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NAPHTHYLISOTHIOCYANATE DISPOSITION IN BILE AND ITS RELATIONSHIP TO LIVER GLUTATHIONE AND TOXICITY

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Abstract—1-Naphthylisothiocyanate (ANIT), but not 2-naphthylisothiocyanate (BNIT), produces cholangiolitic hepatitis in rats after a single, oral administration. The mechanisms responsible for the disparate toxic outcomes for these closely related structural isomers are not fully understood. Recent reports suggest that ANIT-induced hepatotoxicity is dependent upon the formation and biliary excretion of a reversible glutathione–ANIT conjugate. To understand better the relationship between hepatic glutathione, secretion into bile and hepatotoxicity, the bile concentrations and hepatotoxicities of ANIT and BNIT were examined in rats with and without pretreatment with buthionine sulfoximine (BSO). ANIT (100 mg/kg, p.o.) caused a 3-fold elevation of plasma alanine aminotransferase activity (ALT), a 6-fold elevation of total plasma bilirubin, and a >90% reduction in bile flow 24 hr after administration. BNIT, at this same dose and route of administration, did not alter significantly these markers of liver injury. Accumulation of ANIT and BNIT in bile occurred with the same temporal characteristics; however, BNIT accumulated to markedly larger concentrations (292 ± 83 and 235 ± 100 μM BNIT and 78 ± 19 and 29 ± 13 μM ANIT at 1 and 4 hr, respectively). The accumulation of ANIT and BNIT in bile was coincident with a >2-fold elevation of reduced glutathione in bile. Pretreatment of rats with BSO decreased hepatic glutathione concentration and reduced the concentration of naphthylisothiocyanates in bile by 85%. Associated with this reduction was an attenuation of ANIT hepatotoxicity. Altogether, these findings indicate that both ANIT and BNIT accumulate in bile in a glutathione-dependent manner, yet they yield different hepatotoxic outcomes. Therefore, the disparity in hepatotoxicities observed with these isomers is not related to a difference in ability to enter bile. Other differences, such as in metabolism, chemical reactivity, conjugate stability and/or cytotoxic potential to bile duct epithelial cells may be more important determinants of hepatotoxicity.

Key words: glutathione; naphthylisothiocyanate; bile; liver; hepatotoxicity

The pathophysiological changes associated with the development of ANIT†-induced cholangiolitic hepatitis in rats have been well characterized [1–3]. These include bile duct epithelial cell necrosis, hepatic parenchymal cell injury, and the cessation of bile flow. However, the mechanisms involved have not been fully elucidated. Despite its structural similarity to ANIT, BNIT is not hepatotoxic [4, 5].

Recent results have suggested that glutathione may be critically important in ANIT hepatotoxicity, possibly through the formation and secretion into the bile of a reversible glutathione–ANIT conjugate. Exposure of hepatic parenchymal cell suspensions to ANIT resulted in the rapid transport of intracellular reduced glutathione to the extracellular milieu [6]. An unstable glutathione–ANIT conjugate was identified in the cell suspension medium [6]. *In vivo* administration of an hepatotoxic dose of ANIT to rats resulted in the rapid accumulation of ANIT in bile coincident with a 2-fold elevation of biliary glutathione [7]. Moreover, pretreatment of rats with agents that deplete hepatic glutathione protected against an otherwise hepatotoxic dose of ANIT [8]. These findings led to the hypothesis that glutathione-dependent biliary disposition of ANIT is required for ANIT hepatotoxicity. Upon entering hepatic parenchymal cells, it appears that ANIT reacts with glutathione to form a dithiocarbamyl-linked glutathione conjugate [6]. The conjugate may be transported into bile where it decomposes to release reduced glutathione and ANIT. This process may result in the accumulation of ANIT in bile and exposure of bile duct epithelium to cytotoxic concentrations of ANIT.

