ATP-dependent transport of glutathione conjugate of 7 β ,8 α -dihydroxy-9 α ,10 α -oxy-7,8,9,10-tetrahydrobenzo[a]pyrene in murine hepatic canalicular plasma membrane vesicles

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Glutathione (GSH) S-transferases (GSTs) have an important role in the detoxification of (+)-*anti*-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene [(+)-*anti*-BPDE], which is the ultimate carcinogen of benzo[a]pyrene. However, the fate and/or biological activity of the GSH conjugate of (+)-*anti*-BPDE [(+)-*anti*-BPD-SG] is not known. We now report that (–)-*anti*-BPD-SG is a competitive inhibitor (K_i 19 μ M) of Pi-class isoenzyme mGSTP1-1, which among murine hepatic GSTs is most efficient in the GSH conjugation of (+)-*anti*-BPDE. Thus the inhibition of mGSTP1-1 activity by (–)-*anti*-BPD-SG might interfere with the GST-catalysed GSH conjugation of (+)-*anti*-BPDE unless one or more mechanisms exist for the removal of the conjugate. The results of the present study indicate that (–)-*anti*-BPD-SG is transported across canalicular liver plasma membrane (cLPM) in an ATP-dependent manner. The ATP-dependent transport of (–)-*anti*-[³H]BPD-SG followed Michaelis–Menten kinetics (K_m 46 μ M). The ATP dependence of

the (–)-*anti*-BPD-SG transport was confirmed by measuring the stimulation of ATP hydrolysis (ATPase activity) by the conjugate in the presence of cLPM protein, which also followed Michaelis–Menten kinetics. In contrast, a kinetic analysis of ATP-dependent uptake of the model conjugate S-[³H](2,4-dinitrophenyl)-glutathione ([³H]DNP-SG) revealed the presence of a high-affinity and a low-affinity transport system in mouse cLPM, with apparent K_m values of 18 and 500 μ M respectively. The ATP-dependent transport of (–)-*anti*-BPD-SG was inhibited competitively by DNP-SG (K_i 1.65 μ M). Likewise, (–)-*anti*-BPD-SG was found to be a potent competitive inhibitor of the high-affinity component of DNP-SG transport (K_i 6.3 μ M). Our results suggest that GST-catalysed conjugation of (+)-*anti*-BPDE with GSH, coupled with ATP-dependent transport of the resultant conjugate across cLPM, might be the ultimate detoxification pathway for this carcinogen.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BP) are ubiquitous environmental pollutants and are known to produce tumours at various sites in laboratory animals [1,2]. The PAHs are present in cigarette smoke and automobile exhaust, for example, and are believed to be aetiological factors in human chemical carcinogenesis [1]. BP is by far the best studied PAH and is often used to model exposure and biological effects for this class of environmental pollutants. It is well established that PAHs, including BP, require cytochrome P-450-dependent metabolic activation for the generation of their ultimate carcinogenic metabolites, the diol epoxides [3–6]. For example, 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) is believed to be the ultimate carcinogenic metabolite of BP [7–9]. Although BPDE exists as a pair of optical enantiomers of two diastereomers (*syn*- and *anti*-BPDE), (+)-*anti*- and (–)-*syn*-BPDE stereoisomers predominate under physiological conditions [6]. Studies of racemic *anti*- and *syn*-BPDE and their pure optical enantiomers have shown clearly that the (+)-enantiomer of *anti*-BPDE (Scheme 1) is the most potent mutagen *in vitro* and the most potent carcinogen *in vivo* [7–9].

Covalent interaction of (+)-*anti*-BPDE with nucleophilic sites in DNA, which has been shown to occur with high preference at the exocyclic amino group of deoxyguanosine via *trans* addition to the benzylic C-10 position in BPDE, is believed to be a critical step in BP-induced carcinogenesis [10,11]. Even though several different mechanisms can convert (+)-*anti*-BPDE into less harmful species and thus protect DNA [12–14], the toxicologically most important mechanism of (+)-*anti*-BPDE inactivation is its glutathione (GSH) S-transferase (GST)-catalysed conjugation with GSH [15–17]. Thus it has been shown that in the presence of GSH, both rat liver cytosol and purified cytosolic rat liver GSTs decrease the binding of *anti*-BPDE to DNA [18].

