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Possible (enzymatic) routes and biological sites for metabolic reduction of BNP7787, a new protector against cisplatin-induced side-effects

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Abstract

Disodium 2,2′-dithio-bis-ethane sulfonate (BNP7787) is under investigation as a potential new chemoprotector against cisplatin-induced nephrotoxicity. The selective protection of BNP7787 appears to arise from the preferential uptake of the drug in the kidneys, where BNP7787 would undergo intracellular conversion into mesna (2-mercapto ethane sulfonate), which in turn can prevent cisplatin induced toxicities. In the present study, we have investigated whether the reduction of BNP7787 into the reactive compound mesna is restricted to the kidney or whether it can also occur in other organs, cells and physiological compartments, including the cytosolic fraction of the renal cortex, plasma, red blood cells (RBCs), liver and small intestine from rats and several tumors (OVCAR-3, MRI-H-207 and WARD). We also determined whether the endogenous thiols glutathione (GSH) and cysteine and the enzyme systems glutaredoxin and thioredoxin, which are all present in the kidney, can be involved in the BNP7787 reduction. UV detection and micro-HPLC with dual electrochemical detection were used to analyze the various incubation mixtures. Our observations are that, in contrast to plasma, a very large reductive conversion of BNP7787 to mesna was measured in RBC lysate. Intact RBCs, however, did not take up BNP7787. Although BNP7787 could be reduced in cytosol of liver and several tumors, this reduction will not be relevant in vivo, since these tissues do not take up large amounts of BNP7787. Kidney cortex cytosol was, similar to the small intestine cytosol, able to substantially reduce BNP7787 to mesna. The ability to reduce BNP7787 in the presence of the endogenous thiols GSH and cysteine, the glutaredoxin system as well as the thioredoxin system, could at least in part explain the high BNP7787 reductive activity of the kidney cortex cytosol. In conclusion, the high reduction of BNP7787 into mesna in the kidney as well as our earlier observation that the distribution of BNP7787 and mesna was mainly restricted to rat kidney are strong arguments in favor of selective protection of the kidney by BNP7787.

Keywords: BNP7787; Mesna; Reduction; Kidney; Glutaredoxin; Thioredoxin

1. Introduction

Disodium 2,2′-dithio-bis-ethane sulfonate (BNP7787) is a new investigational agent that is being developed as a chemoprotector against cisplatin and taxane-induced toxicities [1–3]. In the case of cisplatin, promising results were obtained in preclinical studies in Beagle dogs and rats in which BNP7787 was able to protect against cisplatin-induced side-effects, i.e. nephrotoxicity [1]. Furthermore, in WARD tumor-bearing rats and OVCAR-3 tumor-bearing nude mice, administration of BNP7787 did not reduce the antitumor activity of cisplatin [1,3]. Our pharmacokinetic study in WARD tumor-bearing rats has shown that after an i.v. bolus injection of BNP7787, high concentrations of BNP7787 and especially of mesna were only obtained in the kidney and not in skeletal muscle, liver or tumor [4]. Thus, the selective nephroprotection of BNP7787 is probably based in part on the preferential uptake of BNP7787 in the renal tubules with local formation of its metabolite mesna by reduction of BNP7787 (Fig. 1). Mesna is more reactive with (hydrated) cisplatin than its disulfide BNP7787 [5,6], and can locally inactivate

Abbreviations: GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; RBC, red blood cell; TrxR, thioredoxin reductase; VUmc, Vrije Universiteit medical center

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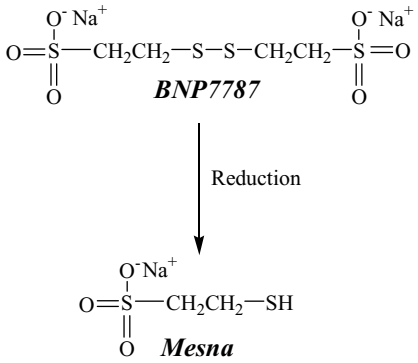

Fig. 1. Reduction of BNP7787 into mesna.

(hydrated) cisplatin by forming non-toxic platinum complexes. Mesna can possibly also protect the kidney by acting as an antioxidant [7,8].

Several investigators have shown that the reduction of BNP7787 in kidney and liver is dependent on the presence of the endogenous thiol glutathione (GSH) [9–11]. Ormstad et al. [11] have suggested that, besides the endogenous thiol GSH, the glutaredoxin system, consisting of the enzymes glutathione reductase and glutaredoxin and NADPH, are involved in the reduction of BNP7787 in the kidney based on experiments with cytosolic extracts from rats. First, BNP7787 reacts with GSH to form mesna and a mesna-GSH complex, which in turn can react with GSH in the presence of glutaredoxin to form GSSG and mesna. The formed GSSG can be reduced back to GSH in the presence of glutathione reductase and NADPH (Fig. 2). In the present study, we investigated the propensity of BNP7787 to undergo reduction to mesna by glutathione reductase in the presence and absence of glutaredoxin and GSH. The thioredoxin system, consisting of thioredoxin reductase, thioredoxin and NADPH, is also expressed in tissues, such as the kidney and liver [12,13] and is known to be able to reduce disulfide species [14,15]. Hence, we also tested the thioredoxin system for its ability to reduce the disulfide BNP7787 in vitro.

In addition to evaluating possible (enzymatic) reduction routes of BNP7787, we also investigated whether the reduction of BNP7787 into the reactive compound mesna is restricted to the kidney or whether it can occur in other physiological and biological systems, such as plasma, red

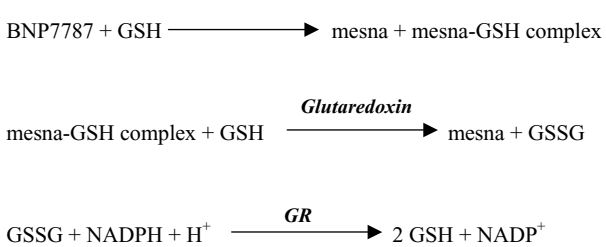

Fig. 2. Proposed scheme for the reduction of BNP7787 by glutathione (GSH) and the glutaredoxin system, which consists of glutathione reductase (GR), glutaredoxin and NADPH.

blood cells (RBCs), liver and small intestine from rats and several tumors. The reduction of BNP7787 was followed by measuring the decrease in BNP7787 concentration and the simultaneous formation of mesna using our recently developed specific and sensitive micro-HPLC assay with dual electrochemical detection [16].

