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ORIGINAL ARTICLE

Mechanistic study of BNP7787-mediated cisplatin nephroprotection: modulation of human aminopeptidase N

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Abstract
Purpose Previous studies from our laboratory have identified a role for gamma-glutamyl transpeptidase (GGT) in BNP7787 (disodium 2,2′-dithio-*bis* ethane sulfonate, dimesna, Tavocept™)-mediated cisplatin nephroprotection. Dekant has proposed that gamma-glutamyl transpeptidase (GGT), aminopeptidase N (APN) and cysteine-conjugate-*β*-lyase (CCBL) comprise a multi-enzyme pathway that acts on xenobiotic-glutathione conjugates converting them to nephrotoxic metabolites. We report modulation of APN activity within this pathway by BNP7787-derived mesna-disulfide heteroconjugates.
Methods A fluorimetric assay was used to determine the effect of BNP7787, BNP7787-derived mesna-disulfide heteroconjugates, and the BNP7787 metabolite, mesna (sodium 2-mercaptoethane sulfonate), on the initial velocity and overall progress curve of the human APN reaction in vitro.
Results Neither BNP7787 nor mesna-cysteinyl-glutamate inhibited human APN. Select BNP7787-derived mesna-disulfide heteroconjugates (mesna-cysteine, mesna-glutathione, mesna-cysteinyl-glycine) and high concentrations of mesna inhibited APN activity. Allosteric effects on the enzyme progress curve outside of the linear initial velocity region were observed for mesna-cysteinyl-glycine, mesna-glutathione and mesna-cysteinyl-glutamate and appeared to be a function of having both mesna and di- or tri-peptide functionalities in one molecule. In situ-generated mesna-cisplatin conjugates were not a substrate for human APN.
Conclusions BNP7787-mediated prevention or mitigation of cisplatin-induced nephrotoxicity may involve APN inhibition by certain BNP7787-derived mesna-disulfide heteroconjugates and appears correlated to the presence of a glycinate moiety and/or an anionic group. Two general mechanisms for BNP7787-mediated nephroprotection of cisplatin-induced nephrotoxicity involving the GGT, APN and CCBL nephrotoxigenic pathway are proposed which acting in a concerted and/or synergistic manner, and thereby prevent or mitigate cisplatin-induced renal toxicity.

Keywords Aminopeptidase N · BNP7787 · Chemoprotective agent · Cisplatin · Dimesna · Mesna-disulfide heteroconjugates · Nephrotoxicity · Nephrotoxigenic · Xenobiotic toxification pathway

Introduction

Cisplatin (CDDP or *cis*-diammine-dichloroplatinum (II)) is one of the most commonly prescribed agents for chemotherapy of solid tumors [1]. Cisplatin has been used for the treatment of germ cell tumors, ovarian and bladder carcinomas, squamous cell tumors of the head and neck and non-small cell lung tumors, either as a single agent or in combination with other chemotherapy drugs [2]. Cisplatin is a highly nephrotoxic drug, and it appears to exert this nephrotoxic effect on the quiescent proximal renal tubule cells that demonstrate characteristic patterns of microscopic histological damage in rats and humans [3–7]. The mitigation or prevention of cisplatin-induced nephrotoxicity is currently achieved by administration of large volumes of hypertonic saline; as well as medically managing an

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iatrogenic saline-enriched forced diuresis with agents such as mannitol and furosemide. Despite the clinical utility of cisplatin, such prophylactic medical interventions represent important limitations and indicate that there is an unmet need for the development of safe, efficacious, convenient and cost-effective cisplatin nephroprotective agents [8].

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 [9]. Currently, the only agent that is approved for prevention of cisplatin-induced nephrotoxicity is amifostine [WR2721: (ethanethiol, 2-[(3-aminopropyl)amino] dihydrogen phosphate ester)]. However, its administration is associated with several, potentially serious side effects, including ototoxicity and hypotension [10, 11]. Other adverse events that limit its clinical utility include dizziness, severe nausea and vomiting, transient decrease in serum calcium levels, and infusion-related flushing [11]. While some of these first generation agents appear to protect against certain toxicities, nearly all platinum-protecting drugs that have been previously developed have demonstrated an unwanted tumor protecting effect; as well as their own intrinsic toxicities following administration [12]. Consequently, there remains an unmet need for a safe, non-toxic nephroprotective agent that can be used to treat cisplatin-induced nephrotoxicity.

The nephrotoxic effects of cisplatin have long been associated with its reaction with thiol-containing molecules found in vivo, such as glutathione [13–16]. Dekant et al. have shown that when halogenated alkenes and quinones (such as trichloroethane or 2-bromohydroquinone) form glutathione-S-conjugates, these conjugates are subsequently metabolized to form nephrotoxins [13, 17]. This observation was extended to cisplatin by Hanigan et al., who showed that a cisplatin-glutathione conjugate formed in situ is a substrate for GGT, the first enzyme of the GGT xenobiotic toxification pathway [18–20]. The product of the GGT catalyzed hydrolysis of glutathione-cisplatin, cysteinyl-glycine-cisplatin, is thought to be further metabolized by APN to ultimately yield a reactive, platinum species that is nephrotoxic; GGT and APN, along with CCBL, work in a stepwise, sequential manner and are part of the GGT xenobiotic toxification pathway (Fig. 1a) [21]. It should be noted that in recent in vivo studies, Wainford et al. have suggested that only GGT is involved in cisplatin nephrotoxicity [22]; however, earlier in vitro and in vivo work by Hanigan and coworkers supports a role for GGT, APN and CCBL in cisplatin-mediated nephrotoxicity [18–21], and earlier work by Hausheer and co-workers proposed that GGT, APN and CCBL were key factors in the pathogenesis of cisplatin nephrotoxicity (FDA IND #51,014; 1996). Physiological thiols are depleted upon cisplatin administration, and concurrent with this thiol depletion, the formation of toxic platinum–thiol or platinum–sulfur species may also occur [23, 24].

