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Preclinical report

Cisplatin combined with tiopronin or sodium thiosulfate: cytotoxicity *in vitro* and antitumor activity *in vivo*

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We have previously reported that the thiol compound tiopronin protects rat kidneys *in vitro* against the toxic activity of cisplatin. The influence of tiopronin and sodium thiosulfate (STS) on the cytotoxicity of cisplatin has been investigated on P388 leukemic cells *in vitro* after 3 days. The combination has also been investigated *in vivo* in BDF1 mice bearing a P388 s.c. tumor. In contrast to STS, tiopronin did not significantly reduce the cytotoxic activity of cisplatin *in vitro* and nor did it affect the uptake of platinum (cisplatin-derived), binding to DNA or the percantage of interstrand cross-links (%ISCL) formation. The co-administration of cisplatin (4 mg/kg) and tiopronin (150 and 300 mg/kg) to BDF1 female mice bearing a s.c. P388 tumor produced a significant reduction in tumor growth similar to that of a single 6 mg/kg dose of cisplatin. Interestingly, pre-incubation *in vitro* of either tiopronin or STS for 2 h with the species formed from cisplatin by hydrolysis demonstrated their ability in inhibiting the cytotoxicity of these reactive platinum products. These results indicate that tiopronin does not reduce the cytotoxicity of cisplatin *in vitro*, as STS does. This may be, at least partly, because of a different effect of the two thiol compounds on the cellular uptake and binding of platinum to DNA. Notably, tiopronin substantially reduced tumor growth in mice treated with a non-toxic dose of cisplatin ($p \leq 0.0277$), suggesting some positive influence of this thiol compound on the antitumor properties of cisplatin. The ability of tiopronin to protect *in vitro* against the cytotoxicity of the aquation products of cisplatin may be related to its nephroprotective effect. [© **1999 Lippincott Williams & Wilkins.**]

Key words: Anti-cancer activity, cisplatin, nephroprotection, tiopronin.

Introduction

Cisplatin is one of the most effective antineoplastic drugs used in several types of cancers including those of testis and ovary.[1-3] However, its clinical usefulness is hampered by its side effects, among which nephrotoxicity is the the most severe and dose-limiting.[4] Although many compounds have been tested as antidotes in order to minimize the toxicity of cisplatin,[5-8] particularly its nephrotoxicity, the preclinical findings have demonstrated for most of them that, besides reducing the nephrotoxic activity of cisplatin, they also reduced its antitumor activity.[9] At present the infusion of NaCl, mannitol or furosemide[10-12] is generally utilized to reduce this dose-limiting nephrotoxicity in a clinical setting.

It has been demonstrated previously that tiopronin [*N*-(2-mercaptopropionyl)-glycine], a SH-containing compound already used in the clinic in the treatment of rheumatoid arthritis and cysteine urolithiasis,[13,14] was able to protect kidneys against the toxic activity of cisplatin both *in vitro*[15] and *in vivo* in rats.[16] Tiopronin can be administrated orally, its pharmacology is known, and its adverse effects are few and non-fatal.[14,17] Overall these characteristics should simplify a possible clinical role for tiopronin as a protective drug.

In the present paper we have evaluated the influence of tiopronin on the cytotoxic and antitumor

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activity of cisplatin both *in vitro* and *in vivo*. *In vitro* experiments were performed using as reference the thiol compound STS, which has previously been reported to reduce both the cytotoxicity of cisplatin *in vitro* and its antitumor efficacy *in vivo*.[18]

Materials and methods

Chemicals

Cisplatin and tiopronin were obtained from Sigma (St Louis, MO), while STS was obtained from Merck (Milano, Italy).

Transformation of cisplatin to its hydrolysis products

The hydrolysis products of cisplatin were obtained by the incubation of cisplatin (50 μg/ml) in distilled water for 72 h at room temperature. Under these conditions, after the incubation, more than 80% of the platinum is present as hydrolysis products with the remainder being cisplatin.[19,20]

Cell line

Murine P388 leukemic cells were maintained in exponential growth in RPMI 1640 medium containing glutamine (2 mmol/l), gentamycin (100 mg/ml) and 10% fetal calf serum (complete medium).

Studies of cytotoxicity *in vitro*

The effects of either cisplatin or its hydrolyzed forms combined with tiopronin or STS were tested *in vitro* using a continuous exposure to the compounds for 72 h. P388 cells were plated in round-bottomed microtiter plates at 800 cells/well and treated with the single compounds (cisplatin, tiopronin and STS were diluted in normal saline, while the hydrolysis products derived from cisplatin were diluted in distilled water) and appropriate combinations of the two compounds used in each specific experiment. The final volume of each well was 200 μl, while drugs were added in a volume of 20 μl, alone or after being mixed at the relevant concentrations. Three different kinds of experiment were performed. In the first, cisplatin and tiopronin or STS were mixed and added to the cells immediately after mixing. In the second, combined drugs were incubated for 2 h at 37°C and then added to the cells. Thirdly, cells were incubated with tiopronin or STS for 2 h at 37°C, then washed with complete medium and cisplatin was then added. Cisplatin and its hydrolysis species were used over the concentration range of 0.0125–0.4 μM, while tiopronin and STS were used in the range of 0.0156–1 mM. After 3 days an aliquot (30 μl) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO) solution (2 mg/ml in PBS) was added to each well and incubated for 4 h at 37°C. At the end of the culture period plates were centrifuged at 275 *g* for 5 min. Then, culture medium was carefully aspirated and 100 μl of 100% dimethylsulfoxide was added. Complete solubilization of the formazan crystals was achieved after 10 min of incubation at 37°C and vigorous mixing of well contents with a multichannel pipette. The absorbance was measured on a microculture plate reader 400 ATC (SLT Labinstruments, Vienna, Austria) at 540 nm.[21] Individual experiments were performed 4–6 times and the IC_{50} values calculated on the basis of probit analysis of single concentration–response curves.

