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ORIGINAL ARTICLE

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The chemical reactivity of BNP7787 and its metabolite mesna with the cytostatic agent cisplatin: comparison with the nucleophiles thiosulfate, DDTC, glutathione and its disulfide GSSG

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Abstract *Purpose*: BNP7787 is a new chemoprotective agent presently under clinical investigation to protect against cisplatin-induced toxicities, especially nephrotoxicity and neurotoxicity. In the kidneys BNP7787 is postulated to undergo selective conversion into mesna, which can locally detoxify cisplatin. The reactivity of cisplatin with this new chemoprotective agent and with its metabolite mesna was investigated at clinically observed plasma concentrations and compared with the nucleophiles thiosulfate (TS) and DDTC, and with the endogenous compounds glutathione (GSH) and oxidized glutathione (GSSG). *Methods*: Reaction kinetics experiments were performed at 37°C and pH 7.4 in the presence of a high chloride concentration (0.15 *M*). The degradation of cisplatin was measured over time using HPLC with off-line flameless atomic absorption spectrophotometry. *Results*: The degradation half-lives of cisplatin (13.5 μ*M*) with 17.2 m*M* BNP7787, 340 μ*M* mesna and 17.2 m*M* mesna were 124 min, about 790 min and 73 min, respectively. Cisplatin reacted at least 9.5 times more slowly with 17.2 m*M* BNP7787 and 5.5 times more slowly with 17.2 m*M* mesna than with 17.2 m*M* of the modulating agents DDTC or TS (i.e. half-lives 11 and 13 min, respectively). The half-lives of cisplatin with 17.2 m*M* GSH and GSSG (i.e. 122 and 115 min, respectively) were comparable with the half-life obtained with BNP7787. The thiol mesna was shown to be a stronger nucleophile than its corresponding disulfide BNP7787. *Conclusions*: The much slower relative reactivity of BNP7787, the short residence of BNP7787 (approximately 2 h) and the much lower concentration of mesna in the circulation following BNP7787 administration precludes chemical inactivation of cisplatin in the circulation, and thus the antitumor activity of cisplatin is maintained.

Keywords Cisplatin · BNP7787 · Mesna · Glutathione · Reaction kinetics

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Introduction

Cisplatin is one of the most active and widely used drugs in the treatment of several types of solid tumors, and it has shown a steep dose-response relationship for antitumor activity [20]. High-dose cisplatin regimens are clinically limited because of dose-limiting nephrotoxicity and neurotoxicity. Several chemoprotectors for cisplatin have been studied in the clinic with a view to preventing or reducing cisplatin-induced nephrotoxicity. Diethyldithiocarbamate (DDTC) has been shown to prevent renal toxicity induced by cisplatin [2]. However, the use of DDTC in the clinic has been hampered by severe, but reversible, sympathetic dysautonomia (flushing, diaphoresis and tachycardia) [2, 21]. Howell and Taetle [12] attempted to reduce cisplatin-induced nephrotoxicity with the reactive and rapidly excreted nucleophile sodium thiosulfate (TS). Because of the high chemical reactivity, TS reacts with cisplatin in the circulation and thereby reduces the antitumor activity of cisplatin [26]. This problem can be partially avoided by administering TS and cisplatin via a two-route regimen, i.e. cisplatin is administered locally (i.p.) to the tumor while TS is given systemically to protect non-tumor tissues [1, 24]. The non-selective tumor protection of TS led to the development of more-selective protectors, such as amifostine which selectively protects normal tissue and not the tumor [10]. However, amifostine administration is associated with hypotension, nausea and vomiting as well as other untoward side effects in some patients [10].

BNP7787 is a new chemoprotective agent presently undergoing clinical development to protect against cisplatin-induced nephrotoxicity and neurotoxicity [3, 8] and taxane-induced neurotoxicity [3, 4, 9]. The selectivity of BNP7787 originates in part from the enzymatic formation of mesna in the kidneys, intestine and liver [7, 18, 19], which can locally inactivate toxic (hydrated) cisplatin species. This mechanism prevents cisplatin-induced nephrotoxicity without reducing the antitumor activity of cisplatin. To obtain more insight into this mechanism of action of BNP7787, the direct chemical interaction of BNP7787 and its metabolite mesna with cisplatin was studied under pharmacological conditions: incubations were performed at 37°C and pH 7.4 with peak plasma drug and metabolite concentrations determined in patients after administration of the compounds. In addition, the reactivities of cisplatin with TS, DDTC and the endogenous compound glutathione (GSH) and oxidized glutathione (GSSG) (Fig. 1) were determined at equimolar concentrations and compared with those of cisplatin with BNP7787 and its metabolite mesna.