2. Materials and methods

2.1. Materials

BNP7787 was provided by BioNumerik Pharmaceuticals. Glutathione reductase (E.C. 1.6.4.2., yeast), thioredoxin (oxidized form, *E. coli*), mesna (sodium 2-mercaptoethanesulfonate), insulin from bovine pancreas, reduced glutathione (GSH), oxidized glutathione (GSSG) and β-NADPH were obtained from Sigma. Glutaredoxin (*E. coli*) and thioredoxin reductase (E.C. 1.8.1.9., mammalian (bovine)) were purchased from IMCO Corporation Ltd. AB. Cysteine was purchased from Merck. Phosphate-buffered saline (PBS; pH 7.4, 10 mM sodium phosphate and 0.14 M NaCl) was prepared by the Hospital Pharmacy Department of the Vrije Universiteit medical center (VUmc). All other chemicals used were of analytical grade. Deionized water from a Millipore Milli-Q system was used throughout the experiments.

2.2. Matrices

A solution consisting of 3 mM EDTA in PBS was used as a buffer (pH 7.4) for the reduction experiments in various matrices. This buffer will be referred to as phosphate buffer A. For the UV measurements, a solution containing 50 mM KH_2PO_4/KOH buffer, 110 mM NaCl and 3 mM EDTA (pH 7.4) was used. This buffer will be referred to as phosphate buffer B.

Human ovarian cancer xenografts OVCAR-3 and MRI-H-207 removed from nude mice and the colorectal tumor WARD removed from rats were obtained as frozen tissues. EDTA-plasma, red blood cells (RBCs), liver, small intestine and kidney cortex were obtained from non-tumor-bearing rats with permission from the local ethical committee on the use of experimental animals. After sampling, blood was centrifuged for 10 min at 3000 × *g* and 4 °C. Plasma was immediately used in the incubation experiment. One volume of RBCs was washed twice with 1 vol. of PBS and centrifuged for 2 min at 9000 × *g* and 4 °C. After removing the PBS, part of the intact RBCs was used immediately for determining the uptake of BNP7787 in RBCs. RBC lysate was prepared by destroying the cell membrane of the RBCs by freezing and thawing the cells twice. The lysed RBCs were suspended in the appropriate volume of phosphate buffer A to obtain a concentration of 250 mg (wet weight)/ml. The RBC lysate was stored at −80 °C until use.

Homogenates of kidney cortex, liver and small intestine and various tumors (OVCAR-3, MRI-H-207 and WARD) were prepared by pulverization with a Braun Microdismembrator (Salm and Kipp; Breukelen, The Netherlands). An appropriate volume of phosphate buffer A was added to obtain a concentration of 250 mg tissue (wet weight)/ml. This suspension was centrifuged at 600 × *g* and 4 °C for 5 min. A cytosolic fraction of the tissue homogenates was prepared by centrifugation of the supernatant for 10 min at 10,000 × *g* at 4 °C. The cytosolic fractions of the tissue homogenates were stored at −80 °C until use.

The protein concentration present in the various matrices was determined using the Biorad Bradford protein assay [17].

2.3. Experimental conditions

(Auto)oxidation of the thiols mesna, GSH and cysteine, was prevented by the presence of EDTA in all experiments and by purging all solutions with nitrogen for at least 20 min before use. All reduction experiments were performed under a nitrogen atmosphere. Under these circumstances, 2 mM of mesna in phosphate buffer A incubated at 37 °C for 60 min, remained unchanged and no formation of BNP7787 could be detected. GSH was also not autooxidized to GSSG under these circumstances. This was shown by incubating GSH (0.5, 2.5 or 5 mM) with glutathione reductase (10 μg/ml) and NADPH (1 mM) in phosphate buffer B at 37 °C for 30 min and measuring the absorption of NADPH at 340 nm. Oxidation of GSH would have led to the formation of GSSG. GSSG is a good substrate for glutathione reductase and would therefore be reduced back to GSH by glutathione reductase under consumption of NADPH. No decrease in NADPH absorption was detected indicating that GSSG was not formed.

All experiments were performed in duplicate at 37 °C and pH 7.4. Just before the experiment started, all solutions were equilibrated at 37 °C for 5 min.

2.4. Reduction of BNP7787 in plasma, RBC lysate and cytosol of various tissues

The reduction of BNP7787 was investigated in plasma, RBC lysate and in cytosol of various tissue homogenates (i.e. kidney cortex, liver, small intestine and OVCAR-3, MRI-H-207 and WARD tumor). The experiments started when BNP7787 was added to plasma, RBC lysate or tissue cytosol to obtain a BNP7787 concentration of 1 mM. To study the BNP7787 reduction ability of enzymes, which use NADPH as cofactor, and the reduction ability of the endogenous thiol GSH, the reduction of 1 mM BNP7787 in kidney cortex cytosol was also measured in the presence of the cofactor NADPH (1 mM) and/or GSH (0.5 mM). The involvement of enzymes in the reduction of BNP7787 was investigated further by heating the cytosol first for 2 min at 100 °C to remove all the proteins present followed by addition of 1 mM BNP7787 to the deproteinized kidney cortex cytosol.

Immediately after addition of BNP7787 to the various matrices ($t = 0$) and at selected time intervals up to 60 min, 70 μl of the solution (initial volume 0.5 ml) was taken and deproteinized with 70 μl 0.33 M sulphuric acid and 70 μl 5% (w/v) sodium hexametaphosphate. After mixing well, the samples were centrifuged at 9000 × *g* for 2 min. If the concentration of BNP7787 or mesna in a sample was above the dynamic range of the assay (i.e. dynamic range in plasma: 3–120 μM for mesna and 15–1200 μM for BNP7787; in tissue: 20–2000 nmol/g for both compounds), the sample was diluted with blank deproteinized plasma or tissue cytosol. Of each (diluted) sample, 150 μl was added to 30 μl of citrate/NaOH buffer (mixture of 1 ml 5.0 M sodium hydroxide and 4 ml 0.5 M tri-sodium citrate buffer, pH 3.0). The concentrations of BNP7787 and mesna were measured by micro-HPLC with a dual electrochemical detector.