If this hypothesis is correct, the lack of hepatotoxicity of BNIT may be due to its inability to accumulate in bile. Therefore, to further our understanding of NIT hepatotoxicity, we examined the dependence of NIT disposition and hepatotoxicity on hepatic glutathione in rats with or without pretreatment with BSO.

MATERIALS AND METHODS

Chemicals

ANIT, γ-glutamylglutamate, iodoacetic acid, BSO, glutathione and glutathione disulfide were purchased from Sigma Chemical Co. (St. Louis, MO). ANIT was supplied as a yellowish crystalline material of >95% purity. BNIT (Chem Service, West Chester, PA) was supplied as a dark brown crystalline material of unknown purity. Therefore, BNIT was recrystallized prior to use. In brief, 0.5 g BNIT was dissolved in 20 mL hot ethanol and decolorized with 0.25 g activated charcoal (NORIT, Baker Chemical Co., Phillipsburg, NJ). The charcoal was removed by passing the BNIT–charcoal mixture through a 0.45 μm filter (GVWP02500, Millipore Corp., Bedford, MA). BNIT crystals were collected

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† Abbreviations: ANIT, 1-naphthylisothiocyanate; BNIT, 2-naphthylisothiocyanate; BSO, buthionine sulfoximine; and NIT, naphthylisothiocyanate.

on Whatman No. 2 filters and dried under vacuum after addition of 8.0 mL cold deionized water to the filtrate and incubation at 4° to force recrystallization. HPLC analysis indicated a purity of >95%. The absorbance and NMR spectra of BNIT were consistent with those previously reported for BNIT [9, 10]. All other reagents were of the highest grade commercially available.

Animals

Male, Sprague–Dawley rats (CF:CD(SD)BR) (Charles River, Portage, MI) weighing 180–250 g were housed in plastic cages on aspen-chip bedding (Northeastern Products Corp., Caspian, MI) under conditions of controlled temperature (18–21°) and humidity (55 ± 5%) with a 12-hr light/12-hr dark cycle. Rats were allowed free access to rat chow (Purina Lab Chow No. 5001, Mills Inc., Richmond, IN) and tap water.

NIT administration and sample collection

Rats were fasted for 24 hr prior to receiving ANIT or BNIT (100 mg/kg; 50 mg/mL corn oil, p.o.) or an equivalent volume of corn oil. At specified times, they were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed onto heating pads to maintain body temperature. An abdominal midline incision was made, and bile was collected for 30 min as described previously [8]. Bile was collected into microcentrifuge tubes containing 250 μL of 1 mM bathophenanthroline disulfonic acid in 10% perchloric acid. Blood (4.5 mL) was drawn from the descending aorta into a syringe containing 0.5 mL sodium citrate (3.8%, w:v) immediately after bile collection. A section of liver (0.4 to 0.6 g) was excised and immediately frozen (liquid nitrogen or dry ice/ethanol bath) and stored at −20° for glutathione analysis. The remaining liver was excised and weighed.

Glutathione depletion

To decrease hepatic glutathione, rats were treated with BSO (890 mg/kg, 89 mg/mL saline, i.p.) 2 hr prior to NIT administration.

