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Mitochondrial permeability transition induced by novel pyridothiopyranopyrimidine derivatives: Potential new antimitochondrial antitumour agents

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ABSTRACT

New pyridothiopyranopyrimidine derivatives (PTP1 and PTP2) were synthesised. Evaluation of the antiproliferative activity showed a significant capacity of the two compounds to inhibit cell growth. Investigation of the mechanism of action reveals that PTP1 interferes with the mitochondrial functions by inducing both swelling of the mitochondrial matrix and collapse of the electrical potential. These phenomena are fully prevented by typical inhibitors of the mitochondrial permeability transition, and are accompanied by the release of cytochrome *c* in the cytosol. The estimation of the redox state of thiol groups and glutathione suggests that the induction of permeability transition mediated by PTP1 is the result of an oxidative stress. The ability of cyclosporin A to prevent the antiproliferative effect of PTP1 indicates the induction of mitochondrial permeability transition as the molecular event responsible for the inhibition of cell growth. PTP1 also induces DNA fragmentation in intact cells.

As regards PTP2, the presence of the *p*-toluensulphonamido group makes the lead chromophore unable to induce any effect on mitochondria.

1. Introduction

In the development of new anticancer agents, DNA has been widely considered the molecular target of preference, due to the crucial role that it plays in the cell division process [1]. As a consequence, most of the currently available anticancer drugs directly affect the macromolecule.

Nevertheless, the need to overcome certain drawbacks associated with conventional chemotherapy has stimulated the requirement to identify novel anticancer agents characterised by a different mechanism of action.

Several traditional anticancer drugs have been thought to exert their antiproliferative effect by inducing apoptosis pathways upstream of the mitochondria.

It has been proved that in mitochondrial-mediated apoptosis, a central role is played by mitochondrial permeability transition (MPT), a process that leads to an increase in inner membrane permeability to solutes with a molecular mass up to 1500 Da. This phenomenon is regulated by the opening of a large conductance channel known as the permeability transition pore. Opening of this non-selective pore provokes the dissipation of inner transmembrane potential ($\Delta\Psi$), matrix swelling and outer membrane disruption, thus leading to the release of a caspase activator, such as cytochrome *c*, which triggers the proapoptotic pathway leading to DNA fragmentation [2].

In this regard, it is to be emphasised that a primary insult, like a DNA adduct, which takes place after chemotherapy, can

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cause apoptotic cell death. In particular, in the absence of DNA repair, an increase in p53 levels occurs. This proapoptotic protein induces an increase in the levels of Bax, a mammalian cell death protein targeting mitochondrial membranes, which moves to mitochondria, causing cytochrome *c* release. Once in the cytoplasm, cytochrome *c* interacts with additional proteins to form the so-called ''apoptosome''. The apoptosome implies the participation, besides cytochrome *c*, of APAF-1 (which binds ATP) and caspase-9, which together activate another component, pro-caspase-3. This induces the activation of caspase-3, which cleaves and activates DNA fragmentation factors (DFF), which in turn subsequently activate endonucleases. All this produces in the cell the biochemical and morphological changes recognised as apoptosis [2,3].

One of the main causes of treatment failure in oncology is the development of chemoresistance. Multiple drug-resistant mechanisms have been identified, including defects in the regulation of apoptosis [4,5].

Chemoresistant cells often demonstrate the ability to neutralise the signals that converge to mitochondria. It has been demonstrated that loss of Bax in small cell lung cancer increases resistance to some chemotherapeutic agents [6], and reduced levels have been associated with poor responses to chemotherapy and shorter overall survival in patients with metastatic breast cancer [7]. Furthermore, loss or mutation of p53 seems to provoke a less favourable response to chemotherapy, at least in some cancers and in response to some drugs [8].

In this connection, the identification of antiproliferative agents, which directly affect mitochondria can assume great interest in the development of improved anticancer strategies [9], and at present a number of studies underline the pivotal role of these organelles in the field of antitumour approaches. Indeed, the ability of promising pre-clinical compounds to affect mitochondrial function has been demonstrated [10], and furthermore, targeting the mitochondria seems to offer new advances in the treatment of multiple myeloma [11].

Alterations of the mitochondria functions are targeted by several chemotherapeutic agents. Interestingly, some reports have demonstrated that certain DNA intercalating agents can inhibit mitochondrial DNA synthesis [12,13] with a mechanism, which might involve a preferential accumulation in mitochondria [14] rather than in the nucleus, leading to the disruption of mtDNA replication. In addition, both type I and II topoisomerases have been identified in mitochondria, and some topoisomerase II-targeted anticancer drugs, such as etoposide or amsacrine, have revealed mitochondrial inhibitory properties [15] with a mechanism which seems to be related to permeability transition pore formation [16]. These classes of anticancer drugs, which exert a determinant action on mitochondria in addition to their primary cytosolic or nuclear effects, are structurally related, since they are all characterized by an appropriately substituted planar polycyclic chromophore. In this field, we have carried out extensive studies on several heterocyclic ring systems, with the aim of developing new DNA intercalating and/or topoisomerase targeting agents [17–19]. As a part of this screening programme, we recently designed the synthesis of a planar pyridothiopyrano[4,3-*c*]pyrazole [20] which, when evaluated on two human tumour cell lines, showed a detectable cytotoxic activity, thus appearing to offer an interesting chromophore system. These results prompted us to synthesise the new tricyclic derivative PTP1 (Fig. 1), characterised by the homologous pyridothiopyranopyrimidine nucleus, bearing an amino group in the 2-position. In this fused system, a pendant *p*-toluensulphonamido group was also introduced (compound PTP2, Fig. 1), to enhance the DNA-binding properties of the scaffold and/or its antiproliferative activity [21,22].

The ability of the new derivatives to exert an antiproliferative activity was evaluated by means of an inhibition growth assay on human tumour cell lines. Linear flow dichroism experiments were performed to assess the occurrence of a molecular complex with DNA. The effect on isolated rat liver mitochondria (RLM) was evaluated and the ability to induce MPT via an oxidative stress is discussed. The effect of cyclosporin A (CsA) on cells makes it possible to correlate the induction of MPT with the antiproliferative activity. Finally, the DNA fragmentation assay was carried out to assess the occurrence of apoptosis.

2. Materials and methods

2.1. *Chemical synthesis*

The synthetic procedure leading to the target 2-amino-5*H*-pyrido [3′,2′;5,6]thiopyrano[4,3-*d*]pyrimidine PTP1 and to the 2-*p*-toluensulphonamido substituted derivative PTP2 (Fig. 1) involved, as the starting key compound, 2,3-dihydro-3-dimethylaminomethylene thiopyrano[2,3-*b*]pyridin-4(4*H*)-one, whose preparation has been previously described by us [23]. Guanidine hydrochloride, *p*-toluensulphonylchloride and triethylamine were commercially available from Sigma–Aldrich.