The GSTs constitute a superfamily of multifunctional isoenzymes that can detoxify a wide variety of electrophilic xenobiotics primarily by catalysing their conjugation with GSH [19,20]. The cytosolic GST activity in mammalian tissues is due to multiple isoenzymes, which, on the basis of their structural and catalytic properties, can be grouped into four major classes, Alpha, Mu, Pi and Theta [21,22]. Kinetic studies have clearly shown that the GST isoenzymes of different classes differ significantly in their catalytic efficiency in the GSH conjugation of (+)-*anti*-BPDE [15,17]. For example, it has been shown that

Abbreviations used: BPDE, 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene; BP, benzo[a]pyrene; (–)-*anti*-BPD-SG, glutathione conjugate of (–)-*anti*-BPDE; (+)-*anti*-BPD-SG, glutathione conjugate of (+)-*anti*-BPDE; CDNB, 1-chloro-2,4-dinitrobenzene; cLPM, canalicular liver plasma membrane; DNP-SG, S-(2,4-dinitrophenyl)-glutathione; GSH, reduced glutathione; GST, glutathione S-transferase; PAH, polycyclic aromatic hydrocarbon.

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Scheme 1 Structures of *anti*-BPDE enantiomers and their resultant GSH conjugates

the Pi-class human and rat GST isoenzyme is more efficient than other classes of GSTs in the GSH conjugation of (+)-*anti*-BPDE [15,23]. Although the importance of the GST-catalysed conjugation of (+)-*anti*-BPDE with GSH in the detoxification of this ultimate carcinogen is indisputable, the fate and/or biological activity, if any, of the (+)-*anti*-BPDE-GSH conjugate [(+)-*anti*-BPD-SG] is not known. In the present paper we report that the GSH conjugate of (+)-*anti*-BPDE is a potent inhibitor of the Pi-class murine GST isoenzyme (mGSTP1-1 in the recently recommended nomenclature for murine GSTs [20]), which among hepatic GSTs seems to be most important in the detoxification of (+)-*anti*-BPDE in the liver of A/J mouse. The results of the present study also show that (-)-*anti*-BPD-SG is transported across canalicular liver plasma membrane (cLPM) in an ATP-dependent manner. In conclusion, our results suggest that the GST-catalysed conjugation of (+)-*anti*-BPDE with GSH coupled with ATP-dependent canalicular transport of the resultant conjugate might be the ultimate detoxification pathway for this carcinogen and possibly other similar carcinogens.

EXPERIMENTAL

Materials

Female A/J mice were purchased from the National Cancer Institute (Frederick, MD, U.S.A.). Racemic *anti*-BPDE was procured from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO, U.S.A.). [^3H]GSH (specific radioactivity 44.8 Ci/mmol) and [γ - ^{32}P]ATP (specific radioactivity 3000 Ci/mmol) were purchased from DuPont-NEN (Boston, MA, U.S.A.). The 96-well multi-screen filtration plates were purchased from Millipore Corporation (Bedford, MA, U.S.A.). Other reagents were of highest purity available.

Synthesis and purification of GSH conjugates of (+)- and (-)-*anti*-BPDE

For the synthesis of non-radiolabelled GSH conjugates of (+)- and (-)-*anti*-BPDE (see Scheme 1 for structures), 11.6 μmol of ($\pm$)-*anti*-BPDE was incubated with 326 μmol of GSH in 50 mM Tris/HCl buffer, pH 7.5, containing 2.5 mM KCl and 0.5 mM EDTA (TKE buffer) in the presence of 1 mg of purified human