Measurement of cellular platinum accumulation

P388 cells were treated for 2 h at 37°C with cisplatin at different concentrations with or without tiopronin or STS. The molar ratio between tiopronin or STS and cisplatin was maintained constant (156), while cisplatin concentration varied (8–32 μM). This molar ratio was chosen because it was representative of the prevalent result of addittivity shown in the cytotoxicity experiments with combinations of tiopronin and cisplatin *in vitro*. Cells were exposed to drugs at a density of 1×10^6 cells/ml and maintained in suspension by continuous agitation for the whole period of drug exposure. Under these culture conditions no significant loss of viability was observed, as determined by the Trypan blue dye-exclusion assay. Cells were then collected by centrifugation and rapidly washed twice with cold normal saline to remove extracellular drug. Collected cells were then treated with 2–3 ml of HNO_3 (65%) at 120°C. The mixture was digested until all the HNO_3 had evaporated and the residue was dissolved in 10 mM HNO_3 (0.1–0.2 ml). Platinum concentrations were determined by flameless atomic absorption spectroscopy (AAS; Atomic Absorption Spectrophotometer, Hitachi Model Z-9000 simultaneous spectrophotometer, W Pabisch Instrument, Milano, Italy).[22] Individual experiments were performed 3 times. The detection limit was 30–50 ng Pt/ml, while intracellular platinum levels were expressed as ng Pt/10^6 cells.

Determination of platinum binding to DNA

P388 cells were exposed to cisplatin (16 μM), with or without tiopronin (the molar ratio between tiopronin and cisplatin was 78, 156 and 312), for 2 h at 37°C and then washed twice with cold normal saline. High molecular weight DNA was isolated by means of the salting out technique.[23] Briefly, cells were pelleted and then 6 ml of a lysis solution (2 mM EDTA, 10 mM Tris-HCl, pH 8, 400 mM NaCl), 240 μl proteinase K (Boehringer Mannheim, Mannheim, Germany) and 800 μl 10% sodium dodecylsulfate (BioRad, Richmond, CA) were added. The solution was mixed gently and left overnight in a waterbath at 37°C. After incubation, 2 ml CH_3COONa saturated solution was added and mixed vigorously for 15 s. Vials were then centrifuged at 750 *g* for 30 min, supernatants recovered and DNA precipitated with 1 volume of isopropyl alcohol. DNA was dissolved overnight in TE solution (10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8) at 50°C. Then 50 μg of DNA, whose yield and purity were measured by absorbance at 260 and 280 nm [the mean A_{260}/A_{280} ratio was 2.16+0.29 (SD)], was kept for the analysis of interstrand cross-links (ISCL), while the rest of DNA was digested in 65% HNO_3 and the residue diluted in 10 mM HNO_3. Bound platinum was determined by AAS. The %ISCL was evaluated by the ethidium bromide fluorescence technique.[24] Briefly, DNA was resuspended in TE, then 3 ml of a solution containing ethidium bromide (0.5 mg/ml in 0.4 mM EDTA, 20 mM K_2HPO_4, pH 11.8) was added to 0.2 ml (20 μg) aliquots of DNA extracted from control and treated cells. The fluorescence was measured before and after heating at 90°C for 10 min (Perkin-Elmer LS-5B spectrofluorimeter; excitation wavelength, 525 nm; emission wavelength 580 nm). The %ISCL was determined by the formula:

$$(f_t - f_n)/(1 - f_n) \times 100$$

Where f_t and f_n are the fluorescence after denaturation divided by the fluorescence before denaturation of treated (f_t) and control (f_n) samples. Single experiments were repeated 6 times. For both parameters data obtained in each experiment from all drug combinations were expressed as percentage relative to cells treated with cisplatin alone.

Animals

C57BL/6 × DBA/2 hybrid female mice (hereafter called BDF1), weighing 18-20 g, were used. Mice were obtained from Charles River (Calco, Italy) and allowed a 7 day rest period before experiments. They were housed six per cage and maintained at 22°C with a 12 h light/dark cycle, and fed on a standard diet (4RF-25; Italiana Mangimi, Settimo Milanese, Italy) and water *ad libitum*.

