Pharmazeutische/Medizinische Chemie, Institut für Pharmazie, Universität Greifswald, Germany

Studies of the inhibitory activities of tiopronin and mercaptosuccinic acid on glutathione peroxidase and their cytotoxic and antioxidant properties

S. Behnisch-Cornwell, G. Laubenstein, P.J. Bednarski*

This paper is dedicated to Prof. Dr. Hans-Hartwig Otto on the occasion of his 80th birthday.

Received March 28, 2019, accepted May 6, 2019

**Corresponding author: Patrick J. Bednarski, Pharmazeutische/Medizinische Chemie, Institut für Pharmazie, Universität Greifswald, Friedrich-Ludwig-Jahn Straße 17, 17489 Greifswald, Germany*
bednarsk@uni-greifswald.de

Pharmazie 74: 536-542 (2019) *doi: 10.1691/ph.2019.9472*

Glutathione peroxidase (GPx), an important antioxidative enzmye, can be inhibited by various thiols, including of tiopronin and mercaptosuccinic acid (MSA). Recently, there has been discussion regarding the combination of tiopronin in anticancer therapy to overcome acquired resistance to anticancer drugs. However, thiols are also known to act as antioxidants, which can be contraindicated in cancer chemotherapy. This article focuses on the inhibitory effects of tiopronin and MSA on bovine and human glutathione peroxidase activities, and their effects on the redox status of cancer cells. IC_{50} values for the inhibition for the bovine erythrocyte enzyme were 356 and 24.7 µM for tiopronin and MSA, respectively, with the corresponding K_i values of 343 µM and 14.6 µM, respectively at pH 7.4 and 25 °C. MSA inhibited human GPx activity in human cancer cell lysates at its IC_{50} while tiopronin did not. Both compounds were cytotoxic to human cancer cell lines GUMBUS and HL-60, with IC_{50} values between 42.7 and 149.4 µM. Neither had an effect on cell cycle. Only MSA induced apoptosis in HL-60 cells but not in GUMBUS cells, while tiopronin resulted in no apoptosis in either cell line. Combination studies of the MSA with hydrogen peroxide in living cells enhanced the production of reactive oxygen species in GUMBUS cells while tiopronin acted as antioxidant in HL-60 cells. MSA and tiopronin antagonized the cytotoxic effect of cisplatin, doxorubicin and methotrexate in combination studies. Our findings indicate that the antioxidant properties of both thiols prevail over their GPx inhibitory activity in human cancer cell lines.

1. Introduction

Oxidative reactions such as oxidative phosphorylation are among the most important biochemical processes. These redox processes must be balanced within the cell or else oxidative or reductive stress arises. This can result in irreversible alterations of membranes, proteins and DNA followed by induction of apoptosis or necrosis leading to cell death (Cerutti 1985; Duan et al. 2016; Lloret et al. 2016). Oxidative stress is characterised by excesses of reactive oxygen species (ROS) like peroxides, superoxide and hydroxyl radicals, and reactive nitrogen species (RNS) like peroxinitrite, to name but a few. Cells have a variety of mechanisms to deal with oxidative stress, such as maintaining adequate levels of antioxidants like glutathione (GSH), tocopherol and ascorbic acid or by expressing antioxidative enzymes like catalase, superoxide dismutase, thioredoxin reductase, glutathione reductase and glutathione peroxidase (GPx).

The GPx enzymes are one of the main removers of peroxides in pro- and eukaryotes, flora and fungi (Brigelius-Flohe et al. 2013; Lubos et al. 2011). GPx reduces organic peroxides to water and corresponding alcohols, or, in the case of hydrogen peroxide to a second molecule of water, by catalyzing the oxidation of two molecules of GSH to the disulphide (GSSG). Eight isoenzymes with different localisations and main functions are known. For GPx isoenzymes a catalytic tetrade is highly conserved, whereas the isoenzymes GPx-1 – GPx-4 and GPx-6 are characterised by the use of the unique amino acid selenocysteine in the catalytic center. Glutamine, tryptophan and asparagine residues of the catalytic tetrade are crucial for enzyme substrate interaction. The most prevalent forms are GPx-1, with localisation in the cytosol, and GPx-4, which is located in the cell membrane.

Inhibiton of GPx-1 could be of therapeutic benefit for diseases like cancer, where resistance to anticancer drugs can result when antioxidative enzyme become over-expressed (Dokic et al. 2012; Jardim et al. 2013; Schulz et al. 2012). However, due to the relatively flat active site of GPx-1, this enzyme is not a readily drugable target. Thus, only a few, relatively weak inhibitors are known to date. Most of these are either thiols (Chaudiere et al. 1984b), acylhydrazone heterocycles (Schulz et al. 2012), misonidazole (Kumar et al. 1986) or mercury- and gold compounds (Chaudiere et al. 1984a; Farina et al. 2009; Roberts et al. 1998) . Among the thiol inhibitors, coenzyme A (Little et al. 1970), tiopronin [(*R,S*)-*N*-2–mercaptopropionylglycine] (Fig. 1a) and mercaptosuccinic

Abbreviations
CDDP (cisplatin); CI (combination index); DOX (doxorubicin); GPx (glutathione peroxidase); GR (glutathione reductase); GSH (glutathione); MSA (mercaptosuccinic acid); MTX (Methotrexate); PI (propidium iodide); PS (phosphatidyl serine); RNS (reactive nitrogen species); ROS (reactive oxygen species); Tiopronin [(*R,S*)-*N*-2–mercaptopropionylglycine]

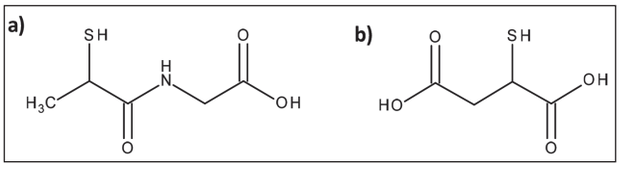

Fig. 1: Structures of a) tiopronin and b) mercaptosuccinic acid (MSA).

acid (MSA) (Fig. 1b) have been described, with MSA being the best characterized GPx-1 inhibitor. It has been reported that MSA and tiopronin oxidize the selenocysteine in the active side of the enzyme, which then undergoes a reactive transfer to the lysine 166. After oxidation of the sulfenamide to a sulfonamide, GPx-1 becomes irreversible inhibited (Hall et al. 2014).