placental GST Pi protein [specific activity 68 $\mu\text{mol}/\text{min}$ per mg for the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 25 °C]. The reaction mixture was incubated for 4 h at 37 °C. The unreacted *anti*-BPDE was removed by extracting the reaction mixture with ethyl acetate. The GSH conjugates of (+)- and (-)-*anti*-BPDE, free from unreacted GSH, were purified by preparative HPLC. In brief, the column (Delta-PrepPak column; 25 mm $\times$ 100 mm) was pre-equilibrated with 78 % solvent I (5 % acetonitrile/0.1 % trifluoroacetic acid)/22 % solvent II (90 % acetonitrile/0.1 % trifluoroacetic acid). The *anti*-BPDE-GSH conjugates were eluted with a linear gradient of 22–24.5 % solvent II over 30 min at a flow rate of 5 ml/min. The purity and concentrations of the GSH conjugates were determined by reverse-phase HPLC as described previously [24]. Figures 1(A) and 1(B) depict the reverse-phase HPLC elution profile and purity of (-)- and (+)-*anti*-BPD-SG respectively. The ^3H -labelled (-)-*anti*-BPD-SG was synthesized and purified as described above for the non-radioactive conjugate.

Synthesis and purification of GSH conjugate of CDNB

Non-radiolabelled GSH conjugate of CDNB (DNP-SG) was synthesized by the procedure described by Awasthi et al. [25]. The purity of the DNP-SG preparation was checked by reverse-phase HPLC. In brief, the column was pre-equilibrated with 100 % solvent I. The DNP-SG was eluted with 100 % solvent I for minutes 0–5 followed by a linear gradient of 0–100 % solvent II for minutes 5–25 at a flow rate of 1 ml/min. The GSH conjugate of CDNB was eluted at a retention time of about 18.8 min (Figure 1C). The radiolabelled DNP-SG was synthesized and purified similarly.

Purification of murine GST Pi

The Pi class isoenzyme mGSTP1-1 of female A/J mouse liver was purified by GSH-affinity chromatography, by the method of Simons and Vander Jagt [26], followed by chromatofocusing. Details of affinity chromatography and chromatofocusing have been described previously [24,27]. The homogeneity of mGSTP1-1 was ascertained by reverse-phase HPLC and Western blot analysis as described previously [24]. Protein content was determined by the method of Bradford [28].

Kinetics of the inhibition of mGSTP1-1 activity by (-)-*anti*-BPD-SG and (+)-*anti*-BPD-SG

The GST activity towards CDNB was determined by the method of Habig et al. [29]. The reaction mixture contained, in 1 ml, 0.1 M potassium phosphate buffer, pH 6.5, 2.5 μg of mGSTP1-1 protein, 1 mM GSH and the desired concentrations of CDNB (0.2–1.0 mM) and (-)- or (+)-*anti*-BPD-SG (2–150 μM). The rate of GSH-CDNB conjugation was monitored at 340 nm. A blank without enzyme was included to account for non-enzymic conjugation of GSH with CDNB.

Preparation of mouse cLPM vesicles

The cLPM vesicles were prepared as described by Meier and Boyer [30], with some modifications. In brief, 50 g of liver was minced, suspended in 200 ml of 1 mM sodium bicarbonate buffer, pH 7.4 (buffer A), and homogenized with a Type A glass homogenizer. The homogenate was diluted to 1 litre with ice-cold buffer A, filtered twice through glass wool and centrifuged at 1500 g for 15 min. The pellet was collected, resuspended in 50 % (w/v) sucrose solution and agitated for 15 min to disrupt

Figure 1 Reverse-phase HPLC analysis of GSH conjugates of *anti*-BPD and CDNB

(A) (–)-*anti*-BPD-SG, which was eluted at a retention time of about 5.5 min; (B) (+)-*anti*-BPD-SG, which was eluted at a retention time of about 6.3 min; (C) DNP-SG, which was eluted at a retention time of about 18.8 min.