In vivo studies

Cisplatin was dissolved in normal saline (i.e. 0.9% NaCl) at a concentration of 0.8 mg/ml, while tiopronin was diluted in 0.9% NaCl at a concentration of 6 and 12 mg/ml. P388 cells were maintained by weekly i.p. transplantation of 10^6 cells, diluted in 0.1 ml of 0.9% NaCl. On day 0 of each experiment, mice were randomized into groups of six and injected s.c. with 10^6 P388 leukemic cells diluted in 0.1 ml of 0.9% NaCl. Drug treatment was started on the next day (day 1). Tiopronin was administered orally (p.o.) at the final doses of 150 and 300 mg/kg. Cisplatin was administered i.p. 1 h later at doses of 4 and 6 mg/kg. In order to avoid unnecessary suffering because of over-developed tumor masses, mice were sacrificed at day 11. Before killing, perpendicular tumor diameters were measured by a caliper and tumor volumes calculated using the formula:[25]

$$\frac{\text{length} \times (\text{width})^2}{2}$$

The weights of animals were recorded during the course of the experiments and the percent change in body weight on day 7 was used as an index of overall drug toxicity.

Data analysis

The cytotoxicity data were analyzed by two different methods according to the cytotoxic effect observed when tiopronin and STS were used alone over the different ranges of concentrations. When these compounds did not show any evident cytotoxic affect (this was only for STS) the reciprocal interference of the two compounds was evaluated by the simple statistical analysis of the differences found between the different survival curves obtained with the different concentrations of STS, combined with cisplatin or its hydrolysis products. When these compounds showed a cytotoxic effect at the concentrations used (this was for tiopronin), then the concentra-

tion-response curves for single compounds were firstly drawn by probit analysis and then the effect of the combination treatment was analyzed by the isobole method.[26] Briefly, for a combination of compounds A and B the combination index D is calculated by the equation:

$$\frac{Ac}{Ae} + \frac{Bc}{Be} = D$$

where Ac and Bc are the concentrations of the compounds in the combination, and Ae and Be are the concentrations of compounds which alone gave the same magnitude of effect. If $D<1$, the effect of the combination was considered synergistic; if $D=1$, the effect was considered simply additive; finally, if $D>1$, the effect was considered antagonistic. Each experiment was performed with six to 10 replicates. Calculation of p values was performed by the unpaired Student's t-test. Experimental D values for additivity (sham combinations) were calculated using combinations of two preparations of cisplatin or tiopronin.

One way analysis of variance (ANOVA) followed by a multiple comparison procedure (Fisher's PLSD test) was used for testing the significance of differences in platinum uptake, platinum-DNA binding and %ISCL of P388 cells *in vitro* and in tumor volumes of animals bearing s.c. tumors.

Results

Cytotoxic effect of cisplatin alone, its hydrolysis products or tiopronin and STS on P388 cells

As expected, cisplatin and its hydrolysis products demonstrated a high cytotoxic activity against P388 cells. The mean IC_{50} found for cisplatin was 0.05 ± 0.02 μM, while for the species formed from cisplatin by hydrolysis the mean IC_{50} was 0.11 ± 0.03 μM. Tiopronin was significantly more cytotoxic than STS, its mean IC_{50} being 0.096 ± 0.024 mM versus a mean IC_{50} for STS much higher than 1 mM (the percent of viable cells at this concentration was about 100%).

Effect of tiopronin and STS on the cytotoxicity of cisplatin

When co-incubated with cisplatin, tiopronin did not generally interfere with the cytotoxic activity of the antineoplastic drug. As shown in Table 1, a moderate antagonism was seen at the lowest cisplatin concentration used in combination with tiopronin at doses ranging from 0.0156 to

Table 1. Cytotoxic activity of combinations of tiopronin plus cisplatin on P388 cells *in vitro*

Tiopronin concentrations (mM)	Cisplatin concentrations (μM)				
	0.2	0.1	0.05	0.025	0.0125
0.25	$D=1.26$ NS[a] add	$D=0.90$ $p<0.05$ syn	$D=0.78$ $p<0.01$ syn	$D=0.72$ $p<0.001$ syn	$D=0.69$ $p<0.001$ syn
0.125	$D=1.47$ $p<0.01$ ant	$D=1.16$ NS add	$D=1.12$ NS add	$D=1.25$ NS add	$D=1.24$ NS add
0.0625	$D=1.32$ NS add	$D=1.19$ NS add	$D=1.16$ NS add	$D=1.34$ NS add	$D=1.69$ $p<0.01$ ant
0.0312	$D=1.23$ NS add	$D=0.95$ NS add	$D=1.09$ NS add	$D=1.30$ NS add	$D=1.76$ $p<0.001$ ant
0.0156	$D=1.14$ NS add	$D=0.95$ NS add	$D=0.94$ NS add	$D=1.35$ NS add	$D=2.35$ $p<0.001$ ant

[a] p values were calculated according to the unpaired Student's t-test by comparing D values for the combinations of cisplatin and tiopronin with the experimental D values for additivity obtained using sham combinations of two cisplatin and tiopronin solutions [D value for additivity: 1.13 ± 0.26 (SD)]; add, additivity; ant, antagonism; syn, synergism; NS, not significant.

