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ORIGINAL ARTICLE

Mechanistic study of BNP7787-mediated cisplatin nephroprotection: modulation of gamma-glutamyl transpeptidase

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Abstract
Purpose The mechanisms for cisplatin-induced renal cell injury have been the focus of intense investigation for many years with a view to provide a more effective and convenient form of nephroprotection. BNP7787 (disodium 2,2′-dithio-bis ethane sulfonate; dimesna, Tavocept™), is a water-soluble disulfide investigational new drug that is undergoing clinical development for the prevention and mitigation of clinically important chemotherapy-induced toxicities associated with platinum-type chemotherapeutic agents. We hypothesized that part of BNP7787's mechanism of action (MOA) pertaining to the potential prevention of cisplatin-induced nephrotoxicity involves the inhibition of gamma-glutamyl transpeptidase (GGT) activity, mediated by BNP7787-derived mesna–disulfide heteroconjugates that contain a terminal gamma-glutamate moiety [e.g., mesna–glutathione (MSSGlutathione) and mesna–cysteinyl-glutamate (MSSCE)].
Methods Inhibition studies were conducted on human and porcine GGT to determine the effect of mesna–disulfide heteroconjugates on the enzyme's activity in vitro. These studies utilized a fluorimetric assay that monitored the hydrolysis of L-gamma-glutamyl-7-amino-4-trifluoromethylcoumarin (GG-AFC) to AFC.
Results Mesna–disulfide heteroconjugates that contained gamma-glutamyl moieties were potent inhibitors of human and porcine GGT. An in situ-generated mesna–cisplatin conjugate was not a substrate for GGT.
Conclusions The GGT xenobiotic metabolism pathway is postulated to be a major toxification pathway for cisplatin nephrotoxicity, and BNP7787 may play a novel and critical therapeutic role in the modulation of GGT activity. We further postulate that there are two general mechanisms for BNP7787-mediated nephroprotection against cisplatin-induced nephrotoxicity involving this pathway. First, the active BNP7787 pharmacophore, mesna, produces an inactive mesna–cisplatin conjugate that is not a substrate for the GGT toxification pathway (GGT xenobiotic metabolism pathway) and, second, BNP7787-derived mesna–disulfide heteroconjugates may serve as selective, potent inhibitors of GGT, possibly resulting in nephroprotection by a novel means.

Keywords BNP7787 · Cisplatin · Gamma-glutamyl transpeptidase (GGT) · Glutathione · Mesna–disulfide heteroconjugates · Nephrotoxicity

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Introduction

Platinum drugs constitute an important class of compounds in the treatment of cancer, and have demonstrated a broad spectrum of anti-cancer activity against a variety of tumors including germ cell tumors, ovarian and bladder carcinomas, squamous cell tumors of the head and neck, esophageal cancers, non-small cell lung tumors and colon carcinomas either as single agent or in combination with other chemotherapy drugs [1]. Cisplatin (*cis*-diamminedichloroplatinum(II)) is an important anti-neoplastic platinum agent that has been an integral part of chemotherapy regimens used in the treatment of cancer patients [2, 3]. Cisplatin is highly nephrotoxic and microscopic damage due to its nephrotoxic effect is evident in quiescent proximal

renal tubule cells [4–16]. The extent of cisplatin-induced nephrotoxicity correlates with the total platinum concentration present in the kidney [16].

Gamma-glutamyl transpeptidase (GGT, EC 2.3.2.2) appears to play a major role in the common adverse pharmacological outcome of cisplatin-induced nephrotoxicity and is thought to promote the conversion of platinum–sulfur conjugates to platinum–sulfur species that contribute to necrotic renal cell death [4] (Fig. 1a). In addition to GGT, the activation and toxification of platinum–sulfur compounds, derived from platinum–glutathione metabolites, is proposed to involve aminopeptidase N (APN) and cysteine-*S*-conjugate-*β*-lyase (CCBL) [4, 17–19]. GGT, APN and CCBL work in a stepwise, sequential manner and are part of what is referred to herein as the GGT xenobiotic metabolism pathway (Fig. 1a). Hanigan et al. have tested mixtures prepared in situ that contain glutathione–cisplatin along with other molecules and have suggested that glutathione–cisplatin (Fig. 1a) is a substrate for GGT [4]. Further support for the concerted involvement of the GGT, APN and CCBL enzymes in cisplatin-mediated nephrotoxicity is evidenced by the fact that mice deficient in GGT are resistant to nephrotoxic effects of cisplatin, and that inhibition of either GGT by acivicin or CCBL by aminooxyacetic acid resulted in abrogation of the nephrotoxic effects of cisplatin [17, 18, 20, 21]. In addition to having an important role in the common adverse pharmacological outcome of cisplatin-induced nephrotoxicity [4, 17, 18, 20, 21], GGT is involved in a wide variety of physiological processes including drug resistance, metastatic activity of cancer cells, cardiovascular disease and lipid peroxidation, as well as being involved in several disorders that involve glutathione homeostasis [22–26].