2.5. Reduction of BNP7787 by GSH or cysteine

To check whether BNP7787 could be reduced by the endogenous thiols GSH and cysteine, 1 mM of BNP7787 was incubated in phosphate buffer A without and with 0.5 or 2.5 mM GSH or with 0.5 mM cysteine. The experiments started when BNP7787 was added. Immediately after vortexing ($t = 0$) and at selected time intervals, 70 μl of the solution was taken and 70 μl 0.33 M sulphuric acid and 70 μl 5% (w/v) sodium hexametaphosphate were added. After mixing well, 150 μl was added to 30 μl of citrate/NaOH buffer. The decrease in BNP7787 concentration and formation of mesna was followed by micro-HPLC with a dual electrochemical detector.

2.6. Reduction of BNP7787 by purified enzymes

2.6.1. Glutathione reductase

The reduction of 1 mM BNP7787 by glutathione reductase (10 μg/ml) without and with 0.5, 2.5 or 5 mM GSH was monitored for 30 min at 340 nm by the coupled oxidation of the cofactor NADPH, which was used in a concentration of 1 mM. As a positive control 1 mM of GSSG was used, as it is a substrate for glutathione reductase. The reaction started when the enzyme was added to the incubation mixture (total volume of 1 ml).

2.6.2. Glutaredoxin

The reduction of 1 mM BNP7787 by glutaredoxin (0.5 μM) without and with 2.5 mM GSH was measured by determining the BNP7787 and mesna concentrations in the incubation solution by micro-HPLC with a dual electrochemical detector. The experiment started when BNP7787 was added to the mixture. Immediately after vortexing ($t = 0$) and at selected time intervals up to 30 min, 70 μl of the incubation solution (initial volume

1 ml) was deproteinized with 70 μl 0.33 M sulphuric acid and 70 μl 5% (w/v) sodium hexametaphosphate. Of each sample, 150 μl was added to 30 μl of citrate/NaOH buffer and the sample was ready to be injected onto the micro-HPLC.

2.6.3. Glutathione reductase + glutaredoxin

The reduction of 1 mM BNP7787 by glutathione reductase (10 μg/ml) plus glutaredoxin (0.5 μM) without and with 0.5 or 2.5 mM GSH was monitored for 30 min at 340 nm by the coupled oxidation of NADPH (1 mM). GSSG (1 mM) was used as a positive control. The incubation solution containing 1 mM BNP7787, 1 mM NADPH and 2.5 mM GSH and the enzymes GSH reductase and glutaredoxin was measured by micro-HPLC with a dual electrochemical detector. At the same time-points as the UV measurement, 70 μl of the solution was deproteinized by adding 70 μl 0.33 M sulphuric acid and 70 μl 5% (w/v) sodium hexametaphosphate. Of each sample, 150 μl was added to 30 μl of citrate/NaOH buffer.

2.6.4. Thioredoxin reductase with and without thioredoxin

The reduction of 1 mM BNP7787 by thioredoxin reductase (8.4 nM) with and without thioredoxin (5 μM) was investigated by monitoring the coupled oxidation of 0.2 mM NADPH at 340 nm. As a positive control 160 μM of insulin was used, as it is a substrate for thioredoxin reductase with thioredoxin.

2.7. Determination of mesna in mixed disulfides and covalently bound to proteins

To investigate whether mixed mesna disulfides (e.g. mesna-GSH or mesna-cysteine complex) and/or covalent binding of mesna to proteins occurred during reduction of BNP7787, the following experiment was performed. The required volume of a 50 mM solution of BNP7787 in phosphate buffer A was added to the cytosol of liver to obtain a BNP7787 concentration of 1 mM. Twenty and 30 min after the start of the incubation, two portions of the solution were taken. One portion was first deproteinized with sulphuric acid and sodium hexametaphosphate followed by reduction with sodium borohydride of all mesna (mixed) disulfides present according to the procedures described by Verschraagen et al. [18]. The second portion first underwent reduction with sodium borohydride followed by deproteinization. BNP7787 and (total) mesna concentrations were determined by injecting the samples onto the micro-HPLC with the dual electrochemical detection system [16].

2.8. Uptake of BNP7787 in RBC

The uptake of BNP7787 in RBC was investigated by incubating 1 mM BNP7787 up to 42 min in a mixture of intact RBCs in phosphate buffer A (250 mg/ml) at 37 °C in a water bath under gentle horizontal shaking. The experiment started when BNP7787 was added to the mixture. Immediately after vortexing ($t = 0$) and at selected time intervals, 400 μl of the mixture (initial volume 4 ml) was taken and was centrifuged at 9000 × g for 2 min at 4 °C. Seventy microliters of the supernatant was deproteinized with 70 μl 0.33 M sulphuric acid and 70 μl 5% (w/v) sodium hexametaphosphate. The RBC pellet was washed with PBS before it was deproteinized. Of each sample, 150 μl was added to 30 μl of citrate/NaOH buffer. The concentrations of BNP7787 and mesna in the surrounding buffer and in the RBC lysate were measured by micro-HPLC with a dual electrochemical detector.

2.9. Analysis

All experiments were performed in duplicate. The reduction of BNP7787 into mesna by various matrices was measured by determining the concentrations of BNP7787 and mesna with micro-HPLC and a dual electrochemical detector according to our earlier developed assay [16].

The reduction of BNP7787 by the purified enzymes glutathione reductase with and without glutaredoxin and thioredoxin reductase with and without thioredoxin was measured indirectly. This was done by measuring the decrease in absorption of the cofactor NADPH at a wavelength of 340 nm with a Hitachi U-2000 double-beam spectrophotometer and temperature controller (Hitachi Ltd.). The UV experiments were performed in 1-cm pathway quartz cuvettes and the incubations were measured up to 30 min. The reference blank contained all compounds, which were also present in the incubation solution except NADPH.

