Alpha-naphthylisothiocyanate-induced elevation of serum bile acids: lack of causative effect on bile acid transport

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Abstract

In recent years chemicals including chlorinated solvents have been found to interfere with the transport of bile acids (BA) by hepatocytes, which probably accounts for the raised serum bile acids (SBA) after exposure. However, the known cholestatic agent, α-naphthylisothiocyanate (ANIT) has never been fully examined for its effect on these processes. Accordingly, the direct effects in vitro and the effects of in vivo treatment on bile acid transport have been investigated in this study. Direct addition of ANIT (5–100 μM) to hepatocytes isolated from untreated rats did not result in any change in uptake or efflux of taurocholic acid (TC), one of the most obviously elevated SBA in ANIT-treated rats. Additionally, accumulation of TC over an extended incubation period was not affected by ANIT. In vivo treatment with ANIT (50 μmol/kg i.p. on each of 3 consecutive days) resulted in a marked elevation of total serum bile acids (TSBA) and a slight increase in the activity of serum alkaline phosphatase (ALP) and a very mild hyperbilirubinemia, while other markers of liver injury were unaltered. In hepatocytes isolated from these rats, $K_{in}$ and $V_{max}$ for uptake and $V_0$ for efflux were no different between ANIT and vehicle-treated animals. In conclusion, ANIT showed no effects

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine amino transferase; ANIT, α-naphthylisothiocyanate; AST, aspartate amino transferase; BA, bile acids; DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; LDH, lactate dehydrogenase; SBA, serum bile acids; TC, taurocholic acid; TSBA, total serum bile acids.

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on transport of BA on in vitro exposure or after treatment in vivo where SBA were clearly elevated. The lack of effects of ANIT on transport of bile acids is consistent with other postulated mechanisms of action. Furthermore, this indicates that the effects noted with solvents are not necessarily replicated by substances known to cause histopathological cholestasis.

**Keywords:** α-Naphthylisothiocyanate; Cholestasis; Bile acids; Uptake; Efflux

### 1. Introduction

Chlorinated solvents have been well recognised as potential hepatotoxic chemicals [1]. Raised concentrations of serum bile acids (SBA) have been reported on exposure to these chemicals [2-5]. Interestingly, increased concentrations of SBA after exposure to low levels of a variety of organic solvents was evident in the absence of any other biochemical or histological alterations [5,6]. Similarly, the known cholestatic agent, α-naphthylisothiocyanate (ANIT), was found to increase the total serum bile acids (TSBA) and some individual bile acids at doses below those at which other parameters of liver function were affected, with taurocholic acid (TC) being elevated at a dose lower than any of the other bile acids [2]. SBA have been suggested as a sensitive test to detect early changes of hepatic function [2,3,5]. Therefore, it is of potential concern when substances are found to increase SBA. However, there is evidence to show that elevated SBA after exposure to chlorinated solvents may be due to the ability of these substances to interfere with the bile acid transport by hepatocytes [7].

To gain further insight into the possibility that the effects noted might be a general effect of solvents due to their high lipid solubility and consequently their ability to dissolve in hepatocyte membrane (the site of bile acid uptake), it was decided to investigate the effects of a non-solvent, with known cholestatic properties, on the same processes. ANIT was considered to satisfy these criteria as it has been used as an experimental cholestatic hepatotoxicant for more than 3 decades. It causes cholestasis and hyperbilirubinemia in rats and mice in a dose dependent and reproducible manner [8]. Acute administration of a single oral dose of ANIT to rats results in an intrahepatic cholestasis within 16–24 h which is marked by necrosis of biliary epithelial cells, focal injury to hepatocytes primarily in the periportal area, reduced biliary excretion of bilirubin and BA, hyperbilirubinemia and elevated SBA [8–10].

The mechanisms of toxicity of ANIT have not been clearly established. In recent years, several attempts have been made to elucidate the pathogenesis of ANIT-induced hepatic injury. Depletion of liver cell glutathione (GSH) and release of cytotoxic mediators such as superoxide have been implicated as factors which may be linked to toxicity of ANIT to hepatocytes. However, the mechanism of action remains to be clarified [11,12]. It has been postulated that ANIT-induced cholestasis may be mediated by increased permeability of hepatic tight junctions [13–19]. More recently it has been suggested that the process may be biphasic with changes in tight junctions associated with an earlier phase, while a later phase is linked to bile duct obstruction [20].
As the mechanism of ANIT-induced cholestasis remains uncertain, our studies were undertaken to investigate any possible role of an inhibition of bile acid transport in the ANIT-induced elevation of serum bile acids.

