rhodanese was assayed as described by Nandi DL et al. [56], following NADPH oxidation spectrophotometrically at 340 nm in the presence of recombinant thioredoxin reductase from *E. coli* (Sigma-Aldrich, Milan, Italy). The assay was performed as follows: the absorbance of a solution containing 50  $\mu$ M NADPH (Sigma-Aldrich), 16.6  $\mu$ M thioredoxin from *E. coli* (Sigma Aldrich) and 0.1 unit of thioredoxin reductase in 50mM Tris-HCl buffer, pH 8.0, in a final volume of 1.0 ml, was read at 37°C against a blank containing no NADPH, after equilibrium rhodanese (4  $\mu$ M) and thiosulfate or 2-PTS were added to the solution and NADPH oxidation was followed. The stock solutions of NADPH, thioredoxin and thioredoxin reductase were in 50 mM Tris-HCl buffer, pH 8.0, containing 1.0 mM EDTA.

Rhodanese activity was measured by discontinuous colorimetric assay described by Sörbo, where the production of thiocyanate from the reaction between thiosulfate and cyanide was followed measuring the absorbance at 460 nm, using a Perkin-Elmer spectrometer. Briefly, 0.5  $\mu$ g of enzyme (rhodanese) was incubated exactly for 1 minute, at 37°C, in presence of 58 mM cyanide and 58 mM sodium thiosulfate, in 0.3 M NaCl and 50 mM Tris-HCl buffer (pH 8.0). The reaction was stopped by addition of 100  $\mu$ L of 15% formaldehyde and 250  $\mu$ L of Sörbo reagent (100 g of ferric nitrate and 200 mL of 65% nitric acid per 1500 mL), to allow the formation of the thiocyanate-Fe complex, and the absorbance at 460 nm was recorded to valuate the amount of thiocyanate produced.



GSTs activity was measured following the conjugation of the CDNB with the GSH, monitoring the increasing of absorbance at 340 nm, with a Perkin-Elmer spectrometer. The assay was conducted in Dulbecco's phosphate buffered saline, using 2 mM of GSH and 1 mM of CDNB, at 37°C. A cuvette without the enzyme was subtracted as blank.

## ***CELL CULTURE***

HuT 78 human T lymphoblastoid cells were purchased from the ISS (Istituto Superiore di Sanità, Italy). HuT 78 ( $0.2 \times 10^6$ ) cells were pre-incubated for 24 h in RPMI medium 1640 (GIBCO, Italy) with 1% glutamine 10% heat-inactivated FCS and antibiotics (1% penicillin and streptomycin sulfate) at 37°C in air supplemented with 5% CO<sub>2</sub>. Then, exposed to 2-PTS or GSSP for 24 and 48 h. The cells were collected and counted after trypan blue staining (0.4% Trypan blue solution, Sigma, Italy) by optical microscopy using a Thoma chamber. The rate of growth inhibition by 2-PTS or GSSP was calculated based on the control culture taken as 100% growth.

## ***CELL CYCLE ASSAY***

The cell cycle distribution of HuT 78 cells was measured by flow cytometry. The harvested cells (about  $0.5 \times 10^6$  cells) were stained with 50 µg/ml propidium iodide (Sigma, Milan Italy) in ice-cold PBS buffer with

0.1% Triton X-100 and 1 mg/ml sodium citrate. Then, they were immediately analyzed using a flow cytometer FACSCalibur (Beckton and Dickinson, San José, CA, USA) and the percentages of cells in each phase of cell cycle were evaluated, according to Nicoletti et al. [59].

### ***ROS DETECTION & GLUTATHIONE DOSAGE***

Intracellular reduced (GSH) and oxidized (GSSG) forms of the glutathione were assayed upon formation of *S*-carboxymethyl derivatives of free thiols with iodoacetic acid, followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives by the reaction with 1-fluoro-2,4-dinitrobenzene [73]. Data are expressed as nanomoles of GSH equivalents per milligram of protein.