Materials and methods

Chemicals

BNP7787 (disodium 2,2′-dithio-bis-ethane sulfonate) was provided by BioNumerik Pharmaceuticals (San Antonio, Tx.). Cisplatin, mesna (sodium 2-mercaptoethanesulfonate), sodium diethyldithiocarbamate (DDTC), GSH and GSSG were obtained from Sigma Chemicals (St. Louis, Mo.). TS was obtained from Brocacef (Maarssen, The Netherlands). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from ICN Biochemicals (Zoetermeer, The Netherlands). All other chemicals used were of analytical grade. Deionized water from a Millipore Milli-Q system (Etten-Leur, The Netherlands) was used throughout this work.

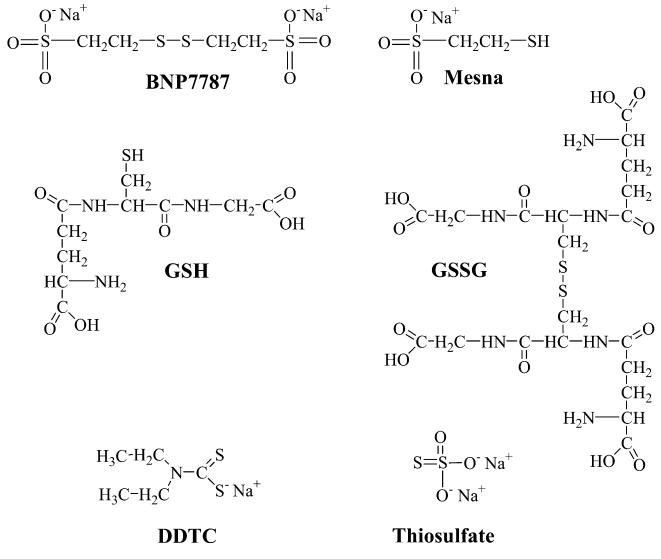

Fig. 1 Structural formulas

Instrumentation

Intact cisplatin was separated from its degradation and reaction products by HPLC and quantified by flameless atomic absorption spectrophotometry (FAAS) according to our assay reported previously [25]. A few optimizations in the HPLC system were made to improve the peak shape and to decrease the analysis time. The FAAS system remained unchanged. In brief, an isocratic HPLC system consisting of a Valco injection valve with a 100-µl loop (Schenkon, Switzerland), a Gynkotek 300 HPLC pump (Germering, Germany), a Spark Holland SPH 99 column thermostat set at 25°C (Emmen, The Netherlands), a degasser model GT-103 and a UV detector ABI spectroflow 759A set at 214 nm (both from Separations Analytical Instruments, H.I. Ambacht, The Netherlands) was used. Separation of intact cisplatin and monohydrated cisplatin was performed on a Phenomenex Nucleosil 5 SB column (150×4.6 mm; Bester, Amstelveen, The Netherlands) preceded by a reverse-phase (C18, 40 µm particle size) guard column (10×2 mm; Chrompack, Bergen op Zoom, The Netherlands). The mobile phase consisted of methanol/0.01 *M* phosphate buffer (pH 5.0; 11:9 v/v) and the flow was 1.5 ml/min. The eluted fraction of intact cisplatin was collected in polypropylene tubes and was evaporated overnight at 80°C using a gentle flow of nitrogen gas. The residue was redissolved by adding 100 µl 0.03 *M* sodium dodecyl sulfate (SDS), 100 µl 0.4 *M* HCl and 100 µl 0.15 *M* NaCl. The platinum concentration in these samples was obtained by injecting 10 µl into the pyro-coated Z-tek graphite furnace (Merck, Amsterdam, The Netherlands) of the FAAS (Spectra AA-300 Zeeman AAS, Varian, Houten, The Netherlands). The measured absorption of the samples had to fall within the linear range of the assay (0.050–0.150 absorption units). Because of expected/measured high absorptions, some of the cisplatin samples were diluted with 0.03 *M* SDS/0.15 *M* NaCl/0.4 *M* HCl (1:1:1 v/v/v). The intact cisplatin samples were measured in duplicate in mirror-wise order to correct for a possible gradual change in signal: $S_1 \ldots S_n – S_n \ldots S_1$.