GGT is a heterodimeric glycoprotein and its heavy subunit is anchored to the cell membrane through its N-terminal segment and the light subunit is non-covalently attached to the heavy subunit [27]. GGT catalyzes the hydrolysis of glutathione to glutamate and cysteinyl-glycine [28, 29] and also catalyzes a transpeptidation reaction where glutamate is transferred to a variety of amino acid acceptor substrates, including L-methionine, L-cystine, L-glutamine, L-alanine, glycinyl-glycine, cysteinyl-glycine or methionyl-glycine [30]. GGT binds its donor substrate (glutathione) and the enzyme is transiently acylated by glutamate, releasing cysteinyl-glycine (i.e., from the substrate, glutathione). The resulting gamma-glutamyl-acyl enzyme intermediate can then react with water releasing either free glutamate or transferring the glutamate to an acceptor substrate forming a transpeptidated product with a gamma-glutamyl bond [29, 30]. After degradation of extracellular glutathione and conjugates, the constituent amino acid and dipeptides are transferred back to the cell and are used in the intracellular de novo synthesis of glutathione [31]. GGT is also capable of hydrolyzing the terminal glutamate from glutathione when

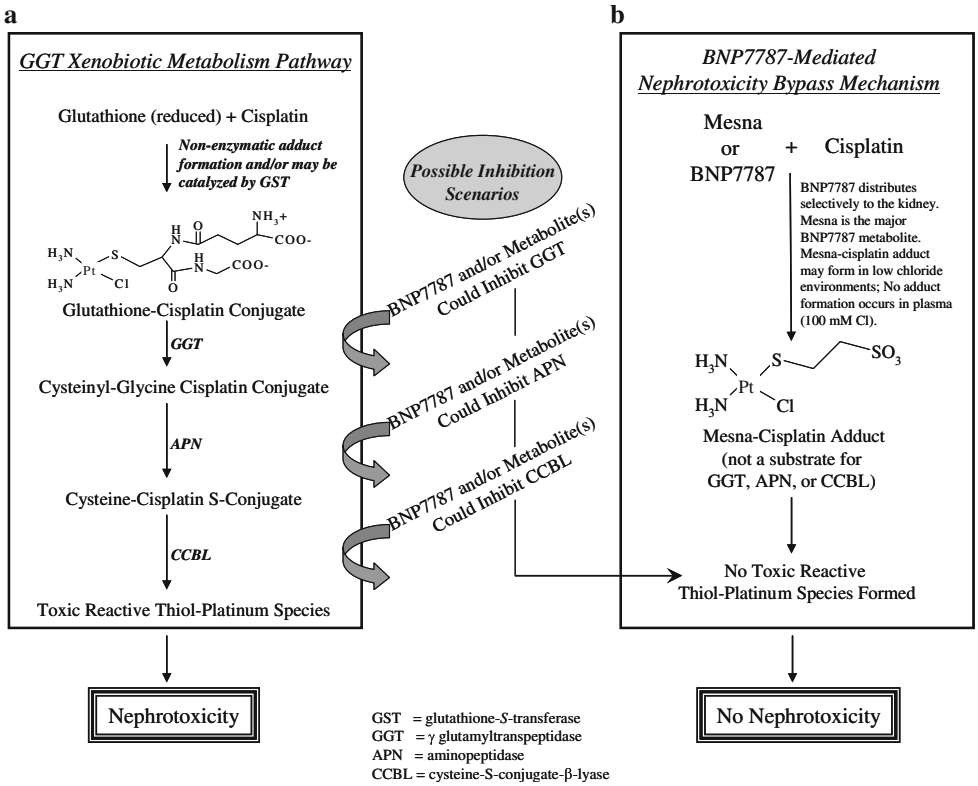

Fig. 1 **a** GGT xenobiotic metabolism pathway based on the work by Dekant, Hanigan et al. [4, 64–67]. Hanigan postulated that cisplatin may enter the proximal renal tubule cells where a glutathione–cisplatin conjugate can be formed. This glutathione–cisplatin conjugate is further metabolized by the enzymes gamma-glutamyl transpeptidase (GGT) or aminopeptidase N (APN), and cysteine-*S*-conjugate-*β*-lyase (CCBL). The product of this pathway is a putative, highly reactive thiol–Pt species, which can interact with and modify proteins leading, ultimately, to renal cell death (nephrotoxicity). **b** Formation of mesna–cisplatin adduct may bypass the GGT xenobiotic metabolism pathway effectively preventing formation of nephrotoxic glutathione–platinum species

glutathione is conjugated to xenobiotics such as halogenated alkenes. The GGT-mediated production of cysteinyl-glycine conjugates has been implicated in nephrotoxicity (Fig. 1a) [4].

We have proposed that disulfide forms of drugs may afford better nephroprotection profiles than thiol compounds [32–35]. The disulfide BNP7787 (disodium-2,2′-dithio-bis-ethane sulfonate, Tavocept™) is an investigational new drug that is undergoing clinical development to prevent or mitigate common and clinically important toxicities associated with taxanes and platinum-based chemotherapeutic agents [35–37]. BNP7787 is chemically and mechanistically distinct from other sulfur-containing drugs, including sodium thiosulfate, diethyldithiocarbamate, glutathione and amifostine, which have been studied for the prevention or reduction of toxicities associated with platinum chemotherapy [36]. It can be given in much higher doses (18.4 g/m^2) without any associated toxicities [33, 35, 38–40]. The mechanisms of action underlying the potential chemoprotective effects of BNP7787 involve several distinct, but interrelated areas. BNP7787 can act as a pharmacological surrogate/modulator of physiological thiols and disulfides (for example, glutathione, cysteine and homocysteine) [34, 41]. Mesna, a metabolite of BNP7787, may react with platinum compounds forming a mesna–cisplatin species that appears to lack susceptibility to the putative toxic, GGT-mediated activation of glutathione–platinum and other thiol modified platinum species (Fig. 1b) [40]; however, it is important to recognize that BNP7787 does not form an adduct with platinum compounds. Additionally, conjugation of mesna with glutathione or cysteinyl-glutamate appears to prevent the toxic activation of glutathione–cisplatin and cisplatin–cysteinyl-glutamate by inhibiting GGT. Such postulated pharmacologically important reactions, if confirmed, could provide helpful information in further elucidating the mechanism(s) of BNP7787-mediated nephroprotection against cisplatin-induced nephrotoxicity.