Tiopronin is approved worldwide for the treatment of cystein-uria, which can lead to the formation of kidney stones; it acts by reacting with cysteine in urine, thus increasing the solubility and renal elimination of the amino acid (Carlsson et al. 1993). In addition, tiopronin is used to treat victims of heavy metal poisoning. It has also found applications in the treatment of acute and chronic hepatitis, fatty liver, cirrhosis of the liver and rheumatoid arthritis. Usually these pharmacological activities of tiopronin have been attributed not to the inhibition of GPx but rather to the antioxidative activity of the thiol (Fetoni et al. 2004; Zhang et al. 1999) and metal chelating ability (Domingo 1995; Fujimoto et al. 1979). Recently, there has been interest in combining tiopronin with anticancer drugs to overcome acquired resistance. One publication reported that tiopronin may act as a GPx inhibitor to selectively kill multi-drug resistant (MDR) cancer cells (Hall et al. 2014). Another reported that MDR overexpressing cancer cells were more sensitive to tiopronin than native cells, and that tiopronin suppressed the expression of the P-gp (ABCB1) and MDR protein MRP1 (ABCC1) in cancer cells, protecting them from the cytotoxicity of doxorubicin (DOX) and paclitaxel (Goldsborough et al. 2011). Thus, tiopronin could find an additional indication in cancer therapy.

The interesting pharmacological features of tiopronin led us to examine the inhibitory activities of tiopronin on GPx-1 from both bovine as well as human origins, which have 87 % amino acid homology and possess the same catalytic tetrade (Wilde et al. 2014). Moreover, we investigated the effects of tiopronin and MSA on the proliferation of cancer cells and their ability to induce apoptosis. The redox state of cells treated with tiopronin or MSA in combination with hydrogen peroxide was also investigated. Finally, we performed drug combination studies of tiopronin and MSA with cisplatin (CDDP), doxorubicin (DOX) or methotrexate (MTX) to probe for synergistic, additive or antagonistic effects on human cancer cells.

2. Investigations and results

2.1. Inhibition of GPx activity by tiopronin and MSA

2.1.1. Inhibitory activity of tiopronin and MSA on isolated bovine glutathione peroxidase 1

To determine the inhibitory activity of MSA and tiopronin on GPx, an enyzme assay that mimics the natural catalytic reaction of the GPx was used. In this assay, GPx-1 reduces tert.-butyl hydroperoxide by converting GSH to GSSG while glutathione reductase (GR) recycles GSSG back to GSH under consumption of NADPH, which can be monitored spectrophotometrically by a decrease of absorption at λ=340 nm. Figure 2 shows concentration dependent inhibition of bovine erythrocyte GPx-1 by both tiopronin and MSA, which were used for the estimation of the IC_{50} values. We obtained IC_{50} values for tiopronin and MSA of 356.2±17.71 and 24.73±1.79 µM at pH 7.4 and 25 °C, respectively (Table 1). Thus, MSA is 14.4 times more potent than tiopronin at inhibiting bovine erythrocyte GPx-1.

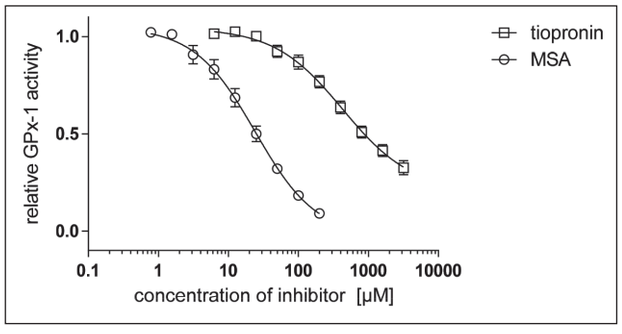

Fig. 2: Concentration-inhibition plots of bovine GPx-1 inhibited by tiopronin and MSA with 1.5 mM GSH at pH 7.4 and 25° C [mean ± SD; n=3].

Table 1: Values (µM) for GPx-1 inhibition at 25 °C [mean ± SD; n>3].

	Tiopronin	**MSA**
IC_{50}	356.2 ± 17.71	24.73 ± 1.79
K_i	343.3 ± 13.77	14.60 ± 0.70

A modified GPx–1 assay with varying concentrations of glutathione was used to determine the maximum conversion rates (V_{max}) and the Michaelis Menten constant (K_m) of the bovine enzyme (Fig. 3). Fitting the data to a parabolic function gave a V_{max} of 143.0 ± 6.35 U/L and a K_m of 1.72±0.17 mM for the substrate GSH. Interestingly, this K_m value lies within the concentration range of 1-12 mM for GSH in many human cancer cells (Bracht et al. 2004).

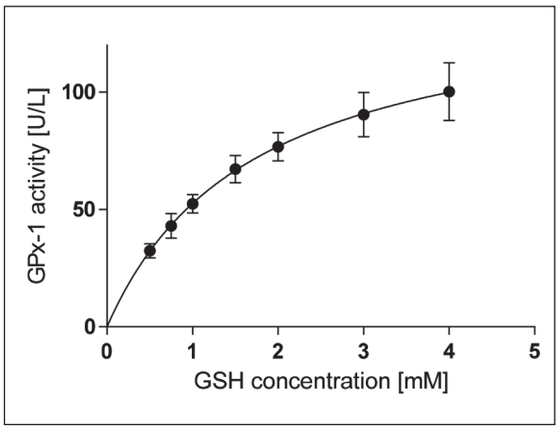

Fig. 3: Concentration-rate plot of bovine GPx-1 activity with various concentrations of GSH at pH 7.4 and 25° C [mean ± SD; n=8].

The inhibition constants (K_i) for tiopronin and MSA were determined with the same assay by measuring enzyme activity at varying concentrations of the substrate GSH and at approximately multiples of the IC_{50}-values of either tiopronin or MSA (Fig. 4). To calculate the K_i-values GraphPad Prism 6.0 software was used. In addition to the calculation of the K_i values, the software also calculates an alpha–value, which gives information on the particular mode of the inhibitor to the enzyme. Alpha values of one indicate that the inhibitor does not change the binding of enzyme and inhibitor, indicating a non-competitive inhibition. Is the alpha–value less than one, the inhibitor would appear to enhance the substrate binding (uncompetitive inhibition). In contrast, very high values mean that the inhibitor displaces the substrate from the active site, indicating a competitive inhibition mode. For tiopronin and MSA, K_i-values of 343.0±13.77 and 14.60±0.70 µM were found, respectively; thus, MSA is 23 times more potent at inhibiting GPx-1 compared to tiopronin, which is consistent with what was found by comparing IC_{50} values. Very high alpha-values of 1.5×10^{12} and 1.4×10^{13} for tiopronin and MSA, respectively, indicate that both inhibitors act by a competitive inhibition mode.