0.0625 mM, as well as after combined treatment of 0.2 μM cisplatin and 0.125 mM tiopronin. Interestingly, the combinations of 0.25 mM tiopronin and cisplatin at concentrations equal to or lower than 0.1 μM resulted in a synergistic effect. In the same culture conditions STS exhibited a significant antagonism when used at concentrations of 0.25, 0.125 and 0.0625 mM (Figure 1).

When drugs were incubated together for 2 h before being added to the cells the behavior of tiopronin and STS was similar. In fact, STS reduced the cytotoxicity of cisplatin in a concentration-dependent manner. Cytotoxicity was nearly completely abrogated when STS was used at a concentration of 0.25 mM (Figure 2) and was still inhibited at the concentration of 0.0156 mM. Similarly, tiopronin significantly inhibited the cytotoxic activity of cisplatin in most of the combinations tested (Table 2).

In a third series of experiments, cells were exposed for 2 h to tiopronin or STS, then washed and incubated with cisplatin. In this case neither tiopronin nor STS inhibited cisplatin cytotoxicity (data not shown).

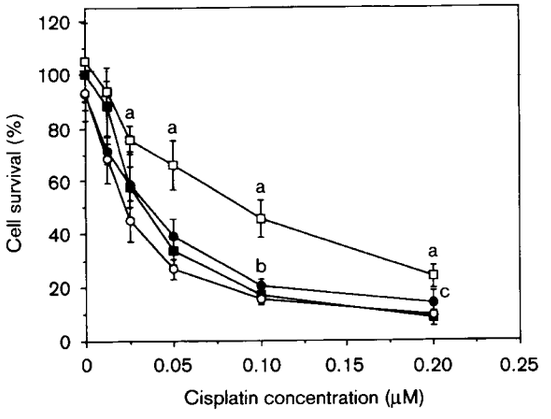

Figure 1. Concentration–response curves for the cytotoxic activity on P388 cells of cisplatin alone (■) or administered together with different concentrations of STS (□, 0.25 mM; ●, 0.0625 mM; ○, 0.0156 mM). Data relative to some STS concentrations (0.125 and 0.0312 mM) were omitted for more clarity. $^{a}p<0.001$; $^{b}p<0.05$; $^{c}p<0.02$. Bars, SD.

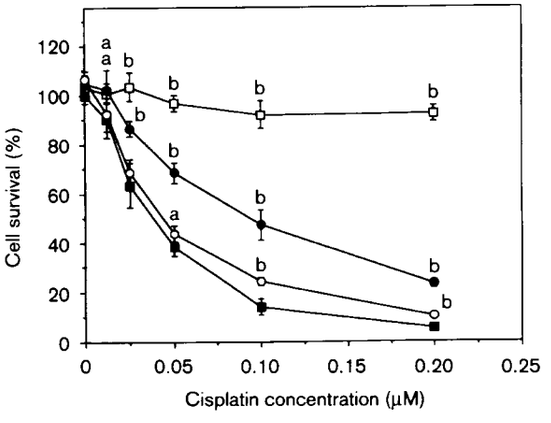

Figure 2. Concentration–response curves for the cytotoxic activity of cisplatin (■) added to P388 cells after being mixed and incubated for 2 h at 37°C with different concentrations of STS (□, 0.25 mM; ●, 0.0625 mM; ○, 0.0156 mM). Data for STS concentrations of 0.125 and 0.0312 mM were omitted. $^{a}p<0.05$; $^{b}p<0.001$. Bars, SD.

Assessment of platinum accumulation into the cells and binding to DNA

To try to explain the differences observed between tiopronin and STS in the inhibition of cisplatin cytotoxicity we evaluated platinum accumulation into P388 cells treated with cisplatin incubated either with or without tiopronin or STS (Table 3). The ratio between tiopronin or STS and cisplatin was maintained constant (156). While STS significantly inhibited platinum uptake by P388 cells (about 50% inhibition after incubation with 16 and 32 μM cisplatin), tiopronin induced only a small, although statistically significant (p=0.0181), reduction of platinum accumulation (about 22%) at a cisplatin concentration of 32 μM.

Assessment of platinum–DNA binding and determination of %ISCL

In order to analyze the influence of tiopronin on the process of DNA platination, platinum-DNA binding and the %ISCL were determined *in vitro* on P388 cells after incubation with cisplatin and either tiopronin or STS. Table 3 also shows that STS, unlike tiopronin, significantly reduced the extent of platinum-DNA binding (p=0.0001). A reduction, although not significant, was also seen in the %ISCL. By the Fisher PLSD multiple comparison procedure following ANOVA a highly significant difference in platinum-DNA binding was also observed when the associations of cisplatin plus 1.25, 2.5 and 5 mM tiopronin were compared to the associations of cisplatin plus 1.25, 2.5 and 5 mM STS (104±9 versus 75±27%, p=0.0142; 87±10 versus 53±22%, p=0.0103; 87±16 versus 39±23%, p=0.0005), respectively (Table 3).

