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CHOLESTATIC POTENTIALS OF α-NAPHTHYLISOTHIOCYANATE (ANIT) AND β-NAPHTHYLISOTHIOCYANATE (BNIT) IN THE ISOLATED PERFUSED RAT LIVER

DAVID C. KOSSOR, JEFFREY A. HANDLER, DEANNE M. DULIK, PAUL C. MEUNIER, THOMAS B. LEONARD and ROBIN S. GOLDSTEIN*

Departments of Toxicology and Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406, U.S.A.

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Abstract—Previous studies in rats have shown that a single oral dose of α-naphthylisothiocyanate (ANIT), but not the regioisomer β-naphthylisothiocyanate (BNIT), results in intrahepatic cholestasis. The present studies were designed to evaluate the intrinsic cholestatic potential of ANIT and BNIT in the isolated perfused rat liver. Livers from male Sprague–Dawley rats (300–450 g) were isolated and perfused with Krebs–Henseleit buffer supplemented with 50 μM taurocholate and ANIT or BNIT (0, 5, 15 or 50 μM). Rates of bile flow, bile acid uptake and bile acid excretion were monitored for up to 70 min. Permeability of tight junctions also was evaluated. At concentrations of 5 μM, neither ANIT nor BNIT altered hepatobiliary function or tight junction permeability. In contrast, perfusion with 50 μM ANIT or BNIT for 35 min resulted in decreases in bile flow rates of 19 ± 8 and 13 ± 4%, respectively. After 70 min of perfusion with ANIT or BNIT, rates of bile flow were decreased by 78 ± 5 and 71 ± 4%, respectively. Bile acid excretion also was decreased following perfusion with 50 μM ANIT or BNIT. Perfusion with 50 μM ANIT or BNIT decreased bile acid uptake by 51 ± 13 and 46 ± 6%, respectively, at 60 min. Bile/plasma (B/P) ratios of [^{3}H]sucrose were not affected by ANIT or BNIT at any time during perfusion, indicating that changes in bile flow and bile acid excretion in the isolated perfused liver were not associated with increased hepatocyte tight junction permeability. These data demonstrate that the direct portal infusion of a 50 μM concentration of either ANIT or BNIT produced marked decreases in bile flow, indicating that these isomers have a comparable intrinsic cholestatic potential in the isolated perfused liver.

Administration of a single oral dose of α-naphthylisothiocyanate (ANIT)† produces hyperbilirubinemia, cholestasis, hepatocellular and biliary epithelial cell necrosis and bile duct obstruction [1]. Structure–activity investigations of ANIT-induced hyperbilirubinemia in mice have demonstrated a requirement for two specific structural features: the presence of sulfur rather than oxygen (α-naphthylisocyanate did not cause hyperbilirubinemia), and an aryl hydrocarbon moiety in a planar configuration [2]. β-Naphthylisothiocyanate (BNIT), which satisfies both of these structural criteria, produces some of the same biochemical changes observed in mice after ANIT treatment, i.e. increased hepatic DT-diaphorase activity and non-protein sulfhydryl content [3, 4] and decreased hepatic mixed-function oxidase activity [5]. In contrast to ANIT, a single oral dose of up to 150 mg/kg of BNIT does not produce hyperbilirubinemia or cholestasis when given orally to mice [2] or rats [1, 4], and does not produce increases in serum activities of aspartate aminotransferase (AST) or γ-glutamyltranspeptidase in rats [4]. Furthermore, the nature of the morphologic changes in the liver of rats given ANIT in the diet for 4 weeks is qualitatively different from those observed in rats treated with dietary BNIT. Specifically, ANIT treatment results in extensive bile duct proliferation accompanied by focal hepatocellular necrosis with a modest inflammatory cell infiltrate [5], whereas hepatobiliary lesions associated with BNIT are limited to multifocal granulomatous inflammatory lesions in the absence of hepatocellular necrosis or bile duct proliferation [6].