BNP7787 (disodium 2,2′-dithio-*bis*-ethane sulfonate, dimesna, Tavocept™) is an investigational drug that is currently undergoing clinical development as a novel chemoprotecting agent [12, 25–35]. BNP7787 can act as a pharmacological surrogate/modulator of physiological thiols and disulfides [24]. Results from previous in vitro studies show that BNP7787 participates in non-enzymatic (S_N2-type) thiol transfer reactions to form BNP7787-derived mesna-disulfide heteroconjugates such as mesna-glutathione (MSSGlutathione), mesna-cysteine (MSSC), mesna-cysteinyl-glutamate (MSSCE) and mesna-cysteinyl-glycine (MSSCG); as well as the BNP7787-derived metabolite, mesna (2-mercapto ethane sulfonate sodium; Fig. 1b) [36]. We have previously evaluated the interactions between human and porcine GGT with BNP7787 and determined that BNP7787 reacts with cisplatin to form a mesna-cisplatin species that is not a substrate for activation to the putative toxic, reactive cisplatin species via the GGT toxification pathway (Fig. 1a) [8]. Accordingly herein, we focus on APN, a key target enzyme immediately downstream in the GGT toxification pathway.

Many peptides and small molecules, including cysteinyl-glycine dipeptides, have been shown to inhibit APN [37, 38]. Compounds with a cysteinyl-glycine group have the potential to: (1) compete with the putative in vivo substrate, cysteinyl-glycine-cisplatin, for binding to the APN active site and/or its turnover [38] and (2) compete with the Leu-AMC substrate for binding to human APN's active site and/or its turnover in the in vitro assays described herein. Since a wide variety of small molecules are known to inhibit APN [37], and given that we have previously reported that several BNP7787-derived mesna-disulfide heteroconjugates also inhibit GGT [8], we evaluated the effects of BNP7787, BNP7787-derived mesna-disulfide heteroconjugates and the BNP7787 metabolite, mesna, on human APN activity in vitro. The hypotheses in this study were as follows: (1) BNP7787, mesna or a cysteine containing mesna-heterodisulfide with a cysteinyl-glycine (i.e., MSSCG or MSSGlutathione) might directly inhibit APN, thus resulting in protection against cisplatin-induced nephrotoxicity, (2) conjugation of mesna with cysteinyl-glycine may prevent the toxic activation of cysteinyl-glycine-cisplatin by competing with these cisplatin conjugates for binding to APN and/or by depleting the amount of cysteinyl-glycine available to form the aforementioned conjugate with the cisplatin and (3) formation of mesna-cisplatin may also reduce the amount of cisplatin available to react with glutathione, this would ultimately reduce the amount of the APN substrate (i.e., cysteinyl-glycine-cisplatin) that is formed.