Antitumor activity against P388 s.c. tumor

A slight (−29%), but statistically significant, reduction in tumor volume was observed when P388 tumor-bearing mice were treated with 300 mg/kg tiopronin alone, compared to untreated controls (1.18±0.23

Table 2. Cytotoxic activity of combinations of tiopronin and cisplatin, after prior incubation together for 2 h, on P388 cells *in vitro*

Tiopronin concentrations (mM)	Cisplatin concentrations (μM)				
	0.2	0.1	0.05	0.025	0.0125
0.25	*D*=1.37 NS[a] add	*D*=1.03 NS add	*D*=1.03 NS add	*D*=0.99 NS add	*D*=0.93 NS add
0.125	*D*=1.62 *p*<0.001 ant	*D*=1.40 *p*<0.05 ant	*D*=1.08 NS add	*D*=1.00 NS add	*D*=1.16 NS add
0.0625	*D*=2.14 *p*<0.001 ant	*D*=2.12 *p*<0.001 ant	*D*=2.14 *p*<0.001 ant	*D*=1.80 *p*<0.001 ant	*D*=2.29 *p*<0.001 ant
0.0312	*D*=2.06 *p*<0.001 ant	*D*=1.98 *p*<0.001 ant	*D*=2.14 *p*<0.001 ant	*D*=2.73 *p*<0.001 ant	*D*=3.75 *p*<0.001 ant
0.0156	*D*=1.88 *p*<0.001 ant	*D*=1.75 *p*<0.001 ant	*D*=1.85 *p*<0.001 ant	*D*=1.74 *p*<0.001 ant	*D*=2.50 *p*<0.001 ant

[a]*p* values were calculated according to the unpaired Student's *t*-test by comparing *D* values for the combinations of cisplatin and tiopronin with the experimental *D* values for additivity obtained using sham combinations of two cisplatin and tiopronin solutions [*D* value for additivity: 1.13±0.26 (SD)]; add, additivity; ant, antagonism; NS, not significant.

Table 3. Effect of tiopronin and STS on platinum uptake, total platinum binding to DNA and the %ISCL formation in P388 leukemic cells treated with cisplatin

	Platinum uptake		
	Cisplatin (μM)[a]		
	8	16	32
Cisplatin alone	1.37±0.20	2.68±0.36	5.31±0.96
Cisplatin+tiopronin	1.12±0.13	2.50±0.44	4.33±0.98[b]
Cisplatin+STS	1.00±0.19	1.35±0.07[c]	2.64±0.87[c]

	Platinum–DNA binding and ISCL[e]					
	Cisplatin (16 μM) plus STS			Cisplatin (16 μM) plus tiopronin		
	1.25 mM	2.5 mM	5 mM	1.25 mM	2.5 mM	5 mM
Platinum–DNA binding	75±27[f]	53±22[g]	39±23[h]	104±9	87±10	87±16
ISCL	73±9[a]	64±29	62±17	87±16	85±15	90±25

[a]Data (mean±SD) are expressed as ng Pt/10^6 cells, while the *p* value for the analysis of variance, evaluated by the ANOVA test followed by Fisher's PLSD test for a multiple comparison between groups, was 0.0001. Tiopronin and STS were used at a molar concentration 156 times higher than that utilized for cisplatin.
[b]*p*=0.0181, [c]*p*=0.0042, [d]*p*=0.0001, as compared to cisplatin alone.
[e]Data (mean±SD) are expressed as percentage relative to cells treated with cisplatin alone (mean platinum–DNA binding, 7.6±2 ng Pt/μg DNA; mean %ISCL, 5.1±1.3%, *n*=6). *The p* values for the analysis of variance, evaluated as before, were 0.0001 for the platinum–DNA binding and 0.1275 for the ISCL formation.
[f]*p*=0.0142, [g]*p*=0.0103, [h]*p*=0.0005, as compared to cells exposed to cisplatin plus the corresponding concentrations of tiopronin.

Table 4. Antitumor activity *in vivo* of cisplatin plus tiopronin in mice bearing s.c. P388 tumors

	Treatment						
	Control[a]	Cisplatin (4 mg/kg)	Cisplatin (6 mg/kg)	Tiopronin (150 mg/kg)	Tiopronin (300 mg/kg)	Cisplatin+ tiopronin (4+150 mg/kg)	Cisplatin tiopronin (4+300 mg/kg)
Tumor volume (cm^3)	1.66±0.58[b]	0.94±0.31[c]	0.41±0.09[d]	1.32±0.31	1.18±0.23[e]	0.52±0.27[f]	0.45±0.11[g]
Percent change in body weight at day 7	+3.8	+0.8	+0.3	+3.3	+4.6	+2.2	−2

[a]Mice treated with normal saline alone.
[b]Tumor volume (mean±SD)=length × (width)^2/2.
[c]$p=0.0009$, [d]$p<0.0001$. [e]$p=0.0156$, as compared to untreated control mice. [f]$p=0.0277$, [g]$p=0.0114$, as compared to mice treated with cisplatin alone. *p* values were calculated by the ANOVA analysis of variance followed by Fisher's PLSD test for a multiple comparison between groups.

versus 1.66±0.58 cm^3, $p=0.0156$). When cisplatin was administered at 4 mg/kg plus tiopronin at 150 and 300 mg/kg, the mean tumor volumes were significantly smaller than in mice receiving cisplatin alone (0.52±0.27 and 0.45±0.11 versus 0.94±0.31 cm^3, $p=0.0277$ and $p=0.0114$, respectively) (Table 4). The combined *in vivo* treatment with cisplatin 4 mg/kg and tiopronin produced a mean reduction in tumor volume similar to that obtained with the highest dose of cisplatin administered as a single agent (6 mg/kg, 0.41± 0.09 cm^3), suggesting a possible potentiation of the cisplatin activity by tiopronin. The percent change in body weight at day 7 was not greatly different between the groups of mice treated with cisplatin and cisplatin plus tiopronin, although a slight decrease in body weight was observed in animals given the combined treatment with the highest dose of tiopronin (Table 4).

