Pharmacological Modulation of Reactive Oxygen Species in Cancer Treatment

Judit Ribas, Paolo Mattiolo and Jacint Boix*

Pharmacology Unit, Departament de Medicina Experimental, Universitat de Lleida / IRBLleida

*Corresponding author: Pharmacology Unit, Departament de Medicina Experimental, Universitat de Lleida / IRBLleida, Edifici de Biomedicina 1, Av. Rovira Roure 80, 25198-Lleida, Catalunya, Spain
Tel: +34 973702404, Fax: +34 973702291
Email: jacint.boix@mex.udl.cat
Abstract: Aerobic metabolism of mammalian cells leads to the generation of reactive oxygen species (ROS). To cope with this toxicity, evolution provided cells with effective antioxidant systems like glutathione. Current anticancer therapies focus on the cancer dependence on oncogenes and non-oncogenes. Tumors trigger mechanisms to circumvent the oncogenic stress and to escape cell death. In this context we have studied 2-phenylethinesulfoxamine (PES), which disables the cell protective mechanisms to confront the proteotoxicity of damaged and unfolded proteins. Proteotoxic stress is increased in tumor cells, thus providing an explanation for the anticancer selectivity of PES. In addition, we have found that PES induces a severe oxidative stress and the activation of p53. The reduction of the cell content in glutathione by means of L-buthionine-sulfoximine (BSO) synergizes with PES. In conclusion, we have found that ROS constitutes a central element in a series of positive feed-back loops in the cell. ROS, p53, proteotoxicity, autophagy and mitochondrial dynamics are interconnected with the mechanisms leading to cell death, either apoptotic or necrotic. This network of interactions provides multiple targets for drug discovery and development in cancer.

Keywords: Cell death, Cell experimental pharmacology, L-buthionine-sulfoximine, Mdivi-1, Oxidative stress, p53, 2-phenylethinesulfoxamine, proteotoxic stress.

BACKGROUND ON REACTIVE OXYGEN SPECIES

Free radicals are a concept of chemistry. A free radical is defined as an atom or molecule characterized by having unpaired valence electrons. Therefore it becomes a high-reactive entity with potential to interact and disrupt biological molecules, either the simplest (carbohydrates and lipids) or the most complex ones (nucleic acids and proteins). Free radicals are present in our environment as the result of chemical processes or ionizing radiation, for instance as a by-product of industrial activity. Alternatively, they can be produced in vivo as the result of the cellular metabolism.