The reasons underlying the different effects of ANIT and BNIT on hepatobiliary function and morphology *in vivo* are not known [1]. However, recent studies have indicated that circulating neutrophils (PMN) may play a role in ANIT-induced liver injury and that ANIT, but not BNIT, activates PMNs to release superoxide [7, 8]. Taken together, these data suggest that isomeric differences in naphthylisothiocyanate-induced hepatotoxicity may be due, in part, to isomeric differences in PMN activation. Alternatively, differences in pharmacokinetics, metabolism, plasma protein binding or the cholestatic potential of these agents may underlie the isomeric differences in naphthylisothiocyanate-induced hepatotoxicity *in vivo*.

* Corresponding author: Robin S. Goldstein, Ph.D., SmithKline Beecham Pharmaceuticals, Department of Toxicology, L64, 709 Swedeland Road, King of Prussia, PA 19406. Tel. (215) 270-7552; FAX (215) 270-7622.

† Abbreviations: ANIT, α-naphthylisothiocyanate; BNIT, β-naphthylisothiocyanate; AST, aspartate aminotransferase; PMN, neutrophil; TC, taurocholate; NAD^+, nicotinamide adenine dinucleotide (oxidized); 3α-HSD, 3α-hydroxysteroid dehydrogenase; and HRP, horseradish peroxidase.

In the rat, oral administration of ANIT, but not BNIT, induces cholestasis. However, the intrinsic cholestatic potential (i.e. the ability to induce cholestasis in the absence of extra-hepatic variables) of these isomers has not been determined. The present studies were designed to evaluate the cholestatic potential of ANIT and BNIT by determining the hepatobiliary effects of these isomers in isolated perfused rat livers where variations due to pharmacokinetics or circulating PMNs are eliminated. In the isolated perfused liver, an equivalent hepatic exposure to ANIT or BNIT was achieved and maintained by a constant portal infusion of each compound.

MATERIALS AND METHODS

Materials. α-Naphthylisothiocyanate (purity >96%), corn oil, taurocholic acid (TC) and oxidized nicotinamide adenine dinucleotide (NAD^+) were purchased from the Sigma Chemical Co. (St. Louis, MO). β-Naphthylisothiocyanate (purity = 98%) was obtained from the Aldrich Chemical Co. (Milwaukee, WI), and 3α-hydroxysteroid dehydrogenase (3α-HSD) from the Worthington Biochemical Corp. (Freehold, NJ). [^{3}H]Sucrose (12.5 mCi/mmol) was purchased from the Amersham Corp. (Arlington Heights, IL), and lecithin (Epikuron 100 PI) from Lucas Meyer, Inc. (Decatur, IL).

Animals. Adult male Sprague–Dawley rats (CD-VAF) (Charles River Laboratories, Raleigh, NC) weighing between 350 and 500 g and approximately 10–12 weeks of age were used in these studies. Rats were housed individually in stainless steel cages in an environmentally controlled room (72 ± 4°F; 50 ± 10% relative humidity) with a 12-hr light–dark cycle. Filtered tap water and food (Certified Rodent Chow No. 5002; Purina Mills, Inc., St. Louis, MO) were provided *ad lib.*

Liver perfusion. Rats were anesthetized with sodium pentobarbital (75 mg/kg, i.p.). The bile duct was cannulated with PE10 tubing (Clay Adams Inc., Parsippany, NJ), and bile was collected into preweighed tubes, without recirculation through the liver. The portal vein and ascending vena cava were cannulated with PE50 tubing. Livers were excised and perfused in a non-recirculating system with Krebs–Henseleit buffer (pH 7.4, 37°) containing 50 μM TC for up to 80 min at a rate of 4 mL·min^{-1}·g liver^{-1} [9]. The perfusate was saturated with 95% O_2/5% CO_2 and oxygen tension in effluent perfusate was monitored using a Clark-type electrode. ANIT or BNIT was dissolved in corn oil and added to an equivalent volume of 15% (w/v) lecithin in deionized water; components were mixed vigorously for 5 min until emulsified. A vehicle control was prepared using corn oil and an equivalent amount of 15% lecithin without ANIT or BNIT. After a 10-min period of bile collection to establish baseline conditions, emulsions were added to perfusate at a ratio of 1:5000 to yield final perfusate concentrations of 0, 5, 15 and 50 μM, and three to seven livers were perfused at each concentration.