Results from previous in vitro studies demonstrate that BNP7787 and mesna undergo non-enzymatic thiol transfer reactions with physiological thiols and disufides to form mesna–disulfide heteroconjugates such as mesna–glutathione (MSSGlutathione), mesna–cysteine (MSSC), mesna–cysteinyl-glutamate (MSSCE) and mesna–cysteinyl-glycine (MSSCG) (Fig. 2a) [35, 41, 42]. Additionally, following intravenous administration in rats, BNP7787 is rapidly and predominantly distributed in the kidney, intestine, bone marrow and major salivary gland [43, 44]. We postulate that BNP7787, mesna and one or more of mesna–disulfide heteroconjugates could interact with GGT to inhibit or modulate its activity resulting in cisplatin nephroprotection (Fig. 1a), and/or that a BNP7787-derived, mesna–cisplatin species may bypass the GGT-mediated xenobiotic metabolism pathway (Figs. 1b, 2b), thereby preventing cisplatin from being metabolized to form a potent mercapturic nephrotoxin [35, 41]. This latter scenario would also reduce the amount of glutathione–cisplatin that can be formed and subsequently metabolized to a nephrotoxic species. Meister et al. have reported studies evaluating GGT activity with various glutamyl-containing dipeptides [45–47]; however, the mesna–disulfide heteroconjugates represent a new class of gamma-glutamyl-based compounds that are generated via a novel pathway involving the in vivo metabolism of BNP7787. Herein, the pharmacological effects of BNP7787 and selected BNP7787 metabolites (mesna–disulfide heteroconjugates) were investigated on human and porcine GGT in vitro using a fluorogenic assay.

Materials and methods

Chemicals and reagents

BNP7787 was synthesized and purified (>97%) at BioNumerik Pharmaceuticals, Inc. The GGT inhibitor, acivicin (α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid) and other general compounds were purchased from Sigma Aldrich (St. Louis, MO). Mesna–disulfide heteroconjugates (mesna–cysteine, mesna–glutathione, mesna–cysteinyl-glutamate and mesna–cysteinyl-glycine; structures available upon request) were synthesized and purified at BioNumerik Pharmaceuticals, Inc. Mesna (≥98% pure by iodine titration) was purchased from Sigma Aldrich and further purified at BioNumerik Pharmaceuticals, Inc., prior to use. The human GGT enzyme, suspended in 0.05 M Tris buffer, was purchased from Scipac. Porcine GGT, approximately 2 units/mL, was purchased from Sigma Aldrich. The buffer (50 mM HEPES, adjusted to pH 8.3 by adding 100 mM NaOH) was purchased from Sigma Aldrich. The substrate (20 mM solution of L-gamma-glutamyl-7-amino-4-trifluoromethylcoumarin, GG-AFC, in DMSO) and the fluorescent standard (80 μM free AFC in DMSO) were purchased from Enzyme Systems Products (now MP Biomedicals). Unless otherwise noted, all compounds were prepared fresh in sterile water immediately before use.

Instrumentation

The GGT assay was performed on a Tecan Ultra fluorescence microplate reader. The standard, default parameters (gain-23, no. of flashes-10 and Z position-8223) for the instrument were used. An excitation filter of 390 nm (±25 nm) and an emission filter of 505 nm (±10 nm) were used. The autoread, autocalibration, automixing and banking were set to "off".