Free radicals derived from oxygen are the most prominent in the cellular context. They are generated by the progressive reduction of molecular oxygen (O2) to finally yield H2O. In Fig. (1A), we show a classical scheme of this process plus the enzymes and reactions involved in vivo [1]. The ionic nature of superoxide anion (O2•−) confers cell membrane impermeability to this entity. It is neutralized and converted to hydrogen peroxide by the superoxide dismutase enzymes (SOD). The hydroxyl radical (OH•) is highly reactive and, therefore, harmful for biological molecules. It is generated by the Fenton and Haber-Weiss reactions that require the participation of transition metals, frequently iron (Fe) in a biological context. Finally, hydrogen peroxide (H2O2) is no strictly a free radical. However, its oxidant capacity added to its efficiency in crossing biological membranes define a highly toxic profile for H2O2. In addition H2O2 becomes the precursor of OH• radical via the Fenton and Haber-Weiss reactions mentioned above. These three molecules are collectively termed reactive oxygen species (ROS). The mitochondrial process of oxidative phosphorylation (OXPHOS) is the main source of ROS in the cells. The ATP yield of the OXPHOS process is greatly advantageous to eukaryotic cells but it implies intracellular toxicity by the generation of ROS. This ambivalent role of oxygen in life has been traditionally referred to as the “oxygen paradox”. However, there are other locations where ROS can be generated. For example, there is a subtle and regulated release of ROS at the cellular membranes mediated by NADPH oxidases (Nox family of proteins) and involved in cell signalling [2].
Massive ROS generation is harmful for the cells. ROS oxidise and disrupt essential molecules like lipids, proteins and nucleic acids. Consequently cells counterbalance ROS by means of detoxifying enzymes or molecules with chemical reducing activity [1]. As shown in Fig. (1A), \( \text{H}_2\text{O}_2 \) is the substrate of catalase (CAT), peroxiredoxins (PR) and glutathione peroxidase (GPx). Vitamin E (VitE) and C (VitC) are molecules with reducing properties in specific intracellular compartments, lipophilic for VitE and hydrophilic for VitC (Fig. 1B). The most relevant antioxidant molecule inside the cell is glutathione and this relevancy is even higher inside the mitochondrial matrix [3]. Because of its high intracellular concentration, reduced glutathione (GSH) determines the intracellular reduction potential. Once oxidised, GSH becomes glutathione disulphide (GSSG). Glutathione reductase (GR) is the enzyme that regenerates GSH. This reduction is coupled to the oxidation of NADPH (Fig. 1B). GSH is a peptide composed of three amino acids, glutamate, cysteine and glycine. The enzyme glutamate-cysteine ligase (GCL), previously named \( \gamma \)-glutamylcisteine synthetase, catalyses the first step in the synthesis of GSH. Then glutathione synthetase catalyses the binding of glycine and, as a result, glutathione (\( \gamma \)-glutamylcisteinylglycine) is generated. Glutathione (GSH) participates in many intracellular processes (Fig. 1B). For instance it reduces the thiol groups of the oxidised proteins, the VitC and, indirectly, the VitE. GSH associates to GPx to transform \( \text{H}_2\text{O}_2 \) into \( \text{H}_2\text{O} \). GSH can be conjugated to drugs, thus defining a specific type of phase II reactions in the metabolism of drugs. Finally, it can also be conjugated to proteins, thus becoming the post-translational modification termed glutathionylation [3]. In a healthy cell, ROS and antioxidant resources are in a homeostatic equilibrium, the imbalance leads to oxidative damage and, eventually, to cell death. The loss of the homeostatic balance is designated as oxidative stress.

Fig. (1). (A) Graphical summary of the chemical process of \( \text{O}_2 \) reduction to \( \text{H}_2\text{O} \). The reaction catalysed by superoxide dismutase enzymes (SOD) is shown. The generation of hydroxyl radical (\( \text{OH}^- \)) by Haber-Weis and Fenton reactions is indicated. Finally, catalase (CAT), peroxiredoxin (PR) and glutathione peroxidase (GPx) are the enzymatic activities devoted to neutralize hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) inside the cells. (B) Scheme of the glutathione (GSH) redox cycle and its coupling to other redox cycles and conjugation reactions inside the cell. The ox- prefix denotes the oxidized form. Glutathione reductase (GR), glutathione peroxidase (GPx), and the superfamily of glutathione transferases (GT) are indicated close to the reactions they catalyse.
CURRENT STRATEGIES IN CANCER TREATMENT

The beginning of the 21st century coincided with the commercialization of Imatinib mesylate (Gleevec®). Imatinib is an inhibitor of ABL tyrosine kinase. The function of ABL is to promote the survival of cells subjected to genotoxic stress. The reciprocal translocation that generates the Philadelphia chromosome is characteristic of chronic myeloid leukemia (CML). The translocation produces a fusion protein (BCR-ABL) and an abnormal increase of ABL activity. The cells of CML become dependent on ABL activity to circumvent cell death by apoptosis. Therefore, Imatinib triggers apoptosis of CML cells and an impressive clinical outcome in CML patients [4,5]. Moreover, the specificity of Imatinib for ABL is not absolute. Imatinib also inhibits the c-KIT kinase that is relevant to the development of the gastrointestinal stromal tumor (GIST). Therefore, Imatinib has also become a successful treatment for GIST [6]. This type of therapeutic strategy has been designated as cancer specific, because the drug targets one or a few types of tumor exclusively, as exemplified by Imatinib. This strategy has also been labelled as personalised therapy of cancer because, for instance, among the patients with gastric cancer, only those with GIST are responsive to Imatinib. The common trait underlying this strategy is the dependence on one specific oncogene of one specific cancer cell (Fig. 2). This evokes the phenomenon of addiction and, consequently, the “oncogene addiction” allegory has met some success to term this concept. Oncogenesis implies many disruptions in the cell homeostasis that incline cells to die, frequently via apoptosis. This cell predisposition uncovers a cellular stress that can be referred to as oncogenic stress. Therefore, the recruitment of antiapoptotic and other pro-survival mechanisms are needed for the progression and success of the oncogenic process. Actually, these pro-survival mechanisms become a druggable weakness, what has lead to the metaphor that “Achilles heels” exist in cancer cells.