ROS measurements were determined as described: cells were incubated with 20  $\mu$ M 2',7'-dichlorodihydrofluorescein diacetate dye (DCF-DA) (Invitrogen-Molecular Probes) for 30 minutes at 37°C before 2-PTS treatment. After treatment cells were immediately washed and resuspended in ice-cold PBS, and analysed by flow cytometry. Detection was performed using a FACSCalibur fluorescence-activated cell sorter at excitation wavelength at 480 nm and an emission wavelength of 525 nm, and for the analysis was used the CELL Quest™ software.

### ***CELL EXTRACT IN ACQUOUS PHASE***

HuT 78 cells were washed twice with ice-cold physiological saline solution and pellets resuspended in 0.5 ml of ice-cold twice-distilled water. Aqueous extracts (from  $20\text{-}30 \times 10^6$  cells/sample) were prepared in EtOH:H<sub>2</sub>O (70:30, v/v). Samples were ultra-sonicated at 20 kHz with an exponential probe, 8  $\mu\text{m}$  peak-to-peak by a MSE ultrasonic disintegrator Mk2 (Crawley, Sussex, UK) and centrifuged at  $14000 \times g$  for 30 min. Supernatants were lyophilized twice in a RVT 4104 Savant lyophilizer (Mildford, Main, USA), and the residue resuspended in 0.7 ml D<sub>2</sub>O (Sigma-Aldrich, Milan, Italy) containing 0.1 mM 3-(trimethylsilyl)-propionic-2,2,3,3-d<sub>4</sub> acid sodium salt (TSP) as internal standard (Merck & Co, Montreal, Canada).

## ***NMR SPECTROSCOPY***

High-resolution NMR experiments (25°C) were performed at 400 MHz (Bruker AVANCE spectrometer, Karlsruhe, Germany). Intact cells were counted, washed three times in PBS, centrifuged at  $600 \times g$  and resuspended in 700  $\mu\text{l}$  of PBS in D<sub>2</sub>O, before their transfer to a 5 mm NMR tube ( $20 \times 10^6$  cells). NMR Analyses on intact cells were carried out using 90° flip angle pulses preceded by 2.50 s presaturation for water signal suppression (interpulse delay 2.50 s, acquisition time 1.86 s, spectral width 11 ppm, 32 K data points, 128 scans). These conditions ensured that the fatty chain (CH<sub>2</sub>)<sub>n</sub>/CH<sub>3</sub> ratio was determined at the magnetization equilibrium (as verified by preliminary experiments). Quantification of individual metabolites in cell extracts was obtained from peak areas using correction

factors determined by experiments at the equilibrium of magnetization (90° pulses, 30.00 s interpulse delay). Metabolite quantification was expressed as nmoles and normalized to the number of extracted cells. Deconvolution of signals under the one-dimensional <sup>1</sup>H NMR spectral profiles were performed using a Bruker WIN-NMR software package.

Others <sup>1</sup>H-NMR assay were performed on a Bruker AVANCE spectrometer(Karlsruhe, Germany), and the compounds analyzed were dissolved as described in the legends.

## ***WESTERN BLOTTING***

Proteins were extracted from HuT 78 cells (5 x 10<sup>6</sup>), in 200 µl of 50 mM Tris-HCl, pH 7.2, containing a protease inhibitors cocktail (Sigma-Aldrich, Milan, Italy) and phosphatase inhibitors (NaI, Na<sub>3</sub>VO<sub>4</sub>), were sonicated with 4 steps of 5 seconds and 1 minute of pause in ice. The samples were centrifuged for 10 minutes at 8000 rpm at 4°C. Proteins were determined by BCA protein assay (Sigma-Aldrich, Milan Italy), cell extracts (60 µg of total protein) were electrophoresed on 13% polyacrylamide gel, electro-blotted on PVDF membrane (Applied Biosystem, Milan, Italy) and proteins levels were analyzed using: anti-rabbit-TST polyclonal antibody (1:250) (ATLAS Antibodies, Stockolm, Sweden), anti-p21 monoclonal antibody (1:200), anti-GSTP1-1 monoclonal antibody (1:2000) (Sigma-Aldrich, Italy), anti-P-p38 monoclonal antibody (1:1000) (Cell Signaling, Danvers, MA). Immunoblot was also probed with anti-GAPDH or anti-β-actin monoclonal antibodies (Sigma-Aldrich, Italy) for controlling the protein loading. The protein