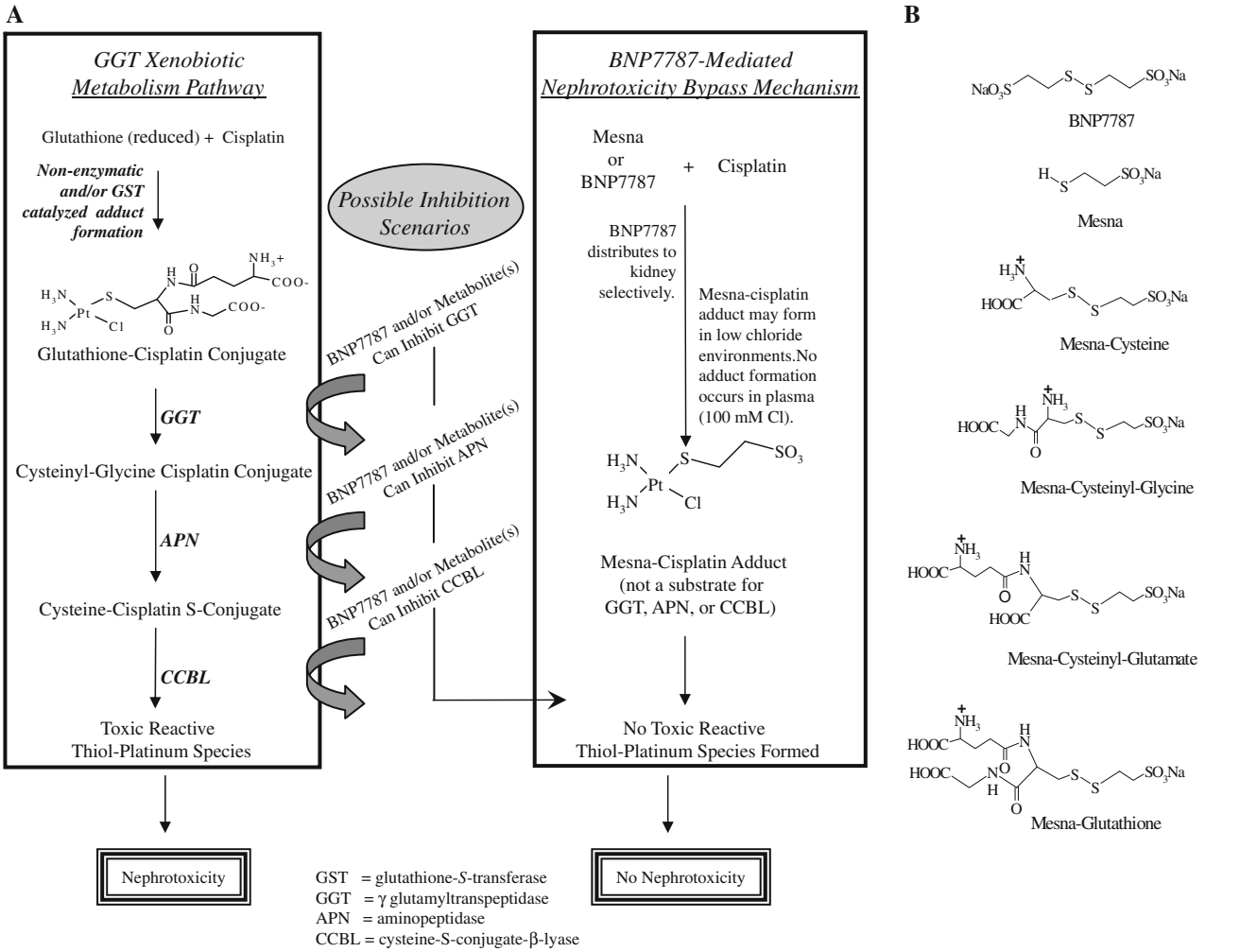

Fig. 1 **a** GGT cisplatin xenobiotic toxification pathway based on work by (1) Dekant, Hanigan and their colleagues [21, 23, 42, 48]; (2) Hausheer and co-workers (FDA IND #51,014; 1996) and BNP7787-mediated nephrotoxicity bypass mechanism proposed herein. Hanigan postulated that cisplatin may enter 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-conjugate-β-lyase (CCBL). The product of this pathway is a putative, highly reactive thiol-platinum species which can interact with and modify proteins leading, ultimately, to renal cell death (nephrotoxicity). In the BNP7787-mediated nephrotoxicity bypass mechanism, formation of a mesna-cisplatin adduct may bypass the GGT xenobiotic toxification pathway effectively preventing formation of nephrotoxic thiol-platinum species. **b** Structures of BNP7787, mesna and mesna-disulfide heteroconjugates

Materials and methods

Reagents and chemicals

BNP7787 was synthesized and purified (≥99%) at BioNumerik Pharmaceuticals, Inc. Mesna-disulfide heteroconjugates (mesna-cysteine, mesna-glutathione, mesna-cysteinyl-glutamate and mesna-cysteinyl-glycine) were synthesized at BioNumerik Pharmaceuticals, Inc. and were ≥93% pure by HPLC peak area. Mesna (≥98% pure by iodine titration) was purchased from Sigma–Aldrich. Bestatin ([(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine hydroxide; Sigma–Aldrich), a slow binding inhibitor of APN, was used as a positive control for inhibition in the APN assays described herein [37]. The APN substrate, leucine-7-amino-4-methylcoumarin (Leu-AMC), was purchased from MP Biomedicals, Inc.

Human recombinant aminopeptidase N (specific activity > 2,500 pmol/min/μg, >95% purity, approximately 0.5 mg/ml), supplied in a 0.2 μm filtered solution of 25 mM MES, 0.15 M NaCl, pH 6.5, was obtained from R&D Systems, Inc. The assay buffer, Tris (50 mM, pH 7.0), was used to dissolve all test articles and to dilute the APN enzyme immediately before use. AMC (50 μM), the product of the APN-mediated hydrolysis of Leu-AMC, was purchased from Sigma–Aldrich and dissolved in DMF and included as a relative fluorescence unit (RFU) standard control on each plate.

Instrumentation

Assays were performed on a Tecan Ultra microtiter plate reader. An optimal gain setting of 25 was determined in pilot experiments and the instrument calculated the optimal Z position for each plate automatically. The user-defined mirror setting was used. Since the APN assay monitored the release of the AMC moiety that was present in the Leu-AMC substrate, an excitation filter of 380 nm (±25 nm) and an emission filter of 465 nm (±10 nm) was used for all assays.

Fluorometric APN assay

The activity of human APN was determined based on enzyme-specific fluorometric biochemical assays described previously by R&D Systems, Inc. The assay monitored the release of fluorescent product, free AMC, that is liberated when human APN cleaves Leu-AMC. Reaction conditions described herein used a vast excess of Leu-AMC (100 μM/assay) in comparison with APN enzyme (~1.9 nM/assay or ~20 ng/assay) in each assay. The initial velocity in reactions was determined during the linear phase of the assay when substrate concentration is saturating (for each experiment we calculated the rate of the reaction during the 2–8 min range of the assay). Additionally, we monitored the progress curve of the assays for at least 30 min.

These assays were performed by two scientists working independently and all assays were run in replicates of 6–8 per plate per test article concentration. For each test article, all assays were conducted on at least 2 different days to ensure reproducibility and quality of the data. Master mixes (75 μl total volume) containing substrate, test article and buffer were aliquoted to wells and plates were equilibrated to 37°C for 5 min inside the fluorimeter. It should be noted that deviations in plate equilibration can lead to lags that are reflected in non-linear behavior during the initial portion of the enzyme progress curve. Next, twenty-five microliters (25 μl) of diluted human APN (8×10^{-4} μg/μl made by diluting 2 μl of 10 μg APN stock from R&D Systems with 1.47 ml of buffer) was added to individual wells to initiate the reaction (no enzyme controls were "initiated" with 25 μl of buffer only) and after addition of APN to equilibrated plates, plates were agitated in the fluorimeter for 10 s prior to the first read. Buffer background control, enzyme only negative control, substrate autohydrolysis control, product only control (AMC) and enzyme activity positive control were also performed. Experiments where assays were initiated with Leu-AMC instead of APN were also conducted for several test articles and gave essentially identical results to the assays initiated with APN.

Assay optimization

Experiments demonstrating assay dependence on APN and substrate concentrations and inhibition using the known inhibitor, Bestatin, were conducted and gave "textbook-type" results (data not shown). These experiments were used to establish experimental conditions for obtaining an appropriate linear range for the APN assay. Leu-AMC at a concentration of 100 μM/well was selected as the substrate concentration and 20 ng/well concentration of APN was selected as the optimal enzyme concentration.

Preparation of the mesna-cisplatin adduct in situ

The mesna-cisplatin conjugate (Fig. 1a) was generated in situ and was expected to be a heterogeneous mixture. Different concentrations of mesna (100–400 μM) were incubated with equimolar 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, without purification, to evaluate the effect of the mesna-cisplatin conjugate on the APN activity assay, as described in preceding sections.