NIT determinations

NIT was measured in bile and plasma. An aliquot (0.1 to 0.15 mL) of bile collected as described above was added to a mixture of acetonitrile:glacial acetic acid:water (40:0.1:59.9 or 99.9:0.1:0, by vol.) to a total volume of 1.5 mL and allowed to stand at 4° for 30 min. Plasma (0.5 mL) was added to 1.0 mL of acetonitrile:glacial acetic acid:water (60:0.1:39.9 or 99.9:0.1:0, by vol.) and allowed to stand at 4° for 30 min. The samples were spun in a centrifuge (15,000 *g*, 2 min) to remove precipitate. Aliquots of supernatant fluid were then injected into a 4 mm × 250 mm, reverse-phase column (5 μm, ODSII, Custom LC, Houston, TX). NITs were eluted (1.0 mL/min) from the column with a linear gradient of acetonitrile:glacial acetic acid:water (51:0.1:48.9 to 90:0.1:9.9, by vol., from 0 to 5 min, 90:0.1:9.9 from 5 to 18 min). Elution of ANIT and BNIT from the column was detected by monitoring the column effluent at 308 and 262 nm, respectively. The concentrations of NIT in bile and plasma were calculated from peak areas obtained for each sample. A standard curve of peak areas was constructed for ANIT and BNIT by injecting a known amount of each dissolved in acetonitrile:glacial acetic acid:water (40:0.1:59.9, by vol.). Collection of bile into perchloric acid as described above was found to reduce the recovery of NITs by a factor of 2.8 compared with direct assessment of bile. Addition of ANIT or BNIT to supernatants of perchloric acid-treated bile resulted in a 6 and 10% loss of detectable NIT, respectively. Plasma samples prepared as described above led to a loss of detectable NIT of 60 and 0% for the acetonitrile:glacial acetic acid:water systems 60:0.1:39.9 and 99.9:0.1:0, respectively. The bile and plasma concentrations of NITs reported herein were corrected for losses during sample preparation.

HPLC analysis did not reveal the presence of additional NIT-derived material in bile or plasma. This may have been the result of sample collection/processing and/or choice of detection wavelength. The possibility exists that NIT-derived metabolites other than those reported were present in bile and plasma but not detected with the methods employed.

Glutathione, bilirubin, and alanine aminotransferase activity determinations

Bile and liver glutathione (reduced and oxidized) were determined according to the method of Fariss and Reed [11]. Plasma total bilirubin concentrations were determined as described previously [8]. Plasma alanine aminotransferase activities were determined spectrophotometrically utilizing a Sigma Kit No. 59-10 (Sigma Chemical Co.).

Analysis of data

SigmaStat Statistical Analysis software (Ver. 1.0, Jandel Scientific, San Rafael, CA) was used for the analysis of all data. A two-tailed Student's *t*-test was used for comparisons between NIT- and corn oil-treated groups when requirements for parametric analysis were met. Comparisons between groups for which variances were not homogenous were made with the Mann-Whitney Rank Sum test. Results are expressed as means ± SEM. A value of $P < 0.05$ was the criterion for significance.

RESULTS

NIT hepatotoxicity

The effects of a single oral administration of 100 mg NIT/kg on markers of liver injury 24 hr after administration are listed in Table 1. ANIT caused a 3-fold elevation of plasma ALT activity and a 6-fold elevation of plasma total bilirubin in comparison with control rats.

Table 1. NIT-induced changes in markers of liver injury 24 hr after administration

Treatment	ALT*	Bilirubin†	Bile flow‡
Corn oil	34 ± 7	0.25 ± 0.05	48 ± 4
ANIT	116 ± 20§	1.55 ± 0.18§	3 ± 2§
BNIT	53 ± 10	0.41 ± 0.02	64 ± 1§

Fasted rats were treated with NIT (100 mg/kg, p.o.) or corn oil 24 hr prior to assessment of liver injury. Values are means ± SEM, N = 4–9.

* Plasma alanine aminotransferase activity is expressed as U/L at 30°.

† Plasma total bilirubin is expressed as mg/dL.

‡ Bile flow is expressed as μL/g liver/30 min.

§ Significantly different from corn oil-treated rats ($P < 0.05$).

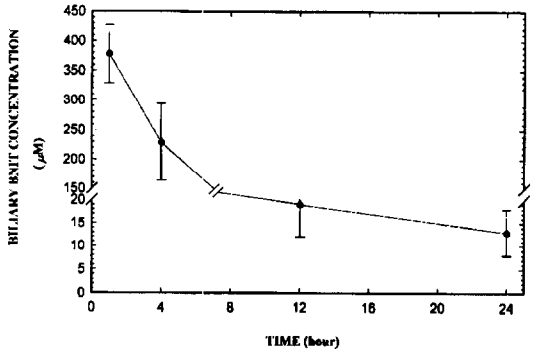

Fig. 1. Concentration of BNIT in bile at various times after BNIT administration (100 mg/kg, p.o.). Values are means ± SEM, N = 4–12.

