PERGAMON

European Journal of Cancer 38 (2002) 1148–1156

European Journal of Cancer

www.ejconline.com

BNP7787, a novel protector against platinum-related toxicities, does not affect the efficacy of cisplatin or carboplatin in human tumour xenografts

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Received 12 September 2001; received in revised form 28 November 2001; accepted 23 January 2002

Abstract

BNP7787 (2′,2′-dithio-bis-ethane sulphonate sodium), a water-soluble disulphide, is chemically and mechanistically different from other sulphur-containing chemoprotective agents. Presently, BNP7787 is under investigation for its protective properties with regard to the side-effects of platinum compounds. In this study, we evaluated BNP7787, mesna and amifostine for their effects on the antitumour activity of platinum compounds. Continuous exposure to BNP7787 did not affect the antiproliferative effects of cisplatin or carboplatin, but the efficacy of both compounds was reduced in the presence of mesna *in vitro* in two human ovarian cancer cell lines. BNP7787 or amifostine combined with cisplatin or carboplatin given in standard schedules for the treatment of nude mice bearing well-established OVCAR-3 xenografts did not interfere with platinum-induced inhibition of tumour growth. Of interest, BNP7787 or amifostine co-administered with carboplatin was significantly more effective than carboplatin alone ($P < 0.01$). In the presence of amifostine, doses of cisplatin and carboplatin could be safely increased by factors of 1.6 and 1.5, respectively. Unlike in a previous study of BNP7787 in tumour-bearing rats, BNP7787 did not protect against additional weight loss following treatment with higher doses of cisplatin in OVCAR-3-bearing mice. Pharmacokinetics of (mixed) disulphides including BNP7787 and extractable mesna in deproteinised plasma revealed a rapid disappearance of BNP7787 and an $AUC^{5\text{-}60}$ value of mesna 9-fold lower than that calculated after an equivalent dose of mesna by weight. We can conclude that BNP7787 does not interfere with the antitumour activity of platinum compounds *in vitro* and *in vivo*. Clinical trials are underway to evaluate the protection of normal tissues by BNP7787 when combined with cisplatin.

Keywords: Cisplatin; Carboplatin; Nephrotoxicity; BNP7787; Mesna; Amifostine

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum) and its analogue carboplatin (*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum) have a broad spectrum of activity against a variety of epithelial tumours. For both drugs, the formation of intracellular DNA cross-links is believed to be the major target mechanism [1]. The platinum compounds differ not only in chemistry and pharmacology, but also in their pattern of toxicities. Where cisplatin has severe emetogenic, renal, neurological and otological side-effects, carboplatin is associated with substantial thrombocytopenia.

Apart from standard vigorous saline hydration required to prevent cisplatin-induced nephrotoxicity, a number of protective agents have been studied in the clinic. Such modulators should not interfere with the antitumour activity, should not be toxic by themselves and should be pharmacologically compatible with platinum compounds. First-generation platinum-protective agents including sodium thiosulphate, diethyldithiocarbamate, and amifostine contain thiol moieties, which are reactive with the nephrotoxic monohydroxy/monoaquo monochloro species of cisplatin [2,3]. Direct

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0959-8049/02/$ - see front matter
PII: S0959-8049(02)00036-9

formulation or admixture of such drugs with cisplatin has shown rapid chemical quenching of cisplatin by the sulphur anion or the sulphhydryl moiety of these agents resulting in the formation of an inactive platinum–thiol conjugate species [3]. It is well known that mesna (2-mercapto-ethane sulphonate) is directly incompatible with cisplatin and, accordingly, the two compounds are administered separately [3]. The most extensively evaluated agent for protection is amifostine (WR2721; *S*-2(3-aminopropylamino)ethylphosphorothioic acid) [4,5]. This agent is rapidly dephosphorylated by membrane-bound alkaline phosphatase in the capillary endothelium to its active metabolite thiol WR1065, which is selectively taken up by normal tissues. Because amifostine is associated with additional side-effects consisting of an increase in nausea and vomiting, as well as transient hypotension, it is not an ideal protector.

Mesna and BNP7787 (2′,2′-dithio-bis-ethane sulphonate sodium; Fig. 1), like all sulphur-containing compounds, are chemically incompatible with cisplatin. The disulphide linkage of BNP7787, however, is inert and stable in plasma which is in contrast to the free thiol-containing drugs that have been introduced into clinical testing in the past. This compound is considered a novel second-generation cytoprotective agent. Following administration, BNP7787 predominates in human plasma in its disulphide form, it rapidly undergoes intracellular transport into certain tissues, such as the renal tubular epithelium, intestines, bone marrow, and other tissues where a fraction of the drug is converted most likely by intracellular thiol transferases and reductases into the water-soluble free thiol species, 2-mercapto-ethane sulphonate (mesna). In contrast to BNP7787, mesna will rapidly react with the toxic monohydroxy/monoaquo monochloro species of cisplatin that are formed in these normal tissues. Preclinical studies in rats have shown that BNP7787 provided complete protection against cisplatin-induced nephrotoxicity and neurotoxicity and did not reduce the antitumour activity of platinum compounds [3]. BNP7787 pretreatment was observed to substantially increase the therapeutic index of cisplatin and to potentiate the anti-tumour activity in tumour-bearing rats. The compound was administered immediately prior to cisplatin with molar ratios of BNP7787 to cisplatin that exceeded 150; the breakpoint for partial nephroprotection by BNP7787 in rats was observed at a molar ratio of 50:1. In Beagle dogs, BNP7787 pretreatment prevented lethal toxicity of high-dose cisplatin and carboplatin due to renal failure (for cisplatin) and myelosuppression (for both drugs) (Hausheer).