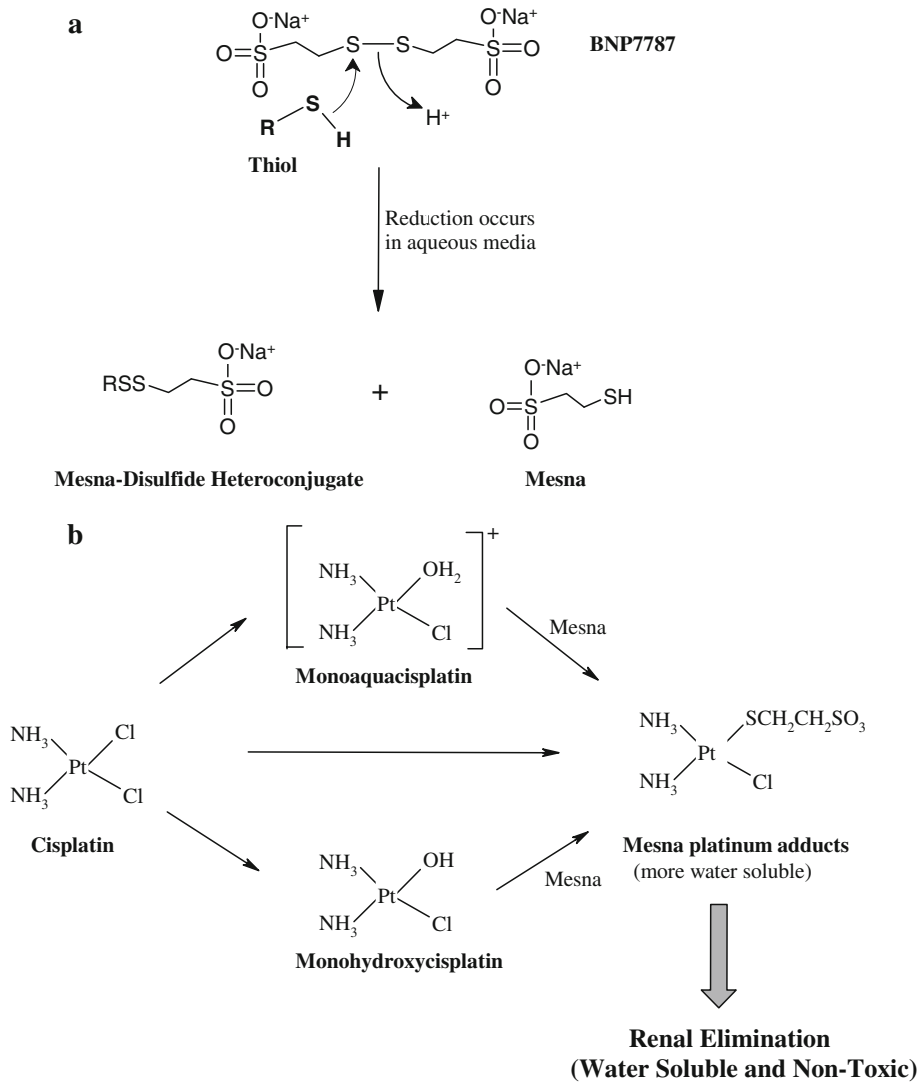

Fig. 2 **a** Postulated S_N2 route of non-enzymatic reduction of BNP7787 to mesna in the kidney [43, 60, 72]. Once BNP7787 is administered, a thiol, RSH (where R is glutathione, cysteine, homocysteine, cys-glu or cys-gly), may attack BNP7787 resulting in the heterolytic cleavage of BNP7787's disulfide bond, producing free mesna and a mesna–thiol mixed disulfide, M–S–S–R, where R is glutathione, cysteine, homocysteine, cysteinyl-glutamate or cysteinyl-glycine. **b** Aquation reactions of cisplatin and subsequent mesna–cisplatin adduct formation

Fluorometric method for GGT assay using crude enzymes

The activity of GGT was determined using a modified fluorometric biochemical assay described previously [48, 49]. The assay monitors the release of fluorescent product, free 7-amino-4-trifluoromethylcoumarin (AFC) that is liberated when GGT cleaves the substrate, GG-AFC. The amount of free AFC and, therefore, the total fluorescence will be reduced if BNP7787, mesna or the other physiological thiols and disulfides inhibit GGT.

Assays were performed in triplicate in a 96-well plate. Five microliters (5 μL) of the crude enzyme (0.01 units) and 180 μL of buffer were added to each well, followed by addition of the test materials (i.e., BNP7787, mesna, or mesna–disulfide heteroconjugates). The plate was covered with a disposable plate sealer and reagents were mixed by inversion and equilibrated to 37°C for approximately 10 min, followed by the addition of 4 μL (500 μM) of the substrate, GG-AFC. Buffer background control, enzyme negative control, substrate autohydrolysis control and enzyme activity positive control were also performed. In addition, reactions containing 0.5 mM acivicin served as inhibition controls. This concentration of acivicin inhibited the GGT rate to 50% and was used to compare with other treatment groups.

The increase in fluorescence over time in a reaction containing both GGT and its substrate, GG-AFC, is an indication of enzyme activity (after correction for autohydrolysis of the substrate), and the magnitude of the increase over time reflects the kinetic rate of GG-AFC hydrolysis by GGT. The linearity across concentrations of standard free AFC was initially evaluated on a cuvette-based, monochromator instrument, the ISS-K2, and a microtiter plate-based, filter instrument, the Tecan Ultra (data not shown). But the plate-based format was used to generate the data shown herein. Data were collected from the linear portion of the

assay over 30 min. Statistical analyses were performed using Excel.

Preparation of the mesna–cisplatin conjugate

The mesna–cisplatin conjugate was generated in situ and was expected to be a heterogeneous mixture. Different concentrations of mesna (100–400 μM) were incubated with equal concentrations of cisplatin in the HEPES buffer for 4 h at 37°C to form the mesna–cisplatin conjugate in situ. This mixture was used to evaluate the effect of the mesna–cisplatin conjugate on the GGT activity assay, as described in preceding sections.

Results

Assay optimization and effects of acivicin on GGT activity

An earlier report by Townsend et al. demonstrated that a 2-h incubation of acivicin (250 μM) at 25°C could inhibit over 89% of the detectable GGT activity in LLC-PK1 cells (1×10^6 cells) as detected by a kinetic assay where the GGT activity was monitored by following the release of 1 μmol of *p*-nitroaniline per min [4]. Treatment of these cells with acivicin resulted in a statistically significant time- and dose-dependent inhibition ($p < 0.05$) [4]. A dose-dependent inhibitory effect by acivicin was also observed in our experiments (data not shown). In our studies, a 50% inhibition in GGT activity relative to untreated enzyme was observed when 500 μM acivicin was incubated with the crude human GGT for 15 min at 25°C. Additionally, the effects of BNP7787, mesna and mesna–disulfide heteroconjugates on GGT activity, in the presence of acivicin, are described herein.

Effect of mesna and BNP7787 on GGT activity

The results of the effects of BNP7787 and its metabolites on GGT are summarized in Table 1. If BNP7787 or one of its metabolites (e.g., mesna or mesna–disulfide heteroconjugates) were either an inhibitor or a substrate of GGT, the observed rate of cleavage (fluorescence increase) would be reduced relative to the untreated control rate of cleavage. Neither BNP7787 nor mesna is a mimic of glutamate and, therefore, it is unlikely that they can directly inhibit GGT. No statistically significant effects on porcine or human GGT activity were observed with BNP7787 or mesna (data not shown); therefore, neither BNP7787 nor mesna acted as substrates or inhibitors of the porcine or human GGT enzymes. When BNP7787 or mesna were incubated with GGT in the presence of acivicin, the GGT inhibition was similar to that observed with acivicin alone (data not shown), suggesting that neither BNP7787 nor mesna had any additive, potentiative or other effects on acivicin inhibition of GGT.