2.1.2. Inhibitory activity of GPx activity in human cancer cell lysates by tiopronin and MSA

Tiopronin and MSA were evaluated for their ability to inhibit GPx activity from human sources. For these studies, cell lysates from human cancer cells were used in place of bovine GPx-1. Not surprisingly, cancer cell lines are characteristic for their varying GPx activity (Fig. 5); e.g., SISO cell lysates have the highest

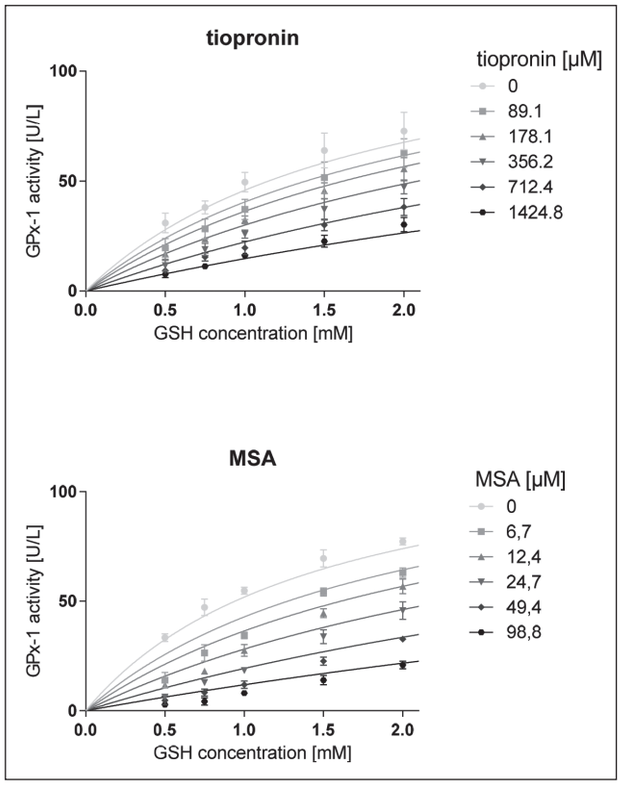

Fig. 4: Concentration-rate plots of bovine GPx-1 activity with various concentrations of GSH and the GPx-inhibitors tiopronin (top) and MSA (bottom) at pH 7.4 and 25° C [mean ± SD; n=3].

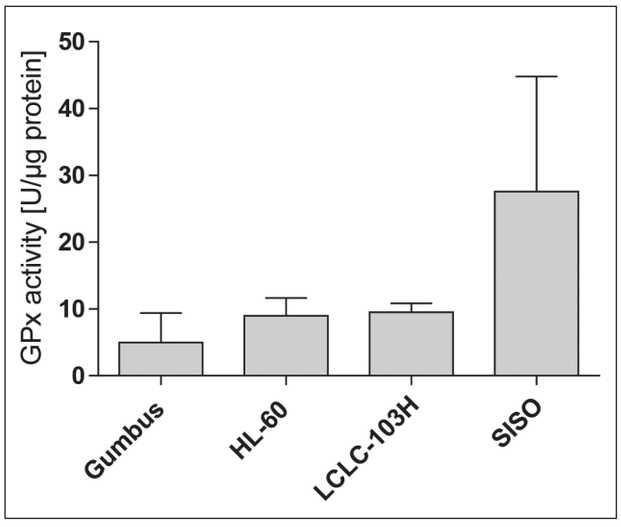

Fig. 5: GPx activity of various cells lysates per µg of total protein at pH 7.4 and 25° C [mean ± SD; n<3].

GPx-activity followed by the cell lysates of HL-60, LCLC-103H and GUMBUS having the lowest activity. The addition of the inhibitors to the lysates of human cancer cells resulted in an inhibitory effect for MSA but not for tiopronin (Fig. 6). Tiopronin showed no GPx-inhibiting effect on GUMBUS, HL-60, LCLC-130H and SISO cell lysates but MSA showed an inhibitory effect in cell lysates of HL-60, LCLC-130H and SISO but not in lysates of GUMBUS cells. On the average of all cell lines, a residual activity of 102.0±8.4 % for tiopronin and 80.4±7.7 for MSA was detected.

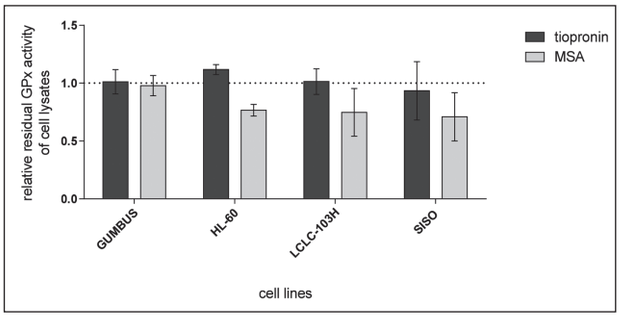

Fig. 6: Relative GPx-activity of cell lysates after exposure to tiopronin (150 µM) and MSA (5.9 µM) at pH 7.4 and 25° C; line marks the untreated control [mean ± SD; n>3; inhibition by MSA was significant with p<0.05].

2.2. Effects of tiopronin and MSA on human cancer cells

2.2.1. Inhibition of viability in GUMBUS und HL-60 cell lines by tiopronin and MSA

Earlier studies reported weak cytotoxic activities of tiopronin and MSA in various human cell lines (Goldsborough et al. 2011; Hall et al. 2014). For our studies, we determined the cytotoxic activity of tiopronin and MSA by the MTT-viability assay with a human B-cell lymphoma (GUMBUS) and a human leukemia (HL-60) cell line for 48 h. Figure 7 shows the concentration-effect curves used in the determination of the IC_{50}-values, which are compiled in Table 2. The IC_{50} values indicate that tiopronin is slightly more toxic to GUMBUS cell line then MSA, with IC_{50} values of 42.7