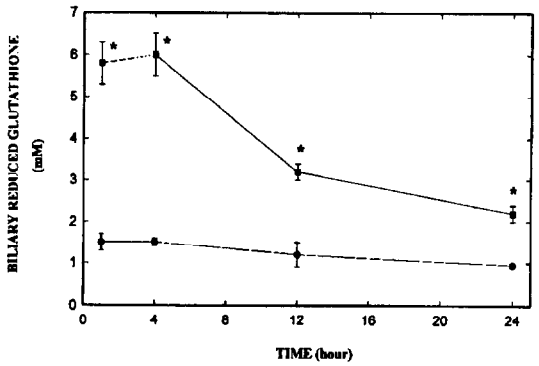

Fig. 3. Concentration of reduced glutathione in bile at various times after administration of BNIT (■) (100 mg/kg, p.o.) or corn oil vehicle (●). Values are means ± SEM N = 3–12. Key: (*) Significantly different from corn oil vehicle ($P < 0.05$).

Bile flow was reduced to <10% of control. BNIT, at this same dose, had no effect on these markers of liver injury.

BNIT disposition

BNIT was detected in bile as early as 1 hr after oral administration (Fig. 1). The largest concentration of BNIT in bile was observed at 1 hr (378 ± 49 μM); concentrations decreased thereafter. BNIT was also detected in plasma at each of the times examined (Fig. 2). The concentrations of BNIT in bile were 20- to 160-fold greater than those in plasma.

Effect of BNIT on bile and liver glutathione status

The concentration of reduced glutathione in bile was increased as much as 3-fold after BNIT administration (Fig. 3). The largest concentrations were observed at 1 and 4 hr with a 50% decline by 12 and 24 hr. In contrast, BNIT caused a modest decrease in the content of reduced glutathione in liver at 1 hr (Fig. 4). This decrease relative to control was more pronounced at 4 hr. Liver reduced glutathione concentrations increased to approximately twice that of control at 12 and 24 hr. In contrast to these effects of BNIT, ANIT has been shown previously [7] to have no marked effect on liver glutathione concentration (reduced or oxidized) during the initial 12 hr post-administration. However, as with BNIT, ANIT did elevate hepatic reduced glutathione concentration by 24 hr after administration.

BNIT had no significant effect on the concentration of oxidized glutathione in bile (Table 2). However, as with reduced glutathione, BNIT caused an initial decrease in liver oxidized glutathione at 1, 4 and 12 hr, which then increased to 360% of control values at 24 hr (Table 2).

Comparison of NIT disposition

Bile and plasma concentrations of ANIT were markedly different from those of BNIT (Table 3). The concentrations of ANIT in plasma were more than 3-fold larger than BNIT at 1 and 4 hr. In contrast, the concentration of ANIT in bile was <30% of that observed for BNIT at these times. The values presented in this table represent data collected from experiments wherein rats were treated with equimolar doses of NIT on the same day.

Effect of NIT on bile glutathione concentration

The concentrations of reduced and oxidized glutathione in bile after ANIT and BNIT administration were evaluated (Table 4). The ANIT-induced increases in reduced glutathione in bile at 1 and 4 hr were not significantly different from those produced by BNIT. The con-

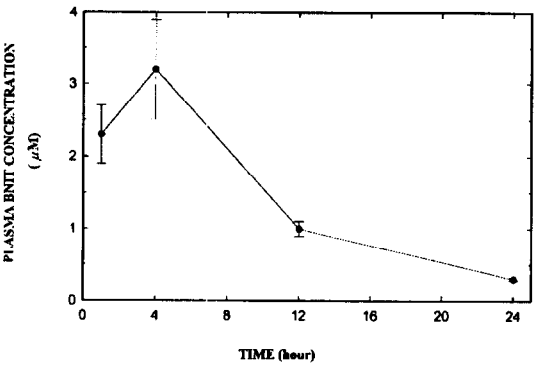

Fig. 2. Concentration of BNIT in plasma at various times after BNIT administration (100 mg/kg, p.o.). Values are means ± SEM, N = 4–12.