Mesna–disulfide heteroconjugates that inhibit GGT activity

Mesna–cysteinyl-glutamate (Fig. 3) and mesna–glutathione (Fig. 4) decreased the rate of cleavage of the GG-AFC substrate by both the porcine and human enzymes in a dose-dependent manner. MSSGlutathione [$p = 9.57\ e^{-16}$ (human); $p = 5.15\ e^{-13}$ (porcine)] was more effective at inhibiting GGT than MSSCE [$p = 8.98\ e^{-9}$ (human); $p = 1.22\ e^{-8}$ (porcine)]. At concentrations of 10 mM, MSSCE resulted in 50% inhibition, whereas MSSGlutathione inhibited about 80% of the activity of GGT. Both MSSGlutathione and MSSCE contain a glutamate that is expected to compete for binding to the GGT active site with the substrate, GG-AFC.

Mesna–disulfide heteroconjugates that enhance GGT activity

Mesna–cysteine enhanced the human and porcine GGT enzyme activity, in a dose-dependent manner, while MSSCG enhanced the rate equally at all concentrations tested herein (Fig. 3a, b). However, the enhancement of MSSCG was about threefold higher relative to the no inhibitor

Table 1 Summary of results of the effects of BNP7787 and its metabolites on GGT hydrolysis of GG-AFC

Compound	Effect on GGT (porcine)	Effect on GGT (human)
BNP7787	No direct inhibition	No direct inhibition
Mesna	No direct inhibition	No direct inhibition
MSSGlutathione	Dose-dependent inhibition	Dose-dependent inhibition
MSSCE	Dose-dependent inhibition	Dose-dependent inhibition
MSSC	Dose-dependent enhancement	Dose-dependent enhancement
MSSCG	Enhanced activity	Enhanced activity
MSSGlutathione + MSSC	Not tested	Inhibited activity
MSSGlutathione + MSSCG	Not tested	Inhibited activity
MSSCE + MSSC	Not tested	Inhibited activity
MSSCE + MSSCG	Not tested	Slightly enhanced activity
Mesna–cisplatin	Not tested	No direct inhibition