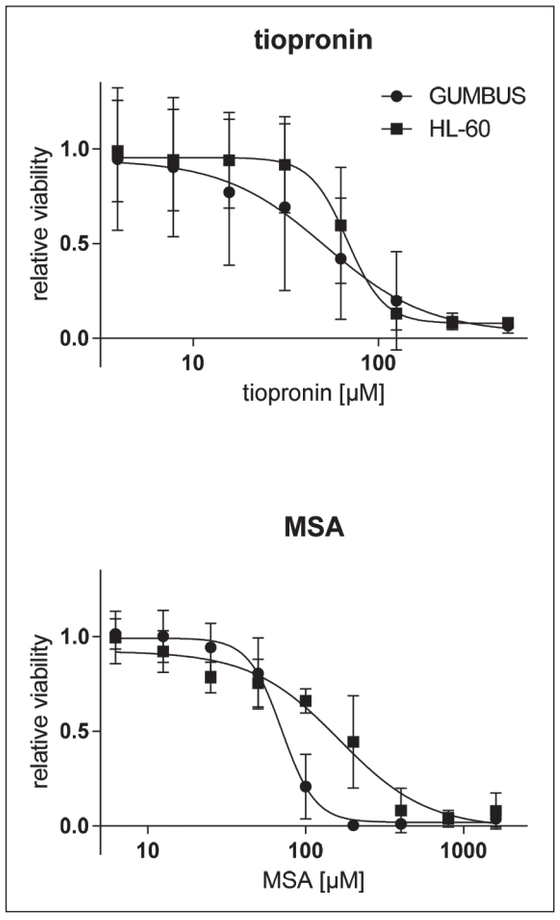

Fig. 7: Concentration-activity plots of the cell viability after incubation with tiopronin (top) and MSA (bottom) for 48 h in GUMBUS and HL-60 cells [mean ± SD; n>3].

and 74.5 µM, respectively. However, the HL-60 cell line is nearly twice as sensitive towards tiopronin as MSA, with IC_{50} values of 64.9 and 149.4 µM, respectively. These IC_{50} values after 48 h for tiopronin are considerably lower than those reported for the wild-type KB-3-1 cell line after 72 h (e.g. 3.9 mM by Hall et al. 2014 or rather 7.5 by Goldsborough et al. 2011). For the ensuring investigations we incubated cells with either the IC_{50} concentration or the IC_{90} ($IC_{90\text{-GUMBUS}}$: tiopronin = 210 µM, MSA = 154 µM; $IC_{90\text{-HL-}60}$: tiopronin = 150 µM, MSA = 668 µM)

Table 2: IC_{50} values (µM) for inhibition of cell viability after 48 h [mean ± SD; n>3].

	tiopronin	MSA
GUMBUS	42.7 ± 18.9	74.5 ± 13.9
HL-60	64.9 ± 20.8	149.4 ± 19.2

2.2.2. Influence of tiopronin and MSA on the cell cycle progression of Gumbus and HL-60 cells

The influence of tiopronin and MSA on the cell cycle was determined by a standard flow cytometric technique based on staining nuclear DNA with propidium iodide (PI). A shift in the distribution of cells from one phase to the previous phase indicates cell cycle arrest, while as shift of cells to the subG phase indicates induction of apoptosis. No influence by either tiopronin or MSA on cell cycle progression in the both cell lines at either the IC_{50} or the IC_{90} of the viability inhibition was detected after 48 h (**Fig.**). However, a strong increase in the subG fraction by MSA at both the IC_{50} and IC_{90} concentrations in HL-60 cells was highly significant compared to control.

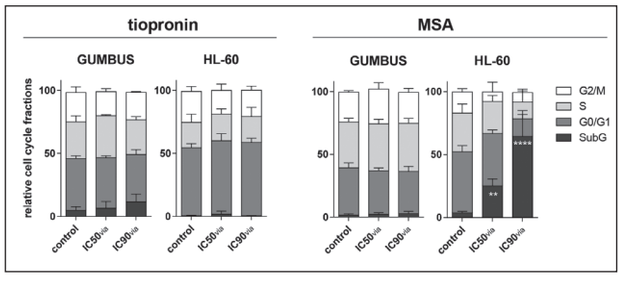

Fig. 8: Fractions of the cell cycle after incubation with tiopronin (left) or MSA (right) in GUMBUS and HL-60 cells for 48 h (tiopronin IC_{50} Gumbus: 42.7 µM, HL-60: 64.9 µM; IC_{90} GUMBUS: 210 µM, HL-60: 154 µM; MSA IC_{50} Gumbus: 74.5 µM, HL-60: 149.4 µM; IC_{90} GUMBUS: 150 µM, HL-60: 668 µM)[mean ± SD; n=2 for tiopronin, n=3 for MSA; **p<0.01, ****p<0.0001 related to control].

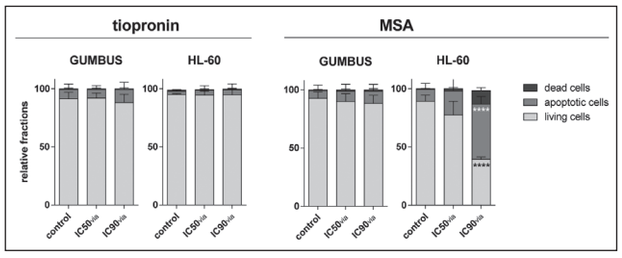

Fig. 9: Fractions after Annexin-V / PI double staining for apoptosis detection after incubation with tiopronin (left) or MSA (right) in GUMBUS and HL-60 cells for 48 h (tiopronin IC_{50} Gumbus: 42.7 µM, HL-60: 64.9 µM; IC_{90} GUMBUS: 210 µM, HL-60: 154 µM; MSA IC_{50} Gumbus: 74.5 µM, HL-60: 149.4 µM; IC_{90} GUMBUS: 150 µM, HL-60: 668 µM) [mean ± SD; n=3; ****p<0.0001 related to control].

2.2.3. Induction of apoptosis in GUMBUS and HL-60 cell lines by tiopronin and MSA

To assess the mechanism of cell death induced by tiopronin and MSA, flow cytometric technique based on FITC linked Annexin–V and PI double staining was used to differentiate between viable, early apoptotic and late apoptotic cells (Martin et al. 1995). Both GUMBUS and HL-60 cells were treated with tiopronin and MSA at their IC_{50} or IC_{90} concentrations of viability inhibition for 48 h. Neither GUMBUS nor HL-60 cells showed an increase in apoptotic cells after a 48 h incubation with tiopronin (see Fig. 9). For MSA, no induction of apoptosis in the GUMBUS cell line was observed but a strong induction in apoptotic cells was noted in the HL-60 cell line, which is consistent with the cell cycle data reported above.