Effect of tiopronin and STS on the cytotoxicity of hydrolysis products of cisplatin

Tiopronin, depending on the concentrations used, significantly inhibited the cytotoxic activity of solutions containing the hydrolysis products of cisplatin (Figure 3). Similarly, STS lowered the cytotoxic activity of the same aquated species, and its activity was particularly evident and significant at their highest concentrations (data not shown).

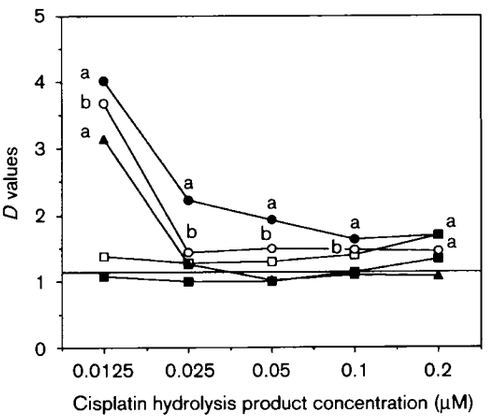

Figure 3. *D* values in the combined experiments on P388 cells involving tiopronin and cisplatin hydrolysis products, previously mixed together and incubated for 2 h at 37°C. The experimental mean *D* values for additivity was calculated using combinations of two serial dilutions of tiopronin and cisplatin (mean *D* value: 1.13±0.26). Concentrations of cisplatin hydrolysis products: (■) 0.25, (□) 0.125, (●) 0.062, (○) 0.0312 and (▲) 0.0156 mM. [a]$p<0.001$, [b]$p<0.05$, as compared to the experimental mean *D* value for the sham combinations.

Discussion

In previous work[15] attention was focused on the protective effect of the SH-containing compound tiopronin on cisplatin-induced nephrotoxicity. The results demonstrated that this drug protected rat kidney slices against enzyme leakage and the decrease in reduction of MTT caused by cisplatin. Preliminary experiments in normal Wistar rats *in vivo* have demonstrated that tiopronin, given p.o. or i.p. 1 h before a cisplatin dose of 6 mg/kg, protected against the nephrotoxicity induced by the platinum compound.[16]

The capability of SH-containing compounds, such as dithiothreitol, sodium diethyldithiocarbamate, reduced

GSH, L-methionine and WR-2721, to counteract the depletion of GSH is essential for the protection they provide. This kind of compound may serve as precursor of glutathione, may maintain glutathione in a reduced state and protect protein -SH groups by the reaction of the nucleophilic sulfur atoms with the hydration products of cisplatin. We have already demonstrated that cisplatin, when co-incubated with tiopronin, lowered the concentration of -SH groups, presumably by forming a complex between these two compounds.[15] In some cases it has been observed that, besides reducing the toxic activity of cisplatin, this kind of reaction may also diminish its antineoplastic efficacy. This is particularly true for STS. This compound inactivates cisplatin in blood,[27] and reduces its cytotoxic and antitumoral activity *in vitro* and *in vivo*.[28,29] These observations also explain the failure of STS to increase the therapeutic index of cisplatin in some rodent tumor models.[18,27] In fact, although STS can protect against the toxicity of cisplatin and allow higher doses of cisplatin to be given, it concomitantly and markedly reduces the antitumor activity of cisplatin. For its clear ability to inactivate cisplatin in such experimental conditions, STS was chosen as the positive reference compound for tiopronin in our experiments *in vitro*.

These experiments clearly show that tiopronin, when added together with cisplatin to P388 cells, inhibited the cytotoxicity of cisplatin only in a few drug combinations and generally less than did STS. It is also noteworthy that in other drug combinations, tiopronin, used at a highly cytotoxic concentration, was able to synergize with cisplatin. Only when tiopronin was incubated for 2 h with cisplatin before being added to the cells was the cytotoxicity of the platinum compound significantly reduced, as was the case when cisplatin was preincubated with STS. This finding indirectly suggests that the rate of inactivation of cisplatin by tiopronin is slower than by the reference compound STS. The observation that both tiopronin and STS have no effects on cisplatin cytotoxicity, when cells were exposed to both compounds for 2 h before being incubated with cisplatin, indicates that the reactivity of these thiol compounds towards cisplatin is a crucial step, but probably not the only one, for the efficacy of these drug combinations.