Reaction kinetics

The experiments were performed using a cisplatin concentration of 13.5 µ*M*, which is the peak plasma concentration in patients receiving 75 mg/m^2 cisplatin as a 1-h i.v. infusion (Verschraagen et al., submitted for publication). The reaction kinetics of cisplatin were determined in the presence or absence of 340 µ*M* mesna and 17.2 m*M* BNP7787, which are the plasma concentrations observed in phase I patients after administration of 41 g/m^2 BNP7787 given as a 15-min i.v. infusion (Verschraagen et al., submitted for publication). For comparison, the reaction kinetics of cisplatin were also determined in the presence of 17.2 m*M* of mesna, GSH, GSSG, TS and DDTC. This comparison was made to determine the relative reactivity of these compounds by using the same concentration as BNP7787.

All stock solutions of the modulating agents were prepared just before use in 0.02 *M* HEPES buffer with 0.15 *M* NaCl (pH 7.4). To prevent oxidation of the thiols (mesna and GSH), the HEPES buffer was purged with nitrogen for at least 20 min before use and the reaction mixtures were kept under an atmosphere of nitrogen. During the experiments, the reaction mixtures were also protected from light to prevent photodegradation of cisplatin.

Immediately prior to commencement of the experiment, tubes containing the required volume of modulating agent in HEPES buffer containing 0.15 *M* NaCl were placed in an Eppendorf Thermomixer 5436 (Merck, Amsterdam, The Netherlands) at 37°C. After 5 min the required volume of a 27 µ*M* solution of cisplatin in HEPES buffer with 0.15 *M* NaCl, which was prepared from a 1 m*M* stock solution of cisplatin in 0.15 *M* NaCl, was added. Immediately after vortexing (t = 0) and at preselected times 400 µl was taken from the reaction mixture (initial volume 3.8 ml). The samples were immediately analyzed for intact cisplatin. All incubations were performed in duplicate.

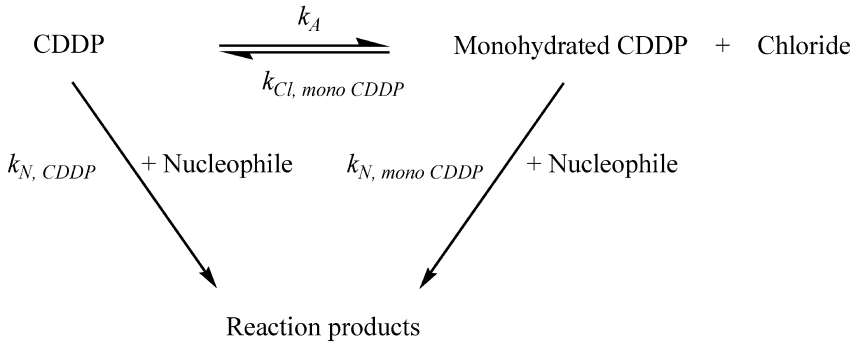

Fig. 2 Schematic representation of the interaction of nucleophiles with cisplatin along parallel pathways, i.e. via aquation and direct displacement of the chloride ligands in cisplatin

Calculations

The reaction of cisplatin with a nucleophilic agent can occur along parallel pathways, i.e. via a direct interaction with cisplatin and via the rate-limiting monoaquation step of cisplatin followed by a rapid reaction of the monoaqua/monohydroxy monohydrated platinum species with the nucleophilic agent (Fig. 2) [16, 17]. When the monohydrated cisplatin concentration is at steady state, the decomposition rate of cisplatin can be described as follows:

$$\frac{-\mathrm{d[cisplatin]}}{\mathrm{dt}} = \left(k_{N,CDDP}\cdot[\mathrm{N}] + \frac{k_{N,monoCDDP}\cdot[\mathrm{N}]}{k_{Cl,monoCDDP}\cdot[\mathrm{Cl^-}] + k_{N,monoCDDP}\cdot[\mathrm{N}]}\cdot k_A\right)\cdot[\mathrm{cisplatin}] \quad (1)$$

in which $k_{\mathrm{N,CDDP}}$ represents the second-order rate constant for the direct reaction of intact cisplatin with the nucleophile N under study, [N] represents the concentration of the nucleophilic agent under study, $k_{\mathrm{N,monoCDDP}}$ represents the second-order rate constant for reaction of N with monohydrated cisplatin, [Cl^-] represents the concentration of chloride, $k_{\mathrm{Cl,monoCDDP}}$ represents the second-order rate constant for the annation reaction of Cl^- with monohydrated cisplatin, k_{A} represents the first-order aquation rate constant of cisplatin and [cisplatin] represents the cisplatin concentration.