Bile acid concentrations in the perfusate and the bile were determined spectrophotometrically according to the method of Koss *et al.* [10], and rates of bile acid uptake were calculated from the equation:

$$\frac{[(\text{TC conc.}_{(in)} - \text{TC conc.}_{(out)})(\text{mL perfusate/min})]}{(\text{g wet weight liver})}.$$

Bile was collected at 5-min intervals and rates of bile flow were determined gravimetrically. The total bile acid content of each sample was determined, and rates of bile acid excretion were calculated from the equation:

$$\frac{(\text{bile acid conc. in bile})(\text{bile flow})}{(\text{g wet weight liver})}.$$

Tight junction permeability was assessed by measuring the bile/perfusate (B/P) ratio of [^{3}H]-sucrose [11]. Briefly, trace amounts of [^{3}H]sucrose (final concentration = 10^4 dpm/mL) were infused throughout the experimental period, and aliquots of perfusate effluent and bile were collected at 5-min intervals and assayed via scintillation spectroscopy. Sucrose was used in lieu of horseradish peroxidase (HRP) in these studies because ANIT and BNIT interfered with the spectrophotometric detection of peroxidase activity (data not shown).

Histology. Immediately after perfusion with either vehicle or vehicle containing a 50 μM concentration of either ANIT or BNIT, sections (4–5 μm) were collected from the left lateral lobe of each liver and were fixed in 10% buffered formalin. Sections were stained with hematoxolin and eosin and were examined using light microscopy.

Statistics. Data are expressed as means ± SEM and were analyzed by the General Linear Models procedure followed by the least-squares means test [12]. The criterion of significance was $P < 0.05$.

RESULTS AND DISCUSSION

The studies reported herein were designed to evaluate the cholestatic potential of ANIT and BNIT in the isolated perfused liver. Perfusion with 5 μM ANIT or BNIT had no effect on bile flow, while perfusion with 15 μM ANIT or BNIT resulted in a modest decrease in bile flow after 70 min of perfusion (Fig. 1). Increasing the concentration of ANIT or BNIT to 50 μM produced a significant time-dependent decrease in bile flow. Following 35 min of infusion, bile flow was reduced to 125 ± 13 and 137 ± 6 μL·(g liver)$^{-1}$·hr^{-1} in ANIT- and BNIT-treated livers, respectively, compared with the control rate of 155 ± 4. By 70 min, bile flow was decreased to 31 ± 7 and 42 ± 5 μL·(g liver)$^{-1}$·hr^{-1}, respectively, compared with the control rate of 143 ± 8 (Fig. 1). These results demonstrate that ANIT and BNIT have a comparable cholestatic potential in isolated perfused rat livers.

No significant change in bile acid excretion was observed following perfusion with 5 or 15 μM ANIT or BNIT. Time-dependent decreases in bile acid excretion rates, which paralleled the decreases in bile flow, were observed following perfusion with 50 μM ANIT or BNIT (Fig. 2). Following 35 min of perfusion, bile acid excretion rates were decreased to 5.1 ± 0.5 and 6.5 ± 0.3 μmol·(g liver)$^{-1}$·hr^{-1} in ANIT- and BNIT-treated livers, respectively,