membrane aggregates. This preparation was layered at the bottom of a sucrose gradient containing 40 ml of 8.5% (w/v) sucrose, 70 ml of 36.5% sucrose and 70 ml of 44% sucrose, and centrifuged at 12500 *g* overnight. The turbid layer of mixed liver plasma membrane fraction at the 36.5%/44% sucrose interface was collected, diluted 1:10 with ice-cold buffer A and centrifuged at 16000 *g* for 30 min. The pellet thus obtained was resuspended in buffer A containing 250 mM sucrose and homogenized by using a Type B glass homogenizer. The homogenate (2 ml) was layered on top of a sucrose gradient containing 2.5 ml of 31% sucrose, 2.5 ml of 34% sucrose and 3 ml of 38% sucrose solution. The samples were centrifuged at 200000 *g* for 3 h in a swinging-bucket rotor. This centrifugation resulted in three distinct bands and a pellet. The band at the top of 31% sucrose represented cLPM [30], which was aspirated by using a Pasteur pipette. The cLPM fraction was diluted 1:10 with 10 mM Tris/HCl, pH 7.4, containing 250 mM sucrose (buffer B), and centrifuged again at 105000 *g* for 1 h. The pellet was resuspended in 2 ml of buffer B, homogenized by using a Type B glass homogenizer, and passed through a 27-gauge needle seven times to prepare cLPM vesicles. The purity of the cLPM preparation was ascertained by

determining Na⁺,K⁺-ATPase, Mg²⁺-ATPase [31] and alkaline phosphatase [32] activities, as described by Meier et al. [33].

Determination of (–)-*anti*-[³H]BPD-SG and [³H]DNP-SG transport

Transport of (–)-*anti*-[³H]BPD-SG and [³H]DNP-SG into cLPM vesicles was measured by a rapid filtration technique, as described previously [34]. In brief, the frozen cLPM vesicles were thawed rapidly by immersing in a water bath at 37 °C; they were kept on ice throughout the experiment. The incubation mixture contained, in a final volume of 0.1 ml, buffer B, 15 µg of cLPM vesicle protein, 10 mM MgCl₂, 10 mM creatine phosphate, 12 units of creatine kinase, 1 mM EGTA, 1 mM ouabain, and the desired concentration of [³H]DNP-SG [specific radioactivity (values given as means ± S.D.) 277545 ± 82985 c.p.m./nmol] or (–)-*anti*-[³H]BPD-SG (specific radioactivity 26914 ± 4496 c.p.m./nmol). The reaction mixture was preincubated for 5 min at 37 °C and the reaction was initiated by the addition of 2 mM ATP (the pH of the ATP solution was adjusted to 7.4 with 0.1 N NaOH). The reaction mixture was incubated at 37 °C for 5 min and aliquots (30 µl) were applied under suction to 0.45 µm pore-size Millipore nitrocellulose filters containing 0.3 ml of ice-cold stop buffer (10 mM Tris/HCl, pH 7.4, containing 250 mM sucrose and 100 mM NaCl). The filters were washed twice with 0.3 ml of stop buffer. The filters were then blotted dry, punched out of the plates and dissolved in scintillation fluid for determination of radioactivity. In parallel controls, ATP was replaced by an equiosmolar concentration of NaCl (3 mM) and ATP-dependent transport was calculated by subtracting the control uptake values from those obtained in the presence of ATP. The kinetic constants were determined by fitting a hyperbolic function to data points through non-linear regression analysis.

Determination of ATPase activity

The stimulation of ATP hydrolysis by (–)-*anti*-BPD-SG and DNP-SG in the presence of cLPM protein was measured by the method of Knowles and Leng [35], with some modifications described previously [36].

RESULTS

We have shown previously that more than 90% of total cytosolic GST activity in the liver of female A/J mouse is accounted for by four isoenzymes with isoelectric points (pI values) of 9.3 (Alpha class mGSTA3-3), 8.8 (Pi class mGSTP1-1), 8.6 (Mu class mGSTM1-1) and 5.9 (Alpha class mGSTA4-4) [24]. However,

Table 1 Kinetic constants for the inhibition of mGSTP1-1 activity by (+)-*anti*-BPD-SG and (–)-*anti*-BPD-SG

mGSTP1-1 activity was measured as a function of CDNB concentration (0.2–1.0 mM) at a fixed concentration of GSH (1 mM) in the absence or presence of (+)-*anti*-BPD-SG and (–)-*anti*-BPD-SG. The nature of inhibition was determined from a double-reciprocal plot. The *K_i* was determined from a replot of the double-reciprocal plot. Results are means ± S.D. for three independent experiments, each performed in triplicate. Abbreviation: n.d., not determined.