Trastuzumab, a humanized monoclonal antibody against HER protein in breast cancer, Gefitinib and Erlotinib in those non-small cell lung cancers (NSCLC) with mutated EGF receptor, or Crizotinib for NSCLC with the fusion protein EML4-ALK, are examples of drugs directed to the “Achilles heel” of specific types of tumors, in clinical use presently [7]. Still in an experimental phase, the possibility of a combined drug therapy emerges. For instance, the synergistic association of ABT-737 and Roscovitine or its R-enantiomer (Selecticlib) [8,9]. Many tumor cells are dependent on the antiapoptotic Bcl-2 gene to circumvent the oncogenic stress. This stress can be caused by the increase of some of the BH3-only proteins, which are pro-apoptotic and antagonistic of Bcl-2 (Fig. 2). In this context, specific inhibitors of Bcl-2 like ABT-737 are of great interest. However, it has been found that cells become resistant to ABT-737 by overexpressing Mcl-1, a short-lived, antiapoptotic protein of the Bcl-2 family. Mcl-1 is not inhibited by ABT-737. In this context, the inhibition of CDK9 and the subsequent transcriptional elongation phenomenon by Roscovitine/Selecticlib promotes an early and fast decay of the Mcl-1 protein [10]. Therefore, ABT-737 and Roscovitine/Selecticlib can be successfully combined to kill cancer cells selectively (Fig. 2).

Stressed cells rely on homeostatic mechanisms such as the chaperone activity of heat shock proteins (HSP). The same holds true for tumors that are naturally exposed to oncogenic stress. These homeostatic mechanisms are not intrinsically oncogenic but a response to the oncogenic stress and, therefore, the concept of non-oncogene dependence or “non-oncogene addiction” of cancer can be proposed (Fig. 2). The best examples of drugs following this strategy are the inhibitors of HSP70 (PES) and HSP90 (Geldanamycin). HSP70 and 90 prevent misfolding and aggregation of proteins due to their chaperone activity. In addition, HSP70 is involved in
the autophagic clearance of proteins either misfolded or aggregated. PES (2-phenylethinesulfonamine) has proven to be quite selective at killing cancer cells by promoting proteotoxic stress and causing a necrotic type of cell death [11,12]. This fact has stimulated the research in this category of innovative drugs [13–15].

There are other cellular phenomena that can be harnessed to fight cancer. Cells can be induced to terminally differentiate or to enter senescence. Both processes imply a cytostatic effect, i.e. cell quiescence and the stop of the cancer growth. Nevertheless killing cancer cells, i.e. a cytocidal effect, seems the most direct approach to achieve cancer regression. There are different types of cell death that are periodically classified and catalogued [16]. In brief, type 1 is apoptosis, which morphological and molecular definition is precise. Type 2 is autophagic cell death, which is considered a misnomer. Autophagy is frequently found in dying cells, but it is rarely involved in causing the cell demise. On the contrary, autophagy is essentially helping the cells to get rid of protein aggregates, damaged organelles and subsequent ROS production. Type 3 is necrosis, which is characterized by membrane disruption and the spillage of the cell content. Because of this spillage, necrosis has the ability to promote immunity, strong inflammatory responses and tissue disruption. This fact has traditionally been considered negative when compared to the silent, self-contained apoptotic process. However, apoptosis is not so self-contained. Some apoptotic cells expose Calreticulin at the cell surface and secrete ATP and HMGB1 protein. These events are highly immunogenic and have proved to be crucial for succeeding in causing tumor regression with apoptosis-inducing agents. This is the case of Doxorubicin, Cyclophosphamide, Bortezomib or γ-irradiation [17,18]. Accordingly, the interest of the agents that induce necrotic cell death should be reconsidered.