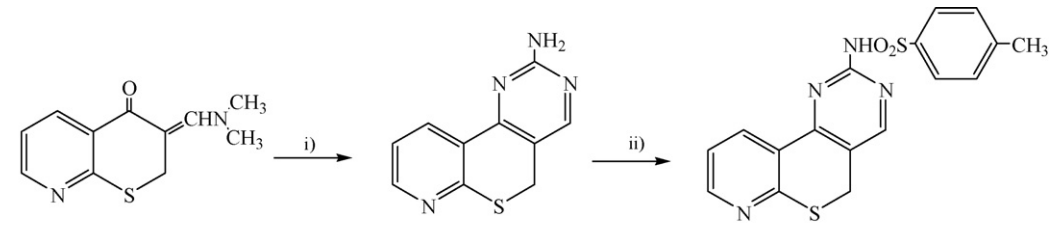

Fig. 1 – Synthetic method used to prepare new pyridothiopyranopyrimidine derivatives PTP1 and PTP2: (i) guanidine hydrochloride, EtONa/EtOH, 100 °C, 6 h; (ii) *p*-toluensulphonylchloride, Et_3N, anhydrous toluene, 110 °C, 21 h.

The crude target compounds obtained (PTP1 and PTP2) were purified by crystallization, and their proposed structures were assigned on the basis of analytical and spectral data, which relied on those of earlier studies performed by our group [23].

Infrared spectra were obtained on a NICOLET/AVATAR, 360 FT spectrophotometer as Nujol mulls. ^{1}H NMR spectra were recorded on a Varian Gemini 200 spectrometer, in dimethyl-d_6 sulphoxide solution, using TMS as the internal standard. Mass spectra were obtained on a Finningan Polaris/GCQ Plus spectrometer using an electron beam energy of 70 eV. Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminium sheets (60 F-254). Elemental analyses were performed by our Analytical Laboratory.

2.1.1. *2-Amino-5H-pyrido[3′,2′;5,6]thiopyrano[4,3-d]pyrimidine (**PTP1**)*

Guanidine hydrochloride (0.160 g, 1.70 mmol) was added, at room temperature, under a nitrogen atmosphere, to a stirred solution of sodium ethoxide (2.55 mmol of sodium in 8 mL of anhydrous ethanol). The resulting suspension was stirred at room temperature for 15 min, and then 2,3-dihydro-3-dimethylamino methylenethiopyrano[2,3-*b*]pyridin-4(4*H*)-one (0.187 g, 0.85 mmol) was added, and the reaction mixture was refluxed for 6 h. After cooling, the resulting suspension was concentrated under a reduced pressure and the residue obtained was washed with water and collected to give crude pyrimidine PTP1, which was purified by recrystallization from ethanol. Yield = 60%; m.p. 195–197 °C; ^{1}H NMR: δ = 4.08 (s, 2H; CH_2S); 6.75 (s, 2H; NH_2 exch. with D_2O); 7.32 (dd, 1H; ArH); 8.27 (s, 1H, ArH); 8.40 (d, 1H, ArH); 8.46 (d, 1H; ArH). IR: ν = 3320, 3180, 1640, 1585, 1570, 1345, 1215, 1060, 790, 760 cm^{-1}; MS: *m*/*z* (%): 216 (60, M^+), 215 (100, $M^+ - H$). Elemental analysis: calculated (%) for $C_{10}H_8N_4S$ (216): C 55.55, H 3.70, N 25.93; found: C 54.95, H 4.01, N 25.76. Purity > 99%.

2.1.2. *N-[5H-Pyrido[3′,2′;5,6]thiopyrano[4,3-d]pyrimidin-2-yl]-p-toluensulphonamide (PTP2)*

The derivative PTP1 (0.216 g, 1.00 mmol) was added, at room temperature, to a stirred solution of *p*-toluensulphonylchloride (0.195 g, 1.00 mmol) in anhydrous toluene (5 mL); subsequently, triethylamine (0.2 mL) was added and the reaction mixture was refluxed, under stirring for 21 h. After cooling, the insoluble triethylamine hydrochloride was filtered off and the solution obtained was concentrated *in vacuo* to dryness. The residue obtained was purified by recrystallization from ethanol, to give compound PTP2. Yield = 49%; m.p. 187–191 °C; ^{1}H NMR: δ = 2.28 (s, 3H; CH_3); 4.15 (s, 2H; CH_2S); 7.11 (d, 2H; ArH); 7.35–7.45 (m, 1H; ArH); 7.47 (d, 2H; ArH); 8.38 (s, 1H; ArH); 8.43–8.49 (dd, 1H; ArH); 8.53–8.56 (dd, 1H; ArH). IR: ν = 3330, 3170, 1670, 1630, 1240, 1165, 1120, 1030, 820, 780 cm^{-1}; MS: *m*/*z* (%): 371 (5, M^+), 91 (100). Elemental analysis: calculated (%) for $C_{17}H_{14}N_4O_2S_2$ (371): C 55.14, H 3.78, N 15.13; found: C 54.21, H 4.25, N 14.87. Purity > 99%.

2.2. *Inhibition growth assays*

HL-60 (human myeloid leukaemic cells) and HeLa (human cervix adenocarcinoma cells) were grown in RPMI 1640 (Sigma Chemical Co.) supplemented with 15% heat-inactivated fetal calf serum (Biological Industries) and in Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal calf serum (Biological Industries), respectively. One hundred units per milliliter penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma Chemical Co.) were added to both media. The cells were cultured at 37 °C in a moist atmosphere of 5% carbon dioxide in air.

HL-60 cells (4×10^4) were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, various concentrations of the test agents were added to the complete medium and incubated for a further 72 h. HeLa (4×10^4) cells were seeded into each well of a 24-well cell culture plate. After incubation for 24 h, the medium was replaced with an equal volume of fresh medium, and various concentrations of the test agents were added. The cells were then incubated in standard conditions for a further 72 h.

For the experiments in the presence of cyclosporin A, after incubation for 24 h, HeLa cells were treated with 2 μM cyclosporin A for 20 min; the medium was replaced with an equal volume of fresh medium, 15 μM of test agent was added and the cells were incubated for a further 48 h.

A trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as IC_{50} values, i.e. the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

For the microphotographs, cell cultures were washed with PBS buffer, fixed with 3.7% formaldehyde in PBS and then examined under an optical microscope (Leitz DMIRB, Leica).