At concentrations of N in large excess over cisplatin the rate law becomes:

$$\frac{-\mathrm{d[cisplatin]}}{\mathrm{dt}} = k_{obs}\cdot[\mathrm{cisplatin}] \quad (2)$$

in which k_{obs} represents the observed first-order rate constant for loss of cisplatin.

Integration of Eq. 2 yields:

$$\ln[\mathrm{cisplatin}]_t = \ln[\mathrm{cisplatin}]_{t=0} - k_{obs}\cdot t \quad (3)$$

Therefore, k_{obs} could be calculated from semilogarithmic plots of the mean measured cisplatin absorption of the reaction samples, expressed as a percentage of the initial absorption (at t = 0), versus time. The plots were fitted with the least-squares method.

The half-life of cisplatin could be calculated by:

$$t_{1/2,\mathrm{obs}} = \frac{\ln 2}{k_{obs}} \quad (4)$$

When the reaction of the nucleophile with monohydrated cisplatin is not competitive with chloride (i.e. $k_{\mathrm{N,monoCDDP}}\cdot[\mathrm{N}] >> k_{\mathrm{Cl,\ monoCDDP}}\cdot[\mathrm{Cl^-}]$) then:

$$k_{obs} = k_A + k_{N,CDDP}\cdot[\mathrm{N}] \quad (5)$$

However, when monohydrated cisplatin reacts exclusively with chloride (i.e. $k_{\mathrm{Cl,monoCDDP}}\cdot[\mathrm{Cl^-}] >> k_{\mathrm{N,monoCDDP}}\cdot[\mathrm{N}]$) then:

$$k_{obs} = k_{N,\mathrm{CDDP}}\cdot[\mathrm{N}] \quad (6)$$

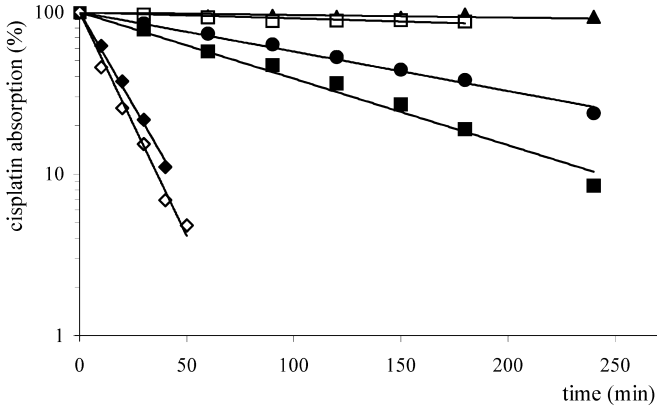

Fig. 3 Degradation of cisplatin alone (▲) and with 340 μ*M* mesna (□), 17.2 m*M* mesna (■), 17.2 m*M* BNP7787 (●), 17.2 m*M* TS (◆) or 17.2 m*M* DDTC (◇) at 37°C in 0.02 *M* HEPES buffer (pH 7.4) containing 0.15 *M* NaCl. The degradation of cisplatin with 17.2 m*M* GSH or 17.2 m*M* GSSG coincided with that with 17.2 m*M* BNP7787

Results

The amount of intact cisplatin, incubated for 4 h in HEPES buffer (pH 7.4) containing 0.15 *M* NaCl for 4 h at 37°C, remained almost unchanged as can be seen in Fig. 3. Thus, no loss of cisplatin by formation of hydrated cisplatin species or by interaction with HEPES buffer was observed.

Semilogarithmic plots of the percentage absorption vs time were found to be linear. Therefore, observed rate constants (k_{obs}) and corresponding observed half-lives ($t_{1/2,\mathrm{obs}}$) could be calculated for the loss of cisplatin reacting with 17.2 m*M* of modulating agent. The correlation coefficients were greater than 0.982. In Table 1, the k_{obs} and $t_{1/2,\mathrm{obs}}$ values are summarized. The thiol mesna, at a concentration of 17.2 m*M*, reacted approximately two times faster with cisplatin ($t_{1/2,\mathrm{obs}}$ 73.3 min) than 17.2 m*M* BNP7787 ($t_{1/2,\mathrm{obs}}$ 123.6 min), GSH ($t_{1/2,\mathrm{obs}}$ 121.8 min) or GSSG ($t_{1/2,\mathrm{obs}}$ 114.9 min). The half-life of cisplatin in the presence of mesna at a concentration of 340 μ*M* was about 793 min (Fig. 3). This half-life could not be calculated reliably because the