Tiopronin, unlike STS, even at a concentration higher than that reached in plasma *in vivo*,[17] did not generally inhibit the platinum uptake, the platinum-DNA binding or the %ISCL in P388 cells exposed to cisplatin. This could explain, at least in part, the different effect of equimolar concentrations of these two -SH compounds on cisplatin cytotoxicity and suggests that the antitumor efficacy of the cisplatin and tiopronin combination is better than cisplatin and STS combination.[28,29] This possibility was verified *in vivo* using the tumor model consisting of P388 leukemic cells implanted s.c. in BDF1 female mice. In accordance with our previous experiments in rats,[16] tiopronin was given p.o. 1 h before i.p. cisplatin. Under our experimental conditions the antitumor efficacy of a non-toxic dose of cisplatin (4 mg/kg) was significantly increased by doses of tiopronin which were either ineffective (150 mg/kg) or effective (300 mg/kg) on tumor progression as single agents. Indeed, a slight (−29%), but significant (p=0.0156) antitumor activity was observed in mice receiving 300 mg/kg tiopronin compared to untreated animals, whereas the single treatment with 150 mg/kg tiopronin did not exhibit a significant antitumor effect. The ability of a non-effective dose of tiopronin to improve the antitumor efficacy of cisplatin was also observed in a preliminary experiment performed on BDF1 female mice bearing i.p. P388 leukemia. The combination of 150 mg/kg tiopronin p.o. and 4 mg/kg cisplatin i.p. exhibited a considerably greater activity than did cisplatin alone. The median survival time of tumor-bearing mice receiving cisplatin alone was increased by tiopronin by 186%, while the percent of survivors at day 60 (period of observation) increased from 16.7 to 50% (unpublished observations).

The possibility that tiopronin, similarly to STS, could inhibit the cytotoxic activity of hydrolysis products derived from cisplatin, which significantly contribute to the adverse effects of the parent drug,[30,31] was investigated *in vitro*. Tiopronin reduced the cytotoxicity of these species in murine P388 cells, presumably by a reaction of the nucleophilic sulfur atom of tiopronin with the hydration products of cisplatin.[15] It is of note that a similar effect was also observed when the human A2780 ovarian cancer cell line was utilized as target (unpublished observations). This observation along with the indication that tiopronin may be actively concentrated in rat kidney suggests a possible mechanism for the previously reported reduction of cisplatin nephrotoxicity.[15]

The results *in vivo* generally confirmed the previous ones obtained *in vitro*, underlining the interesting effect exerted by tiopronin administered in combination with cisplatin. The mechanism(s) of this pharmacological interaction, however, remains an open question. Indeed, although the ability of tiopronin to counteract the cytotoxicity of the hydrolyzed forms of cisplatin may contribute to its nephroprotective potential, it could not, by itself, explain how an improved antitumor efficacy was observed in mice receiving a non-toxic dose of cisplatin. The effect of

tiopronin on platinum-DNA binding and the ISCL formation suggests that this -SH compound, unlike STS, did not counteract the process of DNA platination by cisplatin. Consequently, the antitumor activity of cisplatin was not impaired. Nevertheless, our observations have not yet provided an insight into how an increased antitumor activity *in vivo* may be obtained after co-administration of cisplatin and tiopronin.

Whatever the mechanism(s), the fact that tiopronin did not show acute toxicity in mice at doses which improve the antitumor efficacy of cisplatin in our tumor model, along with the advantages that tiopronin can be administered orally, that its pharmacology is known and that its adverse effects are few and non-fatal, are a considerable encouragement to clinical investigation of this drug in combination with cisplatin.