2.3. *Linear flow dichroism*

Linear dichroism (LD) measurements were performed on a Jasco J500A circular dichroism spectropolarimeter converted for LD and equipped with an IBM PC and a Jasco J interface.

Linear dichroism is defined as:

$$LD_{(\lambda)} = A_{\parallel(\lambda)} - A_{\perp(\lambda)}$$

where $A_\parallel$ and $A_\perp$ correspond to the absorbances of the sample when polarized light is oriented parallel or perpendicular to the flow direction, respectively. The orientation is produced by a device designed by Wada and Kosawa [24] at a shear gradient of 500–700 rpm and each spectrum was accumulated four times.

Salmon testes DNA was purchased from Sigma Chemical Co. (D-1626). The DNA concentration was determined using an extinction coefficient of 6600 M(p)$^{-1}$ cm^{-1} at 260 nm. A solution of salmon testes DNA (1.5×10^{-3} M) in ETN buffer (containing 10 mM Tris, 10 mM NaCl, and 1 mM EDTA, pH 7) was used. Spectra were recorded at 25 °C at [drug]/[DNA] = 0, 0.02, 0.04 and 0.08.

2.4. *Mitochondrial isolation and standard incubation procedures*

Rat liver mitochondria were isolated by conventional differential centrifugation in a buffer containing 250 mM sucrose, 5 mM HEPES (pH 7.4) and 1 mM EGTA [25]; EGTA was omitted from the final washing solution. Protein content was measured by the biuret method with BSA as a standard [26].

Mitochondria (1 mg protein/mL) were incubated in a water-jacketed cell at 20 °C. The standard medium contained 250 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM succinate and 1.25 μM rotenone. Variations and/or other additions are given with each experiment.

2.5. Determination of mitochondrial functions

Membrane potential ($\Delta\Psi$) was calculated on the basis of distribution of the lipid-soluble cation tetraphenylphosphonium (TPP^+) measured across the inner membrane, using a TPP^+-specific electrode [27]. Mitochondrial swelling was determined by measuring the apparent absorbance change of mitochondrial suspensions at 540 nm in a Kontron Uvikon model 922 spectrophotometer equipped with thermostatic control. The protein sulphydryl oxidation assay was performed as in Santos et al. [28]. The oxidation of glutathione was performed as in Tietze [29]. The redox state of endogenous pyridine nucleotides was followed fluorometrically in an Aminco-Bowman 4-8202 spectrofluorometer with excitation at 354 nm and emission at 462 nm.

2.6. Fe^{3+} reducing activity measurements

The ability of compounds PTP1 and PTP2 to reduce Fe^{3+} ions to Fe^{2+} was measured by means of a modified ferrozine method [30]. The reaction mixture consisted of 50 nM Na-acetate buffer (pH 5.4), 10 mM ferrozine, 250 μM compound to be tested (PTP1, PTP2 or quercetin) and 1 mM $FeCl_3$, in a final volume of 1 mL. The reaction was started by the addition of $FeCl_3$ and the increase of absorbance at 562 nm after 3 and 9 min was recorded using a control lacking ferrozine [31], by a Perkin-Elmer Lambda 15 Spectrometer. The Fe^{2+} concentration was determined by using an extinction coefficient for the Fe–$(ferrozine)_3^{2+}$ complex of 27900 M^{-1} cm^{-1} [32].

2.7. Detection of cytochrome c

Mitochondria (1 mg protein/mL) were incubated for 15 min at 20 °C in standard medium with the appropriate additions. The reaction mixtures were then centrifuged at 13,000 × *g* for 10 min at 4 °C to obtain mitochondrial pellets. The supernatant fractions were further spun at 100,000 × *g* for 15 min at 4 °C to eliminate mitochondrial membrane fragments, and were concentrated five times by ultrafiltration through Centrikon 10 membranes (Amicon) at 4 °C. Aliquots of 10 μL of the concentrated supernatants were subjected to 15% SDS-PAGE, and analysed by Western blotting using mouse anti-cyt *c* antibody (Pharmingen, San Diego, CA).

2.8. DNA isolation and electrophoresis

HeLa (5×10^5) cells were incubated in standard conditions for 24 h at 37 °C, and then the medium was replaced with an equal volume of fresh medium, and the test agent was added at the indicated concentration. Control cells were grown under the same conditions with the addition of the solvent alone. After 24 h of incubation, DNA from control and treated cells was extracted according to the procedure described by Sambrook et al. [33]. The isolated DNA was dissolved in TE buffer (Tris 10 mM, pH 8, EDTA 1 mM) and analysed by agarose (1%) gel electrophoresis. The gel was stained with ethidium bromide solution (1 μg/mL), transilluminated by UV light and fluorescence emission visualised using a CCD camera coupled to a Bio-Rad Gel Doc 1000 apparatus.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was applied to the data. Statistical analysis was performed with SPSS 10.0 [34]. All probabilities are two-tailed. Data were checked for normality and homogeneity of variance (Levene test). Differences between means were evaluated for significance by using Duncan's multiple range test (DMRT) ($P < 0.05$).

3. Results

3.1. Effects of the new derivatives on human tumour cell lines

The antiproliferative activity of the newly synthesised derivatives PTP1 and PTP2 was tested on two tumour cell lines, HeLa and HL-60. Table 1 shows the results expressed as IC_{50} values, i.e. the concentration (μM) of the compound causing 50% of cell death with respect to the control culture. Ellipticine and cisplatin were taken into account as reference drugs.

Compounds PTP1 and PTP2 appear to exert a significant and similar antiproliferative effect towards both cell lines taken into consideration. Nevertheless, even though the calculated IC_{50} values are significantly lower than that induced by ellipticine and cisplatin, both PTP1 and PTP2 induce the same maximum effect as reference drugs (data not shown). These results point to the pyridothiopyranopyrimidine PTP1 as a chromophore that possesses an antiproliferative capacity independently of the insertion of the *p*-toluensulphonyl group, since the presence of the latter does not influence the cellular effect.

3.2. Analysis of the ability to interact with DNA

In an attempt to establish the mechanism of action responsible for the antiproliferative activity of compounds PTP1 and

Table 1 – Cell growth inhibition in the presence of examined compounds PTP1 and PTP2

Compound	Cell lines IC_{50} (μM)[a]	
	HeLa	HL-60
PTP1	11.0 ± 0.6	18.2 ± 1.1
PTP2	16.3 ± 1.2	14.4 ± 0.9
Ellipticine	0.31 ± 0.01	0.64 ± 0.02
Cisplatin	0.84 ± 0.06	1.60 ± 0.08

Ellipticine and cisplatin were taken into account as reference compounds.