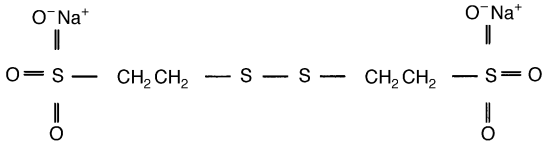

Fig. 1. Chemical structure of BNP7787.

The purpose of the study was to investigate, in parallel with a phase I dose-finding study of BNP7787 given before a fixed-dose of cisplatin in patients with solid tumours, a possible influence of BNP7787 on the antiproliferative effects of cisplatin and carboplatin in human ovarian cancer cell lines *in vitro* and on the growth inhibition induced by platinum compounds in nude mice bearing human ovarian cancer xenografts. Comparative studies were carried out in parallel using mesna and amifostine. Additional experiments included dose escalation of the platinum compounds *in vivo* aiming at an increased therapeutic index in the presence of BNP7787. We also performed pharmacokinetic studies of BNP7787 and mesna in mice.

2. Materials and methods

2.1. Drugs

Cisplatin (Platinol; powder to be dissolved in 1 mg/ml water) and carboplatin (Paraplatin; 10 mg/ml solution) were obtained from Bristol-Myers Squibb (Woerden, The Netherlands). BNP7787 was provided by BioNumerik Pharmaceuticals, Inc., and was dissolved in water at a concentration of 150 mg/ml at pH 7.2. Mesna (Uromitexan) was obtained from Dagra Pharma (Diemen, The Netherlands) as a 100 mg/ml solution. Amifostine (500 mg of lyophilised WR-2721 and 500 mg of mannitol) was obtained from US Bioscience (West Conshohocken, PA, USA) and dissolved in 9.5 ml NaCl 0.9% to a 50 mg/ml solution. For *in vitro* use, drugs were further diluted in tissue culture medium.

2.2. In vitro *sensitivity*

Human ovarian cancer A2780 [6] and OVCAR-3 [7] cells were used to test a possible influence of BNP7787 or mesna on the growth-inhibitory properties of cisplatin and carboplatin. Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Breda, The Netherlands), containing 10% heat-inactivated fetal calf serum (Sebak, Aidenbach, Germany), 50 IU/ml penicillin and 50 μg/ml streptomycin (Flow, Irvine, UK) in an incubator with a humidified atmosphere containing 5% CO_2 at 37 °C.

For growth-inhibition experiments, A2780 cells (3000 cells/well) and OVCAR-3 (5000 cells/well) were plated in 96-well microtitre plates. After 24 h of cell seeding, cells were exposed for 96 h to varying concentrations of cisplatin or carboplatin in the absence or presence of BNP7787 or mesna. The molar ratio of the protective agent:platinum compound was 100:1. Protective agents were added simultaneously or 15–20 min before the

platinum compounds. They were also added for a period of 20 min before the platinum compounds and washed away before the platinum compounds were added. After 96 h, the 3-(4,5-dimethylthiazol-2-yl)-2,6-dimethyl-morpholino-2,5-diphenyl-tetrazolium bromide (MTT) assay was carried out. The cells were stained for 4 h with the tetrazolium salt MTT (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (0.5 mg/ml). MTT was reduced by living cells only to a dark-coloured formazan which was measured on a Labsystems Multiscan Bichromatic plate reader (Helsinki, Finland) at 540 nm. The results were expressed as the IC50, which is the concentration of the drug(s) inducing a 50% inhibition of cell growth of treated cells when compared with the growth of control cells. In control cultures, cell growth was exponential during the assay period. All concentrations were tested in quadruplicate and each experiment was repeated three times.

2.3. In vivo *sensitivity*

Female nude mice (Hsd: athymic nude-*nu*) were purchased at the age of 6 weeks (Harlan, Horst, The Netherlands). The animals were maintained in cages with paper filter covers under controlled atmospheric conditions. Cages, covers, bedding, food and water were changed and sterilised weekly. Animals were handled in a sterile manner in a laminar down-flowhood. Ethical approval was obtained from the 'Dutch Committee for Experimental Animals' for transplantation of human tumour tissue and for treatment experiments in nude mice.

The generation of OVCAR-3 xenografts grown subcutaneously (s.c.) in both flanks of nude mice has been previously described in Refs. [8–10]. For treatment experiments, 8- to 10-week old animals were inoculated s.c. with tumour fragments of 2–3 mm diameter. Upon growth, tumours were measured twice a week in three dimensions with a slide caliper. The volume was calculated by the equation length×width×height×0.5, and expressed in mm^3. Mice were weighed at the time of the tumour measurements.

Treatment was started in groups of six mice with a mean tumour volume of approximately 100 mm^3 in each group (designated as day 0). The schedules of 5 mg/kg cisplatin intravenously (i.v.) weekly×2 and 60 mg/kg carboplatin i.v. weekly×2 were maximum tolerated schedules on the basis of a reversible 10% weight loss established in previous studies [8–10]. Amifostine, used as a reference compound, was given in a dose of 200 mg/kg intraperitoneally (i.p.) weekly×2 and preceded platinum compounds by 5 min [8,9]. The doses of BNP7787 and mesna were 1000 mg/kg i.v. given weekly×2 and preceded cisplatin by 5 min. The molar ratio of BNP7787:cisplatin was 184:1 and for mesna:cisplatin the molar ratio was 368:1. 1000 mg/kg BNP7787 i.v. was also given concurrently with and 30 min after carboplatin reaching a molar ratio for BNP7787:carboplatin of 32:1.