Kinetic parameter	(–)- <i>anti</i> -BPD-SG	(+)- <i>anti</i> -BPD-SG
IC ₅₀ (µM)	51 ± 2	117 ± 12
<i>K_i</i> (µM)	19 ± 7	n.d.
Nature of inhibition	Competitive	n.d.

Figure 2 Kinetics of the inhibition of mGSTP1-1 activity by (–)-anti-BPD-SG

mGSTP1-1 activity was measured as a function of CDNB concentration (0.2–1.0 mM) at a fixed concentration of GSH (1 mM) in the absence (●) and in the presence (■) of 100 μM (–)-anti-BPD-SG. Data points are averages of triplicate determinations.

mGSTP1-1 is 7–69-fold more efficient than mGSTM1-1, mGSTA3-3 and mGSTA4-4 in the GSH conjugation of (+)-anti-BPDE [17]. Recently we demonstrated that the relative

contribution of mGSTP1-1 to the hepatic GSH conjugation of (+)-anti-BPDE (approx. 79%) far exceeds the combined contributions of mGSTM1-1, mGSTA3-3 and mGSTA4-4 (approx. 19%) [37]. Taken together, these results suggest that mGSTP1-1 might have a major role in the hepatic detoxification of (+)-anti-BPDE.

Table 1 summarizes the effects of the GSH conjugates of (+)- and (–)-anti-BPDE [(–)-anti-BPD-SG and (+)-anti-BPD-SG respectively] on the mGSTP1-1-catalysed conjugation of GSH with CDNB. The (–)-anti-BPD-SG was found to be a potent inhibitor of mGSTP1-1-catalysed GSH-CDNB conjugation, with an IC_{50} of $51 \pm 2 \mu\text{M}$. The IC_{50} for (+)-anti-BPD-SG for the inhibition of mGSTP1-1-catalysed GSH-CDNB conjugation was approx. 2.3-fold higher than that for (–)-anti-BPD-SG. It is important to note that the (+)-enantiomer of anti-BPDE is a far more potent carcinogen *in vivo* than (–)-anti-BPDE [8,9]. As shown in Figure 2, (–)-anti-BPD-SG inhibited mGSTP1-1 activity competitively with a K_i of $19 \mu\text{M}$. These results suggest that the inhibition of mGSTP1-1 activity by (–)-anti-BPD-SG might interfere with the GST-catalysed conjugation of (+)-anti-BPDE with GSH unless one or more mechanisms exist for the removal of the conjugate.

ATP-dependent extrusion of GSH-xenobiotic conjugates from hepatocytes across the cLPM into bile is believed to be an important mechanism in the detoxification of xenobiotics [38–40]. To determine whether (–)-anti-BPD-SG is transported across

Figure 3 ATP-dependent canalicular transport of (–)-anti-BPD-SG and DNP-SG

(A) Time course of the uptake of (–)-anti-BPD-SG by cLPM vesicles in the absence (●) and in the presence (▲) of ATP. The incubation mixture contained, in a final volume of 0.1 ml, 10 mM Tris/HCl, pH 7.4, containing 250 mM sucrose, 10 mM MgCl_2 , 2 mM ATP, 10 mM creatine phosphate, 12 units of creatine kinase, 1 mM EGTA, 1 mM ouabain, 15 μg of cLPM vesicle protein and 50 μM (–)-anti-[^3H]BPD-SG. An equiosmolar concentration of NaCl (3 mM) was added to the controls in place of ATP. (B) Osmotic sensitivity of ATP-dependent transport of (–)-anti-BPD-SG into cLPM vesicles. The ATP-dependent uptake of (–)-anti-BPD-SG, after 5 min of incubation, was measured in the presence of 0.25–1.0 M sucrose. The concentrations of ATP and (–)-anti-BPD-SG were kept constant at 2 mM and 50 μM respectively. (C) Lineweaver–Burk plot of the ATP-dependent uptake of (–)-anti-BPD-SG by cLPM vesicles. (D) Lineweaver–Burk plot of the ATP-dependent uptake of DNP-SG by cLPM vesicles. Data points are averages of triplicate determinations.