[a] Trypan blue exclusion was used to calculate the IC_{50} values. The mean values ± S.D. of at least four experiments are reported.

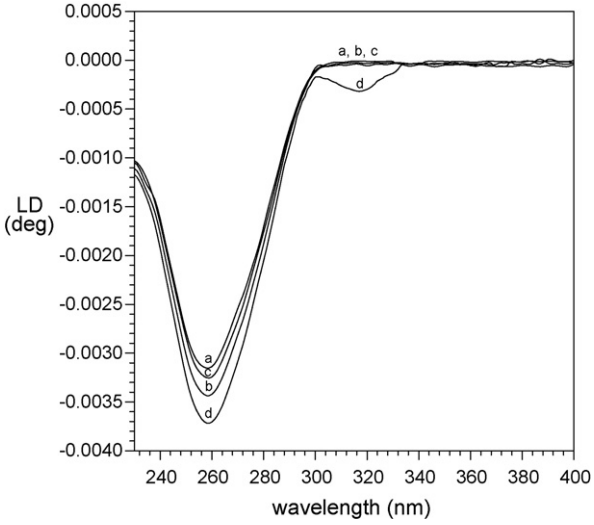

Fig. 2 – Linear flow dichroism spectra for DNA alone (trace a) and in the presence of compound PTP1 (trace b), PTP2 (trace c) and ellipticine (trace d) at [drug]/[DNA] ratio = 0.08. [DNA] = 1.5 × 10^{-3} M. Four other experiments gave identical results.

PTP2, their ability to form a molecular complex with DNA was investigated. For this purpose, flow linear dichroism experiments were performed at different [drug]/[DNA] ratios. Fig. 2 shows the spectra of an aqueous solution of the macromolecule alone (trace a) and in the presence of compounds PTP1 and PTP2 (traces b and c, respectively) at [drug]/[DNA] = 0.08. The spectrum of ellipticine in the same experimental conditions is given as a reference (trace d).

All dichroic spectra show a strong negative signal at 260 nm, typical of the macromolecule. Nevertheless, in the presence of ellipticine, which is known to form an intercalative complex [1], a further negative signal appears at higher wavelengths (300–330 nm). Due to the fact that no contribution from DNA base pairs can be detected in this spectral region, this signal is attributable to the drug chromophore, which undergoes orientation as a result of the formation of a complex with DNA. Furthermore, the negative sign of this signal indicates that the plane of the chromophore lies parallel to the plane of DNA bases, i.e. the mode of binding is intercalative. On the contrary, comparing the spectra obtained in the presence of both PTP1 and PTP2 with that of DNA, no appreciable difference can be detected. These results indicate that the pyridothiopyranopyrimidine derivatives do not give rise to a molecular complex with the macromolecule. Thus, as the observed antiproliferative capacity (Table 1) cannot derive from an interaction with DNA, it is reasonable to assume that it ensues from the effect on a different cellular target.

3.3. *Effects of PTP1 and PTP2 on mitochondria*

Mitochondria play a key role in the apoptosis process, and in particular, the induction of MPT and the consequent physiological features are in several cases established hallmarks of apoptosis [35]. In this connection, it appeared to be of interest to evaluate the occurrence of any effect of the cytotoxic compounds PTP1 and PTP2 on these organelles.

When RLM, incubated in standard medium as described in the experimental section, are treated with 50 μM PTP1 and 30 μM Ca^{2+}, an apparent decrease in absorbance of about 1 unit, at 540 nm, is observed in the suspension. This event is indicative of the occurrence of a large-amplitude swelling of the mitochondrial matrix (Fig. 3A). The effect of PTP1 is dose-dependent, as observable in the inset to the figure, and exhibits an $S_{0.5}$ value of about 25 μM. As the drug induces the maximum effect at about 50 μM, this concentration was used in all the subsequent experiments. The colloid-osmotic alteration observed in Fig. 3A is accompanied by a complete de-energization of the inner membrane, as demonstrated by the collapse of the electrical potential (ΔΨ) (Fig. 3B). Ca^{2+} and PTP1, when present alone, are completely ineffective in inducing either of the above-mentioned effects (Fig. 3A and B).

By contrast, the addition to the mitochondrial suspension of PTP2 at the same concentrations as PTP1 and in the presence of 30 μM Ca^{2+}, does not induce any effect, either on

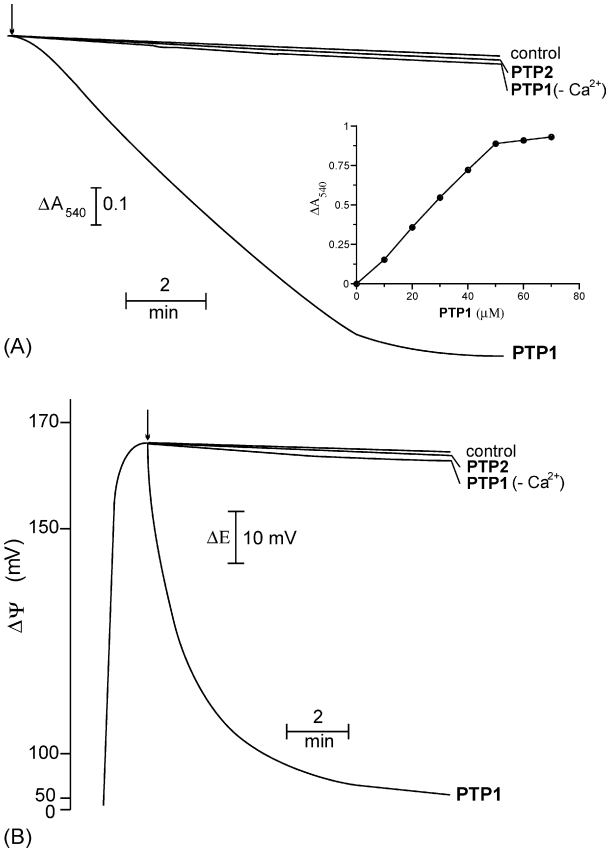

Fig. 3 – Effects of PTP1 and PTP2 on mitochondrial swelling (A) and ΔΨ collapse (B). All incubations were carried out as indicated in standard incubation procedures in the presence of 30 μM Ca^{2+}, except where indicated (-Ca^{2+}). When present, 50 μM PTP1 or PTP2. The assays were performed at least four times with similar results. (A) A downward deflection indicates absorbance decrease. The inset shows the dose-dependent effect of PTP1, calculated as the maximum extent of ΔA after 12 min of incubation at every concentration. (B) ΔE is the electrode potential.