References

1. Sleijfer DT, Meijer S, Mulder NH. Cisplatin: a review of clinical applications and renal toxicity. *Pharmaceutisch Weekblad Scientific Edition* 1985; **7**: 227-37.
2. van Basten JP, Schrafford-Koops H, Sleijfer DT, Pras E, van Driel MF, Hoekstra HJ. Current concept about testicular cancer. *Eur J Surg Oncol* 1997; **23**: 354-60.
3. Thigpen T, Vance R, Puneky L, Khansurt T. Chemotherapy in advanced ovarian carcinoma: current standards of care based on randomized trials. *Gynecol Oncol* 1994; **55**: 597-607.
4. Skinner R. Strategies to prevent nephrotoxicity of anticancer drugs. *Curr Opin Oncol* 1995; **7**: 310-5.
5. Esposito M, Fulco RA, Collecchi P, *et al.* Improved therapeutic index of cisplatin by procaine hydrochloride. *J Natl Cancer Inst* 1990; **8**: 677-84.
6. Heidemann HT, Muller S, Mertins L, Stepan G, Hoffmann K, Ohnhaus EE. Effect of aminophylline on cisplatin nephrotoxicity in the rat. *Br J Pharmacol* 1989; **97**: 313-8.
7. Sleijfer DT, Offerman JJG, Mulder NH, *et al.* The protective potential of the combination of verapamil and cimetidine on cisplatin-induced nephrotoxicity in man. *Cancer* 1987; **60**: 2823-8.
8. Bird JE, Walser MM, Quebbeman J. Protective effect of organic cation transport inhibitors on *cis*-diamminedichloroplatinum(II)-induced nephrotoxicity. *J Pharmacol Exp Ther* 1984; **231**: 752-8.
9. Aamdal S, Fodstad O, Pihl A. Some procedures to reduce *cis*-platinum toxicity reduce antitumour activity. *Cancer Treat Rev* 1987; **14**: 389-95.
10. Anand AJ, Bashey B. Newer insights into cisplatin nephrotoxicity. *Ann Pharmacother* 1993; **27**: 1519-25.
11. Pinzani V, Bressolle F, Haug IJ, Galtier M, Blayac JP, Balmer P. Cisplatin-induced renal toxicity and toxicity-modulating strategies: a review. *Cancer Chemother Pharmacol* 1994; **35**: 1-9.
12. Dumas M, de Gislain C, d'Athis P, *et al.* Evaluation of the effect of furosemide on ultrafilterable platinum kinetics in patients treated with *cis*-diamminedichloroplatinum. *Cancer Chemother Pharmacol* 1989; **23**: 37-40.
13. Amor B, Lery C, De Gery A. Tiopronin (*N*-[2-mercaptopropionyl]glycin) in rheumatoid arthritis. *Arth Rheum* 1982; **25**: 698-703.
14. Reynolds JEF, ed. Tiopronin. In: *Martindale. The extra pharmacopoeia*, 31st edn. London: Royal Pharmaceutical Society 1996: 1761.
15. Zhang JG, Lindup WE. Tiopronin protects against the nephrotoxicity of cisplatin in rat renal cortical slices *in vitro*. *Toxicol Appl Pharmacol* 1996; **141**: 425-33.
16. Zhang JG, Lindup WE. Tiopronin protects against the nephrotoxicity of cisplatin in the rat *in vivo*. *Naunyn Schmiedeberg's Arch Pharmacol* 1998; **358** (suppl 2): R532, abstr P45.9.
17. Carlsson MS, Denneberg T, Emanuelsson BM, Kagefdal B, Lindgren S. Pharmacokinetics of oral Tiopronin. *Eur J Clin Pharmacol* 1993; **45**: 79-84.
18. Catino JJ, Francher DM, Schurig JE. Evaluation of *cis*-diamminedichloroplatinum(II) combined with metoclopramide or sodium thiosulphate on L1210 leukemia *in vitro* and *in vivo*. *Cancer Chemother Pharmacol* 1986; **18**: 1-4.
19. Fulco RA, Vannozzi M, Collecchi P, *et al.* Effect of normal saline on cisplatin pharmacokinetics and antitumor activity in mice bearing P388 leukemia. *Anticancer Res* 1990; **10**: 1603-10.
20. Hincal AA, Long DF, Repta AJ. Cisplatin stability in aqueous parenteral vehicles. *J Parent Drug Ass* 1979; **33**: 107-16.
21. Hussain RF, Nouri AME, Oliver RTD. A new approach for measurement of cytotoxicity using colorimetric assay. *J Immunol Methods* 1993; **160**: 89-96.
22. Cafaggi S, Esposito M, Parodi B, *et al.* Synthesis and antitumor activity of a new *cis*-diammineplatinum(II) complex containing procaine hydrochloride. *Anticancer Res* 1992; **12**: 2285-92.
23. Gao X, Fernandez-Vina M, Shumway W, Stastny P. DNA typing for class II HLA antigens with allele-specific or group-specific amplification. I. Typing for subsets of HLA-DR4. *Hum Immunol* 1990; **27**: 40-50.
24. Coluccia M, Boccarelli A, Mariggiò MA, *et al.* Platinum(II) complexes containing iminoethers: a *trans* platinum antitumor agent. *Chem-Biol Interact* 1995; **98**: 251-66.
25. Arakawa H, Iguchi T, Morita M, *et al.* Novel indolocarbazole compound 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl) 5H-indolo [2,3-*a*]pyrrolo-[3,4-*c*]carbazole-5,7(6*H*)-dione (NB-506): its potent antitumor activities in mice. *Cancer Res* 1995; **55**: 1316-20.
26. Berembaum MC. What is synergy? *Pharmacol Rev* 1989; **41**: 93-141.
27. Abe R, Akiyoshi T, Baba T. Inactivation of *cis*-diamminedichloroplatinum(II) in blood by sodium thiosulphate. *Oncology* 1990; **47**: 65-9.
28. Aamdal S, Fodstad O, Pihl A. Sodium thiosulphate fails to increase the therapeutic index of intravenously administered *cis*-diamminedichloroplatinum(II) in mice bearing murine and human tumors. *Cancer Chemother Pharmacol* 1988; **21**: 129-33.
29. Uozumi J, Ishizawa M, Iwamoto Y, Baba T. Sodium thiosulphate inhibits *cis*-diamminedichloroplatinum(II) activity. *Cancer Chemother Pharmacol* 1984; **13**: 82-5.

30. Daley-Yates PT, McBrien DCH. Cisplatin metabolites in plasma: a study of their pharmacokinetics and importance in the nephrotoxic and antitumour activity of cisplatin. *Biochem Pharmacol* 1984; **33**: 3063–70.

31. Jones MM, Basinger MA, Beaty JA, Holscher MA. The relative nephrotoxicity of cisplatin, *cis*-$[Pt(NH_3)_2(guanosine)_2]^{2+}$, and the hydrolysis product of cisplatin in the rat. *Cancer Chemother Pharmacol* 1991; **29**: 29–32.

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