Table 2 Kinetic constants for the ATP-dependent canalicular transport of (–)-*anti*-BPD-SG and DNP-SGResults are means $\pm$ S.D. for three independent experiments, each performed in triplicate.

Conjugate	K_m (μ M)	V_{max} (nmol/min per mg)
(–)- <i>anti</i> -BPD-SG	46 ± 9	0.440 ± 0.22
DNP-SG, high affinity	18 ± 5	0.020 ± 0.012
DNP-SG, low affinity	500 ± 200	0.230 ± 0.150

cLPM, the ATP-dependent uptake of (–)-*anti*-[3 H]BPD-SG by purified cLPM vesicles was measured. As shown in Figure 3(A), the incubation of cLPM vesicles with (–)-*anti*-[3 H]BPD-SG resulted in a time-dependent uptake of the conjugate, which was significantly increased in the presence of ATP. The ATP-dependent uptake of (–)-*anti*-[3 H]BPD-SG by cLPM vesicles increased with increasing vesicle protein concentration, and was linear up to 20 μ g of cLPM protein (results not shown). To confirm that the accumulation of (–)-*anti*-[3 H]BPD-SG by cLPM vesicles represented transport rather than non-specific binding of the conjugate to the vesicle surface, the osmotic sensitivity of the ATP-dependent canalicular transport of (–)-*anti*-BPD-SG was examined. It is well known that an increase in extravesicular osmolarity decreases intravesicular volume, owing to shrinkage of the vesicles. We reasoned that if the ATP-dependent uptake of (–)-*anti*-BPD-SG by cLPM vesicles was due to transport, then the uptake of this conjugate should be inhibited by increasing the osmotic pressure. The effect of osmolarity on transport was examined by measuring the ATP-dependent uptake of (–)-*anti*-BPD-SG in the presence of different concentrations of sucrose; the results are shown in Figure 3(B). The ATP-dependent uptake of (–)-*anti*-BPD-SG by cLPM vesicles decreased with increasing concentration of the sucrose in the incubation medium, in a dose-dependent manner. These results indicate clearly that the uptake of (–)-*anti*-BPD-SG by cLPM vesicles is a transport process with very little binding to the vesicles (Figure 3B).

When (–)-*anti*-[3 H]BPD-SG uptake by cLPM vesicles was measured as a function of conjugate concentration at a fixed concentration of ATP (2 mM), a linear Lineweaver–Burk plot was observed (Figure 3C). The kinetic constants for the transport of (–)-*anti*-BPD-SG into cLPM vesicles are summarized in Table 2. The V_{max} for the uptake of (–)-*anti*-BPD-SG was 0.44 ± 0.22 nmol/min per mg, with a corresponding K_m of 46 ± 9 μ M (Table 2). Unlike (–)-*anti*-BPD-SG, however, uptake studies with various concentrations of DNP-SG, a model compound of GSH conjugates, revealed biphasic kinetics (Figure 3D) and suggested the presence of a high-affinity and a low-affinity component for the transport of DNP-SG. The K_m values for the high-affinity and low-affinity components were 18 ± 5 μ M (V_{max} 0.020 ± 0.012 nmol/min per mg) and 500 ± 200 μ M (V_{max} 0.230 ± 0.150 nmol/min per mg) respectively.