For the evaluation of response, tumours <20 mm^3 at the start of treatment were excluded. Animals dying within 2 weeks after the final drug injection were considered toxic deaths and were also excluded. Relative tumour volumes were used which were calculated using the formula V_T/V_0, in which V_T is the volume on any given day and V_0 is the volume at the start of treatment. The mean relative volume of treated tumours and that of control tumours on day 31 of the experiment were used to calculate differences between the groups. In addition, the specific growth delay was chosen to express drug efficacy which was calculated as $(T_{treated}-T_{control})/T_{control}$. In this formula, T represents the number of days required for a tumour to double in volume from the start of treatment.

2.4. *Plasma concentrations and pharmacokinetics of BNP7787 and mesna*

Non-tumour-bearing nude mice were injected i.v. with 1000 mg/kg BNP7787 or mesna i.v. to determine the pharmacokinetics of BNP7787 and mesna in nude mice. At 5, 15, 30 and 60 min after administration, blood was taken from groups of three mice each per time-point. Plasma was immediately obtained and deproteinised with an equal volume of 0.33 M sulphuric acid and an equal volume of sodium hexametaphosphate (5% wt./vol.). The deproteinised plasma was stored at −20 °C until analysis.

Mesna was determined by high-performance liquid chromatography (HPLC) according to Verschraagen and colleagues [11]. In short, a Phenomenex Customsil 5 ODS-4 column (100×4.6 mm; Bester, Amstelveen, The Netherlands) was used protected by a refillable guard column (20×2 mm) with pellicular C18 refill (35–50 m Alltech, Deerfield, USA). The mobile phase was an aqueous solution of tri-sodium citrate dihydrate (0.1 M), tetrabutylammonium dihydrogenphosphate (1.0 mM) and cysteamine (0.1 μM), adjusted to pH 3.5 with 85% *o*-phosphoric acid. The flow-rate used was 1 ml/min and the column was kept at a temperature of 36 °C. The electrochemical detector (Antec Leyden, Leiden, The Netherlands), provided with a wall-jet Au electrode, was set at an operating potential of +1.00 V relative to an Ag/AgCl reference electrode. The plasma containing (mixed) disulphide(s) was analysed as mesna after reduction with sodium borohydride to free mesna, subsequent HPLC analysis and calculated as $([mesna]_{after\ reduction}-[mesna]_{before\ reduction})/2$ [11]. It should be noted that this analytical method cannot discriminate between BNP7787 itself and a mixture of possible disulphide heteroconjugates formed from mesna with cysteine, homocysteine or glutathione. In

addition, because of deproteinisation of plasma, the non-covalently protein-bound fraction of mesna will also be measured as extractable mesna.

Areas under the curve (AUC^{5-60}) were calculated from the semilogarithmic plots of the mean concentrations versus time by using the trapezoidal rule (WinNonLin in standard edition version 1.5, Pharsight Corporation, Mountain View, USA).

2.5. Statistics

Differences in the antiproliferative effects *in vitro*, as well as in the antitumour effects *in vivo*, were evaluated by the Student's *t*-test.

3. Results

3.1. In vitro *sensitivity*

The influence of BNP7787 and mesna on the antiproliferative effects of cisplatin and carboplatin in A2780 and OVCAR-3 cells was measured by adding the protective agents simultaneously or 15–20 min before the platinum compounds. A molar ratio of 100:1 was used (Table 1). The mean concentration at which BNP7787 itself showed growth-inhibiting properties when given for 96 h was $\geqslant 2\times10^3$ μM. The simultaneous presence of BNP7787 for 96 h did not affect the IC50 values of cisplatin or carboplatin in either of the two cell lines. Mesna alone inhibited cell growth at higher concentrations than BNP7787, i.e. $\geqslant 6.4\times10^3$ μM present for 96 h. In contrast to BNP7787, a 96-h concurrent exposure to mesna increased the IC50 of cisplatin 1.4- to 4.1-fold and of carboplatin 1.5- to 2.4-fold in all experiments. Reduction of the antiproliferative effects of cisplatin >3-fold reached statistical significance ($P<0.05$).

Another experiment was performed in which cells were exposed to BNP7787 and mesna for 20 min, whereafter the cells were washed and exposed to platinum compounds for 96 h. Pretreatment did not result in an increase of the IC50 values (Table 1).

3.2. In vivo *sensitivity*

In the first experiment, cisplatin was studied at the standard dose of 5 mg/kg i.v. weekly×2 in nude mice bearing OVCAR-3 xenografts. At this dose, a mean maximum weight loss of 9.0±12.8% was observed (Table 2). Pretreatment with BNP7787, mesna or amifostine did not increase the weight loss induced by cisplatin, but in the case of mesna one out of six mice died from toxicity. Protective agents given alone did not affect the weight of the animals or inhibit tumour growth. Cisplatin by itself was clearly effective (Fig. 2a) and a specific growth delay of 3.80 was calculated. Specific growth delay values in all groups treated with protective agents preceding cisplatin were slightly shorter, with the shortest (2.74) being observed with mesna. From the calculated relative tumour volumes on day 31, cisplatin was clearly effective at reducing the relative tumour volume when compared with control tumour growth ($P<0.001$). No significant differences were present in the inhibition of tumour growth when mice were treated with cisplatin in the absence or presence of protective agents.