2.10. Calculations

The mean measured absorption or mean measured BNP7787 or mesna concentration calculated from the duplicate measurements obtained in the various incubation experiments at the different time-points were expressed as percentage of the initial absorption at $t = 0$ or the total mesna concentration at $t = 0$ (1 mM BNP7787 = 2 mM total mesna), respectively.

The reductive activity of the cytosol of the various tissue homogenates was determined as follows. Semilogarithmic plots were made of the BNP7787 concentration vs. time. The plots were fitted with the least-squares method. If the plots were linear, the first-order rate law was obeyed for loss of BNP7787:

$$-\frac{\mathrm{d}[\mathrm{BNP7787}]}{\mathrm{d}t} = k_{\mathrm{obs}}[\mathrm{BNP7787}]$$

in which k_{obs} represents the observed first-order rate constant for loss of BNP7787.

k_{obs} was divided by the protein concentration of the cytosol of various tissue homogenates to determine the BNP7787 reductive activity per mg of protein.

The observed half-life of BNP7787 ($t_{1/2,obs}$) could be calculated by:

$$t_{1/2,obs} = \frac{\ln 2}{k_{obs}}$$

3. Results

The reduction of BNP7787 into mesna by various matrices was determined by measuring the decline in BNP7787 concentration and formation of mesna in time during incubation with 1 mM BNP7787 at 37 °C. Semi-logarithmic plots of the BNP7787 concentration vs. time were found to be linear over the first 10 min of incubation. Therefore, observed rate constants (k_{obs}) and corresponding observed half-life times ($t_{1/2,obs}$) could be calculated for the loss of BNP7787 in the various matrices. In Table 1, these k_{obs} and $t_{1/2,obs}$ values are summarized.

3.1. Reduction of BNP7787 in plasma, RBC lysate and RBCs

A very limited amount of BNP7787 was reduced to mesna when BNP7787 was incubated in plasma, i.e. $t_{1/2,obs}$ = 201.6 min (Table 1). In contrast, mesna was rapidly formed when BNP7787 was incubated in RBC lysate ($t_{1/2,obs}$ = 8.3 min; Table 1).

Uptake of BNP7787 in intact RBCs was limited at every measured time-point up to 42 min of incubation. The total amount of BNP7787 and mesna present in the RBCs was only approximately 0.6% of the total added BNP7787. In the buffer surrounding the RBC a small decrease was observed of the BNP7787 concentration with time (i.e. less than 15% during 42 min incubation). An almost equivalent amount of mesna was formed in the surrounding buffer. When BNP7787 was incubated in buffer alone at 37 °C, the concentration of BNP7787 remained unchanged for at least 60 min and no mesna was detected during this time period. Since BNP7787 is not reduced to mesna in buffer, the small reduction of BNP7787 in the buffer is probably caused by reducing substances originating from damaged RBCs. Small amounts of RBCs were probably damaged by the horizontal shaking of the tube.

3.2. Reduction of BNP7787 in the cytosol of various tissue homogenates

The results of the incubation experiments of 1 mM BNP7787 in the renal cortical cytosolic fraction of kidney cortex homogenate (250 mg/ml) are shown in Fig. 3A and B. A decrease in the BNP7787 concentration ($t_{1/2,obs}$ =

Table 1
Reduction of BNP7787 in various matrices determined after incubation with 1 mM BNP7787 at 37 °C[a]

Matrix	k_{obs}[b] (min^{-1})	$t_{1/2,obs}$ (min)	Protein concentration (mg/ml)	BNP7787 reductive activity[c] ($\times 10^{-3}$ min^{-1} mg^{-1})
Phosphate buffer				
No addition[d]	0.0020	355.5		
0.5 mM cysteine	0.0210	33.0		
0.5 mM GSH	0.0112	62.0		
2.5 mM GSH	0.0273	24.3		
2.5 mM GSH + glutaredoxin	0.0285	25.4		
2.5 mM GSH + GR + glutaredoxin + NADPH	0.1134	6.1		
Blood of rat				
Plasma	0.0034	201.6	67.2	0.05
RBC lysate	0.0836	8.3	92.6	0.90
Cytosol of rat				
Kidney cortex	0.0186	37.2	15.1	1.23
Kidney cortex + GSH	0.0342	20.3		
Kidney cortex + NADPH	0.0491	14.1		
Kidney cortex + GSH + NADPH	0.0615	11.3		
Kidney cortex after heating the cytosol first[d]	0.0056	123.8		
Liver	0.0579	12.0	21.6	2.68
Small intestine	0.0067	103.6	5.8	1.16
Cytosol of tumor				
OVCAR-3 (ovarian cancer xenograft)	0.0113	61.2	12.6	0.90
MRI-H-207 (ovarian cancer xenograft)	0.0058	119.5	13.1	0.44
WARD (colon tumor)	0.0066	104.5	13.1	0.51

GSH: glutathione; GR: glutathione reductase.

[a] The precise conditions and concentrations used are described in Section 2.

[b] k_{obs} is determined over the first 3 time-points after the start of the incubation of 1 mM BNP7787 in the various matrices, i.e. t = 0, 5 and 10 min.

[c] BNP7787 reductive activity was expressed as the k_{obs} per mg of protein per min.

[d] The correlation coefficients for the data of these experiments were very low (<0.35). Therefore, k_{obs} and $t_{1/2,obs}$ are not accurate, but just indicative.