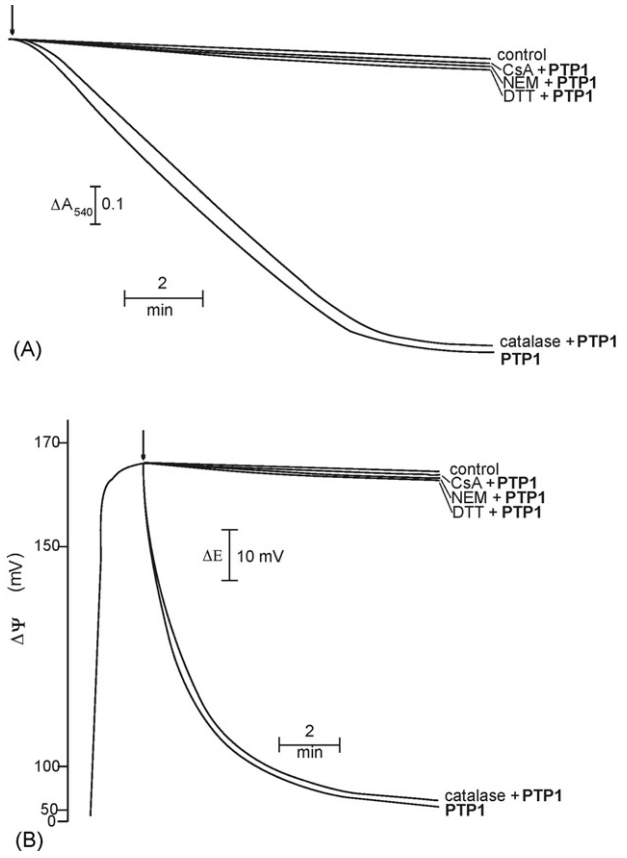

Fig. 4 – Effects of different MPT inhibitors and catalase on mitochondrial swelling (A) and ΔΨ collapse (B) induced by PTP1. Experimental conditions as indicated in the legend to Fig. 3. When present, 1 μM CsA, 10 μM NEM, 5 mM DTT and 1000 U/mg prot catalase. Four additional experiments gave similar results.

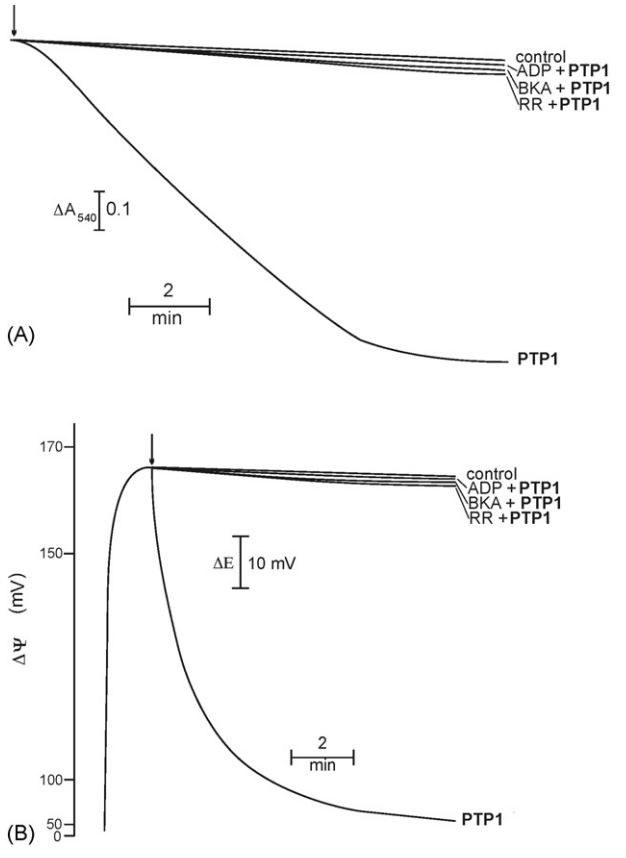

Fig. 5 – Effects of different MPT inhibitors on mitochondrial swelling (A) and ΔΨ collapse (B) induced by PTP1. Experimental conditions as indicated in the legend to Fig. 3. When present, 0.5 mM ADP, 5 μM BKA and 1 μM RR. Data are representative of five similar experiments.

the swelling of the matrix or on the electrical transmembrane potential (Fig. 3A and B). These results indicate for this derivative an inability to affect mitochondrial functions.

The mitochondrial swelling and ΔΨ collapse induced by PTP1 are fully prevented by CsA, *N*-ethylmaleimide (NEM) and dithiothreitol (DTT) (Fig. 4A and B), as well as ADP, bongkrekic acid (BKA) and ruthenium red (RR) (Fig. 5A and B), which are typical MPT inhibitors [36]. Catalase, on the other hand, does not exhibit any appreciable effect (Fig. 4A and B). The inhibition induced by the alkylating agent, NEM, and the reductant, DTT, points to the hypothesis that the events observed are the result of an oxidative stress, even if catalase is ineffective. Of some interest in this connection are the histograms of Fig. 6, which refer to the effect of Ca^{2+} and PTP1 on the redox state of the thiol groups and glutathione, respectively. The results demonstrate that Ca^{2+} or PTP1, when incubated alone, decrease the total content of SH groups by about 11 or 13.5%, respectively. When both the agents are incubated together, the decrease in the SH groups is about 17–18% (Fig. 6A). The decrease in SH content accounts for a corresponding formation of oxidised dithiol groups. The changes in the redox state of glutathione shown in Fig. 6B reveal a trend almost identical to that observed for thiol groups. The presence of NEM in the incubation medium prevents the decrease in reduced sulphydryl groups and glutathione, both in the absence and in the presence of Ca^{2+} (Fig. 6A and B). Also the reducing agent dithioerythritol (DTE) exhibits the same effect (results not reported). An event which very often accompanies MPT induction is the release from mitochondria of cytochrome *c*. The Western blot analysis documented in Fig. 7 shows that Ca^{2+} and PTP1, when incubated alone (lanes 1 and 2, respectively) induce a reduced loss of cytochrome *c*, almost identical to that observed with the medium alone (not reported). PTP1 in the presence of Ca^{2+}, by inducing pore opening, provoked a marked loss in the medium of cytochrome *c* (lane 3). The presence of CsA inhibited the loss of this soluble factor at the same level observed with Ca^{2+} and PTP1 alone (lane 4).

All the experiments were performed in a sucrose medium in order to compare the results with those obtained with other pro-oxidant agents [37,38]. However, results obtained with saline media gave identical results.