Results

Effect of BNP7787 and mesna on human APN

Although BNP7787 is structurally distinct from cysteinylglycine, APN can accept a wide variety of small molecules as substrates and this includes several compounds that contain disulfide moieties (e.g., Psammaplin A and RB101) [37]; therefore, it was important to evaluate the effect of BNP7787 on APN activity. In comparison with NaCl controls for the sodium content in BNP7787, BNP7787 did not affect human APN significantly at the physiologically relevant concentrations (Fig. 2a; P values were >0.05, see figure legend). However, both the NaCl controls and BNP7787 slightly stimulated APN activity in comparison reactions that contained only enzyme and substrate and lacked the additional sodium (Fig. 2a). At 5 and 10 mM concentrations, mesna significantly inhibited APN activity (Fig. 2b). It should be noted, however, that in vivo concentrations of BNP7787-derived mesna in plasma when BNP7787 is administered to patients is generally 200–250 μM [47]. Following BNP7787 administration, mesna does not reach millimolar concentrations in human plasma in vivo but it does reach millimolar concentrations in the kidney and/or urine in both humans and rats [31, 33, 39]. Indeed, BNP7787 is eliminated primarily through the kidney and in situ generation of mesna may reach millimolar concentrations in the renal tubules which is also the site of

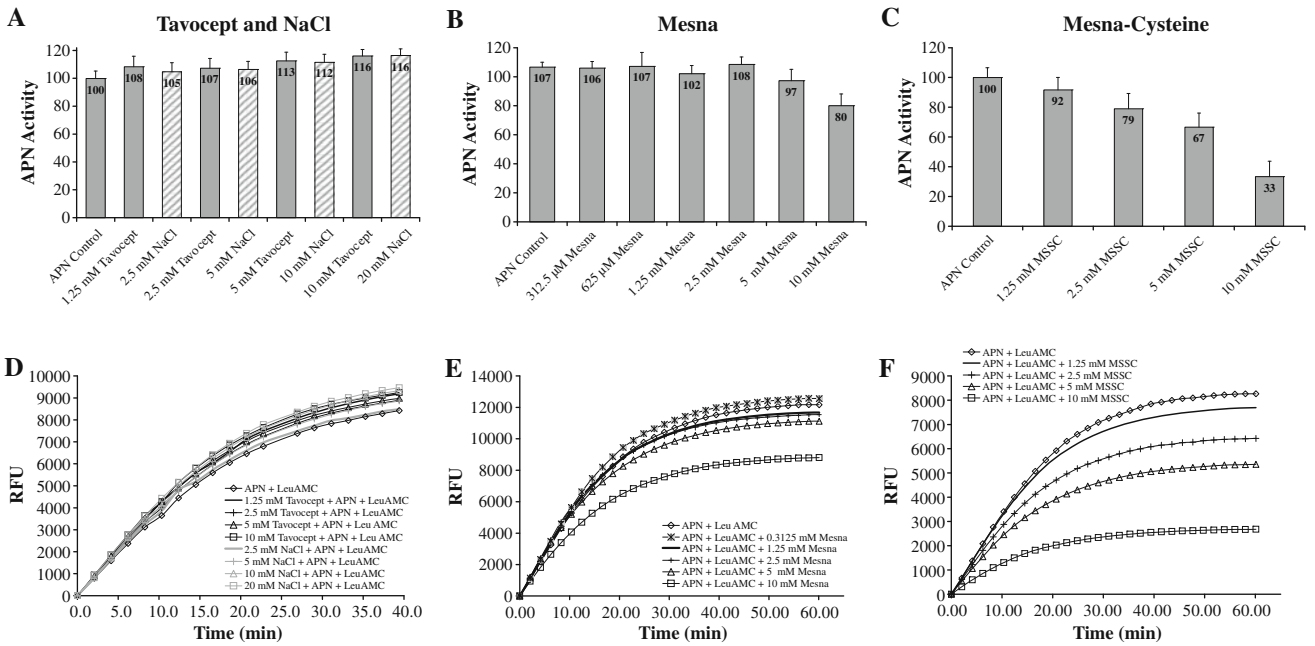

Fig. 2 Effects of **a** BNP7787 and NaCl; **b** mesna and **c** MSSC on initial velocity and the overall progress curve of human APN. The APN assay consisted of monitoring the fluorescence due to free AMC produced from the reaction of APN with the substrate, Leu-AMC. *P* values for 1.25, 2.5, 5.0 and 10 mM BNP7787 versus control were *0.0328*, *0.0399*, *0.00164* and $2.8e^{-5}$, respectively. *P* values for 2.5, 5.0, 10 and 20 mM NaCl versus control were 0.147, *0.0380*, *0.00211* and $2.81e^{-5}$, respectively. *P* values for 1.25 BNP7787 versus 2.5 mM NaCl, 2.5 mM BNP7787 versus 5.0 mM NaCl, 5 mM BNP7787 versus 10 mM NaCl, and 10 mM BNP7787 versus 20 mM NaCl were 0.395, 0.814, 0.784 and 0.918, respectively. Statistically significant *P* values are italicized. Note that both BNP7787 and NaCl have significant effects in comparison with a control APN reaction containing only substrate, but that this effect is likely due to the salt counterion since 10 and 20 mM NaCl have essentially the same effect as the corresponding 5 and 10 mM BNP7787. (*Note*: BNP7787 contains 2 mol of sodium counterion per mole; therefore, we compare 5 mM BNP7787 to 10 mM NaCl, etc.) BNP7787 did not have a statistically significant effect when compared to NaCl. *P* values for 0.3125, 1.25, 2.5, 5 and 10 mM mesna were 0.805, 0.920, 0.165, 0.530, *0.034* and $9.97e^{-5}$, respectively. *P* values for 1.25, 2.5, 5 and 10 mM MSSC were 0.0898, *0.00123*, $1.27e^{-5}$ and $4.13e^{-8}$, respectively

cisplatin-induced nephrotoxicity and where APN is localized within the kidney. Accordingly, such high localized concentrations of mesna may be inhibitory to APN. Lower concentrations (2.5 mM or less) of mesna had no discernable effect on APN activity (Fig. 2b).

Effect of mesna-disulfide heteroconjugates on human APN initial velocity

We observed that mesna-cysteine (MSSC) inhibited APN activity in a dose-dependent manner that was significant at concentrations above 1.25 mM (Fig. 2c). MSSC exhibits classical dose-dependent inhibition, and we postulate that this molecule is active site directed (orthosteric). It is possible that other mesna-disulfide heteroconjugates also bind at the active site; however, inhibition observed with other mesna-disulfide heteroconjugates was not as strikingly dose-dependent.