In the past, we have shown that when amifostine 200 mg/kg i.p. was given 5 min before cisplatin the dose could be safely increased by a factor of 1.6 to 8 mg/kg cisplatin in OVCAR-3 bearing mice [8]. Thus, 200 mg/kg amifostine i.p. + 8 mg/kg cisplatin i.v. weekly×2 was included as a treatment group. During the experiment, the second injection in the mesna-pretreated group had to be postponed for 1 week because of excessive weight loss. From Table 3, it is clear that when compared with

Table 1
Antiproliferative effects of BNP7787 or mesna added to cisplatin or carboplatin in a molar ratio of 100:1 in human ovarian cancer cell lines

BNP7787 or mesna added to Pt-drug	BNP7787 alone IC50[a,b]	Mesna alone IC50[a,b]	Cisplatin IC50 + S.D.[a]			Carboplatin IC50 + S.D.[a]		
			Alone	+ BNP7787	+ Mesna	Alone	+ BNP7787	+ Mesna
A2780								
Simultaneous	$\geqslant 6.3\times10^3$	$\geqslant 9.4\times10^3$	19.7±1.5	19.3±4.5	54.7±31.0	9.0±1.2	8.7±2.2	15.3±14.7
15–20 min pre	$\geqslant 3.1\times10^3$	$\geqslant 6.4\times10^3$	25.3±16.6	38.3±24.2	80.0±24.3[c]	13.7±9.0	15.8±10.8	21.0±16.1
20 min + wash			21.3±1.5	12.7±4.9	17.7±2.1	10.8±2.0	7.3±0.9	11.0±4.6
OVCAR-3								
Simultaneous	$\geqslant 2.0\times10^3$	$>10^4$	22.0±2.6	20.7±2.1	90.0±17.3[c]	12.7±1.5	13.7±2.5	23.7±20.3
15–20 min pre	$\geqslant 3.2\times10^3$	$>10^4$	32.0±1.7	24.3±13.8	45.3±47.4	15.0±0.0	17.7±1.5	36.7±3.5
20 min + wash			24.3±5.5	15.5±4.4	21.0±2.6	13.5±2.6	11.7±1.5	13.3±1.2

S.D., standard deviation.
[a] IC50 in μM.
[b] Some values $>10^4$ (highest concentration tested).

amifostine-pretreated mice maximum weight loss was higher for BNP7787 and even more pronounced for mesna. On day 28, recovery was not yet complete. No toxic deaths occurred in combination with BNP7787, but three out of six mice died in the mesna group. Specific growth delay of amifostine preceding 8 mg/kg cisplatin was 5.40 which was better than 3.80 reached in the case of 5 mg/kg cisplatin. Specific growth delay values were longer in BNP7787 and mesna pretreatment groups, but these may be influenced by their poor recovery from weight loss. The antitumour activity of 8 mg/kg of cisplatin preceded by protective agents was significantly different from control tumour growth ($P<0.001$) (Fig. 2b). There were no significant differences in the inhibition of tumour growth among the three treatment groups.

Table 2
Weight loss and antitumour activity of cisplatin without/with protective agents in nude mice bearing OVCAR-3 xenografts

Treatment weekly×2	Maximum weight loss %±S.D.	Weight day 14 %±S.D.	Toxic deaths	SGD[a]	RTV±S.E.M.[b] day 31 (tumours)
Control	0	105.4±6.7	0/6	n.a.	27.44±5.68 ($n=10$)
5 mg/kg cisplatin i.v.	9.0±12.8	92.9±13.2	0/6	3.80	1.29±0.38[d] ($n=12$)
1000 mg/kg BNP7787 i.v./5 mg/kg cisplatin i.v.[c]	6.6±6.1	95.8±8.3	0/6	3.48	2.55±0.42[d] ($n=12$)
1000 mg/kg mesna i.v./5 mg/kg cisplatin i.v.[c]	6.7±2.1	94.5±4.7	1/6	2.74	3.73±1.57[d] ($n=10$)
200 mg/kg amifostine i.p./5 mg/kg cisplatin i.v.[c]	6.1±5.0	97.6±4.7	0/6	2.94	2.97±0.68[d] ($n=12$)
1000 mg/kg BNP7787 i.v.	0.0±3.2	106.0±5.3	0/6	0.22	29.28±4.38 ($n=9$)
1000 mg/kg mesna i.v.	2.4±1.9	103.7±1.8	0/6	0.15	26.51±3.19 ($n=10$)
200 mg/kg amifostine i.p.	1.2±2.0	102.1±2.9	0/6	0.32	21.68±4.86 ($n=12$)

S.D., standard deviation; S.E.M., standard error of the mean; n.a., not applicable.
[a] SGD, specific growth delay.
[b] RTV, relative tumour volume.
[c] Protective agents given 5 min before cisplatin.
[d] $P<0.001$ when compared with control tumour growth.