2.3. Combination experiments with tiopronin or MSA in cancer cell lines

2.3.1. Influence of tiopronin and MSA on the ROS production by hydrogen peroxide in GUMBUS and HL-60 cells

If tiopronin and MSA were effective, irreversible GPx-1 inhibitors in cells, then one would expect that a pre-incubation of cells with either of these would result in an increase in reactive oxygen species (ROS) when the cells were exposed to a peroxide such as hydrogen peroxide. To determine whether tiopronin and MSA have an effect on the intracellular levels of (ROS), induced by the addition of hydrogen peroxide, cells were pre-incubated for 24 h with either of the two thiols to ensured their cellular uptake and inhibition of GPx. Neither tiopronin nor MSA affected the basal

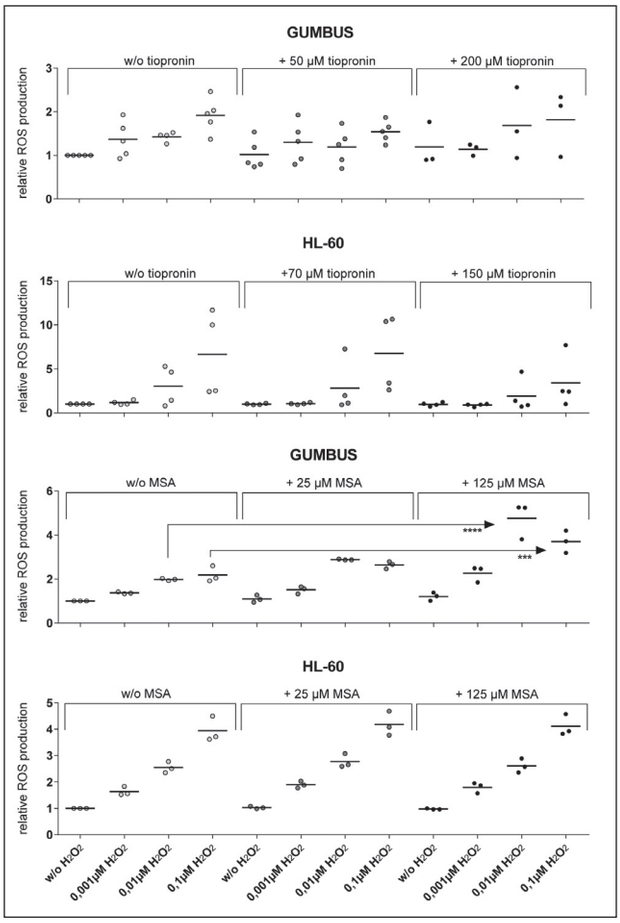

Fig. 10: Relative ROS accumulation induced by hydrogen peroxide after a 24 h pre-incubation with tiopronin or MSA [mean and each measured sample; n>3; ***p<0.001, ****p<0.0001 related to corresponding control].

levels of ROS in GUMBUS and HL-60 cells after 24 h (Fig. 10). The incubations of cells with just hydrogen peroxide resulted in a significant concentration dependant increase in intracellular ROS levels (significance not shown in Fig. 10). In the GUMBUS cell line, only MSA led to a statistically significant increase in ROS levels compared to controls with just hydrogen peroxide incubation, consistent with our hypothesis and suggesting that MSA can act intracellularly as a GPx inhibitor. On the other hand, no effect on ROS levels was observed in the GUMBUS cell line with tiopronin pre-treatment. However, in the HL-60 cell line, MSA was unable to change the ROS levels. Contrary to our hypothesis, tiopronin actually led to a decrease in ROS levels in HL-60 cells, suggesting that this thiol may act directly as an antioxidant and not as a GPx inhibitor in cells.

2.3.2. Combinations of tiopronin or MSA with cisplatin, doxorubicin or methotrexate in GUMBUS and HL-60 cells

Anticancer drugs such as DOX, CDDP and MTX have been reported to increase oxidative stress in cells as one of their mechanism of cytotoxicity (Herman et al. 2005; Miyajima et al. 1997; Yang et al. 1996). Thus, a combination of such an anticancer drug with a GPx-1 inhibitor might lead to a further increase in oxidative stress and thus a synergistic killing of cancer cells. To test this hypothesis, experiments with combinations of anticancer drugs with either tiopronin or MSA were performed and analysed by the method of Chou-Talalay to detect synergistic, additive or antagonistic effects in combination treatments (Chou 2006, 2010). For these experiments, a constant combination ratio of tiopronin or MSA with either DOX, CDDP or MTX in multiple concentrations (0.25×, 0.5×, 1×, 2× and 4×) of the IC_{50} for the viability inhibition were added to cultures of either GUMBUS or HL-60 cells. Cytotoxicity was determined by the MTT assay. The underlying IC_{50} values of the anticancer drugs are reported in Table 3. The combination index (CI) was calculated by CompuSyn 1.0 software, which gives information on the effect of the combination; i.e, is the CI near one, the combination acts additively, is the CI smaller than one the two compounds act synergistically together, is the CI much greater than one, then an antagonistic action of the two compounds is indicated (Chou TC 2006). Figure 11 show the CI plots for the combination assays as a function of combination ratios. In this figure each point is a calculated result of the dose-response curve for the viability determination under combination of both drugs and the effect of the combination related to the effect of each drugs alone.

The combination of tiopronin with CDDP resulted in an antagonistic effect in both cell lines while the combination of MSA with CDDP resulted in an additive toxicity effect in GUMBUS cell line for high doses and a slight antagonistic effect in HL-60 cells. The CI values increases with decreasing concentrations of the anticancer drug, indicating a stronger antagonistic effect at higher residual viabilities. CI values for the combinations of tiopronin or MSA with DOX indicate additive or antagonistic effects for the entirety combination ratios. The CI values vary between 1 and 3.36 under combined drug exposure for residual viabilities under 75 %. Only at very high concentrations of tiopronin and DOX in the HL-60 cell line does the CI values fall below 1: i.e., to 0.68 at residual viability of 10 %. The combination of MSA with MTX in HL-60 cell was the only example of a synergistic effect with CI values below one. For the combination of MTX with tiopronin in GUMBUS and HL-60, and with MSA in GUMBUS cells we detected CI values greater than 1, indicating antagonistic combination effects.

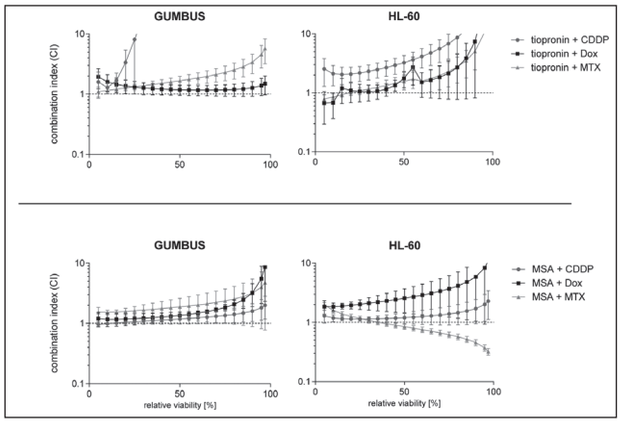

Fig. 11: CI-plots for drug combinations of tiopronin (top) or MSA (bottom) with CDDP, DOX and MTX in GUMBUS (left) and HL-60 cells (right); plotted as relative residual viability under combination versus combination index (CI=1 additive effect; CI<1synergistic effect; CI>1 antagonistic effect) [mean ± SEM; n>3].