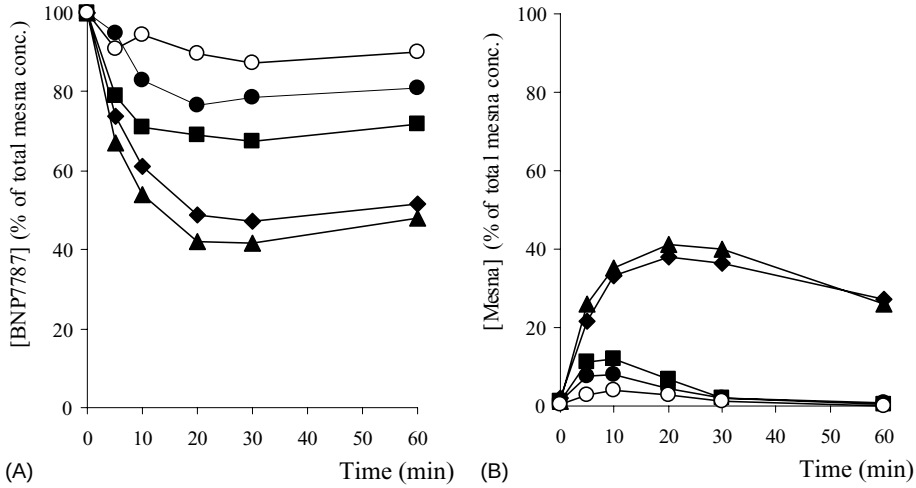

Fig. 3. BNP7787 (A) and mesna (B) concentration vs. time curves obtained during incubation of 1 mM BNP7787 in the cytosol of kidney cortex homogenate (250 mg/ml) at 37 °C without addition (●), plus 0.5 mM GSH (■), plus 1 mM NADPH (◆), plus 0.5 mM GSH and 1 mM NADPH (▲) or after heating the cytosol for 2 min at 100 °C before incubation with BNP7787 (○). BNP7787 and mesna concentrations are expressed as percentage (%) of the total mesna concentration at $t = 0$. The experiments were performed in duplicate and results are mean values.

37.2 min; Table 1) and simultaneous formation of mesna were observed. After removing the proteins from the cytosol before incubation by heating for 2 min, reduction of BNP7787 to mesna was limited ($t_{1/2,obs}$ is approximately 123.8 min; Table 1). The decrease in reduction of BNP7787 to mesna indicates that proteins, i.e. enzymes, might be involved in the reduction of BNP7787. The ability of enzymes to reduce BNP7787 was further investigated by adding NADPH, which is a cofactor for various enzymes, to the renal cortical cytosolic homogenate. Addition of NADPH led to an increased reduction of BNP7787 ($t_{1/2,obs}$ = 14.1 min; Table 1). The endogenous thiol GSH was also able to reduce BNP7787 as expressed in the decreased $t_{1/2,obs}$ of 20.3 min after addition of GSH to the kidney cortex cytosol (Table 1). The highest reduction rate of BNP7787 was obtained when GSH as well as NADPH were added to the renal cortical cytosolic homogenate ($t_{1/2,obs}$ = 11.3 min; Table 1). A very limited decrease in BNP7787 concentration ($t_{1/2,obs}$ = 103.6 min; Table 1) and simultaneous mesna formation was observed when 1 mM BNP7787 was incubated in the small intestine cytosolic homogenate (250 mg/ml) (Fig. 4A and B). In contrast, a large reduction rate of BNP7787 ($t_{1/2,obs}$ = 12.0 min; Table 1) was measured during incubation in the liver cytosolic homogenate (250 mg/ml) (Fig. 4A and B).

Notably, the amount of mesna generated during the incubation of BNP7787 in cytosol of kidney cortex, small intestine and especially in cytosol of liver homogenate did not account for the reduction observed in the concentration BNP7787. Therefore, we performed an additional experiment in cytosol of liver homogenate to investigate the missing fraction of mesna. The outcome revealed that

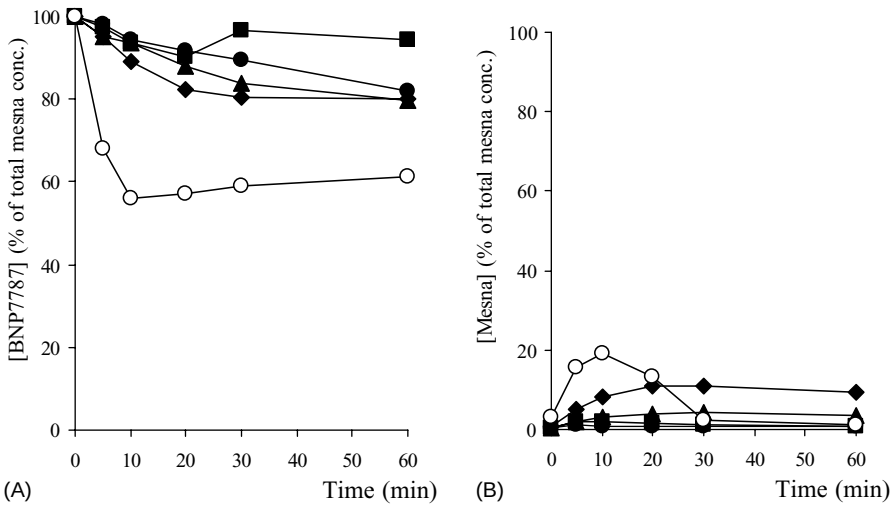

Fig. 4. BNP7787 (A) and mesna (B) concentration vs. time curves obtained during incubation of 1 mM BNP7787 in cytosol of small intestine (●), liver (○), OVCAR-3 tumor (◆), MRI-H-207 tumor (▲) or WARD tumor (■) homogenate (250 mg/ml) at 37 °C. BNP7787 and mesna concentrations are expressed as percentage (%) of the total mesna concentration at $t = 0$. The experiments were performed in duplicate and results are mean values.

the missing amount of mesna was principally present as mixed mesna disulfides (27% of total mesna at 20 min and 33% at 30 min after the start of incubation). Only 4 and 7% of total mesna were present as covalently protein-bound mesna at 20 and 30 min after the initiation of the incubation, respectively.

The cytosolic fractions of OVCAR-3, MRI-H-207 and WARD tumor homogenate (250 mg/ml) were all able to reduce BNP7787 (1 mM) to mesna (Fig. 4A and B). The reduction of BNP7787 in the cytosol of the OVCAR-3 tumor homogenate ($t_{1/2,obs} = 61.2$ min) was approximately two times faster than the reduction in cytosol of MRI-H-207 or WARD tumor ($t_{1/2,obs} = 119.5$ and 104.5 min, respectively).