3.4. *DNA fragmentation induced by PTP1*

Cytochrome *c* is a well-known caspase activator that triggers the pro-apoptotic pathway. During the apoptotic process,

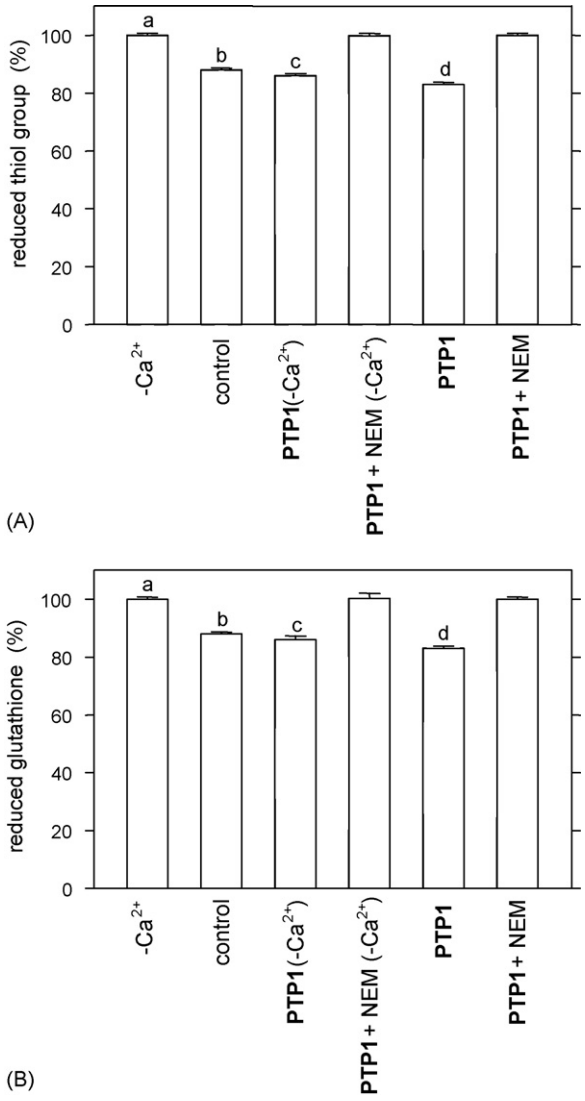

Fig. 6 – Mitochondrial thiol (A) and glutathione (B) oxidation induced by PTP1. All incubations were carried out as indicated in standard incubation procedures in the presence of 30 μM Ca^{2+}, except where indicated (-Ca^{2+}). When present, 50 μM PTP1, 10 μM NEM. The data are expressed as a percentage of the thiol or glutathione reduction, and represent the average ± mean S.D. from four independent experiments. Values followed by different letters are significantly different ($P < 0.05$) as determined by DMTR.

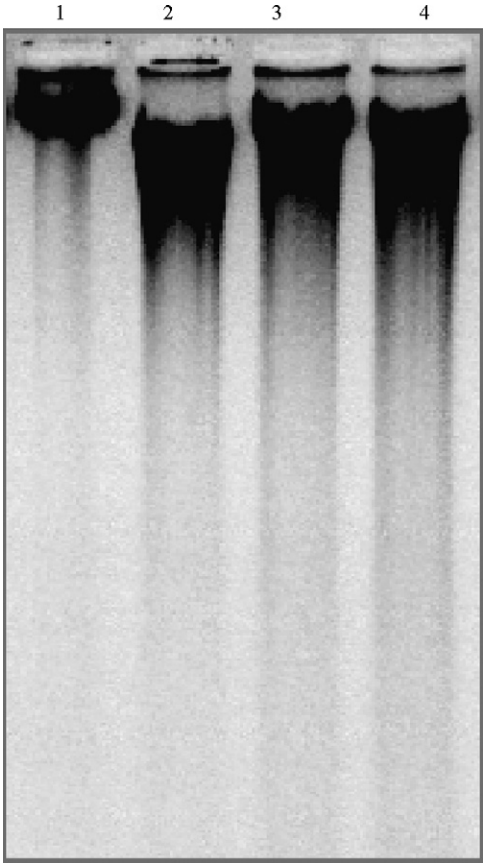

Fig. 8 – Agarose gel electrophoresis of DNA extracted from HeLa cells. Lane 1: untreated control; lane 2: 50 μM cisplatin; lane 3: 100 μM PTP1; lane 4: 200 μM PTP1.

activated endonucleases degrade the higher order chromatin structure of DNA into mono- and oligonucleosomal DNA-fragments, giving rise to a ''ladder'' of nucleosomal-sized multimers [39]. To investigate more in depth whether the mechanism of cell death induced by PTP1 was associated with apoptosis following mitochondrial membrane disruption, isolation and gel electrophoresis of DNA extracted from HeLa cells treated with PTP1 were carried out. Cells incubated in the presence of cisplatin were taken as reference. Fig. 8 shows the gel electrophoresis of DNA after treatment with 50 μM cisplatin (lane 2) and 100 and 200 μM PTP1 (lanes 3 and 4, respectively). The results show, also for the cells incubated in the presence of PTP1, the typical DNA fragmentation that is

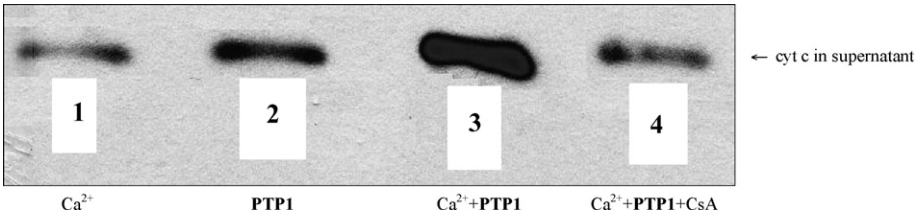

Fig. 7 – Release of cytochrome c (cyt c) induced by PTP1 in the presence of Ca^{2+} and CsA. The result of Western blotting of supernatant fractions is shown. RLM were incubated in standard medium. Lane 1: 30 μM Ca^{2+}; lane 2: 50 μM PTP1; lane 3: 30 μM Ca^{2+} and 50 μM PTP1; lane 4: 30 μM Ca^{2+}, 50 μM PTP1 and 1 μM CsA. The assays were performed four times with almost identical results.

Table 2 – Fe^{3+} reducing activity of compounds PTP1, PTP2 and quercetin as reference compound

Compound	μmol Fe^{2+}/μmol compound after 3 min[a]	μmol Fe^{2+}/μmol compound after 9 min[a]
PTP1	0.0955	0.156
PTP2	0.0611	0.0811
Quercetin	0.1471	0.1481

[a] Values represent means of four independent experiments.

used to characterise cell death by apoptosis, thus indicating the ability of the test compound to induce the apoptotic process.