Table 3
Weight loss and antitumour activity of a higher dose of cisplatin with protective agents in nude mice bearing OVCAR-3 xenografts

Treatment every 2 weeks×2	Maximum weight loss %±S.D.	Weight day 28 %±S.D.	Toxic deaths	SGD[a]	RTV±S.E.M.[b] day 31 (tumours)
Control	0	116.6±9.2	0/6	n.a.	22.10±4.16 ($n=9$)
1000 mg/kg BNP7787 i.v./8 mg/kg cisplatin i.v.[c]	24.8±9.2	89.3±12.3	0/6	6.34	0.39±0.08[d] ($n=12$)
1000 mg/kg mesna i.v./8 mg/kg cisplatin i.v.[c]	33.3±8.8	71.4±11.2	3/6	6.95	0.26±0.11[d] ($n=6$)
200 mg/kg amifostine i.p./8 mg/kg cisplatin i.v.[c]	14.6±4.2	98.2±4.8	0/6	5.40	0.43±0.09[d] ($n=12$)

S.D., standard deviation; S.E.M., standard error of the mean; n.a., not applicable.
[a] SGD, specific growth delay.
[b] RTV, relative tumour volume.
[c] Protective agents given 5 min before cisplatin.
[d] $P<0.001$ when compared with control tumour growth.

Table 4
Weight loss and antitumour activity of carboplatin without/with protective agents in nude mice bearing OVCAR-3 xenografts

Treatment weekly×2	Maximum weight loss %±S.D.	Weight day 14 %±S.D.	Toxic deaths	SGD[a]	RTV±S.E.M.[b] day 31 (tumours)
Control	0	106.5±5.5	0/6	n.a.[e]	20.55±3.25 ($n=10$)
60 mg/kg carboplatin i.v.	0.7±4.3	103.8±1.6	0/6	4.01	0.93±0.32[e] ($n=10$)
1000 mg/kg BNP7787 i.v. 2×/60 mg/kg carboplatin i.v.[c]	9.9±11.9	92.4±14.1	0/6	4.72	0.50±0.14[e,f] ($n=8$)
200 mg/kg amifostine i.p./60 mg/kg carboplatin i.v.[d]	4.3±5.4	99.5±9.1	0/6	5.25	0.32±0.05[e,f] ($n=12$)
1000 mg/kg BNP7787 i.v. 2×/90 mg/kg carboplatin i.v.[c]	11.4±3.1	93.7±3.8	2/6	8.44	0.17±0.03[e,f] ($n=7$)
200 mg/kg amifostine i.p./90 mg/kg carboplatin i.v.[d]	9.3±3.9	97.3±3.9	0/6	5.69	0.28±0.06[e,f] ($n=12$)

S.D., standard deviation; S.E.M., standard error of the mean; n.a., not applicable.
[a] SGD, specific growth delay.
[b] RTV, relative tumour volume.
[c] BNP7787 given concurrently with carboplatin and 30 min after carboplatin.
[d] Amifostine given 5 min before carboplatin.
[e] $P<0.001$ when compared with control tumour growth.
[f] $P<0.01$ when compared with 60 mg/kg carboplatin i.v.-treated tumours.

Earlier, we have demonstrated that the standard dose of 60 mg/kg carboplatin i.v. in OVCAR-3 bearing mice could be increased by a factor of 1.5 to 90 mg/kg i.v. when preceded by 200 mg/kg amifostine i.p. given 5 min before [9]. Therefore, these treatment groups were included to compare the influence of 1000 mg/kg BNP7787 i.v.×2 on the efficacy of 60 or 90 mg/kg carboplatin i.v. (Table 4). As shown previously, amifostine could be safely combined with 60–90 mg/kg carboplatin i.v. BNP7787 pretreatment caused slightly more weight loss and slower recovery than amifostine pretreatment. In the group of BNP7787 + 90 mg/kg carboplatin i.v., there were two out of six toxic deaths. 60 mg/kg carboplatin i.v. was clearly effective (Fig. 3a and b) and a specific growth delay of 4.01 was observed. Specific growth delay values for groups treated with protective agents were longer being 4.72 in the BNP7787-pretreated group and 5.25 in the amifostine-pretreated group. By calculating differences between the mean relative tumour volumes on day 31, the mean relative