The BNP7787 reductive activity of the various matrices per mg of protein was expressed as the k_{obs} divided by the amount of protein present in the various matrices (Table 1). The highest reductive activity per mg protein was obtained in cytosol of liver homogenate (2.68×10^{-3} min^{-1} mg^{-1}), which was approximately 2.2-fold higher than the activity of the cytosol of kidney cortex homogenate (1.23×10^{-3} min^{-1} mg^{-1}) and small intestine (1.16×10^{-3} min^{-1} mg^{-1}). In plasma, a very low BNP7787 reductive conversion rate of 0.05×10^{-3} min^{-1} mg^{-1}was observed.

3.3. Reduction of BNP7787 by GSH or cysteine

No formation of mesna or decrease in BNP7787 concentration was detected when BNP7787 was incubated in phosphate buffer A for 60 min. Addition of 0.5 mM of cysteine led to a decrease in BNP7787 ($t_{1/2,obs} = 33.0$ min; Table 1), which was approximately two-fold faster than the reduction of BNP7787 by GSH at 0.5 mM ($t_{1/2,obs} =$ 62.0 min; Table 1). The reduction of BNP7787 by GSH and the simultaneous formation of mesna increased substantially at a higher GSH concentration (2.5 mM) ($t_{1/2,obs} =$ 24.3 min; Table 1).

3.4. Reduction of BNP7787 by various purified enzymes

No consumption of NADPH was detected when 1 mM of BNP7787 was incubated with glutathione reductase (10 μg/ml) for 30 min at 37 °C and pH 7.4 (Fig. 5). When BNP7787 was incubated with glutathione reductase in the presence of 2.5 mM GSH, however, an almost complete decrease in NADPH absorption was measured which was slow in onset (Fig. 5). The decrease in NADPH absorption was faster in onset in combination with a higher GSH concentration of 5 mM (Fig. 5). GSSG was a good substrate of glutathione reductase as reflected by the immediate, fast and complete decrease in absorption of the cofactor NADPH during incubation (data not shown). The reduction in NADPH absorption observed during the incubation of glutathione reductase and GSH with BNP7787 was not caused by auto-oxidation of GSH to GSSG, because during incubation of 0.5, 2.5 or 5 mM GSH with glutathione reductase without BNP7787 no consumption of NADPH was observed.

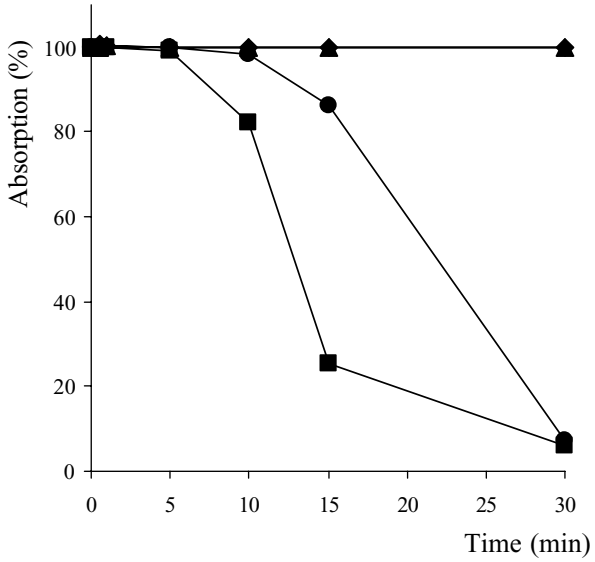

Fig. 5. Absorption at 340 nm vs. time curves obtained during incubation of 1 mM BNP7787 with the enzyme glutathione reductase (10 μg/ml) and cofactor NADPH (1 mM) (◆), plus 0.5 mM GSH (▲), plus 2.5 mM GSH (●) or plus 5 mM GSH (■) in phosphate buffer at 37 °C. The absorption is expressed as percentage (%) of the initial absorption at $t = 0$. The experiments were performed in duplicate and results are mean values.

Glutathione reductase (10 μg/ml) combined with glutaredoxin (0.5 μM) was not able to reduce 1 mM BNP7787 during 30 min of incubation at 37 °C. When 2.5 mM of GSH was added to the mixture, an almost complete reduction of the NADPH absorption was measured (Fig. 6A), which was faster in onset as compared to the solution of 1 mM BNP7787, 1 mM NADPH, 10 μg/ml glutathione reductase and 2.5 mM GSH without glutaredoxin (Fig. 5). The BNP7787 concentration in the solution of 2.5 mM GSH, 10 μg/ml glutathione reductase and 0.5 μM glutaredoxin decreased immediately from the start of the incubation ($t_{1/2,obs} = 6.1$ min; Table 1) and simultaneous formation of mesna was measured (Fig. 6B). In the absence of glutathione reductase, glutaredoxin (0.5 μM) could not increase the reduction of BNP7787 by 2.5 mM GSH as reflected by the $t_{1/2,obs}$ of 25.4 min, which was comparable to the value obtained by 2.5 mM GSH without glutaredoxin ($t_{1/2,obs} = 24.3$ min).

Thioredoxin reductase (8.4 nM) could not reduce BNP7787 (1 mM) during the 30 min incubation in the presence of NADPH (0.2 mM) (data not shown). Addition of thioredoxin (5 μM) to the mixture, however, led to a linear decrease in NADPH absorption. This consumption of NADPH with 1 mM of BNP7787 as a substrate was larger and faster than the NADPH consumption with 160 μM insulin, which is a known good substrate for thioredoxin reductase with thioredoxin [14,22] (i.e. 90 and 56% decrease in NADPH absorption at $t = 30$ min, respectively).