3.5. *Fe^{3+} reducing activity measurements of compounds PTP1 and PTP2*

Oxidative stress seems to play a key role in MPT induction; on this basis, we investigated whether the activity exerted by compound PTP1 on mitochondria is really related to redox phenomena. The involvement of the oxidation process was evaluated by quantifying the ability of compound PTP1 to reduce Fe^{3+} ions to Fe^{2+} using the Ferrozine-based colorimetric assay. Ferrozine is a chromophoric chelator that strongly binds Fe^{2+}, forming a stable complex, which absorbs light at 562 nm, with a high extinction coefficient [30,32]. After application of a 1 mM $FeCl_3$ solution to the reaction mixture containing ferrozine and the samples, the absorbance of the Fe^{2+}–ferrozine complex rapidly increased to maximal values within 9/10 min (Table 2). The spectral readings were recorded taking into account quercetin as a reference compound [40]. The results obtained were expressed as Fe^{2+}/compound μmoles ratios, since the absorbance of the Fe^{2+}–ferrozine complex is proportional to the iron concentration. The data showed that the 2-amino derivative PTP1 reduces Fe^{3+} effectively, giving values almost identical to those of quercetin after 9 min of incubation.

As regards compound PTP2, the Ferrozine-based colorimetric assay indicates a practically negligible reductant capacity. This result could account for the inability of PTP2 to exert any effect on mitochondrial functions, and suggests a critical functional role for the 2-amino group of the pyridothiopyranopyrimidine chromophore.

3.6. *Cyclosporin A antagonises the antiproliferative effect of PTP1 on HeLa cells*

In order to correlate the antiproliferative ability of compound PTP1 with the effect noted on isolated mitochondria, we performed a cell inhibition growth assay in the presence of CsA.

Fig. 9 reports the results expressed in terms of the percentage of the number of viable cells compared with untreated culture. Incubation for 48 h in the presence of PTP1 induces, as expected, a decrease of about 70% in cell number (hatched bars). Pretreatment for 20 min with CsA (empty bars) itself provokes a weak decrease in cell number (about 4%) and clearly inhibits the antiproliferative effect induced by PTP1. In particular, in the presence of PTP1 the calculated percentage is

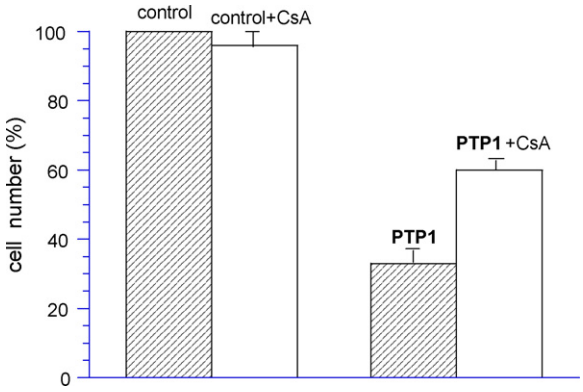

Fig. 9 – Effects of cyclosporin A (CsA) on cell growth inhibition induced by PTP1. HeLa cells were incubated for 48 h in the absence or in the presence of 15 μM PTP1 without pretreatment (hatched bars) and after pretreatment with 2 μM CsA for 20 min (empty bars). Values are mean ± S.D. of four different experiments. Statistical analysis was carried out using Student's *t*-test (*$P < 0.05$ vs. control).

about 30% lower with respect to controls, thus indicating a significant lessening of the effect of the test compound after treatment with CsA. These results are further supported by the microphotographs of the cell cultures (Fig. 10).

4. Discussion

The results reported in Figs. 3–7 unequivocally demonstrate that PTP1 behaves like a typical MPT inducer: the induction of a colloid-osmotic swelling paralleled by a full drop in $\Delta\Psi$ and the inhibition of these events by typical inhibitors of MPT such as CsA, ADP, BKA and RR, are a clear confirmation that the transition pore is involved in the effect exhibited by PTP1. The further observations regarding the inhibitory effects of NEM and DTT convincingly support the hypothesis that MPT may be the result of an oxidative stress provoked by the drug. This is confirmed by the oxidation of thiol groups and glutathione illustrated in Fig. 6A and B. It should be recalled that, to open the pore, it is necessary for two main events to take place: (i) Ca^{2+}, at a supraphysiological concentration, must interact at the level of critical sites on adenine nucleotide translocase (AdNT), in order to favour the subsequent interaction of cyclophilin D with this protein to predispose the opening of the pore [41]. (ii) Two critical thiol groups belonging to two cysteins located on AdNT must be oxidised to form a disulphide bridge [42]. If only one of these events takes place, the pore remains closed. Ca^{2+} alone, besides interacting with AdNT, also oxidises 11% of thiol groups (Fig. 6), by a mechanism explained below. However, the pore remains closed (see control curves, Figs. 3–5) because, even though a substantial amount of thiols are oxidised, the critical cysteins remain reduced. PTP1, alone, oxidises 13.5% of sulphydryl groups, 2.5% higher than Ca^{2+} (Fig. 6A); however, also in this case, the pore remains closed because, even if the cysteins are oxidised, the absence of Ca^{2+} does not permit the interaction of