Table 1 Reaction kinetics of 13.5 μ*M* cisplatin with 17.2 m*M* BNP7787, mesna, GSH, GSSG, TS and DDTC

Modulating agent	$k_{obs} \times 10^3$ (min^{-1})	$t_{1/2,obs}$ (min)	r^2	$k_{N,CDDP}$ ($M^{-1}min^{-1}$) (Eq. 6)
BNP7787	5.6	123.6	0.991	0.33
Mesna	9.5	73.3	0.982	0.55
GSH	5.7	121.8	0.994	0.33
GSSG	6.0	114.9	0.967	0.35
TS	52.9	13.1	0.994	3.07
DDTC	63.7	10.9	0.989	3.70

duration of the experiment was shorter than the observed half-life of cisplatin. TS and DDTC reacted very rapidly with cisplatin, i.e. with half-lives of 13.1 and 10.9 min, respectively (Table 1).

The reaction experiments were performed at 37°C and pH 7.4 in the presence of a high chloride concentration (0.15 *M*) compared to the modulating agent concentration (17.2 m*M* or 340 μ*M*). Under these conditions which approximate normal physiological chloride concentrations and pH, we can assume that it is unlikely that the aquation of cisplatin to hydrated species followed by reaction with modulating agent will play a major role in the degradation of cisplatin. Thus, the second-order rate constant ($k_{N,CDDP}$) for direct chloride displacement by a modulating agent was estimated by using Eq. 6. The real $k_{N,CDDP}$ value is likely to be somewhat lower. High $k_{N,CDDP}$ values were obtained for the direct reaction of cisplatin with TS and DDTC, whereas the $k_{N,CDDP}$ values for BNP7787, mesna, GSH and GSSG were 5- to 11-fold lower (Table 1).

Discussion

The validity of our assay was tested by determining the reaction kinetics of cisplatin with TS and DDTC, which are strong nucleophiles with known $k_{N,CDDP}$ values of 3.4 $M^{-1}min^{-1}$ and 3.7 $M^{-1}min^{-1}$, respectively [6, 15, 23]. In the presence of 17.2 m*M* TS or DDTC, cisplatin degradation occurred very rapidly (i.e. $t_{1/2,obs}$ 13.1 and 10.9 min, respectively; Table 1). This was also reflected by the calculated $k_{N,CDDP}$ values of both compounds, which were higher than 3 $M^{-1}min^{-1}$. The calculated $k_{N,CDDP}$ values of TS and DDTC were in good agreement with the values reported in the literature [6, 23], indicating that our assay could be used to determine the reaction kinetics of cisplatin with other nucleophiles.

The second-order rate constant, $k_{N,CDDP}$, is an important reactivity-indicating parameter to estimate the effect of a modulating agent on the inactivation of cisplatin. The high $k_{N,CDDP}$ value of TS ($k_{TS,CDDP}$ 3.1 $M^{-1}min^{-1}$) indicates that TS is able to react quickly and directly with cisplatin. In animals and patients, TS has been shown to reduce the plasma concentrations of cisplatin [1, 13], thereby reducing the antitumor activity of cisplatin [26]. The observed decrease in AUC of cisplatin (i.e. from 8.1 to 5.5 μg·h/ml [13]) in patients can be explained by a calculated 25% reduction of the plasma half-life of cisplatin [half-life 28.2 min (Verschraagen et al., submitted for publication)] at a steady-state concentration of 2.6 m*M* TS ($k_{TS,CDDP}$ 3.1 $M^{-1}min^{-1}$). Because TS is so highly reactive with cisplatin, it has been used in a two-route regimen with cisplatin, i.e. cisplatin is locally administered for residual ovarian cancer by i.p. administration, while TS is given i.v. to protect non-tumor tissues [1, 24].