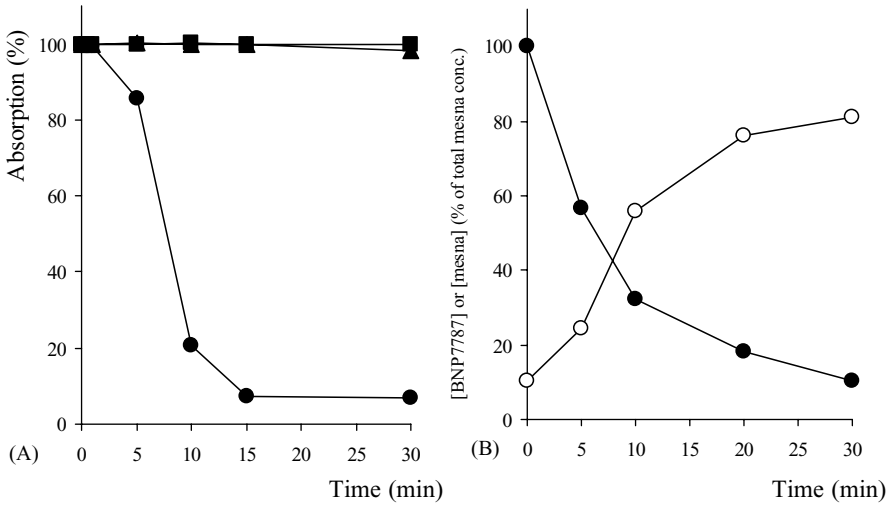

Fig. 6. (A) Absorption at 340 nm vs. time curves obtained during incubation of 1 mM BNP7787 with the enzymes glutathione reductase (10 μg/ml) and glutaredoxin (0.5 μM) and cofactor NADPH (1 mM) (■), plus 0.5 mM GSH (▲) or plus 2.5 mM GSH (●) in phosphate buffer at 37 °C. (B) Concentration vs. time curves of BNP7787 (●) and mesna (○) during incubation of 1 mM BNP7787 with glutathione reductase (10 μg/ml), glutaredoxin (0.5 μM), 1 mM NADPH and 2.5 mM GSH in phosphate buffer at 37 °C. The absorption (A) and BNP7787 and mesna concentrations (B) are expressed as percentage (%) of the initial absorption and as percentage of initial concentration of total mesna at $t = 0$, respectively. The experiments were performed in duplicate and results are mean values.

4. Discussion

BNP7787 is currently undergoing clinical development to study its potential as a chemoprotector against platinum and taxane-induced side-effects. In rats and dogs, BNP7787 administration could completely protect against cisplatin-induced nephrotoxicity [1]. We now investigated several (tissue) matrices and reductive routes that might be involved in the reduction of BNP7787 to form mesna for local detoxification of cisplatin. The results in this study showed that the cytosol of kidney cortex was indeed able to substantially reduce BNP7787 into mesna, as was the case for cytosol of liver and small intestine. A high BNP7787 reductive activity was also measured in RBCs lysate, which is in contrast with the very limited reduction of BNP7787 in plasma. The reduction of BNP7787 to mesna was not restricted to normal tissues, because formation of mesna could be detected in the cytosol of MRI-H-207, WARD and OVCAR-3 tumors. Besides the endogenous thiols GSH and cysteine, BNP7787 could be reduced by the thioredoxin system. The glutaredoxin system was able to accelerate the reduction of BNP7787 by GSH. The possible clinical relevance of these findings will be discussed in the next paragraphs.

After an i.v. bolus injection of 1000 mg/kg BNP7787 in rats, the concentrations of BNP7787 in the kidney ranged from 0.2 to 7.6 mM for BNP7787 and from 1.8 to 6.4 mM for mesna over the first 90 min following administration [4]. The concentration of BNP7787 used in this in vitro study was within this range and is therefore representative for approximating the in vivo situation. The high BNP7787 reductive activity observed in the cytosol of the kidney cortex and the fact that BNP7787 and mesna distribution was mainly restricted to the kidney in rats [4] supports our hypothesis that BNP7787 protects the kidney against cisplatin-induced toxicity by preferential uptake of BNP7787 and its local conversion into the thiol mesna. Mesna can in turn inactivate (hydrated) cisplatin [5,6] before the occurrence of toxicity and/or protect the kidney by acting as an antioxidant [7,8].

The reduction rate of BNP7787 was relatively slow in the cytosol of the small intestine. When the BNP7787 reductive activity of the small intestine was expressed per mg protein present, however, it was high and comparable to the reductive activity of the kidney cortex. This finding and the fact that BNP7787 can be taken up by intestinal epithelial cells [19] indicates that BNP7787 could potentially be reduced to mesna in the small intestine in vivo. This is in agreement with the results from Ormstad et al. [11], who showed that BNP7787 undergoes reduction to mesna during oral administration and intestinal absorption in rats. The high BNP7787 reductive activity observed in the liver is probably not relevant in vivo with respect to formation of large amounts of mesna, because BNP7787 was observed to undergo minimal uptake and distribution into the liver of rats after i.v. administration [4]. Furthermore, after oral administration of BNP7787, the high BNP7787 reductive capacity of the liver will probably also not be relevant, because of the high reductive activity of the small intestine through which the drug must first pass.

Mesna formation was limited in the cytosol of the MRI-H-207 and WARD tumor. The BNP7787 reductive activity of the OVCAR-3 tumor was two-fold higher than that of the MRI-H-207 and WARD tumors. These results indicate that the reduction of BNP7787 to mesna was not restricted to normal tissue. Considering the low concentrations of BNP7787 in WARD tumor after i.v. injection of BNP7787

in tumor-bearing rats [4] and the limited formation of mesna in WARD and MRI-H-207 tumor in vitro, no substantial formation of mesna is expected to occur in the WARD and MRI-H-207 tumor in vivo. The fact that BNP7787 did not reduce the antitumor activity of cisplatin in OVCAR-3 tumor-bearing nude mice [3] also indicates that it is very unlikely that substantial amounts of BNP7787 are reduced to the reactive thiol mesna in the OVCAR-3 tumor in vivo.