Table 3: IC_{50} values (mean in µM) of viability inhibition by anticancer drug in GUMBUS and HL-60 cell line used in multiples for combination assay

	Cisplatin	Doxorubicin	Methotrexat
GUMBUS	0.92	0.020	0.034
HL-60	3.3*	0.046*	0.017*

* from Bracht et al. (2006)

3. Discussion

Tiopronin and MSA have been reported to be inhibitors of GPx, however, to the best of our knowledge, enzyme inhibitory IC_{50} or *Ki* values have never been published for either of these compounds. Only two publications (Chaudiere et al. 1984b; Hall et al. 2014) reported inhibitory activities for tiopronin, but these were at just one concentration; e.g., they detected a residual activity of bovine GPx after incubation with 200 µM at 60 % for tiopronin. By means of concentration-inhibition curves we detected a residual GPx activity of 37 % at 200 µM tiopronin (Fig. 2). For MSA, Chaudiere et al. (1984b) and Michiels et al. (1988) reported total GPx inhibition at quite high concentrations of MSA (0.2 mM and 50 µM, respectively), while Wilde et al. (2014) detected a residual activity of 17 % at 40 µM. Under the conditions of our experiments (e.g. at 25 °C), we found residual activities of 11 % and 0 % at 22.2 and 66.6 µM, respectively, similar inhibitory activity of MSA was reported by Chaudiere, Hall and Wilde.

We note an approximate 20 times greater potency for MSA compared to tiopronin based on the determined IC_{50} und K_i values. Interestingly, the K_i for MSA is approx. 100 times lower than the K_m value for GSH, leading us to conclude that the affinity of MSA to GPx is much higher than to its physiological substrate GSH. The very high calculated alpha-values for tiopronin and MSA confirm the studies of Hall et al. (2014) that MSA acts through a competitive inhibition mode.

In studies of GPx-1 inhibition, the bovine enzyme is often used because it is commercially available, with a high homology of 87 %, and 93 % homology in the active site compared to human source (Wilde et al. 2014). We examined whether tiopronin and MSA are able to inhibit human GPx activity and found a significant inhibition of GPx activity by MSA but not by tiopronin. Hall et al. (2014) identified the MSA and tiopronin binding residue for bovine GPx, a lysine near to members of the catalytic tetrade (asparagine and tryptophan – WN motif). A replacement of an alanine to a serine next to the WN – motif in human GPx–1 could explain the loss on the inhibitory activity for tiopronin compared to bovine enzyme.

A more potent toxic effect of MSA compared to tiopronin was detected in both cell lines by the MTT viability assay. There were no effects on the cell cycle progression, indicating a non-selective mechanism of action for both compounds. Measurements of apoptosis by Annexin-V and PI double staining gave negative results for tiopronin with both cell lines and for MSA in GUMBUS cells. However, the exposure of HL–60 cells to MSA produced a significant increase in apoptotic cells. A similar significant increase in

the subG population of the cell cycle in HL-60 cells treated with MSA support this finding.

For drug combination studies we used anticancer agents that are known to partially mediate their cytotoxicity through ROS production. We hypothesized that a synergistic combination effect would be found for both thiols because the inhibition of GPx would be expected to enhance the toxicity of these anticancer drugs by reducing the antioxidative capacity of the cells. Instead, combinations between DOX, CDDP or MTX and tiopronin or MSA showed in most cases either additive or even antagonistic effects. Only in the combination of MTX with MSA in the HL-60 cell line was a synergistic cytotoxic effect apparent.

It is well known that antioxidants can cause reduced toxicity of DOX or MTX leading to the presumption that tiopronin and MSA might be acting as antioxidants (Barros et al. 2013; Burcu et al. 2016; Herman et al. 2005). An antagonistic combination effect between CDDP and tiopronin was not unexpected because CDDP is known to be inactivated by thiols and it is also known that tiopronin can reduce oto- and nephrotoxicity of CDDP by acting as antioxidant *in vivo* (Fetoni et al. 2004; Zhang et al. 1999). However, if protection against side effects were to compromise drug efficacy, then such combinations should be avoided in the practice.

We investigated the effect of tiopronin and MSA on the ROS induction by hydrogen peroxide. If both were acting as effective GPx inhibitors within cells, then an increase in ROS production by hydrogen peroxide could be expected. We found that a preincubation of GUMBUS cells with MSA before hydrogen peroxide exposure significantly enhances ROS levels. In the case of HL–60 cells, which have 10-times greater catalase activity than GUMBUS cells (Bracht et al. 2007), no enhancement of ROS by hydrogen peroxide in combination with MSA was observed. A pre-incubation with tiopronin for 24 h in GUMBUS cells had no effect on ROS levels induced by hydrogen peroxide while in HL-60 cells a reduction in ROS levels was observed. This result indicates that tiopronin may be acting as antioxidant in the HL-60 cell line.

In conclusion, the inhibiton of GPx-1 by tiopronin and MSA was confirmed, with MSA being 20-times more potent asan inhibitor than tiopronin. We also showed that MSA is able to inhibit GPx activity of human source by investigations with cell lysates and by combination studies with living cells in dependence to hydrogen peroxide exposure. In contrast, tiopronin had none of these effects. While both thiols are cytotoxic to cancer cells, only MSA induces apoptosis in the HL-60 cell line. Furthermore, our results indicate that tiopronin can antagonize the cytotoxic effects of various anticancer drugs, presumably because it acts as an antioxidant in human cancer cells. Thus, combination therapies of tiopronin with anticancer drugs should be reconsidered in light of this data.

4. Experimental

4.1. Chemicals

The following chemicals were purchased from Sigma Aldrich (Taufkirchen, Germany): 2´,7´-dichloro fluorescein diacetate (DCFDA), fetal bovine serum, glutathione, glutathione disufilde, bovine glutathione peroxidase-1, glutathione reductase, mercaptosuccinic acid, methotrexat, propidium iodide, ribonuklease A, *tert*-butylhydroperoxide, tiopronin, whereas both RPMI 1640 and penicillin/streptomycin were purchased from PAN Biotech (Aidenbach, Germany), MTT from Alfa Aesar (Haverhill, MA, USA), NADPH from Carl Roth (Karlsruhe, Germany), cisplatin from Chempur (Karlsruhe, Germany) and doxorubicin from Pharmacia & Upjohn (Stockholm, Sweden). The Annexin-V kit was purchased from Miltenyi Biotech (Teterow, Germany). For spectroscopic measurements a Spectramax 384 Plus plate reader from Molecular Devices (Sunnyvale, CA, USA) was used. Flow cytometric analytics were performed with a MACSQuant Analyzer 10 from Miltenyi Biotech (Teterow, Germany). Data were calculated and analysed with GraphPad Prism Software 6.0.