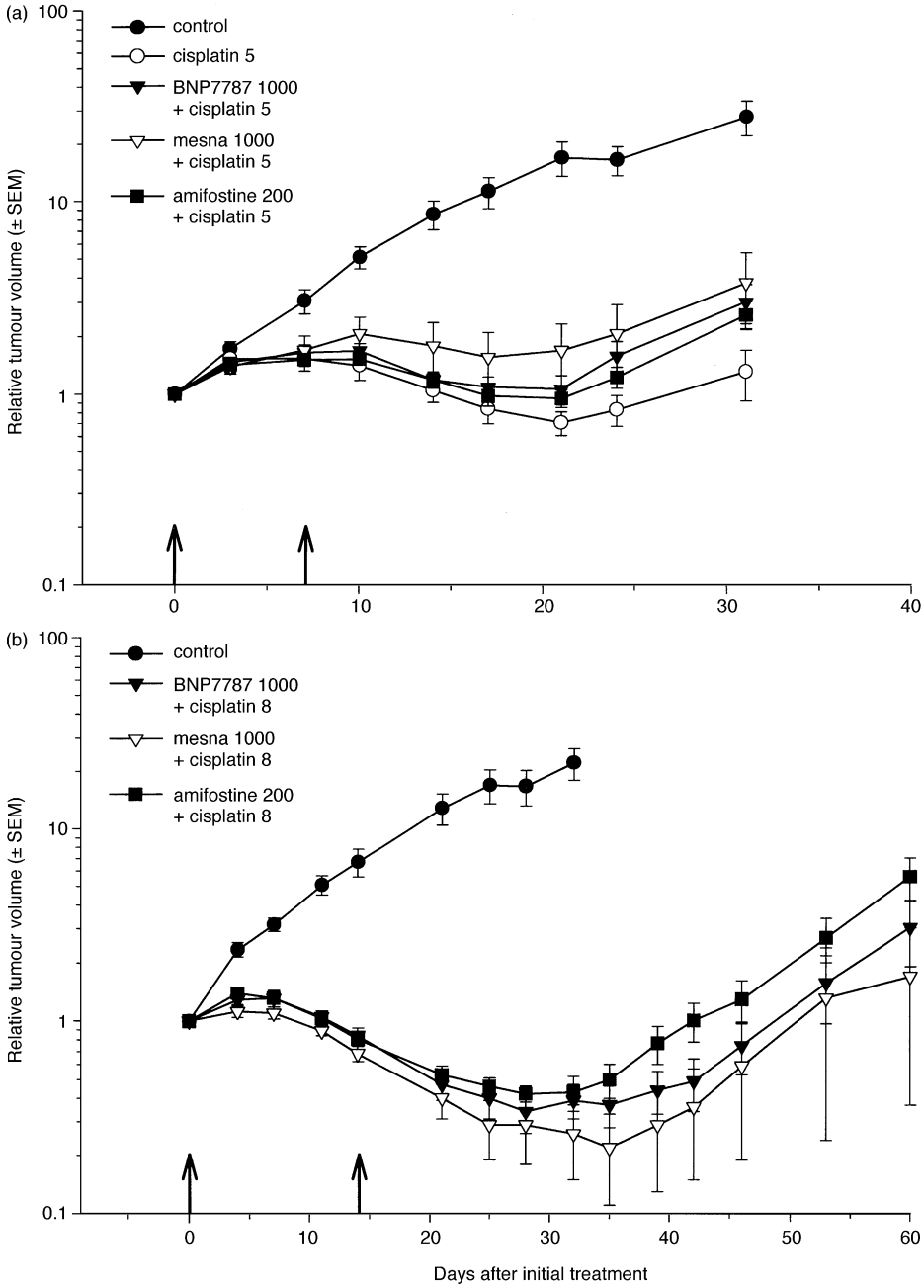

Fig. 2. (a) OVCAR-3 xenografts treated weekly×2 with 5 mg/kg cisplatin i.v., 1000 mg/kg BNP7787 i.v. + 5 mg/kg cisplatin i.v., 200 mg/kg amifostine i.p. + 5 mg/kg cisplatin i.v., 1000 mg/kg mesna i.v. + 5 mg/kg cisplatin i.v., or control tumours. (b) OVCAR-3 xenografts treated every 2 weeks×2 with 1000 mg/kg BNP7787 i.v. + 8 mg/kg cisplatin i.v., 200 mg/kg amifostine i.p. + 8 mg/kg cisplatin i.v., 1000 mg/kg mesna i.v. + 8 mg/kg cisplatin i.v., or control tumours. Arrows represent days of the injection.

tumour volumes of groups that received protecting agents with 60 mg/kg carboplatin i.v. were significantly smaller than for the group given carboplatin alone ($P < 0.01$). Poor weight loss recovery may be the reason that the specific growth delay of 90 mg/kg BNP7787 + carboplatin i.v. (8.44) was slightly better than that of 90 mg/kg amifostine + carboplatin i.v. (5.69). There were no significant differences, however, in the inhibition of tumour growth between the latter two groups.

3.3. Plasma concentrations and pharmacokinetics of BNP7787 and mesna

The concentration–time curves of the compounds after the i.v. administration of 1000 mg/kg BNP7787 and 1000 mg/kg mesna in nude mice are shown in Fig. 4a and b, respectively. The concentration of BNP7787 at 5 min after the BNP7787 injection was 4.6 mM which rapidly decreased to 0.3 mM at 60 min. Mesna also appeared in the plasma and a maximum