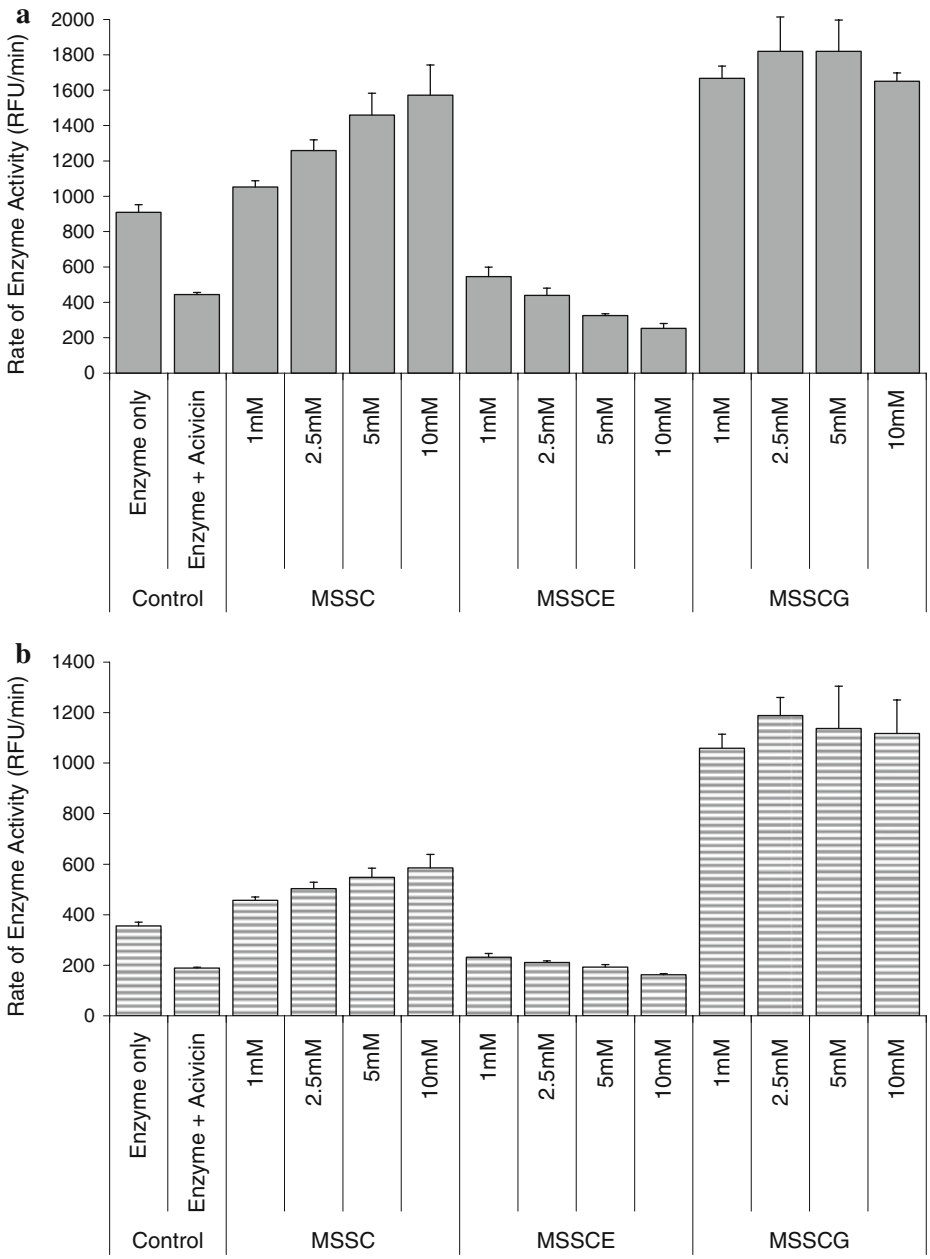

Fig. 3 Effect of mesna–cysteine (MSSC), mesna–cysteinyl-glutamate (MSSCE) and mesna–cysteinyl-glycine (MSSCG) on GGT activity. Different concentrations of MSSC, MSSCE and MSSCG in the presence and absence of acivicin (500 μM) were tested on porcine (**a**) and human GGT (**b**). Pure MSSCE [$p = 8.98\ e^{-9}$ (human); $p = 1.22\ e^{-8}$ (porcine)] decreased the rate of cleavage of substrate, while pure MSSC [$p = 4.93\ e^{-5}$ (human); $p = 6.12\ e^{-5}$ (porcine)] and pure MSSCG [$p = 9.21\ e^{-6}$ (human); $p = 2.16\ e^{-5}$ (porcine)] enhanced the rate of cleavage of the substrate for both the human and porcine enzymes in a statistically significant manner. This enhancement was not dose dependent at the concentrations studied herein; millimolar concentrations were selected and evaluated because both glutathione and BNP7787 are present in vivo at millimolar levels

control, while there was less pronounced enhancement by MSSC, compared to the no inhibitor control.

Effect of combinations of enhancers and inhibitors on GGT activity

Previous studies have shown that when BNP7787 reacted with thiols, formation of mesna–disulfide heteroconjugates (e.g., MSSCG, MSSCE, MSSC and MSSGlutathione) occurred (Fig. 2a) [35, 41, 42]. Therefore, it was necessary to determine the effect on GGT activity by combinations of mesna–disulfide heteroconjugates that, as single agents, either enhanced or inhibited GGT activity. The results indicated that when the enhancers and inhibitors were mixed together, the inhibitory effect usually predominated (Fig. 5). However, when MSSCE (5 mM) was added to MSSCG (10 mM), a small but statistically insignificant enhancement of GGT activity was observed ($p = 0.19$).

Effects on GGT activity by in situ formed mesna–cisplatin conjugate

A fraction of cisplatin is spontaneously converted to a monoaquated species in vivo that may react with the metabolites of BNP7787, such as mesna and/or mesna–disulfide heteroconjugates (Fig. 1b). However, cisplatin does not react directly with BNP7787. For the in vitro studies in this work, the putative mesna–cisplatin conjugate was formed

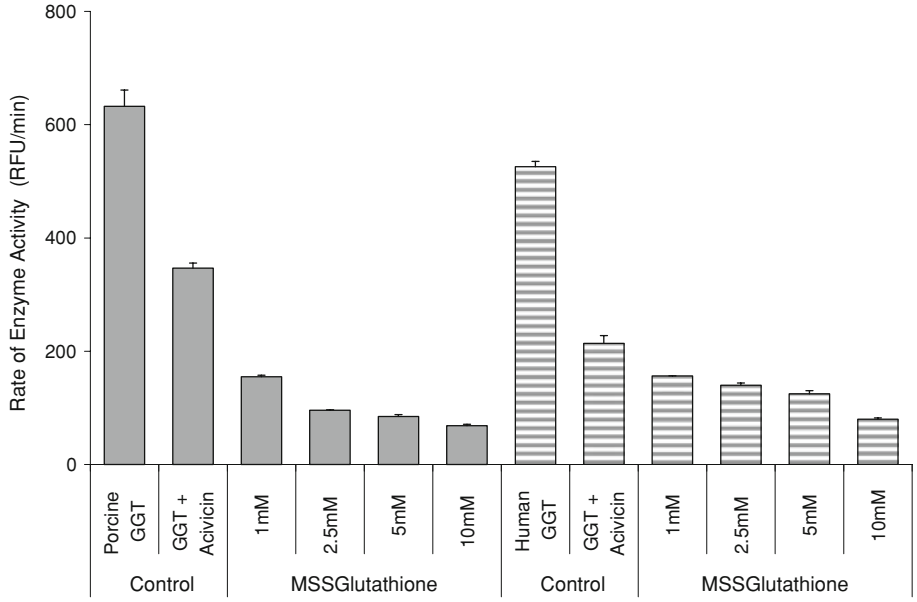

Fig. 4 Effect of mesna–glutathione (MSSGlutathione) on GGT activity. Different concentrations of mesna–glutathione (MSSGlutathione) in the presence and absence of acivicin (500 μM) on porcine and human GGT. Porcine GGT data are shown in the *solid grey bars*; human GGT data are shown in the hashed bars. MSSGlutathione (1 mM) inhibited both the human and porcine enzymes as effectively as acivicin (500 μM). At all MSSGlutathione concentrations there was statistically significant inhibition, and a correlation between MSSGlutathione concentration and inhibition was evident [$p = 9.57\ e^{-16}$ (human); $p = 5.15\ e^{-13}$ (porcine)]. Millimolar concentrations of MSSGlutathione were used to reflect the likely physiological levels of this species based on the concentration of glutathione and BNP7787 in vivo; however, reported levels of cysteine, homocysteine, cysteinyl-glycine, and cysteinyl-glutamate are lower and would not be expected to exceed high micromolar ranges [70, 71]