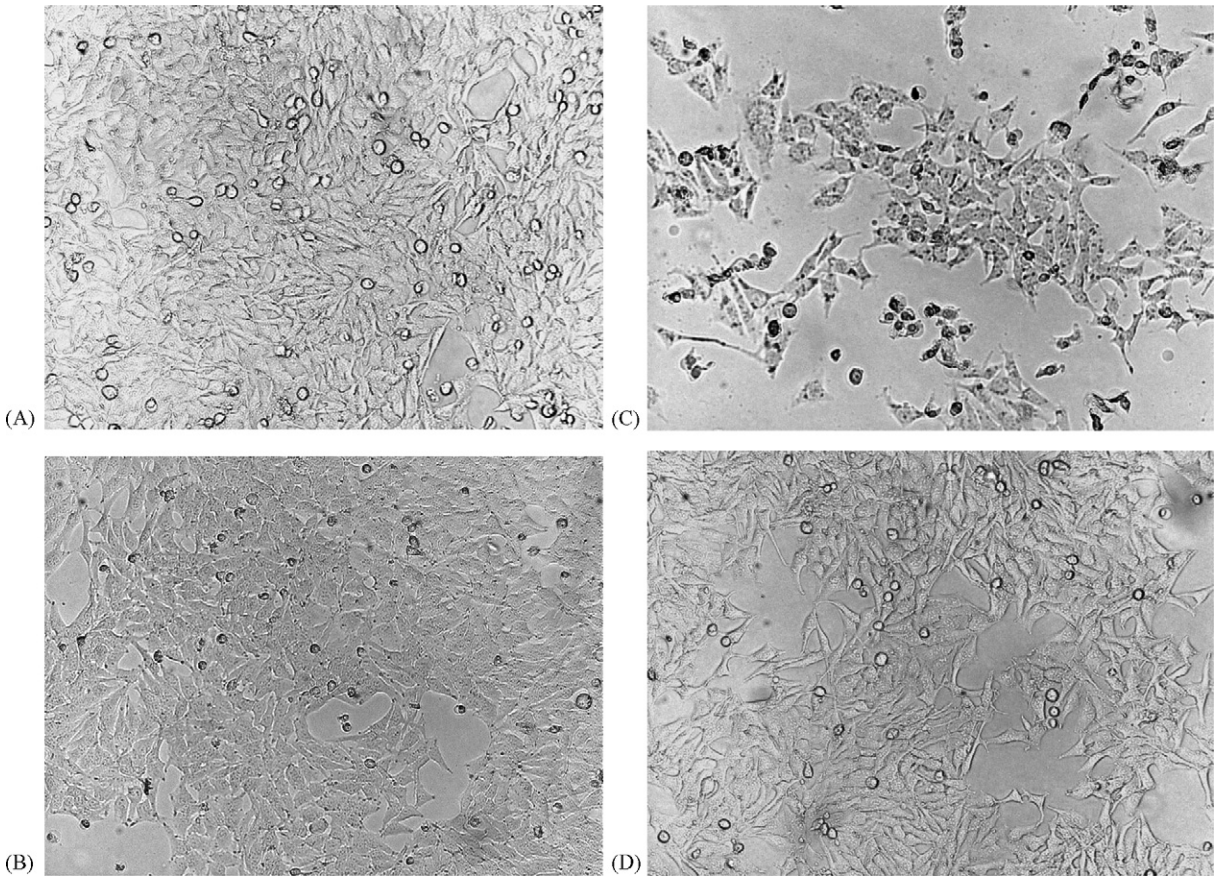

Fig. 10 – Optical microphotographs of the protective effect of cyclosporin A (CsA) on HeLa cells incubated in the presence of PTP1. Photomicrographs: (A) incubation for 48 h in standard conditions; (B) pretreatment with 2 μM CsA for 20 min and further incubation for 48 h; (C) incubation in the presence of 15 μM PTP1 for 48 h; (D) pretreatment with 2 μM CsA for 20 min and further incubation in the presence of 15 μM PTP1 for 48 h.

cyclophilin with AdNT. The pore opens only when Ca^{2+} and PTP1 are present together (Figs. 3–5), that is when the above-mentioned events take place. In this regard, it should be underlined that the critical thiol groups belonging to the cysteins whose oxidation is responsible for pore opening are included in the 2.5%, which differentiate the oxidation between Ca^{2+} and PTP1. This small difference, supported by statistical analysis, is justified by the very low number of thiols, that is two for a molecule of AdNT, involved. It should be pointed out that the apparently low amount of oxidised thiols (~18%, Fig. 6A) and glutathione (~19%, Fig. 6B) induced by PTP1, is very similar to that induced by other pro-oxidant agents such as aluminium (17%) [43], tyramine (25%) [43], carbenoxolone (19%) [44] in thiols. The further oxidation of 4% obtainable with both the agents would thus be due to the increase in oxygen uptake following pore opening, with consequent ROS production. The oxidation of glutathione (Fig. 6B) accounts for the involvement of hydrogen peroxide and/or its derivatives in pore opening. The lack of effects due to catalase is explainable considering that the enzyme cannot enter the mitochondria, and that H_2O_2, probably, does not diffuse out of the organelles, as observed with other pro-oxidants [45]. Another possibility is that H_2O_2 is immediately transformed into other ROS.

The oxidation of thiols and glutathione by Ca^{2+}, is most probably, attributable to its interaction with membrane phospholipids, the consequence of which should be a disorganization of the membrane and an alteration of ubiquinone mobility, leading to ROS production [46]. However, the generation of ROS by Ca^{2+} is not involved in the oxidation of critical SH groups responsible for pore opening, presumably because ROS are formed away from these thiols.

The permeability transition induced by PTP1 is most probably the result of an oxidative stress, a hypothesis, which is reinforced by the fact that compound PTP1 is able to reduce Fe^{3+} ions to Fe^{2+}. The proposed mechanism is that PTP1 acts as a pro-oxidant, affecting electron transport at the level of complexes I and III of the respiratory chain. Even if it has not been well-established which part of the molecule is responsible for the redox effect, the most important structural feature seems to be the presence of the 2-amino group, since the 2-*p*-toluensulphonamido analogue PTP2 does not show any appreciable redox activity. In this connection, it can be hypothesised that PTP1 provokes an accumulation of imino radicals, which by interacting with molecular oxygen would give the superoxide anion and subsequently other ROS. In this case, the pathway of ROS generation should be addressed to reaching the critical thiol groups responsible for MPT pore opening.

The results shown in Fig. 7 demonstrate that PTP1, in the presence of Ca^{2+}, by inducing MPT also provokes the release of cytochrome c, a pro-apoptotic soluble factor that causes the activation of the caspase cascade, and consequently cell death. The occurrence of DNA fragmentation in HeLa cells treated with PTP1 (Fig. 8) confirms the ability of the derivative to activate the apoptotic pathway. The inhibitory effect exhibited by CsA on mitochondrial release of cytochrome c (Fig. 7) demonstrates the close correlation between MPT induction by PTP1 and its possible pro-apoptotic and anti-proliferative activities. The experiments of Figs. 9 and 10 are aim to validate this proposal at the cellular level. The results illustrated in these figures showing that CsA significantly inhibits the antiproliferative effect induced by PTP1 strongly support the proposal. In conclusion, PTP1 can be considered as an anti-proliferating agent whose mechanism of action mainly involves mitochondria-mediated apoptosis through a pro-oxidant induction of transition pore opening. Experiments are in progress in our laboratory in order to verify the toxicity of the compound.

In conclusion, new pyridothiopyranopyrimidine derivatives carrying an amino (PTP1) or a *p*-toluensulphonamido (PTP2) group were prepared. PTP1 and PTP2 show a significant antiproliferative activity on human tumour cell lines. Studies on the mechanism of action reveal PTP1 to be an inducer of mitochondrial permeability transition via a pro-oxidant pathway. Conversely, PTP2 acts through a mechanism which definitely does not involve mitochondria, and which is under investigation in our laboratory. On the basis of these results, compound PTP1 appears to offer an interesting lead chromophore to be further developed to obtain antiproliferative agents targeting mitochondrial functions, and thus proposable for new more effective anti-cancer therapies.

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