Statistically significant inhibition of APN initial velocity was seen with high mesna-cysteinyl-glycine (MSSCG) (10 mM) and low MSSCG (0.312 and 0.625 mM), while intermediate concentrations had no significant effect on initial velocity (Fig. 3a). While we cannot completely explain this effect, it is experimentally reproducible and could be due to: (1) altered binding conformations of MSSCG on APN at both low and high MSSCG concentrations and/or (2) a consequence of allosteric modulation of low and high affinity binding sites. We anticipated that MSSCG would inhibit, as cysteinyl-glycine has been previously shown to inhibit APN activity [37, 38] and in the present work, cysteinyl-glycine was found to inhibit APN activity at all concentrations that were tested (Fig. 4a). However, experiments to evaluate formation of MSSC in situ (formed from MSSCG that had been incubated with APN) did not result in a progress curve similar to MSSC, but rather gave a progress curve identical to MSSCG. This suggests that MSSCG is not cleaved to MSSC and glycine by APN in situ (data not shown). This result could be due to the influence of the mesna moiety on MSSCG and supports the concept that MSSCG binds to the APN enzyme in an allosteric position, instead of at the active site. Alternatively, these findings support that MSSCG may bind to the active site in a manner that does not position it properly for enzymatic turnover, and such binding is distinct from cysteinyl-glycine's (a known APN substrate) binding to the active site.

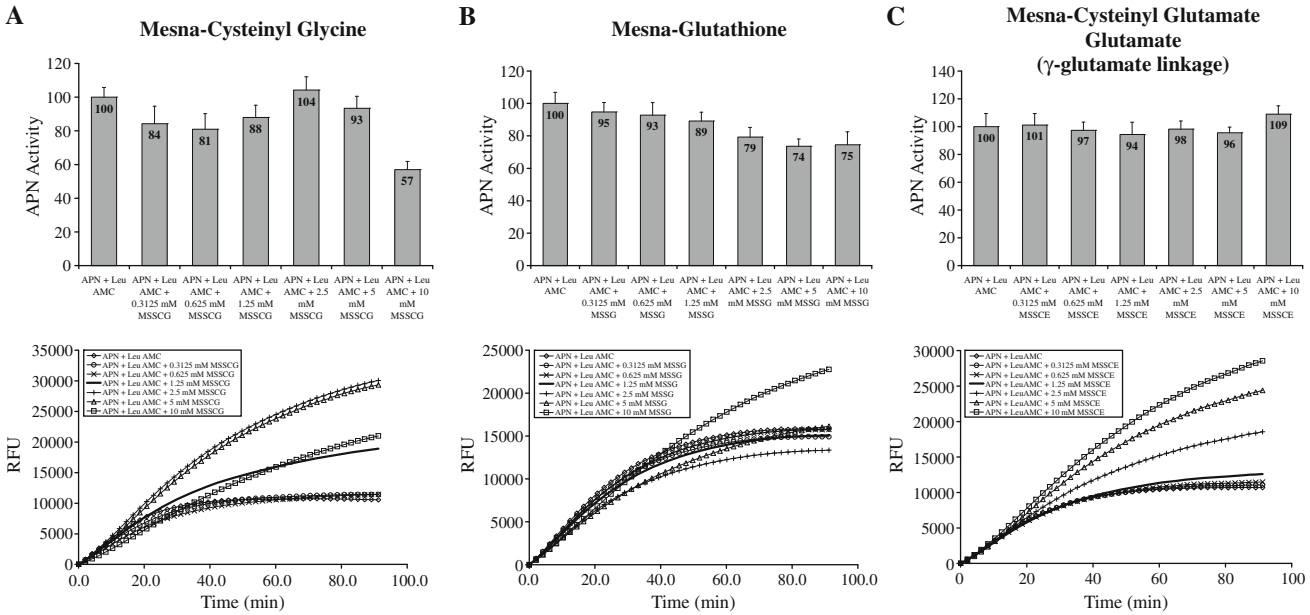

Fig. 3 Effects of **a** MSSCG; **b** MSSCE and **c** mesna-glutathione (MSSGlutathione) on initial velocity and the overall progress curve of human APN. *P* values for 0.3125, 0.625, 1.25, 2.5, 5 and 10 mM MSSCG versus control were *0.04*, *0.0301*, 0.0915, 0.176, 0.500 and *1.97*e^{-5}, respectively. *P* values for 0.3125, 0.625, 1.25, 2.5, 5 and 10 mM MSSCE versus control were 0.840, 0.566, 0.288, 0.691, 0.302, 0.0832, respectively. *P* values for 0.3125, 0.625, 1.25, 2.5, 5 and 10 mM MSSGlutathione versus control were 0.172, 0.103, *0.0099*, *0.0001*, *6.5*e^{-5}, *4.2*e^{-5}, respectively (statistically significant *P* values are italicized.)