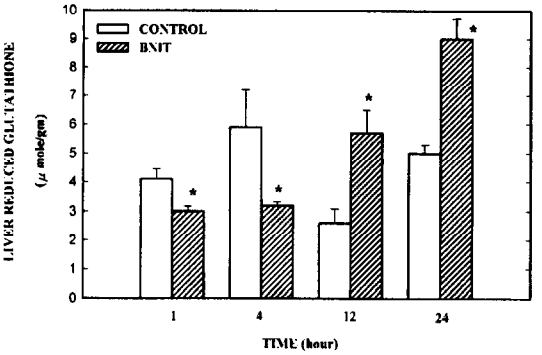

Fig. 4. Time course of BNIT-induced changes in reduced glutathione concentrations in liver. Liver reduced glutathione determinations were made at various times after administration of BNIT (100 mg/kg, p.o.) or corn oil vehicle. Values are means ± SEM, N = 3–12. Key: (*) significantly different from control ($P < 0.05$).

Table 2. Effect of BNIT on oxidized glutathione in bile and liver

Time (hr)	Oxidized glutathione in bile (mM)		Oxidized glutathione in liver (μmol/g liver)	
	Corn oil	BNIT	Corn oil	BNIT
1	0.13 ± 0.03	0.21 ± 0.05	1.33 ± 0.18	0.59 ± 0.15*
4	0.15 ± 0.01	0.29 ± 0.03	0.84 ± 0.44	0.44 ± 0.16
12	0.09 ± 0.02	0.10 ± 0.02	2.75 ± 1.64	1.64 ± 0.19
24	0.12 ± 0.02	0.14 ± 0.03	0.80 ± 0.30	2.88 ± 0.24*

Oxidized glutathione in bile and liver were determined at various times after administration of BNIT (100 mg/kg, p.o.) or corn oil to fasted rats. Values are means ± SEM, N = 3–12.

* Significantly different from corn oil-treated rats ($P < 0.05$).

centration of oxidized glutathione in bile 4 hr after ANIT administration differed significantly from that after BNIT.

Effect of hepatic glutathione depletion on NIT disposition and hepatotoxicity

BSO pretreatment protected against ANIT-induced elevation of total plasma bilirubin and reduced bile flow but was without effect on elevation of plasma ALT (Table 5). Coincident with this protection, BSO markedly reduced the concentration of ANIT in bile (Table 6). BSO administration similarly reduced the concentration of BNIT in bile but was without effect on the plasma concentration of either NIT.

DISCUSSION

ANIT and BNIT are almost identical in structure but differ markedly in their abilities to cause cholangiolitic hepatitis: ANIT administration causes pronounced injury to bile duct epithelial cells, whereas BNIT does not. It is not known if ANIT injures bile duct epithelial cells directly, or whether a metabolite of ANIT or a cytotoxic mediator produced in response to ANIT is involved. In this regard, we have reported that ANIT accumulates in bile within 1 hr after administration [7]. The rapid accumulation of ANIT in bile and consequent exposure of bile duct epithelial cells to large concentrations of ANIT may be of key importance in its ability to injure bile ducts. This led to the hypothesis that BNIT is not hepatotoxic because it does not accumulate in bile. However, as reported herein, BNIT does indeed accumulate in bile and to concentrations larger than those observed for an equimolar dose of ANIT. Therefore, the different hepatotoxic responses of ANIT and BNIT cannot be attributed to an inability of BNIT to accumulate in bile.