![Scheme illustrating how the oncogenic process sets the cells in a state of stress that predisposes to cell death, for example by an elevated ROS content and oxidative stress. To avoid their demise, cells follow two different strategies: (1) Oncogene dependence. (2) Non-oncogene dependence. This allows the identification of targets for drug development. This approach is supported by the success of drugs such as Imatinib, Crizotinib and other, presently and routinely used in patient treatment.](image)

**OXIDATIVE STRESS, p53 AND CANCER THERAPEUTICS**

In Fig. (2), elevated ROS are part of the cellular stress derived from oncogenesis. In other words, oxidative stress becomes part of the oncogenic stress of a cell. Is this true for all cancer cells? Is this an “Achilles heel” to be exploited in the treatment of a few, many or most tumors? The answers are subject to controversy. For
instance, inactivating mutations and deletions of p53 are the most common event in human cancer [19,20]. As a tumor suppressor, p53 senses many different stresses of the cell and triggers a plethora of responses, ranging from cell cycle stop to apoptotic cell death [21]. In spite of being a transcription factor, p53 can activate either transcriptional or non-transcriptional responses [22]. For instance, its translocation to mitochondria to directly activate the mitochondrial outer membrane permeabilisation (MOMP) and apoptosis [23]. The search for a drug with the ability to block this translocation was the aim of the research that led to the discovery of PES. This fact explains the alternative designation of PES as pifithrin-μ [24]. Among the genes regulated by p53 is TIGAR (TP53-induced glycolysis and apoptosis regulator). This gene connects p53 and the regulation of glucose metabolism and ROS. TIGAR promotes the redirection of glucose towards the pentose phosphate shunt and, therefore, increases the production of reduced NADPH [25]. Consistently, a reduced function of TIGAR provides a good explanation for the increased ROS phenotype of tumor cells with a defective function of p53. In this context, the ROS threshold hypothesis for cancer therapy was formulated. In brief, tumor cells suffer from oxidative stress to a greater extent than non-tumor ones and, as a consequence, they are closer to the threshold of cell death induction by ROS. Accordingly, the therapeutic agents with the ability to increase ROS will preferentially harm the tumor cells [26]. Indeed, broadly-use therapies such as irradiation and anthracyclines act, in part, by increasing ROS in cancer cells. Particularly, the glycopeptide bleomycin has a mechanism of action directly based on ROS generation and subsequent DNA damage and fragmentation. The recently approved arsenic trioxide is also a direct producer of ROS in cancer cells. Regarding cancer therapy, several potential new drugs with the ability to promote oxidative stress have reached clinical phases of development (Table 1).

**Table 1. ROS modulating agents undergoing clinical trials in oncology [27]**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOV-002</td>
<td>A form of oxidised glutathione (GSSG) that promotes intracellular GSH/GSSG imbalance, therefore mild oxidative stress and protein glutathionylation</td>
</tr>
<tr>
<td>BSO (L-buthionine-sulfoximine)</td>
<td>Inhibitor of glutamate cysteine ligase (GCL) that causes the synthesis of GSH to be impaired. In this inhibition context, GSH can be depleted by oxidative stress</td>
</tr>
<tr>
<td>Canfosfamide</td>
<td>Inhibitor of glutathione transferase (GT) activity</td>
</tr>
<tr>
<td>Ezatiostat hydrochloride</td>
<td>Inhibitor of glutathione transferase (GT) activity</td>
</tr>
<tr>
<td>Imexon</td>
<td>Pro-oxidant molecule able to deplete cells from GSH</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>Acetaldehyde dehydrogenase inhibitor, classical inducer of alcohol intolerance and pro-oxidant of GSH</td>
</tr>
<tr>
<td>PX-12</td>
<td>Inhibitor of thioredoxin-1, which is overexpressed in tumors with an aggressive phenotype</td>
</tr>
<tr>
<td>Dimesna</td>
<td>Dual inhibitor of thioredoxin-1 and glutaredoxin by an ill-defined mechanism</td>
</tr>
<tr>
<td>Motexafin gadolinium</td>
<td>Dual inhibitor of thioredoxin reductase and ribonucleotide reductase</td>
</tr>
<tr>
<td>Darinaparsin</td>
<td>Arsenic derivative</td>
</tr>
</tbody>
</table>

None of the above mentioned compounds displays the type of anticancer specificity observed in the treatments with Imatinib, Crizotinib, etc. They are promising agents but behave as non-selective chemotherapeutic drugs. There are two important caveats concerning this therapeutic approach. First, cancer cells are heterogeneous inside
a tumor and, as a consequence, some cells can be far away from the lethal threshold of ROS. Second, the hypoxic and nutrient-poor tumor environment selects for cells with increased antioxidant capacity, i.e. elevated GSH content. This GSH-rich phenotype is associated with the proficiency of the cancer cells to metastasize [28]. In conclusion, a ROS-inducing treatment could be overtly partial and promote a more aggressive phenotype. In our opinion, the possibility to be specific or succeed in cancer treatment simply by increasing ROS is scarce. However, we are not so reluctant about the possibility to combine drugs that encompass oxidative stress, as we will discuss below.