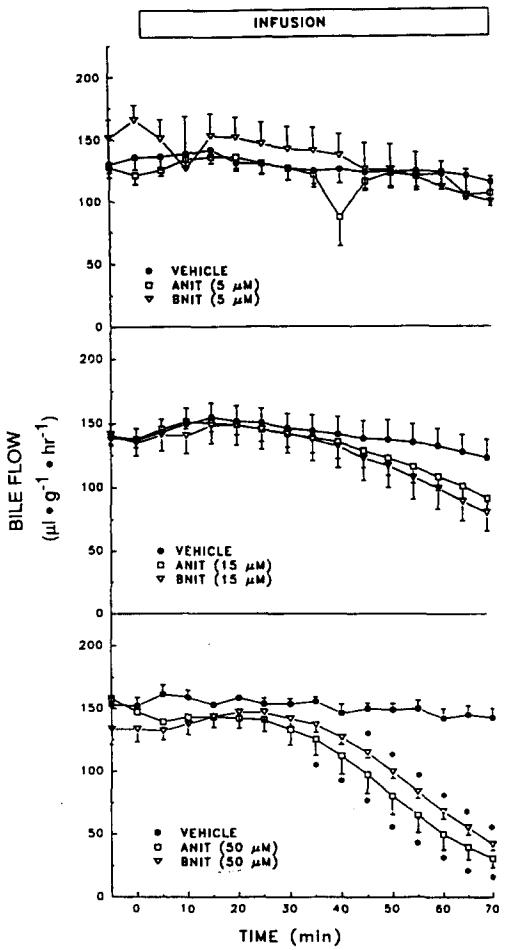

Fig. 1. Time–course of ANIT and BNIT effects on rates of bile flow. Isolated perfused rat livers were perfused for 10 min with Krebs–Henseleit buffer as described in Materials and Methods prior to the infusion of emulsion containing ANIT or BNIT. The final perfusate concentrations of ANIT or BNIT were 5 (top), 15 (middle) or 50 μM (bottom). Values are means ± SEM of 3–7 livers per time point, and asterisks denote significant differences compared with time-matched vehicle controls ($P < 0.05$).

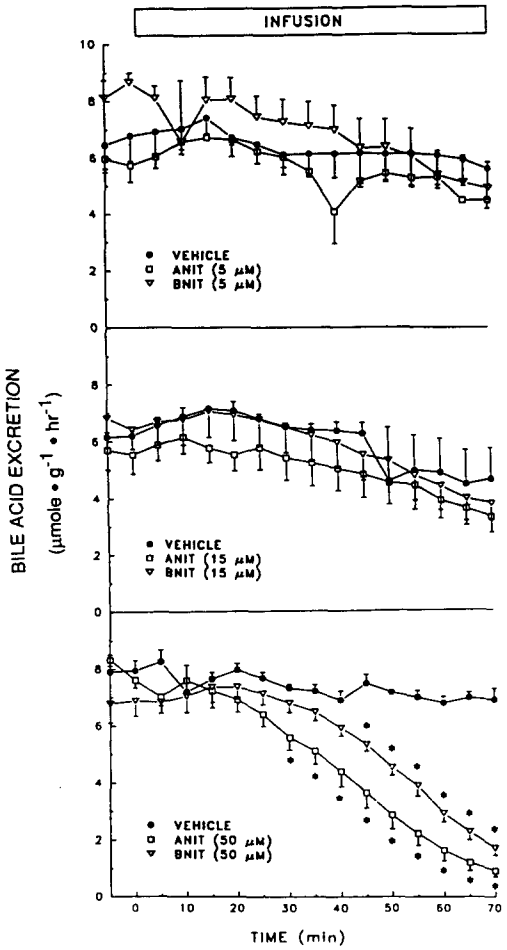

Fig. 2. Time–course of ANIT and BNIT effects on rates of bile acid excretion. Bile was collected at 5-min intervals, and bile acid concentrations were determined as described in Materials and Methods. Bile was collected for 10 min prior to the infusion of emulsion containing ANIT or BNIT. The final perfusate concentrations of ANIT or BNIT were 5 (top), 15 (middle), or 50 μM (bottom). Values are means ± SEM of 3–7 livers per time point, and asterisks denote significant differences compared with time-matched vehicle controls ($P < 0.05$).

compared with control rates of 7.2 ± 0.2 μmol·(g liver)$^{-1}$·hr^{-1}. By 70 min, bile acid excretion rates were reduced to 0.9 ± 0.2 and 1.7 ± 0.2 μmol·(g liver)$^{-1}$·hr^{-1}, respectively, compared with the control rate of 6.9 ± 0.4 μmol·(g liver)$^{-1}$·hr^{-1}. ANIT- and BNIT-induced decreases in bile acid excretion may have been due to decreased hepatocellular uptake of bile acids, decreased canalicular transport of bile acids, regurgitation of bile acids across tight junctions and/or bile duct obstruction. The initial decreases in bile acid excretion were observed at 35 min of perfusion with ANIT or BNIT, at which time the rates of bile acid uptake were comparable in control, ANIT- and BNIT-treated livers (Fig. 3). Reductions in bile acid uptake were only apparent after 50–70 min of perfusion with either ANIT or BNIT (Fig. 3). These results support the conclusion that the onset of ANIT- and BNIT-related decreases in bile flow and bile acid excretion were not due to a decreased hepatocellular uptake of bile acids.