4.2. Cell culture

The human acute myeloid leukemia HL-60, the human large cell lung cancer LCLC-103H and the human cervix cancer SISO cell lines were obtained from Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ, Braunschweig, Germany) while the GUMBUS cell line was derived from a patient with chemotherapy refractory aggressive B-Non-Hodgkin's lymphoma at the University Hospital in Greifswald (Bracht et al. 2007), but is now also available at the DSMZ. Cells were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum, 1 % penicillin (10,000 U/ml) + streptomycin (10 mg/ml) at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

4.3. GPx-Assay

GPx inhibitory effects were measured with microtiter-based modification of the GPx-assay by Paglia et al. (1967). All regents were dissolved in a phosphate buffer (50 mM, pH = 7.4) containing 1.1 mM EDTA and 0.01 % Triton X. UV-translucent Well plates were loaded with reagents to a final volume of 300 µL containing bovine GPx-1 (0.015 U/ml), NADPH (0.2 mM), GR (0.2 U/ml), GSH (1.5 mM), tert-butyl hydroperoxide (0.2 mM) and appropriate inhibitor concentrations and incubated at a temperature 25 °C. For determination of IC_{50} values, inhibitor in serial dilutions were added. For the determination of K_i values, multiple concentrations of the IC_{50} were added as well as varying concentrations of GSH between 0.5–4 mM. For the testing of human GPx activity, bovine enyzme was replaced by the total protein cell lysates (100 µg/well), with protein quantified by the Bradford method (Bradford 1976). The decreasing rate of absorption of NADPH at λ=340 nm was measured every 15 s for 30 min with a 5 s shaking period at a temperature of 25 °C. The negative rate (in min) of the absorption reduction multiplied by the dilution factor and divided by the extinction coefficient for NADPH (6.22 x 10^{-3} l µmol^{-1} cm^{-1}) is the GPx activity in U/L.

4.4. Cell viability assay

To assess cytotoxicity a microtiter-based modification of the MTT assay was used (Mosmann 1983). Briefly, cells were seeded out in a density of 10.000 cells per well for HL-60 or 20.000 cells per well for GUMBUS in 96-well plates and exposed to tiopronin or MSA, or to various combinations with anticancer drugs in serial dilutions in a volume of 100 µL per well. Plates were returned to the cell incubator for 48 h at 37 °C. Then 20 µl of MTT solution (2.5 mg/ml in phosphate buffered saline (PBS)) was added to wells and incubated in cell incubator for 4 h. The MTT crystals that formed were dissolved in 0.04 N HCl in isopropanol by sonification and the optical density (OD) was measured at λ=570 nm. Relative viability is the quotient between $OD_{treated}$ and $OD_{untreated}$, multiplied by 100.

4.5. Cell cycle assay

To detect an influence of either tiopronin or MSA on cell cycle progression, cells were seeded into 6-well plates at a density of 250,000 cells per 3 ml medium. After exposure of cells to either tiopronin or MSA, cells were washed with PBS by centrifugation at 500 x g and resuspension in ice-cold ethanol for 30 min on ice. Ethanol was then replaced by 500 µL PBS containing PI (0.025 µg/ml) and ribonuclease A (0.1 µg/ml) and stored for 30 min at room temperature, followed by flow cytometry determination of PI at $\lambda_{excitation}/\lambda_{emission}$ = 488/655–730 nm. Agglutinated cells were discriminated by method of Wersto et al. (2001).

4.6. Apoptosis determination

Apoptosis was determined by an Annexin-V kit based on an Annexin-V / PI double staining method (Koopman et al. 1994) according to the manufacturer's instructions. Briefly, cells were seeded out into 6-well plates in a density of 125,000 cells per 3 ml medium and exposed to either tiopronin or MSA for 48 h. Cells were then washed with 500 ml binding buffer (included in the kit), centrifuged and resuspension into 50 ml binding buffer. After addition of 5 ml Annexin-V solution cell suspension were incubated in the dark for 15 min at room temperature. After washing the suspension with 500 ml binding buffer, cells were resuspended in 250 µl binding buffer. Before measurement 2.5 µl of PI solution was added and cytometry determination was performed at $\lambda_{excitation}/\lambda_{emission}$ = 488/525±50 nm for Annexin-V (FITC labelled) and $\lambda_{excitation}/\lambda_{emission}$ = 488/655–730 nm for PI. Normal living cells show no fluorescence, apoptotic cells show an increased in FITC emission only, while both necrotic cells and late apoptotic cells show both FITC emission as well as an increased PI signal.

4.7. ROS determination

To detect the effect of either tiopronin or MSA on ROS cellular levels induced by hydrogen peroxide, 250,000 cells in 3 ml in 6-well plates were incubated with either tiopronin or MSA for 24 h at 37 °C. Afterwards, the cells were washed with 1.0 ml PBS and exposed to 2.0 µM 2´,7´-dichloro fluorescein diacetate (DCFDA) in PBS (500 µl) for 30 min at 37 °C. After a repeated washing step, cells were incubated at 37 °C with various concentrations of hydrogen peroxide in PBS for 10 min. After a washing step with PBS, the flow cytometric determinations at $\lambda_{excitation}/\lambda_{emission}$ = 488/525±50 nm in PBS were performed.

4.8. Statistics

For statistical evaluation a two statistical tests performed via GraphPad Prism 6.0 software was used. One way ANOVA: Dunnett´s multiple comparison test were used for the comparison of IC_{50} values and for ROS accumulation assays. Two way ANOVA: Dunnett´s multiple comparison tests were used for cell cycle analyses, apoptosis induction and inhibition of human GPx-activity.

Conflicts of interest: None declared.

References

Barros S, Mencia N, Rodriguez L, Oleaga C, Santos C, Noe V, Ciudad CJ (2013) The redox state of cytochrome c modulates resistance to methotrexate in human MCF7 breast cancer cells. PLoS One 8: e63276.