ANIT-induced hepatotoxicity is dependent upon hepatic glutathione. In a previous study [8], ANIT caused a modest increase in serum aspartate aminotransferase activity, a marked elevation of serum total bilirubin, and cholestasis 24 hr after administration. BSO administration completely protected against these changes. Similar results were obtained in the current study with the exception of plasma ALT elevation (Table 5). BSO treatment had no effect on this marker of hepatic parenchymal cell injury, suggesting that hepatic parenchymal cell injury may be independent of hepatic glutathione status. Another possibility may be that BSO treatment had sensitized hepatic parenchymal cells to ANIT by virtue of reduced levels of intracellular glutathione. The difference between these two studies may relate to the choice of marker enzymes used to assess hepatic parenchymal cell injury. Further study will be required to resolve this discrepancy. However, both studies demonstrated protection by BSO against hyperbilirubinemia and cholestasis, observations consistent with reduced bile duct epithelial cell injury.

Studies *in vitro* have identified a reversible glutathi-

Table 3. Comparison of NIT disposition

	NIT (μM)	
	1 hr	4 hr
Plasma		
ANIT	10.5 ± 1.3*	7.9 ± 1.3*
BNIT	2.0 ± 0.8	2.1 ± 0.4
Bile		
ANIT	78 ± 19*	29 ± 13*
BNIT	292 ± 83	235 ± 100

Plasma and bile NIT concentrations were determined 1 and 4 hr after administration of NIT (100 mg/kg, p.o.) to fasted rats. Values are means ± SEM, N = 5.

* Significantly different from BNIT at this time ($P < 0.05$).

Table 4. Concentration of glutathione in bile after NIT administration

Time (hr)	NIT	Glutathione (mM)	
		Reduced	Oxidized
1	ANIT	5.2 ± 0.5	0.40 ± 0.04
	BNIT	7.1 ± 0.7	0.32 ± 0.09
4	ANIT	5.1 ± 0.6	0.59 ± 0.10*
	BNIT	5.5 ± 0.5	0.31 ± 0.02

Glutathione concentrations were determined 1 and 4 hr after administration of ANIT or BNIT (100 mg/kg, p.o.). Values are means ± SEM, N = 4–5. Bile glutathione concentrations in rats treated with corn oil vehicle routinely range between 1.5 and 3.0 mM reduced glutathione and 0.1 and 0.3 mM oxidized glutathione within 4 hr of administration.

* Significantly different from BNIT at this time ($P < 0.05$).

Table 5. Effect of BSO on ANIT hepatotoxicity

Treatment	BSO	ALT*	Bilirubin†	Bile flow‡
Control	–	24 ± 4	0.16 ± 0.03	46 ± 4
Control	+	25 ± 4	0.22 ± 0.06	40 ± 4
ANIT	–	115 ± 20	1.55 ± 0.18§	3 ± 2§
ANIT	+	118 ± 36	0.24 ± 0.04	42 ± 2

Fasted rats received BSO (890 mg/kg, i.p.) or saline vehicle 2 hr before and 12 hr after administration of ANIT (100 mg/kg, p.o.) or corn oil vehicle. Markers of liver injury were examined 24 hr after ANIT administration. Values are means ± SEM, N = 4–8.

* Alanine aminotransferase is expressed as U/L at 30°.

† Plasma total bilirubin is expressed as mg/dL.

‡ Bile flow is expressed as μL/g liver/30 min.

§ Significantly different from BSO treatment ($P < 0.05$).

Table 6. Effect of BSO on NIT disposition

NIT	BSO	NIT (μM) Bile	NIT (μM) Plasma
ANIT	–	79 ± 20*	12 ± 1.5
ANIT	+	11 ± 3	15 ± 1.3
BNIT	–	430 ± 99*	1.8 ± 0.2
BNIT	+	69 ± 14	2.1 ± 0.6

Fasted rats were given BSO (890 mg/kg, i.p.) 2 hr prior to receiving NIT (100 mg/kg, p.o.). Samples were collected 1 hr after NIT administration. Values are means ± SEM, N = 4–6.