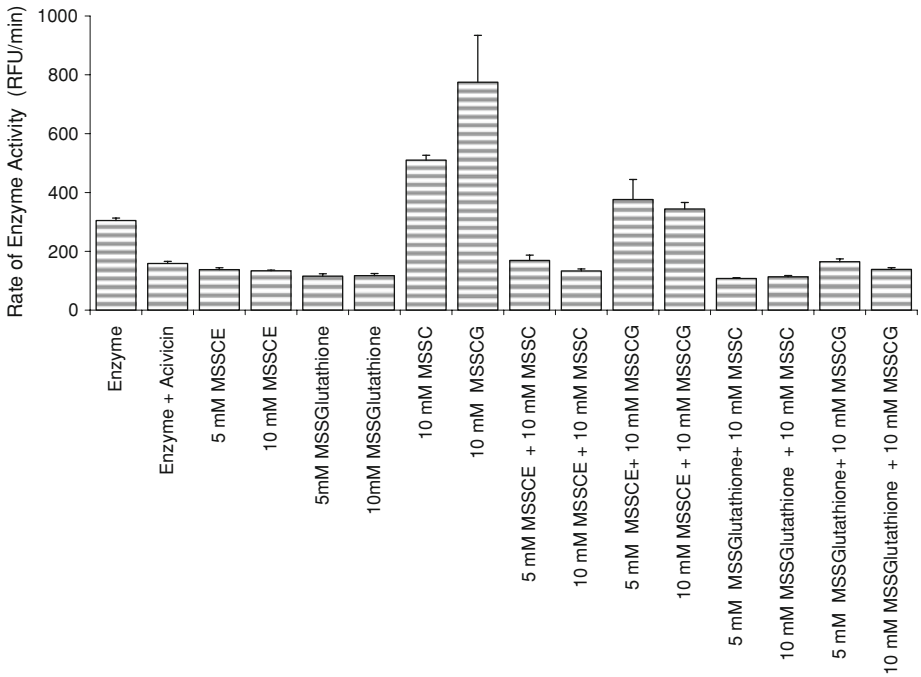

Fig. 5 Effects of the mixture of inhibitors (MSSGlutathione, MSSCE) and enhancers (MSSC, MSSCG) on human GGT. The results indicate that even in the presence of the enhancers, such as MSSC and MSSCG, there was statistically significant inhibition of human GGT when MSSGlutathione was added [$p = 5.71\ e^{-7}$ (MSSGlutathione + MSSCG); $p = 1.97\ e^{-8}$ (MSSGlutathione + MSSC)]. However, when MSSCE and MSSCG were incubated together, there was a slight, but statistically insignificant ($p = 0.19$), enhancement of the GGT activity

by incubating cisplatin with mesna for 4 h and then evaluating the effect of this post-reactant mixture on GGT activity. Relative to the control (enzyme only), there was no significant difference among the test groups consisting of different concentrations of cisplatin and mesna (Table 2). The implications of this observation are discussed in the following sections. The controls of mesna alone or cisplatin alone had no effect on the activity of GGT.

Table 2 Effects of the mesna–cisplatin conjugate on human GGT activity (all concentrations are micromolar)

Cisplatin concentration[a]	Mesna concentration[b]	Enzyme activity (%)
0	0	100 ± 2.06
1	200	104 ± 1.84
10	200	109 ± 1.65
100	200	101 ± 1.96
1	400	101 ± 2.45
10	400	104 ± 1.61
100	400	110 ± 1.34

[a] 1, 10 and 100 μM cisplatin alone did not inhibit enzyme activity

[b] 200 and 400 μM mesna alone did not inhibit enzyme activity

Discussion

Nephrotoxicity is a prevalent and serious side effect of many chemotherapeutic agents, including cisplatin, a widely used chemotherapeutic agent [50]. Several first-generation drugs such as thiol-generating cytoprotective agents have undergone clinical evaluation (e.g., sodium thiosulfate, diethyldithiocarbamate, amifostine and reduced glutathione) in an attempt to reduce the toxicity of platinum drugs like cisplatin [51]. The only agent that is approved for prevention of cisplatin-induced nephrotoxicity is amifostine (WR2721, (ethanethiol, 2-[(3-aminopropyl)amino] dihydrogen phosphate ester)), and this agent has been an important advance; however, its administration is associated with several reported side effects, including ototoxicity, hypotension, dizziness, severe nausea and vomiting, transient decrease in serum calcium levels and infusion-related flushing [52–54]. Despite the ability to protect against certain toxicities, nearly all platinum-protecting drugs have demonstrated tumor protection as well as their own intrinsic toxicities following administration [33, 35]. Consequently, there remains a real, unmet need for a safe, nontoxic nephroprotective agent that can be used to prevent and mitigate cisplatin-induced nephrotoxicity. BNP7787 (Tavocept™) is a water-soluble disulfide containing investigational new drug that is undergoing clinical development for the prevention and mitigation of clinically important chemotherapy-induced toxicities associated with platinum and taxane chemotherapeutic agents [32–37, 44, 55–60]. Previous nonclinical and early human clinical trials have shown that BNP7787 has strong potential to protect against cisplatin-induced nephrotoxicity [33, 38–40, 61, 62] without interfering with the antitumor activity of platinum agents, paclitaxel or other chemotherapy agents [33, 38–40, 61, 62]. BNP7787 can be given to humans at doses (up to 41 g/m^2) that correspond to millimolar concentrations in vivo, and, in contrast to thiol-based reagents, BNP7787 does not perturb the plasma thiol–disulfide balance [35]. BNP7787 demonstrates chemically, pharmacologically and toxicologically distinct behavior in vitro, in vivo and in human subjects compared to free thiol-containing agents, including mesna or other sulfur-containing compounds that have been evaluated for cisplatin nephroprotection.