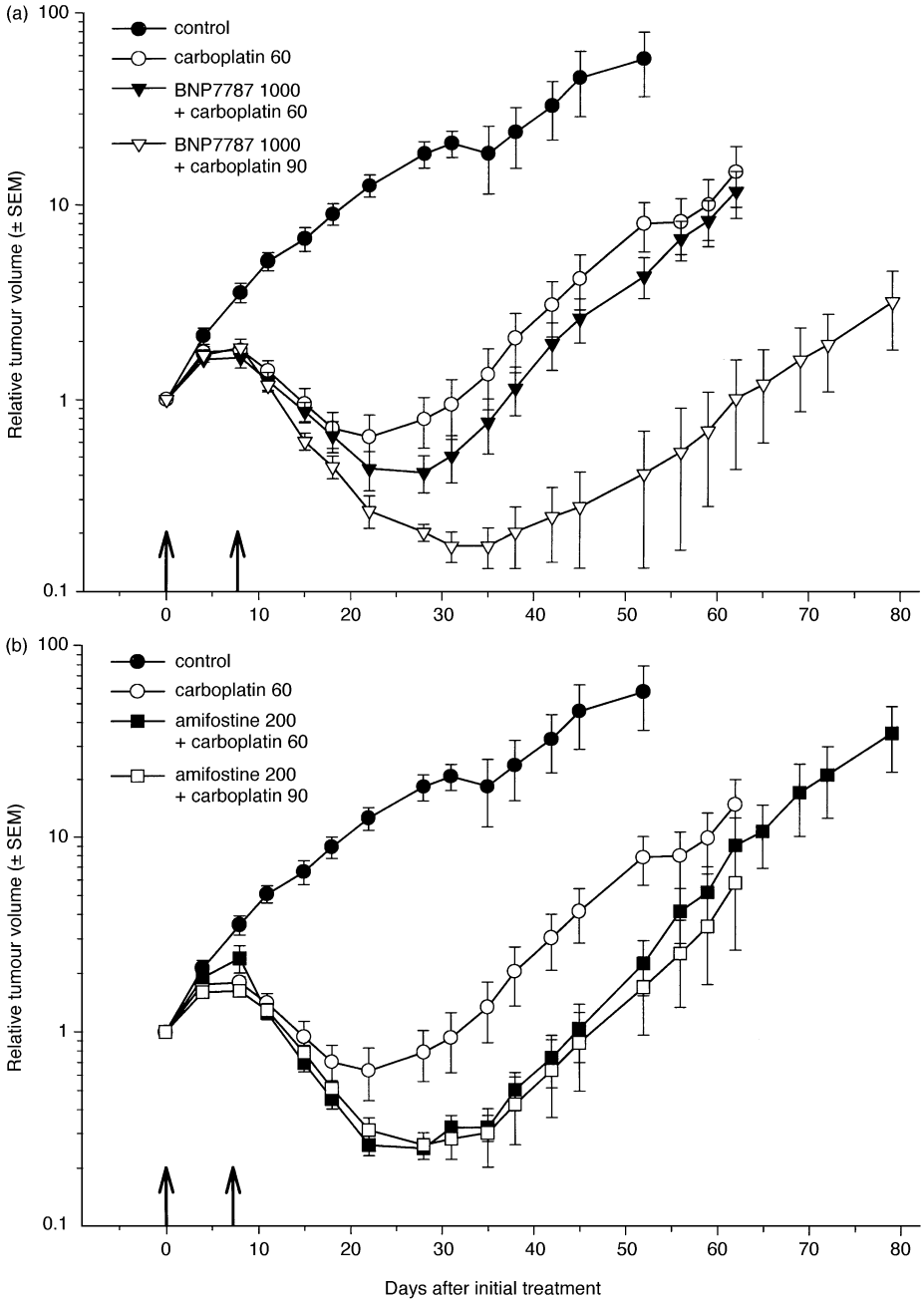

Fig. 3. (a) OVCAR-3 xenografts treated weekly ×2 with 60 mg/kg carboplatin i.v., 1000 mg/kg BNP7787×2 + 60 mg/kg carboplatin i.v., 1000 mg/kg BNP7787×2 + 90 mg/kg carboplatin i.v., or control tumours. (b) OVCAR-3 xenografts treated weekly×2 with 60 mg/kg carboplatin i.v., 200 mg/kg amifostine i.p. + 60 mg/kg carboplatin i.v., 200 mg/kg amifostine i.p. + 90 mg/kg carboplatin i.v., or control tumours. Arrows represent the days of injection.

concentration of 0.5 mM mesna was already obtained at 15 min, which declined to 0.06 mM at 60 min. After the i.v. administration of an equal dose by weight of mesna, the plasma concentrations of mesna were much higher than following BNP7787 injection. After 5 min, the mesna concentration was 5.7 mM which decreased to 0.9 mM at 60 min. The generation of (mixed) disulphides following mesna administration was very rapid and much more pronounced than the generation of mesna following the administration of BNP7787. Following mesna administration, the maximal concentration of (mixed) disulphides was 5.5 mM observed after 5 min and this decreased to 0.8 mM at 60 min. To compare the concentration–time curves after the administration of mesna and BNP7787, of which the doses were equimolar with respect to the total amount of mesna administered or that could theoretically be maximally generated, ratios of AUC^{5-60} values were calculated. The AUC ratio for mesna after injection of mesna and mesna after injection of BNP7787 was 9:1. This observation indicated that the exposure of the mice to mesna after injection of mesna was 9 times higher than that following injection of BNP7787.

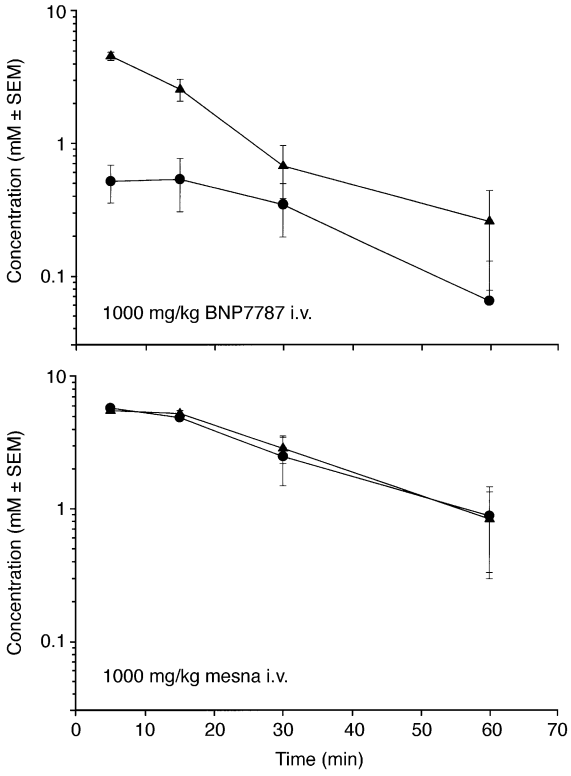

Fig. 4. Plasma concentration–time curves in nude mice ($n = 3$ per time-point) of (mixed) disulphides including BNP7787 (▲) and extractable mesna (●) concentrations after (a) 1000 mg/kg BNP7787 or (b) 1000 mg/kg mesna i.v. injection at time-point 0 min.