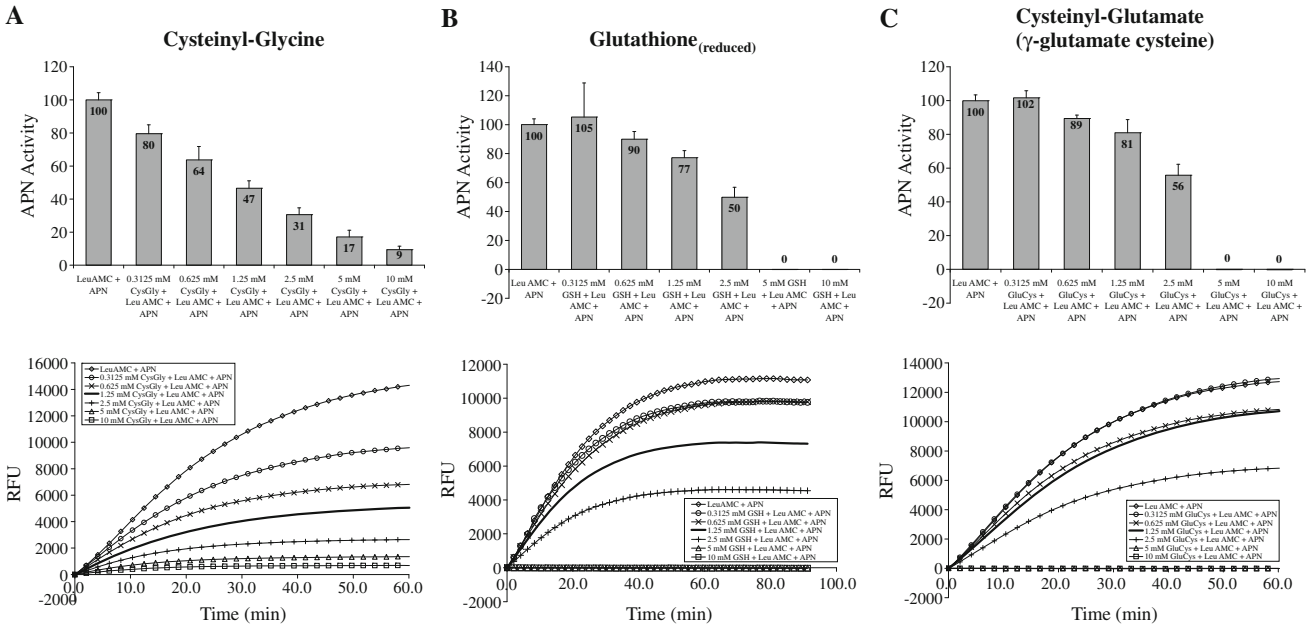

Fig. 4 Effects of **a** cysteinyl-glycine; **b** cysteinyl-glutamate and **c** glutathione (reduced) on initial velocity and the overall progress curve of human APN. *P* values for 0.3125, 0.625, 1.25, 2.5, 5 and 10 mM cysteinyl-glycine versus control were *9.84*e^{-6}, *1.29*e^{-7}, *1.14*e^{-10}, *4.63*e^{-12}, *6.07*e^{-13}, *2.24*e^{-13}, respectively. *P* values for 0.3125, 0.625, 1.25, 2.5, 5 and 10 mM cysteinyl-glutamate versus control were 0.473, *5.89*e^{-5}, *7.27*e^{-5}, *1.0*e^{-9}, *7.7*e^{-15}, *7.6*e^{-15}, respectively. *P* values for 0.3125, 0.625, 1.25, 2.5, 5 and 10 mM glutathione versus control were 0.625, *0.00258*, *1.29*e^{-6}, *4.38*e^{-10}, *2.78*e^{-14}, *2.75*e^{-14}, respectively

Mesna-glutathione at concentrations of 1.25 mM and higher inhibited APN initial velocity with statistical significance (Fig. 3b); whereas reduced glutathione inhibited at concentrations of 0.625 mM and higher (Fig. 4b). Again, consistent with a novel effect due to the presence of a mesna moiety on the di-peptide mesna-cysteinyl-glutamate, MSSCE did not statistically affect the initial velocity of APN at any concentration tested (Fig. 3c); whereas

cysteinyl-glutamate (gamma-glutamyl-cysteine) inhibited at all concentrations ≥0.312 mM (Fig. 4c).

Proposed allosteric effects on APN by mesna-disulfide heteroconjugates

In addition to the above-described effects on APN initial velocity, for several of the mesna-disulfide heteroconjugates (MSSCG, MSSCE and MSSGlutathione), we observed interesting effects on the APN reaction progress curve outside of the linear range where the initial velocity was calculated (and where the substrate is saturating). We propose that these effects that occur later in the APN progress curve are the result of allosteric interactions between the test articles and APN. MSSCG (1.25 mM and higher), MSSCE (2.5 mM and higher) and MSSGlutathione (10 mM) had notable effects on later portions of the APN progress curve (i.e., non-linear regions of the progress curve where initial velocity can no longer be calculated and where the substrate is no longer saturating). Experiments demonstrated that the parent di- and tri-peptides that lacked the mesna moiety (i.e., cysteinyl-glycine, cysteinyl-glutamate and glutathione) did not elicit these allosteric effects and instead gave reproducible progress curves reflecting inhibition, indicating that both the mesna moiety and the di- or tri-peptide must be present in the same molecule for these effects to be observed.

The observed effects on human APN by MSSCG, MSSCE and MSSGlutathione (Fig. 3) may be due to several factors. In the case of MSSCG and MSSGlutathione, initial inhibition (see effects on initial velocity) was observed, but as the progress curve develops (and substrate becomes rate limiting) a notable increase in relative fluorescence units (RFU) occurs that could either reflect: (1) enhanced APN activity; (2) relief of AMC-mediated feedback inhibition of the APN reaction as the AMC product is formed or (3) solution effects that offset photodegradation and/or quenching of the AMC product as the APN reaction proceeds (most fluorescent molecules undergo photodegradation over time). Similarly while MSSCE does not significantly impact initial velocity, at high concentrations (i.e., 10 mM) it has a striking effect on total RFU. Alternatively, these effects on total RFU as the progress curve develops could be a consequence of unusual orthosteric binding directed primarily by the mesna moiety, but again requiring a molecule with both the di- or tri-peptide and mesna moieties. For example, it is possible that MSSGlutathione binds in or around the active site in a distinctly different orientation (or in multiple orientations) in comparison with the parent glutathione molecule, thus triggering the effects that become more prominent as substrate is depleted and no longer saturating. Additionally, we note that there is debate about the possibility of two active sites in *E. coli* APN, and while 3D structural information regarding human APN is not available, the possibility of multiple binding sites exists (discussed briefly in [40]).

Effects on APN activity by in situ formed mesna-cisplatin conjugate

In vivo, cisplatin is converted to an aquated species through the chloride displacement by water, and while this species does not react rapidly with BNP7787, it may react with the BNP7787-derived metabolite, mesna, thus forming mesna-cisplatin. We have previously reported that mesna-cisplatin is not an inhibitor or a substrate for GGT [8]. Accordingly, herein we evaluated the effects of mesna-cisplatin on the enzyme immediately downstream of GGT in the xenobiotic toxification pathway (i.e., APN) using methods described previously [8]. When the mesna-cisplatin mixture was used in APN assay, no effect on human APN activity was observed (Fig. 5).