Bracht K, Boubakari, Grunert R, Bednarski PJ (2006) Correlations between the activities of 19 anti-tumor agents and the intracellular glutathione concentrations in a

panel of 14 human cancer cell lines: comparisons with the National Cancer Institute data. Anticancer Drugs 17: 41-51.
Bracht K, Kiefer T, Dolken G, Bednarski PJ (2007) Characterization of three B-cell lymphoma cell lines from chemotherapy resistant patients with respect to in vitro sensitivity to 21 antitumor agents, ABC-transporter expression and cellular redox status. J Cancer Res Clin Oncol 133: 957-967.
Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.
Brigelius-Flohe R, Maiorino M (2013) Glutathione peroxidases. Biochim Biophys Acta 1830: 3289-3303.
Burcu B, Kanter M, Orhon ZN, Yarali O, Karabacak R (2016) Protective effects of vitamin E on methotrexate-induced jejunal mucosal damage in rats. Anal Quant Cytopathol Histpathol 38: 87-94.
Carlsson MS, Denneberg T, Emanuelsson B-M, K»gedal B, Lindgren S (1993) Pharmacokinetics of oral tiopronin. Eur J Clin Pharmacol 45: 79-84.
Cerutti PA (1985) Prooxidant states and tumor promotion. Science 227: 375-381.
Chaudiere J, Tappel AL (1984a) Interaction of gold(I) with the active site of selenium-glutathione peroxidase. J Inorg Biochem 20: 313-325.
Chaudiere J, Wilhelmsen EC, Tappel AL (1984b) Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans. J Biol Chem 259: 1043-1050.
Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58: 621-681.
Chou TC (2010) Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res 70: 440-446.
Dokic I, Hartmann C, Herold-Mende C, Regnier-Vigouroux A (2012) Glutathione peroxidase 1 activity dictates the sensitivity of glioblastoma cells to oxidative stress. Glia 60: 1785-1800.
Domingo JL (1995) Prevention by chelating agents of metal-induced developmental toxicity. Reprod Toxicol 9: 105-113.
Duan X, Wen Z, Shen H, Shen M, Chen G (2016) Intracerebral hemorrhage, oxidative stress, and antioxidant therapy. Oxid Med Cell Longev 2016: 1203285.
Farina M, Campos F, Vendrell I, Berenguer J, Barzi M, Pons S, Sunol C (2009) Probucol increases glutathione peroxidase-1 activity and displays long-lasting protection against methylmercury toxicity in cerebellar granule cells. Toxicol Sci 112: 416-426.
Fetoni AR, Quaranta N, Marchese R, Cadoni G, Paludetti G, Sergi B (2004) The protective role of tiopronin in cisplatin ototoxicity in Wistar rats. Int J Audiol 43: 465-470.
Fujimoto T, Fuyuta M, Kiyofuji E, Hirata S (1979) Prevention by tiopronin (2-mercaptopropionyl glycine) of methylmercuric chloride-induced teratogenic and fetotoxic effects in mice. Teratology 20: 297-301.
Goldsborough AS, Handley MD, Dulcey AE, Pluchino KM, Kannan P, Brimacombe KR, Hall MD, Griffiths G, Gottesman MM (2011) Collateral sensitivity of multidrug-resistant cells to the orphan drug tiopronin. J Med Chem 54: 4987-4997.
Hall MD, Marshall TS, Kwit AD, Miller Jenkins LM, Dulcey AE, Madigan JP, Pluchino KM, Goldsborough AS, Brimacombe KR, Griffiths GL, Gottesman MM (2014) Inhibition of glutathione peroxidase mediates the collateral sensitivity of multidrug-resistant cells to tiopronin. J Biol Chem 289: 21473-21489.
Herman S, Zurgil N, Deutsch M (2005) Low dose methotrexate induces apoptosis with reactive oxygen species involvement in T lymphocytic cell lines to a greater extent than in monocytic lines. Inflamm Res 54: 273-280.
Jardim BV, Moschetta MG, Leonel C, Gelaleti GB, Regiani VR, Ferreira LC, Lopes JR, Zuccari DA (2013) Glutathione and glutathione peroxidase expression in breast cancer: an immunohistochemical and molecular study. Oncol Rep 30: 1119-1128.
Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84: 1415-1420.
Kumar KS, Weiss JF (1986) Inhibition of glutathione peroxidase and glutathione transferase in mouse liver by misonidazole. Biochem Pharmacol 35: 3143-3146.
Little C, Olinescu RM, O'Brien PJ (1970) Inhibition of glutathione peroxidase by coenzyme A. Biochem Biophys Res Commun 41: 287-293.
Lloret A, Fuchsberger T, Giraldo E, Vina J (2016) Reductive Stress: A New Concept in Alzheimer's Disease. Curr Alzheimer Res 13: 206-211.
Lubos E, Loscalzo J, Handy DE (2011) Glutathione peroxidase-1 in health and disease: from molecular mechanisms to therapeutic opportunities. Antioxid Redox Signal 15: 1957-1997.
Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 182: 1545-1556.
Michiels C, Remacle J (1988) Use of the inhibition of enzymatic antioxidant systems in order to evaluate their physiological importance. Eur J Biochem 177: 435-441.
Miyajima A, Nakashima J, Yoshioka K, Tachibana M, Tazaki H, Murai M (1997) Role of reactive oxygen species in cis-dichlorodiammineplatinum-induced cytotoxicity on bladder cancer cells. Br J Cancer 76: 206-210.
Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63.
Paglia DE, Valentine WN (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70: 158-169.
Roberts JR, Shaw CF, 3rd (1998) Inhibition of erythrocyte selenium-glutathione peroxidase by auranofin analogues and metabolites. Biochem Pharmacol 55: 1291-1299.
Schulz R, Emmrich T, Lemmerhirt H, Leffler U, Sydow K, Hirt C, Kiefer T, Link A, Bednarski PJ (2012) Identification of a glutathione peroxidase inhibitor that reverses resistance to anticancer drugs in human B-cell lymphoma cell lines. Bioorg Med Chem Lett 22: 6712-6715.
Wersto RP, Chrest FJ, Leary JF, Morris C, Stetler-Stevenson MA, Gabrielson E (2001) Doublet discrimination in DNA cell-cycle analysis. Cytometry 46: 296-306.
Wilde F, Chamseddin C, Lemmerhirt H, Bednarski PJ, Jira T, Link A (2014) Evaluation of (S)- and (R)-misonidazole as GPX inhibitors: synthesis, characterization including circular dichroism and in vitro testing on bovine GPx-1. Arch Pharm 347: 153-160.
Yang M, Nazhat NB, Jiang X, Kelsey SM, Blake DR, Newland AC, Morris CJ (1996) Adriamycin stimulates proliferation of human lymphoblastic leukaemic cells via a mechanism of hydrogen peroxide (H2O2) production. Br J Haematol 95: 339-344.
Zhang JG, Viale M, Esposito M, Lindup WE (1999) Tiopronin protects against the nephrotoxicity of cisplatin in the rat. Hum Exp Toxicol 18: 713-717.