Initially observed in 1924 by Otto Warburg, the Warburg effect has lately attracted a great attention in oncology [29]. The effect consists in the shift of glucose metabolism from OXPHOS to lactate production in an oxygen-rich context. Therefore, it has been described as aerobic glycolysis. This phenomenon has provided the basis of an imaging technique for the diagnosis and follow-up of some tumors. This technique comprises cell labelling with $^{18}$F-deoxyglucose and positron emission tomography (PET) scanning. Unfortunately, to date, no similar applications have succeeded in the area of cancer therapy. One obvious question is how the Warburg effect impinges on the intracellular ROS content. The answer is that the amount of ROS generated by the OXPHOS process will be greatly reduced. Therefore, the Warburg effect does not help to explain why many cancer cells display increased ROS. Moreover, it seems to oppose the drugs that induce oxidative stress thus easily explaining the development of resistance. Finally, as mentioned above, it can be associated with the GSH-rich phenotype found in metastasis.

The relationship of p53 and ROS is paradoxical because, in opposition to TIGAR, some of the genes regulated by p53 generate ROS. This is the case of PIG3 (quinone oxidoreductase) and PIG6 (proline oxidase). Under mild p53 activation, cell cycle stop and antioxidant activity (TIGAR) would be promoted to ease the reparation of the damage in cellular DNA. This is consistent with the antioxidant role of the Warburg effect, that has been conceived as a strategy to increase the efficiency of DNA replication [29]. Conversely, under strong p53 activation, cell death and ROS would prevail [30]. The possibility to reactivate p53 in cancer cells has traditionally been approached with great interest [31]. Some drugs act by counteracting some inactivating mutations of p53. Others, like Nutlin-3, increase p53 activity in tumors like human neuroblastoma that are usually characterized by a preserved p53 response [32]. Drugs acting as agonists of p53 will be expected to cause MOMP, apoptosis and ROS production. On the other hand, the generation of ROS will cause DNA damage and p53 activation. In conclusion, ROS and p53 are engaged in a positive feed-back loop. Moreover, ROS can trigger either MOMP and apoptosis or the opening of the mitochondrial permeability transition pore (MPTP) and necrosis. Both, apoptosis and necrosis are inducers of ROS. Again, this is a positive feed-back that drives the cells to their demise. The phenomenon of MOMP is under the control of the Bcl-2 family of proteins, consequently the pharmacological modulation of this event is possible by means of drugs like ABT-737, which neutralises some of the antiapoptotic members. Finally, in some experimental paradigms, p53 is involved in triggering necrotic cell death and ROS are clearly involved [33]. Taken these facts altogether, a complex net of mutual interactions can be considered (see Fig. 5).