The ATP dependence of the canalicular transport of (–)-*anti*-BPD-SG was further confirmed by measuring the stimulation of ATP hydrolysis (ATPase activity) by (–)-*anti*-BPD-SG in the presence of cLPM protein. We reasoned that if the transport of (–)-*anti*-BPD-SG were an ATP-dependent process, this conjugate would stimulate ATP hydrolysis in the presence of cLPM protein. When ATPase activity was measured as a function of (–)-*anti*-BPD-SG concentration, a linear Lineweaver–Burk plot was observed (Figure 4A). The K_m and V_{max} values for (–)-*anti*-BPD-SG-stimulated ATP hydrolysis were 108 ± 36 μ M and 27 ± 3 nmol/min per mg respectively. In contrast, two inde-

Figure 4 Kinetics of the stimulation of ATP hydrolysis (ATPase activity) by (–)-*anti*-BPD-SG and DNP-SG in the presence of cLPM protein

(A) Lineweaver–Burk plot of the stimulation of ATP hydrolysis by (–)-*anti*-BPD-SG. The reaction mixture contained, in a final volume of 0.5 ml, 50 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, 2 mM EGTA, 1 mM ouabain, 0.8 mM sodium phosphate, pH 7.4, 3 mM 2-mercaptoethanol, 1.6 mM [32 P]ATP, 20 μ g of cLPM protein and the desired concentration of (–)-*anti*-BPD-SG (10–160 μ M). A separate incubation without (–)-*anti*-BPD-SG was performed to determine non-specific ATP hydrolysis. (B) Lineweaver–Burk plot of the stimulation of ATP hydrolysis by DNP-SG. Data points are averages of triplicate determinations.

pendent components, a high-affinity and a low-affinity component, were observed for the stimulation of ATP hydrolysis by DNP-SG (Figure 4B). The K_m values for the high-affinity and low-affinity components were 18 ± 9 and 484 ± 21 μ M respectively, and the corresponding V_{max} values were 20 ± 9 and 83 ± 23 nmol/min per mg. These results indicate clearly that, whereas two independent components exist for the ATP-dependent transport of DNP-SG in murine cLPM vesicles, ATP-dependent canalicular transport of the GSH conjugate of (+)-*anti*-BPDE is mediated by a single component.

To determine the functional interrelation between DNP-SG and BPD-SG transporters, the effect of DNP-SG on the ATP-dependent uptake of (–)-*anti*-BPD-SG by cLPM vesicles and vice versa were examined; the results are shown in Figure 5. The ATP-dependent canalicular transport of (–)-*anti*-BPD-SG was competitively inhibited by DNP-SG with a K_i of 1.65 ± 0.28 μ M (Figure 5A). Similarly, (–)-*anti*-BPD-SG was found to be a potent competitive inhibitor of the high-affinity component of the ATP-dependent canalicular transport of DNP-SG (K_i 6.3 ± 0.18 μ M) (Figure 5B). The (–)-*anti*-BPD-SG did not affect the low-affinity component of the ATP-dependent transporter for DNP-SG (results not shown in Figure 5B).

Figure 5 Kinetics of the inhibition of ATP-dependent canalicular transport of $(-)$ -anti-BPD-SG by DNP-SG and vice versa

(A) ATP-dependent canalicular transport of $(-)$ -anti-BPD-SG in the absence ($\bullet$) and in the presence ($\blacktriangle$) of 5 μM DNP-SG. The concentration of $(-)$ -anti-BPD-SG in the reaction mixture was varied between 16 and 100 μM ; the ATP concentration was kept constant at 2 mM. The nature of the inhibition was determined by double-reciprocal plots. The K_i was determined from replots of the double-reciprocal plots. (B) ATP-dependent canalicular transport of DNP-SG in the absence ($\bullet$) and in the presence ($\blacktriangle$) of 30 μM $(-)$ -anti-BPD-SG. Even though the concentration of DNP-SG was varied between 18 and 150 μM , the data points corresponding to the low-affinity component of the ATP-dependent canalicular transport for DNP-SG are not shown.

DISCUSSION

The (+)-enantiomer of *anti*-BPDE has been shown to be the most potent carcinogen in laboratory animals [8,9]. More recently it has been demonstrated that, within the *P53* tumour suppressor gene, *anti*-BPDE preferentially modifies guanine residues in the same mutational hotspots that are frequent in human malignancies [41–43]. Because *anti*-BPDE is found in cigarette smoke as well as other industrial emissions, these findings provides a mechanistic, rather than a statistical, explanation for the well-established epidemiological correlation between exposure to PAHs and cancer. Therefore an understanding of the mechanism(s) of the detoxification of *anti*-BPDE seems to be essential for developing preventive measures against cancer induced by BP.