Direct effects of BNP7787 and BNP7787-derived mesna–disulfide heteroconjugates on GGT hydrolysis of GG-AFC

Dekant et al. have described a general nephrotoxigenic mechanism wherein a xenobiotic conjugates with glutathione and is converted to a toxic metabolite by GGT, APN and CCBL (the GGT xenobiotic metabolism pathway) [63–67]. Hanigan et al. proposed that cisplatin and glutathione form a glutathione–cisplatin conjugate, which is metabolized through the GGT xenobiotic metabolism pathway resulting in the formation of a toxic, reactive platinum species (Fig. 1a) [4, 17, 18, 21]. GGT, APN and CCBL are very important in clearing toxic platinum conjugates of glutathione and cysteine from the kidney; unfortunately, the platinum–thiol conjugates, produced by the action of GGT, APN and CCBL, may result in toxic species that cause cellular damage [4, 17, 18, 20, 21]. We hypothesized that mesna–disulfide heteroconjugates, formed when BNP7787 reacts with physiological thiols containing cysteinyl-glutamate moieties, could potentially inhibit GGT toxification and also reduce the amount of glutathione–cisplatin that is formed in the renal tubule. Additionally, the mesna–cisplatin adduct could limit or prevent the amount and proportion of glutathione–cisplatin to be subsequently metabolized to toxic, reactive platinum species by the GGT pathway in the renal tubule (Fig. 1a, b).

Neither BNP7787 nor mesna had any statistically significant effect on the observed activity of the porcine or human GGT enzymes (data not shown) and this is likely due to a lack of structural similarity between mesna and GGT's preferred substrate, glutathione. However, thiol-glutamate analogs are effective inhibitors of GGT [29, 30, 46] and competing with the binding of the glutamate moiety in glutathione may result in GGT inhibition. Consistent with this hypothesis, MSSGlutathione and MSSCE, inhibited human and porcine GGT (Figs. 3, 4) (cysteinyl-glutamate or γ-glutamyl-cysteine are formed during the biosynthesis of glutathione) [68, 69]. In contrast, we observed that species lacking glutatmate moieties (i.e., MSSC and MSSCG) enhanced the rate of the GGT hydrolysis of GG-AFC; these species probably serve as acceptors of the glutamate group that is cleaved from the GG-AFC substrate (Fig. 3a, b; [68, 69]).

Effects of combinations of inhibitory and enhancing mesna–disulfide heteroconjugates on GGT hydrolysis of GG-AFC

In vitro, we observed that millimolar concentrations of MSSC and MSSCG resulted in statistically significant GGT enhancement (Fig. 3), while statistically significant inhibition was observed for MSSGlutathione and MSSCE (Figs. 3, 4). In vivo, a mixture of the mesna–disulfide heteroconjugates would be present and would probably not exceed micromolar concentrations (based on reported concentrations of physiological thiols and disulfides [70, 71]). Consequently, we examined combinations of enhancers and inhibitors with GGT simultaneously (Fig. 5), to determine the predominant effect on GGT activity when mixtures of the mesna–disulfide heterconjugates were present. When MSSGlutathione and MSSCG were co-incubated, the net pharmacological effect was GGT inhibition (Fig. 5). The glutamate moiety of MSSGlutathione is expected to compete for binding to the active site where GG-AFC normally binds and, since MSSCG is structurally similar to the preferred substrate glutathione, but lacks a glutamate moiety, MSSCG would be expected to influence the catalysis less than MSSGlutathione when both are present (the cysteinyl-glycine moiety of MSSCG probably binds peripherally to the active site pocket). Similar effects were observed when MSSC was incubated with either MSSGlutathione or MSSCE (Fig. 5); however, when MSSCE was incubated with MSSCG, there was a very slight enhancement of GGT activity and it is possible that the cysteinyl-glycine of MSSCG may serve as a glutamate acceptor preferentially driving the GGT reaction forward, but this enhancement was slight and was not significantly different from levels of uninhibited GGT. In summary, the data suggest that in the presence of mixtures of heteroconjugates, the GGT reaction would either be inhibited or essentially unaffected; net inhibition of GGT is further strongly supported by animal and human data with cisplatin and BNP7787 co-administration [35–37].

Indirect effects on GGT by BNP7787 or BNP7787-derived species

It was important to determine if mesna–cisplatin was able to bypass the GGT xenobiotic metabolism pathway entirely (i.e., mesna–cisplatin might not be a substrate for the xenobiotic pathway) (Figs. 1b, 2b). Previous studies have shown that glutathione–cisplatin is a substrate of GGT and toxic to LLC-PK1 cells [4]. In contrast to glutathione–cisplatin, we observed that mesna–cisplatin had no effect on GGT activity and appears not to be a GGT substrate (Table 2). If this putative mesna–cisplatin conjugate supplants a significant amount of the corresponding glutathione–cisplatin conjugate in vivo and is not processed through the xenobiotic pathway, the amount of toxic platinum species produced would be decreased, affording nephroprotection. This is an important finding and is thought to be one key mechanism through which BNP7787 may have a nephroprotective effect against cisplatin-induced nephrotoxicity.

Conclusions

BNP7787 administered in combination with cisplatin appears to have potential advantages over other compounds that have previously been evaluated for cisplatin nephroprotection. Results in this study further elucidate the mechanisms behind potential BNP7787-mediated cisplatin nephroprotection with a specific focus on understanding how BNP7787 and BNP7787-derived mesna–disulfide heteroconjugates may impact GGT-mediated cisplatin toxification activity and/or formation of nephrotoxins as a consequence of xenobiotic metabolism involving GGT. Assuming that the GGT xenobiotic metabolism pathway is the major toxification pathway for cisplatin nephrotoxicity, we postulate that there are two general mechanisms (Fig. 1), either or both of which may result in significant cisplatin nephroprotection: (1) the active pharmacophore (mesna) produces an inactive mesna–cisplatin conjugate that is not a substrate for, and therefore completely bypasses, the GGT toxification pathway (GGT xenobiotic metabolism pathway); and (2) cysteinyl-glutamate containing mesna–disulfide heteroconjugates can serve as selective, potent inhibitors of GGT (e.g., MSSCE, MSSGlutathione) possibly resulting in nephroprotection by a novel means.

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