The $k_{N,CDDP}$ values of the new protector against cisplatin-induced toxicities, BNP7787 (0.33 $M^{-1}min^{-1}$), and its metabolite mesna (0.55 $M^{-1}min^{-1}$) were much lower than the values of the strong nucleophiles TS and DDTC. At clinically relevant concentrations (i.e. 13.5 μ*M* cisplatin, 17.2 m*M* BNP7787 and 340 μ*M* mesna), the reaction of BNP7787 and mesna with cisplatin was slow (i.e. $t_{1/2,obs}$ 123.6 and about 790 min, respectively). In patients the elimination of BNP7787 from the circulation is rapid [final $t_{1/2}$ 1.4 h (Verschraagen et al., submitted for publication)]. Therefore, no or a very limited interaction with cisplatin is expected to occur. This corresponds with the original proposal of BNP7787 as a new chemoprotective agent for cisplatin based on the understanding that a disulfide would not undergo rapid chemical reaction with platinum drugs or their respective monohydrated species relative to free thiol chemoprotective compounds [3, 8]. Our pharmacokinetic results in patients receiving cisplatin immediately preceded by BNP7787 showed that BNP7787 and the formed mesna did not influence the plasma concentrations of cisplatin at the highest BNP7787 dose levels (i.e. 41 g/m^2) (Verschraagen et al., submitted for publication).

Studies in tumor-bearing rats and nude mice have demonstrated that BNP7787 administration protects against cisplatin-induced toxicity without interfering with the antitumor activity of cisplatin [3, 8]. In contrast, reduction of the antitumor activity of cisplatin is seen in tumor-bearing rats and nude mice receiving mesna [3, 8]. In these species, the plasma concentrations of BNP7787 are comparable when the same dose (by weight) of BNP7787 or mesna is given [3, 19]. The plasma mesna concentrations, however, are much higher following mesna administration than after the same dose of BNP7787 [3, 19]. The increased free thiol concentration due to the administration of mesna and the fact that mesna reacts approximately two times faster with cisplatin than BNP7787 possibly explain why concurrent mesna administration interferes with the antitumor activity of cisplatin, whereas BNP7787 administration does not, which is consistent with the original hypothesis.

After administration of BNP7787, the possibility exists that mixed disulfides of mesna (e.g. mesna-GSH) are being formed. Since the disulfides BNP7787 ($k_{BNP7787,CDDP}$ 0.33 $M^{-1}min^{-1}$) and GSSG ($k_{GSSG,CDDP}$ 0.35 $M^{-1}min^{-1}$) have shown a low reactivity towards cisplatin, no rapid thiol-platinum interaction would be expected to occur between mixed disulfides of mesna-GSH and cisplatin in the circulation.

GSH had a low reactivity towards cisplatin ($k_{GSH,CDDP}$ 0.33 $M^{-1}min^{-1}$). The $k_{N,CDDP}$ value of GSH was approximately two times lower than that reported previously (i.e. $k_{GSH,CDDP}$ 0.79 $M^{-1}min^{-1}$ [6]). The higher $k_{GSH,CDDP}$ value of GSH found in the literature might be secondary to a direct interaction of cisplatin with the phosphate ligand [22], which was used as a buffer in that study. To circumvent this problem we performed our experiments in HEPES buffer, which does not interact with cisplatin.

Considering the low plasma concentrations of GSH and GSSG (3.6 μM total GSH [14]) and the low $k_{N,CDDP}$ values of both compounds, it is unlikely that a direct interaction between cisplatin and these endogenous compounds will occur in the circulation after administration of cisplatin. The reactivity of two other endogenous sulfur-containing compounds, i.e. cysteine and methionine, with cisplatin are much higher than the reactivity of GSH ($k_{cysteine,CDDP}$ 2.32 $M^{-1}min^{-1}$ and $k_{methionine,CDDP}$ 2.38 $M^{-1}min^{-1}$ [6]). Their plasma concentrations are also substantially higher than that of total GSH, i.e. 201 μM total cysteine [14] and 25 μM methionine [11]. Therefore, cysteine and/or methionine are good candidates to form platinum-complexes in plasma by a direct interaction with cisplatin after administration. Indeed, Daley-Yates and McBrien [5] have presented evidence that a platinum-methionine complex is present in the circulation of rats after cisplatin administration.

In conclusion, BNP7787 is a weak nucleophile and it reacts with cisplatin substantially more slowly than TS, DDTC, methionine and cysteine. As expected, mesna was shown to be a stronger nucleophile than its corresponding disulfide BNP7787. The relative reactivity of cisplatin with the investigated nucleophiles was DDTC > TS > > mesna > GSSG≈GSH≈BNP7787. Considering the much lower relative reactivity of BNP7787, the short residence time of BNP7787 and the very low mesna concentrations in the circulation after BNP7787 administration, no inactivation of cisplatin by BNP7787 or mesna would be expected to occur. Thus, coadministration of BNP7787 would not result in tumor protection by inactivation of cisplatin in the circulation.

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