Several different mechanisms of biotransformation of *anti*-BPDE compete for its interaction with nucleophilic sites in DNA, which include spontaneous hydrolysis to tetrols and oxo diols [6], conversion into triols and triolepoxides [12,14], hydration by epoxide hydrolase [13] and GST-catalysed conjugation with GSH [15–17,23]. Because *anti*-BPDE is a poor substrate for

epoxide hydrolase [44], the toxicologically most important mechanism of inactivation of this ultimate carcinogen seems to be its conjugation with GSH. The results of the present study indicate that $(-)$ -*anti*-BPD-SG is a potent inhibitor of Pi-class mouse GST isoenzyme, which is the most efficient of the murine hepatic GSTs in the detoxification of this ultimate carcinogen [17,37]. In contrast, GSH conjugate of $(-)$ -*anti*-BPDE, which is a weak carcinogen [8,9], is also a poor inhibitor of mGSTP1-1. These results suggest that by inhibiting mGSTP1-1 activity, $(-)$ -*anti*-BPD-SG might interfere with the GST-catalysed conjugation of this carcinogen with GSH unless one or more mechanisms exist for the removal of the conjugate.

The energy-dependent efflux of GSH conjugates of endogenous reactive metabolites as well as exogenous xenobiotics from the hepatocyte across the cLPM into bile is considered an important cellular defence mechanism [38–40]. In addition to an energy-dependent efflux mechanism, the presence of an ATP-independent system for the canalicular transport of GSH conjugates has been documented in rat cLPM [45]. These studies suggest that, whereas low-molecular-mass and relatively hydrophilic GSH conjugates are transported by the ATP-independent system, high-molecular-mass GSH conjugates are substrates for both ATP-independent and ATP-dependent systems [45]. Although the present study has focused on characterizing the ATP-dependent transport of $(-)$ -*anti*-BPD-SG, the results presented in Figure 3(A) clearly indicate that $(-)$ -*anti*-BPD-SG might also, to some extent, be transported across mouse cLPM in an ATP-independent fashion. Even though further studies are needed to determine whether the canalicular transport of $(-)$ -*anti*-BPD-SG is mediated by one or more transporters, the results of the present study suggest that the ATP-dependent transport of $(-)$ -*anti*-BPD-SG in mouse cLPM vesicles might be mediated by one component. Consistent with the results of conjugate transport studies, kinetic analysis of the $(-)$ -*anti*-BPD-SG-dependent stimulation of ATP hydrolysis (ATPase activity) also indicates the presence of only one component for the transport of this conjugate across mouse cLPM. In contrast, two ATP-dependent transport components, one with high affinity (K_m 19 μM) and the other with low affinity (K_m 500 μM), seem to mediate the hepatocellular excretion of DNP-SG in mouse cLPM. Further support for the presence of two components for DNP-SG transport in mouse cLPM derives from the kinetic analysis of the ATPase activity towards DNP-SG. Even though the precise structural interrelation between the canalicular transporters for $(-)$ -*anti*-BPD-SG and DNP-SG remains to be determined, our results suggest that $(-)$ -*anti*-BPD-SG and DNP-SG might have a common transport mechanism. This is because the transport of $(-)$ -*anti*-BPD-SG across cLPM is competitively inhibited by DNP-SG. Similarly, $(-)$ -*anti*-BPD-SG is a potent competitive inhibitor of the high-affinity component of the ATP-dependent canalicular transporter for DNP-SG.

In conclusion, the results of the present study indicate that the GSH conjugate of (+)-*anti*-BPDE is a potent inhibitor of mGSTP1-1, and that the GSH conjugate of (+)-*anti*-BPDE is transported across cLPM mainly in an ATP-dependent manner. Thus the GST-catalysed conjugation of (+)-*anti*-BPDE with GSH, coupled with the energy-dependent efflux of the resultant conjugate across cLPM, might be the ultimate detoxification pathway for this and similar carcinogens.

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