**CELL DEATH INDUCTION BY THE ASSOCIATION OF PROTEOTOXIC AND OXIDATIVE STRESS**
The screening of chemical libraries to find specific inhibitors of the translocation of p53 to mitochondria allowed the discovery of PES [24]. Consistently, PES protected cells from some death stimuli mediated by this translocation. Surprisingly, PES proved to be very lethal for neoplastic cells [11,12]. PES is a small molecule with chemical traits for being a highly reactive and oxidant agent (Fig. 3A). A characterization of its mode of action revealed its ability to block HSP70 function in vivo and as a consequence: i) the secondary inhibition of HSP90 and the proteasome; ii) the blockage of the chaperone-mediated type of autophagy, which results from HSC70 inhibition; iii) the impairment of the completion of macroautophagy (named autophagy hereafter) [12,34]. The implications for the cells are severe because four pivotal mechanisms to eliminate misfolded and aggregated proteins are lost simultaneously. We have consistently observed how multilamellar structures accumulate in the cells treated with PES, as the result of blocking the autophagic flux and the subsequent lack of autophagosome clearance [35,36]. The process ends up in a necrotic phenotype with the rupture of the cell membrane [36]. At earlier stages we can see the depositions of a proteinaceous material, named aggresomes, which indicates damaged proteins in aggregated clumps (Fig. 3B). We can also observe different stages of mitochondrial damage, i.e. a variable degree of cristae disruption in swollen mitochondria (Fig. 3B). In conclusion, proteotoxic stress is a clear outcome of PES treatment but the downstream mechanisms leading to cell death remain ill-defined. The hypothesis about cancer cells being closer to the lethal threshold of proteotoxicity than normal ones provides a good explanation for the higher susceptibility of cancer cells to PES. Consistently, we have experimentally determined that cells genetically deficient in autophagy are more sensitive to PES (unpublished results). Undoubtedly, the deficit in autophagy raises the proteotoxic stress in these cells.
Fig. (3). (A) Chemical structure of 2-phenylethynesulfonamide (PES), named also Pifitrin-µ. (B) Transmission electron microscopy of HCT116 cells treated for 48 hours with PES (25 µM). Nuclear chromatin (Nu). Mitochondria at successive stages of cristae disruption (m). Deposits of proteinaceous material in the cytoplasm defined as aggresomes (delimited by arrowheads). (C) HCT116 cells were treated as indicated in the graph with PES, the antioxidant N-acetylcysteine (NAC) or the combination of both for 1 hour. ROS detection by means of the DCF-DA reagent was performed. Cells were incubated with DCF-DA, treated afterwards and flow cytometry was finally used to quantify the percentage of cells that were fluorescent by containing the oxidised DFC. The bar values are the mean ± SEM of several independent determinations.

The cellular effects of PES were consistent with an early induction of ROS, which was reversed by a reducing agent like N-acetylcysteine (Fig. 3C). This reversion translated into an increased cell survival at longer times of treatment [36]. The quantification of intracellular ROS is routinely performed by the reagent 2′,7′-dichlorofluorescein diacetate (DCF-DA). The compound enters the cells and is processed to DCF by the intracellular esterases, thus becoming captured inside the cell. Then DCF fluoresces upon oxidation and in proportion to the amount of intracellular ROS. In conclusion, a remarkable oxidative stress was involved in the mode of action of PES. This fact is important because ROS react with proteins and cause proteotoxic stress. The relative amount of proteotoxicity caused by ROS or, alternatively, by HSP70 inhibition in a cell treated with PES is unknown and not easy to discern. The concentrations of PES required to inhibit HSP70 in vitro are higher than those required in vivo. Therefore, the molecular details of HSP70 inhibition by PES remain unclear [37]. The early rise of ROS supports our speculation about a role for PES-triggered oxidative stress in HSP70 inhibition in vivo. In addition we have demonstrated that p53 is involved in the necrotic cell death triggered by PES [36]. Moreover, we conclude that a positive feed-back between ROS and p53 is leading the cells to their demise.

BSO (L-buthionine-sulfoximine) is a small molecule with an amino acid structure (Fig. 4A). BSO is a pharmacological inhibitor of the enzyme GCL and, therefore, it is able to block the synthesis of GSH in the cells. As a consequence, the cells lose their reduction potential and become more susceptible to oxidative stress. The combination of PES and BSO potentiates the generation of ROS (Fig. 4B). The combination of PES and BSO is synergic at inducing death in several cell lines [36]. This synergism provides another evidence of the involvement of ROS in the PES mode of action. Furthermore, it suggests a therapeutic opportunity. BSO is characterized by being minimally toxic for cells in culture unless other cellular insults coincide. Consistently, in clinical trials, BSO shows minimal toxicity for humans [38]. The association of BSO and conventional chemotherapy has been investigated in the past. For instance, in ovarian cancer BSO shows synergism with melphalan and cisplatin [39,40]. Similarly, in colon and hepatic carcinoma, BSO and azathioprine synergise [41]. In spite of these reported synergisms, BSO has still not reached clinical use. The synergism of BSO and PES is promising. It will allow the reduction of the doses of PES without reducing the ratios of cell death. Undoubtedly, a reduced dose of PES will translate into minimising its side effects. However, many questions remain. Will the tumor selectivity of PES be maintained in association with BSO? What are the toxic effects of PES in humans? How severe can these side effects be? Notwithstanding these questions, published evidence supports the interest of combining two drugs that cause proteotoxic and oxidative stress respectively [42].