Although incubation of BNP7787 in plasma in vitro resulted in a very limited decrease in BNP7787 concentration and, in parallel, a very limited formation of mesna, in vivo relatively large amounts of mesna were present in plasma of rats after receiving BNP7787, i.e. the AUC of mesna was approximately 50% of the AUC of BNP7787 [4]. It is unlikely that the amount of mesna detected in the circulation after BNP7787 administration resulted from BNP7787 reduction in plasma. A possible explanation for the presence of mesna in plasma after BNP7787 administration might be that mesna is reintroduced into the circulation after reduction of BNP7787 to mesna in kidney. In this study, we investigated whether the RBCs could be involved in the release of mesna to plasma after BNP778 administration. A large extent of reduction of BNP7787 to mesna was observed after incubation in RBC lysate at 37 °C for 30 min. No substantial uptake of BNP7787 in RBCs could be detected, which was consistent with our in vivo observation after BNP7787 administration in rats [4] and with a previous in vitro study [20]. Thus, RBCs do not act as drug reservoirs for mesna.

The endogenous thiols cysteine and GSH were able to reduce BNP7787 to form mesna and mesna mixed disulfides. Cysteine was able to reduce BNP7787 about twofold faster than a comparable concentration of GSH. GSH, however, is present in much higher concentrations in tissues, such as kidney, liver and intestine than cysteine (i.e. for GSH range 0.5–10 mM and cysteine range 30–200 μM [21]). Therefore, GSH might be involved in the reduction of BNP7787 to mesna in tissues, whereas the in vivo reduction by cysteine in tissues will be limited. The fact that GSH can reduce BNP7787 confirmed the earlier observations [9–11] that the reduction of BNP7787 in kidney and liver was GSH dependent. During incubation of BNP7787 in the cytosol of liver, mixed mesna disulfides were formed besides mesna and covalently bound mesna to proteins. Taking into account the reducing ability of GSH and high concentrations of GSH in liver (4.5–6.5 mM [21]), the formed mesna mixed disulfides probably (partly) consisted of mesna-GSH complexes. Mesna-GSH complexes will also be formed in kidney and small intestine, where high concentrations of GSH are present [21], but likely to a lesser extent than in liver.

BNP7787 reduction in the cytosol of kidney cortex decreased substantially after removing the proteins before incubation, which indicates that proteins, i.e. enzymes, might be involved in the reduction of BNP7787 to mesna in the kidney. Ormstad et al. [11] have postulated that the reduction of BNP7787 to mesna in cytosol of kidney cortex is mediated by GSH and the enzymes glutathione reductase, which uses NADPH as a cofactor, and glutaredoxin. Our results showed that GSH and especially NADPH substantially enhanced BNP7787 reduction when added alone or together to the cytosol of kidney cortex homogenate. Glutathione reductase or glutaredoxin itself were not able to reduce BNP7787 in vitro. Only in combination with high GSH concentrations, reduction of BNP7787 occurred in the presence of NADPH and glutathione reductase with or without glutaredoxin. The concentration of BNP7787 in the solution containing glutathione reductase, glutaredoxin, NADPH and GSH, decreased immediately and, in parallel, mesna was generated. The consumption of NADPH in this solution, however, was slower in onset than the decrease in BNP7787 concentration. These findings are in agreement with the reduction pathway suggested by Ormstad et al. [11] in which BNP7787 reacts with GSH to form mesna and a mesna–GSH complex, which in turn can react with GSH in the presence of glutaredoxin to form GSSG and mesna. The formed GSSG can be reduced back to GSH in the presence of glutathione reductase and NADPH (Fig. 2).

The thioredoxin system, consisting of thioredoxin reductase, thioredoxin and NADPH, is, similar to the glutaredoxin system, present in different tissues, including kidney and liver [12,13]. The thioredoxin system was able to reduce BNP7787. Thioredoxin reductase itself, however, could not directly reduce BNP7787 in the presence of NADPH. The reduction of BNP7787 by the thioredoxin system will probably be achieved by a reaction sequence similar to that suggested for reduction of insulin [14,22]. First, oxidized thioredoxin is reduced by thioredoxin reductase in the presence of NADPH, followed by the reduction of BNP7787 by reduced thioredoxin (Fig. 7). We found that addition of NADPH to kidney cytosol enhanced the reduction of BNP7787 substantially. Since it is known that thioredoxin reductase and thioredoxin are highly expressed in liver and kidney of rats [12,13], the thioredoxin system could be involved in the BNP7787 reduction in vivo.

In conclusion, although BNP7787 could be reduced in RBCs lysate, cytosol of liver and several tumor tissues, it is unlikely that these tissues will contribute to the formation of mesna in vivo since they are hardly able or not able to take up BNP7787 [4]. The use of BNP7787 as an oral drug might be limited because of the high BNP7787 reductive activity of the small intestine cytosol. Intestinal absorption of BNP7787 might lead to high concentrations of mesna in the circulation that could inactivate cisplatin. The ability to reduce BNP7787 in the presence of the endogenous thiol GSH, the glutaredoxin system and the thioredoxin system could at least in part explain the high BNP7787 reductive activity of the kidney cortex cytosol. The existence of other possible (enzymatic) reduction pathways for BNP7787

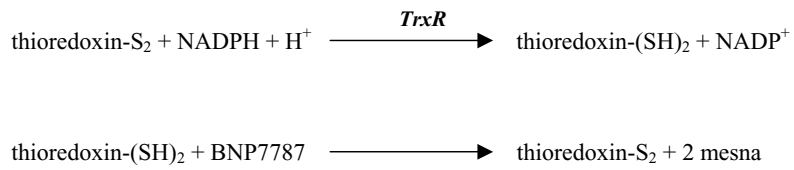

Fig. 7. Proposed scheme for the reduction of BNP7787 by the thioredoxin system, which consists of thioredoxin (oxidized form, i.e. thioreodixin-S_2), thioredoxin reductase (TrxR) and NADPH.

cannot be excluded and remains to be investigated. The high reduction of BNP7787 into mesna in the kidney together with our earlier observation that the BNP7787 and mesna distribution was mainly restricted to the kidney in rats after i.v. injection [4] can explain the selective protection of the kidney by BNP7787. Future studies must reveal which transporter protein is responsible for the selective uptake of BNP7787 by the kidney.

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