Rates of oxygen uptake of approximately 100 μmol·(g liver)$^{-1}$·hr^{-1} were comparable among livers perfused with either 50 μM ANIT, 50 μM BNIT or emulsion alone (Fig. 4). The rate of oxygen uptake was constant throughout the 70 min of perfusion. When compared with baseline values, the addition of a corn oil/lecithin emulsion to the influent perfusate resulted in a slight increase in oxygen uptake that was not statistically significant (Fig. 4).

Light microscopic evaluation of livers perfused with either vehicle or 50 μM ANIT or BNIT indicated that the lumens of the bile ducts were free of debris (data not shown), suggesting that ANIT and BNIT cholestasis was not secondary to bile duct obstruction

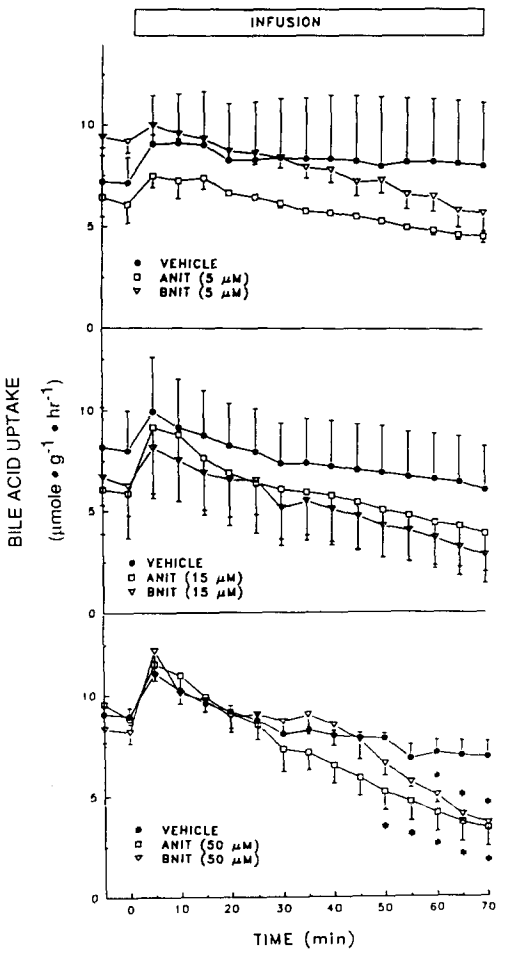

Fig. 3. Time–course of ANIT and BNIT effects on rates of bile acid uptake. Bile and effluent perfusate were collected at 5-min intervals, and bile acid concentrations were determined as described in Materials and Methods. Samples were collected for 10 min prior to the infusion of emulsion containing ANIT or BNIT. The final perfusate concentrations of ANIT or BNIT were 5 (top), 15 (middle), or 50 μM (bottom). Values are means ± SEM of 3–7 livers per time point, and asterisks denote significant differences compared with time-matched vehicle controls ($P < 0.05$).