Discussion

Nephrotoxicity is a prevalent and serious side effect of many chemotherapeutic agents, including cisplatin, a widely used chemotherapeutic agent [2, 5, 8, 41]. One proposed mechanism for cisplatin-induced nephrotoxicity involves formation of a glutathione-cisplatin conjugate which is metabolized through a xenobiotic toxification pathway, thus resulting in intrarenal formation of a toxic, reactive platinum species [18, 19, 21, 42]. This xenobiotic toxification pathway includes the enzymes, GGT, APN and CCBL, and has been demonstrated to be highly important

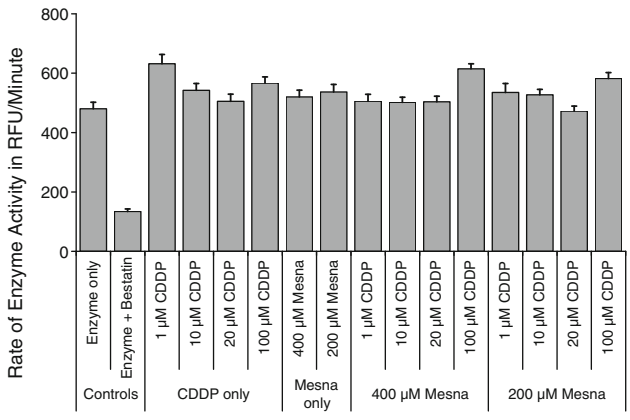

Fig. 5 The mesna-cisplatin conjugate had no detectable effect on human APN activity. Formation of a putative BNP7787-derived mesna-cisplatin adduct could potentially lead to nephroprotection by (1) reducing the amount of cisplatin-glutathione formed and subsequently metabolized into a nephrotoxin via the xenobiotic pathway or by (2) providing an alternative cisplatin adduct in vivo that cannot be metabolized by the GGT, APN and CCBL enzymes into a nephrotoxin

in clearing toxic platinum conjugates of glutathione and cysteine from the kidney [16, 19]. However, the platinum–thiol conjugates produced by the aforementioned pathway may result in the production of toxic platinum thiol species that cause cellular damage. Previously, we have reported studies evaluating the effect of BNP7787 and BNP7787-derived mesna-disulfide heteroconjugates on the first enzyme in this pathway, GGT [8], and the experimental focus herein was on APN, the enzyme immediately downstream of GGT. Studies indicate that APN has a major role in the common adverse pharmacological outcome of cisplatin-induced nephrotoxicity [21, 42] and, consequently, inhibitors of APN may have potential utility in treating or managing cisplatin-induced nephrotoxicity.

APN is a zinc-dependent metalloenzyme and catalyzes the hydrolysis of peptide substrates which often contain neutral residues such as Ala, Leu, Met and Phe at the N-terminus. APN is a membrane associated homodimer composed of two non-covalently associated subunits and is found in many different cell types and tissues [37]. The N-terminal domain of the enzyme is anchored to the cell membrane, while the catalytic domain is exposed to the extracellular surface [38]. APN catalyzes the hydrolysis of cysteinyl-glycine conjugates (including cysteinyl-glycine-cisplatin) to cysteine-conjugates and glycine [43]. The cysteine-cisplatin conjugate (i.e., platinum-cysteinyl-glycine) is reported to result in the production of toxins that are detrimental to kidney cells [21]. Many peptides and small molecules, including cysteinyl-glycine dipeptides, inhibit APN and may be turned over by the enzyme [37, 38]. Compounds with a cysteinyl-glycine group have the potential to compete with the putative in vivo toxification substrate, cysteinyl-glycine-cisplatin, for binding to the APN active site and turnover [38]. Accordingly, these compounds could also compete with the Leu-AMC substrate for binding to APN's active site in our in vitro assays. Since a wide variety of small molecules are known to inhibit APN [37], it was possible that BNP7787, the BNP7787 metabolite, mesna, or BNP7787-derived mesna-disulfide heteroconjugates might inhibit APN.

Previous non-clinical and early human clinical trials have shown that BNP7787 may have potential to protect against cisplatin-induced nephrotoxicity [12, 25–29, 32, 44–46]. BNP7787-derived, mesna-disulfide heteroconjugates are known to form in vitro in both phosphate buffer and plasma [29, 36]. These mesna-disulfide heteroconjugates might contribute to the potential nephroprotection afforded by BNP7787 by either serving as inhibitors of APN (Fig. 1a) or through bypass-related mechanisms where either mesna-cysteinyl-glycine cisplatin is not a substrate for APN and is not converted to a toxic platinum metabolite or where the amount of cysteinyl-glycine-cisplatin that is formed is reduced due to the presence of the various mesna-disulfide heteroconjugates, thus yielding less cysteinyl-glycine cisplatin toxic precursor. In each of these bypass-related mechanisms, the amount of cysteinyl-glycine-platinum that is subsequently metabolized to a toxic, reactive platinum species would be substantially limited or prevented (Fig. 1a).

We observed that the BNP7787-derived mesna heteroconjugates MSSC, MSSGlutathione and MSSCG are inhibitors of human APN affecting the initial velocity of the enzyme in assays (Figs. 2, 3), and we propose that this may represent an important and novel mechanism involved in potential BNP7787-mediated nephroprotection against platinum-based chemotherapeutic agents in humans. A proposed model of possible MSSC interactions with APN based on the crystal structure of APN (pdb code: 2ZXG.pdb) is shown in Fig. 6. The carboxylate group of cysteine provides a coordination site for Zn^{2+}. Additional computational studies are planned and may provide more information about the binding of mesna-disulfide heteroconjugates to APN.

The putative allosteric effects of the BNP7787-derived mesna-disulfide heteroconjugates are more complicated, as there is no available 3D structure of human APN. While the human and *E. coli* enzymes appear to have conserved active site residues, overall sequence identity is low (<25%, data not shown) and in the absence of reliable 3D structure information, it is difficult to speculate about what allosteric effects might occur between human APN and either MSSCG, MSSCE or MSSGlutathione. We also note that the presence of a reactive mesna moiety on MSSCG, MSSCE and MSSGlutathione results in the presence of an additional negative charge (the sulfonate of mesna) and that these mesna-disulfide heteroconjugates may assume distinct, novel binding orientations (with the possibility of multiple binding orientations existing for each molecule) on the APN protein in comparison with cysteinyl-glycine, cysteinyl-glutamate and glutathione that lack the mesna moiety.