In our studies on PES, we found the paradoxical result of Bax protein displaying a pro-survival function [35]. Bax and Bak are pro-apoptotic members of the Bcl-2 family of proteins. Both proteins are pivotal elements
in the MOMP that leads to apoptosis. Bax is known to be involved in the regulation of mitochondrial dynamics, i.e. the state of fusion or fission of mitochondria [43]. This fact prompted us to explore this issue. As a tool we used the compound Mdivi-1, which promotes mitochondrial fusion. Mdivi-1 is an inhibitor of the GTPase activity of DRP-1, the protein in charge of the mitochondrial fission process [44]. We found that mitochondrial fusion had a protective effect in cells treated with PES [35]. It is known that mitochondria respond to oxidative stress by fusion. Mitochondrial fusion is interpreted as a mechanism to mix the contents of healthy and damaged mitochondria. This allows the compensation of the damaged content, such as oxidised mitochondrial DNA, by undamaged one. [45]. Our results are coherent with this explanation since PES was inducing oxidative stress and the promotion of mitochondrial fusion was mitigating the toxicity of PES.

\[ \text{Fig. (4). (A) Chemical structure of L-buthionine-sulfoximine (BSO). (B) U87MG cells were treated as indicated in the graph with PES, BSO or the combination of both for 6 hours in 96 multiwell plates. ROS detection was performed by the DCF-DA procedure, as before. However, the fluorescence of oxidised DFC was recorded by means of a multiwell plate reader. The readings were referred to the untreated condition, which was assigned the unit value. The y-axis is expressed as folds over the unit value. The bar value is the mean ± SEM of 5-6 determinations.} \]

In conclusion, we envisage ROS as a converging node susceptible of direct (BSO) and probably indirect (PES) pharmacology. We have previously commented the positive feed-back between ROS and p53, ROS and apoptosis, and ROS and necrosis (Fig. 5). PES illustrates another positive feed-back between proteotoxicity and ROS. On one hand, ROS causes chemical modifications, subsequent misfolding and aggregation of proteins. On the other hand, proteotoxic stress associated to PES translates into oxidative stress. Moreover, the failure of autophagy is also a facet of the PES mode of action. Autophagy is considered a mechanism of cell defence. Autophagy alleviates cells from protein aggregates and damaged mitochondria. Finally, mitochondrial dynamics adds another dimension to the scheme. Mitochondria fuse to minimise the effects of ROS. However, giant fused mitochondria elude autophagy. Apoptosis is associated to mitochondrial fission by stimulating the complete proteolysis of the protein OPA-1 and OPA-1 is necessary to accomplish the fusion of the inner mitochondrial membrane [45]. Taken these facts altogether, a complex network of interactions is envisaged (Fig. 5). This
scheme indicates several targets to be investigated by experimental pharmacologists. In addition, the network summarises the mode of action of PES and highlights the involvement of ROS.

**Fig. (5).** Scheme illustrating how ROS are a central node that establish feed-back positive loops with prototoxic stress, p53 and the mechanisms leading to cell death, either apoptotic or necrotic. Mitochondrial outer membrane permeabilisation (MOMP) is mediating apoptosis and under regulation by the Bcl-2 family of proteins. Based on our results, the participation of autophagy and mitochondrial dynamics (state of mitochondrial fission or fusion) has been included in the network of interactions. The existence of drugs targeting these events, like PES, is symbolized by thunderbolts. These drugs are potential subjects for experimental pharmacology of cancer. Activation is indicated by arrows. Inhibition is depicted as a bar capped line. The dashed line means no conclusive evidence.

**CONFLICT OF INTEREST**
No conflicts of interest are disclosed.

**ACKNOWLEDGEMENTS**
Research in the Pharmacology Unit is funded by project SAF2011-29730, MINECO (Ministerio de Economía y Competitividad), Spanish Government. Paolo Mattiolo is supported by a FI fellowship from AGAUR (Generalitat de Catalunya).

**REFERENCES**