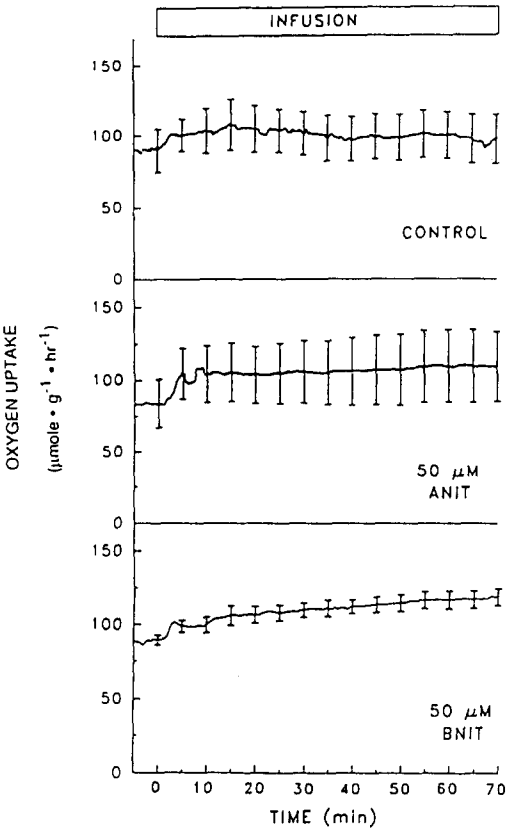

Fig. 4. Time–course of hepatic oxygen uptake. Perfusate contained either vehicle (top), 50 μM ANIT (middle) or 50 μM BNIT (bottom), and oxygen uptake was calculated from the difference in oxygen tension measured in influent and effluent perfusate. Values are means ± SEM of 3–7 livers per time point.

and was likely due to hepatocanalicular dysfunction. Previous *in vivo* studies in rats treated with a single oral dose of ANIT also demonstrated that the onset of cholestasis was not accompanied by morphologic evidence of bile duct obstruction and thus was a consequence of decreased hepatocanalicular function [1, 13].

In the present studies, neither ANIT nor BNIT, at concentrations that produced profound cholestasis (50 μM), altered tight junction permeability as assessed by the biliary excretion of [^{3}H]sucrose (Fig. 5). Although previous studies have demonstrated that increased tight junction permeability precedes the onset of cholestasis, suggesting a causal relationship [14–17], other studies have suggested that cholestasis and tight junction permeability may be unrelated [13, 18]. The observation that ANIT-induced cholestasis in the isolated perfused rat liver occurred in the absence of any effect on tight junction permeability further suggests that an increase in tight junction permeability is not obligatory for the production of ANIT-induced cholestasis.

The present studies provide the first indication that the cholestatic potential of BNIT is comparable with that of ANIT in the isolated perfused liver. The reason BNIT produces cholestasis in the isolated perfused liver but not in the intact rat may be explained in part by *in vivo/in vitro* differences in the hepatic exposure to BNIT and/or its metabolites. ANIT-induced cholestasis is dependent upon the extent of hepatic exposure to the parent compound and/or its metabolites, since interruption of enterohepatic circulation is known to prevent ANIT-induced cholestasis [19]. The oral pharmacokinetics, protein binding and *in vivo* metabolism of ANIT and BNIT have not been reported. Consequently, it is not known if hepatic exposure to unbound ANIT or BNIT or their metabolites in the intact rat is equivalent to that achieved in the isolated perfused liver. If the difference in the cholestatic potential of