4. Discussion

In the present study in human ovarian cancer cell lines, continuous exposure of BNP7787 with cisplatin showed that BNP7787 did not reduce the antiproliferative effects, but that mesna clearly antagonised cisplatin activity. This reduction in cisplatin activity by mesna *in vitro* is most likely due to the known chemical incompatibility of the two agents. Continuous exposure to mesna also resulted in a slight, but not significant reduction in the antiproliferative effects of carboplatin *in vitro*. Pretreatment for a period of 20 min did not interfere with platinum-induced cell-growth inhibition. *In vitro*, BNP7787 alone showed inhibition of cell growth at concentrations of $\geqslant 2$ mM for 96 h in A2780 and OVCAR-3 cells, whereas this was also found for mesna at 3-fold higher concentrations of $\geqslant 6.4$ mM for 96 h. It has been previously reported that mesna can completely inhibit proliferation of sensitive cell lines exposed to 0.1 mM for 72 h [12]. Whether BNP7787 is cytotoxic by itself has not been observed in any system tested to date. It appears that the cell growth inhibition in our cell lines may be due to generated mesna in the tissue culture. Other thiols containing a free SH-group, such as L-cysteine, *N*-acetyl-L-cysteine and glutathione, have also been found to inhibit cell proliferation [13].

No negative effect of BNP7787 was observed on tumour growth inhibition *in vivo* at standard schedules of 5 mg/kg cisplatin i.v. weekly×2 and 60 mg/kg carboplatin i.v. weekly×2 used for treatment of OVCAR-3 xenografts. The same was found for amifostine in the past [8–10]. Of interest, amifostine, and to a lesser extent also BNP7787, can increase the efficacy of the standard schedule of carboplatin in mice (Table 4). For amifostine, this finding has been reported before by our group [9,10]. A possible explanation might be the increase of the platinum concentration in mouse plasma ultrafiltrate, liver, kidney and OVCAR-3 tumour tissue by amifostine. Platinum–DNA adduct levels were slightly increased in the tumour tissue [10]. Comparable pharmacokinetic studies of 60 mg/kg carboplatin i.v. with and without BNP7787 are necessary to explore a possible drug interaction, which might explain enhanced efficacy.

Mesna has been previously studied in combination with cisplatin in mice and rats. In these animals, it protected against nephrotoxicity [14], or it did not prevent renal damage [15]. In our experiment in mice receiving the standard schedule of cisplatin of 5 mg/kg i.v. weekly×2 preceded by mesna, an indication was found that cisplatin antitumour activity was slightly, but not significantly, reduced (Fig. 2). This confirmed the finding that mesna administration prior to cisplatin in tumour-bearing rats had a protective effect on tumour growth inhibition [3]. We observed that cisplatin in combination with mesna was also more toxic than cisplatin combined with a same dose of BNP7787 (Tables 2 and

3). It is believed that the amount of the free thiol in plasma, i.e. mesna, is related to the degree of toxicity induced in the mouse. This is supported by our pharmacokinetic data showing that following mesna administration in mice the exposure to mesna was much higher than that after a same dose of BNP7787 (AUC^{5-60} ratio of 9). It should be kept in mind that, similar to earlier methods [16], our analytical procedure does not discriminate between the free and non-covalently protein-bound mesna. In addition, the direct and simultaneous identification of mesna, BNP7787, glutathione, cysteine, homocysteine and the corresponding mixed disulphides pose stability problems which limit accurate assessment [17].

Higher doses of platinum compounds could safely be given when preceded by 200 mg/kg amifostine i.p. 5 min earlier. This observation confirmed our previous experiments [8,9]. We could not safely increase the dose of cisplatin from 5 to 8 mg/kg or carboplatin from 60 to 90 mg/kg when preceded by 1000 mg/kg BNP7787 because of increased weight loss. Increased toxicity was especially noted when mesna preceded a higher dose of cisplatin. In contrast, in rats bearing well-established WARD colon tumours, the dose of cisplatin could safely be increased from 6 to 9 mg/kg when preceded by 750 or 1500 mg/kg BNP7787 [3]. In both cases, the antitumour activity was potentiated by BNP7787 pretreatment in comparison to cisplatin administration alone. The difference in protection by BNP7787 between mice and rats may have a pharmacological and/or toxicological basis. In addition, there appears to be a substantial difference between murine species and rats, dogs and patients with respect to the toxicity of higher doses of BNP7787. This differential sensitivity may be due to the relatively high osmolarity and volume of BNP7787 when administered i.v. Thus, at higher doses of BNP7787 that are representative of administrations given to rats, dogs and patients, (nude) mice appear to be limited as a model to study changes in the therapeutic index and potentiation of the antitumour activity of platinum compounds. Pharmacokinetic studies of BNP7787 in rats are planned to determine the concentrations of BNP7787 and mesna not only in plasma, but also in tissues (kidney and tumour), to verify the selectivity, i.e. the protection in the kidney and not in the tumour.

In summary, BNP7787 does not interfere with the antitumour activity of platinum compounds *in vitro* and in our experimental nude mouse–human tumour model. In contrast to earlier observations in rats, in mice the dose of cisplatin and carboplatin could not be increased under protection with BNP7787. If the current clinical trial of BNP7787 with cisplatin continues to show evidence of protection of BNP7787 against nephrotoxicity and other side-effects, such as neurotoxicity, it will allow investigations of whether higher doses of cisplatin can be safely given to cancer patients.

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