For the first time, we also report that mesna-cisplatin-containing mixtures (Fig. 5) do not inhibit the activity of human APN. We propose that BNP7787-mediated nephroprotection, occurring via the formation of a BNP7787-derived mesna-cisplatin complex, may be a result of: (1) the inability of APN to metabolize mesna-cisplatin into a nephrotoxin (Fig. 1a) and/or (2) a result of a concomitant decrease in the formation of cysteine-cisplatin species, which are thought to be metabolized via GGT, APN and CCBL to nephrotoxins, as the mesna-cisplatin complexes are formed. While BNP7787 in comparison with a NaCl control did not directly inhibit APN activity, its metabolite, mesna, inhibited significantly at 5 and 10 mM concentrations (Fig. 2). The C_{max} for mesna in plasma when BNP7787 is administered to patients is less than 400 μM

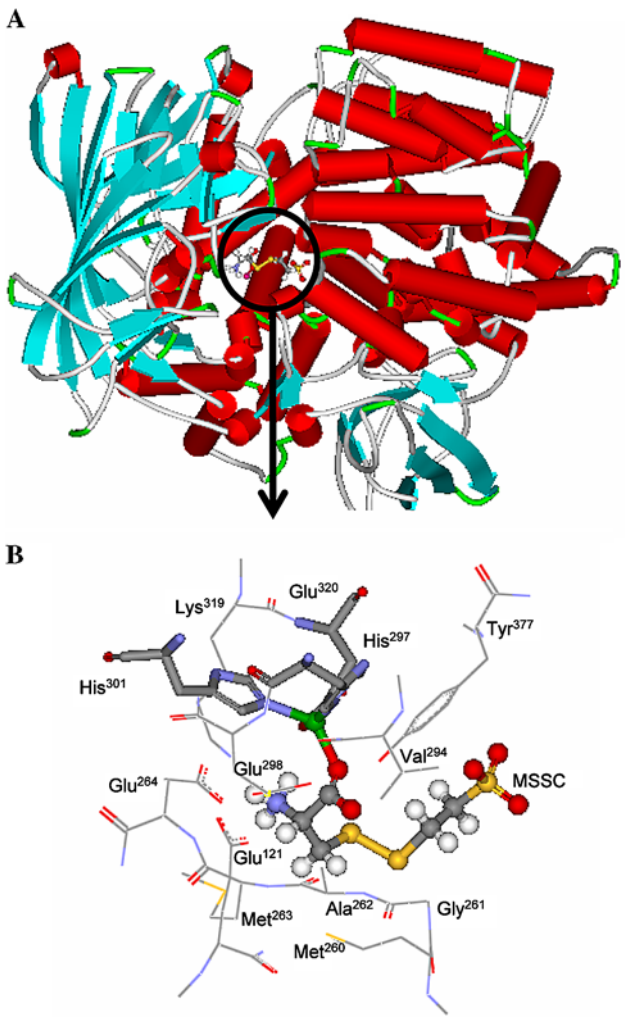

Fig. 6 Proposed model of MSSC interacting with APN (color key for atoms: sulfur = *yellow*; oxygen = *red*; nitrogen = *blue*; carbon = *gray* and hydrogen = *white*). **a** APN shown in secondary structure mode (helices: *red cylinders*, *β*-strand: *aqua arrows*) highlighting MSSC in the active site region. **b** A portion of APN with MSSC is shown with MSSC docked in an orientation similar to that of PL250, an aminophosphinic inhibitor of APN [49], to maintain some of the key interactions present in APN with PL250. The tetrahedral coordination around Zn (*green*) is completed by His^{301}, His^{297} and Glu^{320} (all three residues in stick mode) and MSSC (in ball and stick mode). The structure of APN with MSSC was obtained by gas-phase minimization (500 steps) with distance-dependent dielectric using the Sander module of Amber 6 [50]

[47]. However, since BNP7787 is eliminated primarily through the kidney, the concentration of the BNP7787 metabolite, mesna, may reach millimolar (mM) levels via in situ formation of mesna within the renal tubule, the site of cisplatin-mediated nephrotoxicity and APN localization. For example, concentrations of BNP7787 and mesna reached 84.9 and 21.6 mM, respectively, in urine from patients receiving 41 g/m^2 BNP7787 in a phase 1 trial [39] (subsequent phase 2 and 3 trials used 18.4 g/m^2 which would still be predicted to yield concentrations of BNP7787 and mesna in excess of 5 mM in the kidney). Additionally, data from rat studies where rats received an IV bolus injection of 1,000 mg/kg BNP7787 indicated that BNP7787 concentrations in the kidney ranged from 0.2 to 7.6 mM and mesna concentrations ranged from 1.8 to 6.4 mM [31, 33]. In accord, such high local concentrations of BNP7787-derived mesna could inhibit APN. Further, we also report herein for the first time that all BNP7787-derived mesna-disulfide heteroconjugate compounds that contain a cysteinyl-glycine moiety and the BNP7787-derived mesna-cysteine molecule, directly inhibit APN. The concentration of cystine/cysteine in human plasma is ca. 200–300 μM [39], and therefore would provide a significant amount of cysteine for reaction with BNP7787 to form mesna-cysteine heteroconjugates. These findings support the hypothesis that the glycinate moiety is a key requirement for disulfide-mediated inhibition of APN and that the combination of a disulfide moiety with an anionic group appears to augment inhibition against APN. In summary, two possible mechanisms for BNP7787-mediated nephroprotection have been identified: (1) direct inhibition of human APN activity by the BNP7787-derived mesna heteroconjugates: MSSGlutathione, MSSC and MSSCG and (2) formation of mesna-cisplatin, which is a cisplatin conjugate that is not a substrate for the GGT/APN xenobiotic toxification pathway.

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