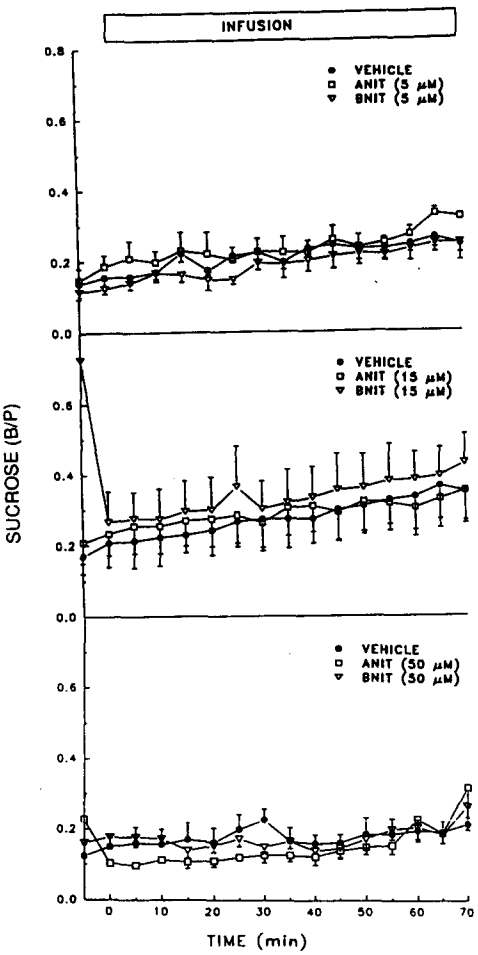

Fig. 5. Time-course of ANIT and BNIT effects on tight junction permeability. Tight junction permeability was assessed by measuring the B/P ratio of [^{3}H]sucrose as described in Materials and Methods. The infusion of [^{3}H]-sucrose in Krebs–Henseleit buffer was initiated 10 min prior to the infusion of emulsion containing ANIT or BNIT. The final perfusate concentrations of ANIT or BNIT were 5 (top), 15 (middle), or 50 μM (bottom). Values are means ± SEM of 3–7 livers per time point.

these isomers *in vivo* is due to differences in metabolism, disposition and/or pharmacokinetics, those differences would not be observed in the isolated perfused liver where hepatic exposure to BNIT was comparable with that of ANIT. Alternatively, the route of exposure may contribute to differences in the hepatic exposure to these compounds. Although ANIT and BNIT produce different effects on hepatobiliary function when given orally, no studies have been done to compare their cholestatic potential following intravenous administration. Thus, it is possible that the cholestatic effects of ANIT or BNIT observed in the isolated perfused liver may be produced *in vivo* following intravenous administration of either ANIT or BNIT.

Although previous studies have indicated that oral BNIT treatment does not produce cholestasis in mice or rats 24 hr after treatment [3, 4], detailed dose–response and time–response studies on the hepatobiliary effects of BNIT *in vivo* have not been reported. Since the cholestatic potentials of ANIT and BNIT are comparable *in vitro*, the differences in the cholestatic effects of these isomers *in vivo* may be related to the more delayed intrahepatic effects produced by ANIT, but not BNIT, in the intact rat, that do not occur in the isolated perfused liver. Previous *in vivo* studies have suggested that ANIT-induced cholestasis is mediated by increased tight junction permeability [14–17], hepatocanalicular dysfunction [13, 20, 21] and bile duct obstruction [13, 22–26]. In contrast to ANIT cholestasis *in vivo*, the cholestatic effects of ANIT (or BNIT) in the isolated perfused rat liver were due solely to hepatocanalicular dysfunction in the absence of an increase in tight junction permeability or bile duct obstruction. Thus, cholestasis produced by ANIT or BNIT *in vitro* was mediated by only one of the three mechanisms identified for ANIT-induced cholestasis *in vivo*. The differences in the cholestatic potential of ANIT and BNIT *in vivo*, therefore, may be related to the fact that treatment with ANIT, but not BNIT, results in biliary epithelial cell necrosis leading to bile duct obstruction [1, 3, 4], an effect that augments the severity and/or duration of cholestasis. Thus, one of the important differences in the ability of ANIT and BNIT to induce cholestasis 24 hr after treatment may be related to differential cytotoxic effects of these chemicals and/or their metabolites on biliary epithelial cells leading to bile duct obstruction.

The comparable cholestatic effects of ANIT and BNIT *in vitro* also suggest that extrahepatic factors such as circulating neutrophils (PMNs) may contribute to ANIT, but not BNIT, cholestasis *in vivo*. ANIT cholestasis *in vivo* has been reported to depend upon PMN activation [7]. Since PMNs are activated by ANIT, but not by BNIT [8], PMN-dependent hepatobiliary damage may contribute to the isomeric differences in cholestatic potential *in vivo*, but not *in vitro* where there are negligible (if any) PMNs. *In vivo/in vitro* differences in the cholestatic potential of ANIT and BNIT therefore may be due, in part, to differences in hepatic exposure, PMN activation, and/or potential cytotoxic effects on the biliary epithelium.

In summary, these *in vitro* data indicate that 50 μM ANIT or BNIT causes hepatocanalicular dysfunction and cholestasis in the isolated perfused rat liver in the absence of altered tight junction permeability or bile duct obstruction. These data suggest that ANIT and BNIT have a comparable intrinsic cholestatic potential.

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