* Significantly different from respective BSO-treated rats ($P < 0.05$).

one–ANIT conjugate in medium from ANIT-treated hepatic parenchymal cell suspensions [6]. It was postulated that the export of this conjugate may be responsible for the rapid movement of intracellular glutathione to the extracellular space. Our recent observation that reduced glutathione concentrations in bile increase in concert with the rapid accumulation of ANIT suggested that a glutathione–ANIT conjugate may be involved in accumulation of ANIT in bile *in vivo* [7]. As for ANIT, BNIT accumulation in bile was coincident with a marked increase in biliary reduced glutathione concentration. The use of BSO to decrease hepatic glutathione prior to NIT administration was associated with markedly reduced bile concentrations of both NITs. This observation supports the hypothesis that hepatic glutathione is directly involved in NIT translocation into bile. The involvement of a reversible glutathione conjugate is suspected but remains unproven for BNIT.

The concentration of reduced glutathione in bile was increased by approximately 3 mM at 1 and 4 hr after administration of ANIT or BNIT. The magnitude of this increase greatly exceeded the concentration of either NIT in bile. The underlying cause for this disparity is not known. One possibility is that NIT accumulation in bile promotes, by secondary mechanisms, the export of large quantities of glutathione. Another is that ANIT and BNIT are transported into bile as reversible glutathione conjugates that decompose to yield free NIT and glutathione. Because ANIT and BNIT are lipophilic and chemically reactive species, they are likely to be reabsorbed into bile duct epithelium or bind to it. In this regard, the concentrations of NIT in bile collected from the common bile duct are likely lower than that of bile within the intrahepatic biliary tree. In contrast, glutathione, being highly hydrophilic, would be expected to remain in bile and be excreted or ultimately metabolized by γ-glutamyltranspeptidase. Accordingly, even if NIT and glutathione in bile arose from transport of a reversible glutathione–NIT conjugate, differences in the concentrations of NIT and glutathione in bile might be expected.

It is notable that significantly less ANIT was detected in bile with respect to BNIT, even though both elevated glutathione to similar concentrations. Of what importance this is to toxic outcome is uncertain. However, it is interesting to speculate that the lower concentrations of ANIT indicate a greater partitioning of ANIT into bile duct epithelial cells and that this ultimately gives rise to injury.

The temporal character of biliary BNIT concentration was similar to that reported previously for ANIT [7]. That bile NIT concentrations decrease without a concomitant decrease in plasma concentration suggests a reduction in the hepatic translocation of NIT into bile. The early decrease in bile concentrations of NIT cannot be attributed to a reduction in bile flow because decreases in bile flow were not observed within the first 12 hr after NIT administration (data not shown). Furthermore, reductions in NIT accumulation in bile were not likely due to limited availability of glutathione because hepatic glutathione concentration remained unchanged by ANIT [7] and was affected only modestly by BNIT (Fig. 4). It may be that, as reactive isothiocyanates, ANIT and BNIT inactivate glutathione *S*-transferases and/or canalicular membrane transport processes responsible for NIT translocation into bile and thereby produce the time-dependent reduction in bile NIT concentrations. Whether time-dependent decreases in NIT concentrations result from overt hepatotoxicity is doubtful. Plasma markers of hepatic parenchymal cell injury are not apparent until 12 hr after ANIT administration and not at all after BNIT administration.

In conclusion, we found that ANIT and BNIT accumulate in bile in a glutathione-dependent manner. BNIT accumulated in bile to significantly larger concentrations than ANIT, yet each produced a similar elevation of reduced glutathione. These data demonstrate that bile duct epithelial cells are exposed to large concentrations of BNIT and that its lack of hepatotoxicity cannot be attributed to an inability to accumulate in bile.

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