complex formed upon incubation with specific secondary antibodies (1:10000) (Sigma-Aldrich, Italy) was identified using a Fluorchem Imaging system (Alpha Innotech Corporation-Analitica De Mori, Milan, Italy) after incubation with ChemiGlow chemiluminescence substrate. Densitometry analysis of Western blots were performed using Quantity One Software (Bio-Rad, Italy).

### ***CONFOCAL MICROSCOPY***

Confocal microscopy experiments, about  $1 \times 10^6$  cells were incubated with 50 ng/ml of doxorubicin, and in presence or absence of GSSP 0.5 mM, and after 2 hours were washed twice with ice-cold PBS. Cell pellets were resuspended in 100  $\mu$ l of ice-cold PBS and transferred to glass microscope slides coated with poly-lysine, and incubated for 60 min. Then, the excess of cell suspension was removed and washed with PBS, and 200  $\mu$ l 4% paraformaldehyde (PFA) was added. After 15 min PFA was removed and washed twice with PBS. The samples were analyzed with a confocal laser scanning microscopy (Olympus FV1000-IX81 equipment), and the excitation light source was set at 488 nm, and emission was acquired between 500 and 550 nm.

### ***STATISTICAL ANALYSIS***

All experiments were carried out at least four times ( $n= 4$ ) unless

otherwise indicated. Data were expressed as mean  $\pm$  S.D. Comparisons between control and treated cells were made using Student's *t* test. Statistical significance was defined as  $P < 0.05$ .

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## ***Short CV of the Candidate***

2006 (1/3) graduated in Chemistry, University Tor Vergata of Rome, with votation 102/110, discuting a sperimental thesis: “Rhodanese from *Azotobacter vinelandii* a model of structure-function relationship of the sulfurtransferases: structural and functional characterization.”. Relators: Prof. M. Paci, PhD. S. Melino.

2006 (August - November) scholarship winner for research activity: “Biochemical interaction studies of natural and synthetic compounds”.

2006 (November) scholarship winner for Research Doctorate in Biochemistry and Molecular Biology.

2003 frequentation (February - July) of faculty of Chemistry of Universitat de Barcelona, like ERASMUS scholarship winner.

2005 frequentation during thesis period (January 2005 - March 2006) of NMR laboratory of Prof. M. Paci group of

University Tor Vergata of Rome (Italy), under supervision of PhD. S. Melino.

2006 participation at training course: “Chemistry and biology risk”.

2006 participation at training course: “Course of proteome study”.

2007 (March - May) guest in the Department of Cellular Biology and Neurosciences of Prof. F. Podo, National Institute of Heath, Rome, Italy.

2008 (25/2) partecipation at training courses:”Dall’ estrazione dell’ RNA all’ analisi dei dati: ottimizzazione del flusso di lavoro di un esperimento di espressione genica”.

2008 (4-5/12) partecipation at training courses:”Spettrometria di massa

quantitativa, tecniche di immagine e di screening per la determinazione di proteine e farmaci”.

### **TECNICAL KNOWLEDGE:**

- DNA recombinant techniques: manipulation, site-specific mutation, DNA purification and sequence analysis (PCR, agarose gel electrophoresis)
- transformation and growth recombinant bacterial cells
- expression, purification and characterization functional and structural of recombinant proteins, and production of recombinant proteins labelled with stable isotope  $^{15}\text{N}$ ,  $^{13}\text{C}$ ,  $^2\text{H}$  (HPLC, FPLC, SDS-PAGE, native electrophoresis, fluorescence, circular dichroism, and Magnetic Resonance homo/etero-nuclear mono/bidimensional)
- synthesis and purification of chemical natural compounds (thin layer chromatography, silica chromatography, HPLC) and their interaction with proteins (NMR 1D and 2D, fluorescence)
- practical application of enzymatic kinetic for constant calculation
- software utilization of for data analysis (Kaleidagraph, GraphPad), for 3-D visualization of proteins (VMD, PyMol), for NMR-data elaboration (NMRview), and utilization of protein and nucleic acid databases
- cellular biology techniques: manipulation of eukaryotic cells, analysis of cytotoxic activity of drugs and cellular viability,



cytofluorimeter uses and applications, Western Blotting, enzymatic assays on cell lysates

- mass spectrometry (ESI) and relative software (Xcalibur)

### **FOREINGES LANGUAGES:**

Knowledge of Spanish (Castilian and Catalan)

Knowledge of English

### **CONGRESS PARTECIPATIONS AND COMUNICATIONS**

2005 GDIRM congress participation 2005, Rome.

p1. Sabelli R., Melino S., Melis R., Cicero O.D., Gallo M., Paci M.; “Investigation on two domains of rhodanese from *Azotobacter vinelandii* and its interactions with sulfane sulfur compounds.”; PROTEINE 2006, Novara.

p2. Sabelli R., Melino S., Melis R., Cicero O.D., Paci M.; “Role of the sulfurtransferase in the metabolism of sulfane sulfurcompounds: possible implication in the apoptosis induction and cancer suppression.”; SIB 2006, Riccione.

p3. Sabelli R., Iorio E., De Martino A., Ricci A., Brunetti V., Podo F., Paci M., Melino S.; “Allyl-thiosulfide and rhodanese interaction has a key role in apoptosis induction”; SIB 2007, Riccione.

p4. S. Melino, D.O. Cicero, M. Gallo, R. Sabelli, R. Melis and M. Paci;

“New investigation on the sulfurtransferases enzyme rhodanese by NMR and its selenium binding” 2nd European Conference on Chemistry for life Sciences (2007).

p5. Sabelli R., Melino S., Viticchie G., Iorio E., Podo F., Paci M.; “Allyl thiosulfate, a natural apoptotic inducer affects the cell detoxification system”; 4th Central European Conference Chemistry towards Biology, 2008, Dobogókő (Hungary).

c1. Sabelli R., Iorio E., De Martino A., Ricci A., Brunetti V., Podo F., Paci M., Melino S.; “Allyl-thiosulfide and rhodanese interaction has a key role in apoptosis induction”; SIB 2007, Riccione.

c2. Sabelli R., Melino S., Viticchie G., Iorio E., Podo F., Paci M.; “Allyl thiosulfate, a natural apoptotic inducer affects the cell detoxification system”; 4th Central European Conference Chemistry towards Biology, 2008, Dobogókő (Hungary).

## ***List of Publications of the Candidate***

Sabelli R., Melino S., Melis R., Cicero O.D., Paci M.; “Role of the sulfurtransferase in the metabolism of sulfane sulfur compounds: possible implication in the apoptosis induction and cancer suppression.”; *IJB* 55 (2006), 1-2, 91.

Sabelli R., Iorio E., De Martino A., Ricci A., Brunetti V., Podo F., Paci M., Melino S.; “Allyl-thiosulfide and rhodanese interaction has a key role in apoptosis induction”; *IJB* 56 (2007), 3, 109.

S. Melino, D.O. Cicero, M. Gallo, R. Sabelli, R. Melis and M. Paci; “New investigation on the sulfurtransferases enzyme rhodanese by NMR and its selenium binding” 2nd European Conference on Chemistry for life Sciences (2007).

Sabelli R., Iorio E., De Martino A., Podo F., Ricci A., Viticchie G., Rotilio G., Paci M., and Melino S.; “Rhodanese-Thioredoxin system and allyl-sulfur compounds: implication in the apoptosis induction” *FEBS Journal* 275 (2008) 3884–3899.