2. Materials and methods

2.1. Chemicals

Taurocholic acid (TC) \([^3\text{H(G)}]\) (2 Ci/mmol) was purchased from Du Pont (Sydney, Australia). ANIT (>95% purity), corn oil and DMSO were purchased from Sigma Chemical Co. (St Louis, MO, USA). Percoll was obtained from Pharmacia Biotech AB (Uppsala, Sweden). Collagenase (144 U/mg solid, Type CLS2) was supplied by Worthington Biochemical Corporation, Freehold, NJ, USA. HEPES was purchased from Calbiochem-Behring, San Diego, CA, USA. All other chemicals were of the highest purity commercially available which were purchased from local agents and distributors.

2.2. Animals and treatment

Male Sprague-Dawley rats weighing 250-350 g (9-12 weeks of age) from the University of Sydney Animal House were used as experimental animals. They were fed with normal laboratory diet (Rodent cubes, Allied Stock Feeds, Sydney) and water ad libitum. Animals were housed in plastic cages with a stainless steel cover in an environmentally controlled room which was automatically maintained at a temperature of about 25°C and a relative humidity of 50 ± 10% and a daily cycle of 12 h light/12 h dark. For in vivo experiments, ANIT (50 μmol/kg i.p.) suspended in corn oil in a final volume of 1 ml/kg was administered to rats at about 09:00 h on each of 3 consecutive days. The dose was selected on the basis of a previous study [2]. All of the control rats received the same amount of vehicle in the same manner. Immediately after the last treatment, food was removed from the animals and 4 h later under halothane anaesthesia either blood samples were taken from the abdominal aorta for liver function tests or in situ perfusion of liver for isolation of hepatocytes was performed. In vitro experiments were conducted with cells from both untreated and treated animals.

2.3. Isolation of hepatocytes

Hepatocytes were isolated from untreated and treated rats by the collagenase digestion method as originally described by Berry and Friend [21], with a few modifications [22] plus an additional percoll centrifugation step [23]. After isolation, hepatocytes were resuspended in Hanks-Hepes buffer at a concentration of \(1.4 \times 10^6\) cells/ml for uptake and \(1 \times 10^7\) cells/ml for efflux. Initial viability, as determined by the Trypan blue exclusion test, was in the range of 90-95%.

2.4. Determination of intracellular potassium ion (K+⁰) and hepatocyte enzyme activities

Twenty min after incubation of the cells with vehicle (4 μl DMSO) or ANIT (final concentration in the flask 5-100 μM) dissolved in 4 μl DMSO at 37°C, intracellular potassium ion content and leakage of cytosolic enzymes, as indices of cell viability,
were determined. Aliquots of 200 μl of cell suspension were centrifuged for 5 s in a Beckman microfuge (Fullerton, CA) for separation of the cells from incubation medium using the silicone oil layer method [24]. Assays for ALT, AST and LDH were carried out on the supernatants with a Roche Centrifichem (Model 400) and appropriate kits (Roche Diagnostic System for ALT and AST and Trace Scientific Pty. Ltd. Melbourne, Australia for LDH). The bottom part was used for the determination of intracellular potassium ion, employing a Corning Flame-Photometer (Model 405, Essex, England), as previously described [22].

2.5. Total serum bile acids and total bilirubin
These parameters were determined with the enzymatic method using a PYE Unicam PU 8800 UV/Vis spectrophotometer and the appropriate kits. TSBA were assayed using an Enzabile Kit supplied by Nycomed AS (Oslo, Norway). Total serum bilirubin was assayed using Unimate 5 from F-Hoffmann-La Roche Ltd. (Basel, Switzerland).

2.6. Serum enzyme assays
ALT, AST, ALP and LDH were assayed enzymatically with a Roche Centrifichem (Model 400) and appropriate kits (Roche Diagnostic System for ALT, AST and ALP and Trace Scientific Pty. Ltd. Melbourne, Australia for LDH).

2.7. In vitro uptake of TC
TC was selected as the model substrate as it is known to enter hepatocytes by active transport across the sinusoidal membrane and it is secreted by hepatocytes across the canalicular membrane in the process of efflux [25,26] and because it is the major elevated bile acid in the serum of ANIT-treated rats [2,27]. To study the in vitro uptake of this substrate, 1.99 ml of a suspension of freshly isolated hepatocytes (1.4 × 10^6 cells/ml) were preincubated (in 25-ml capped Erlenmeyer flasks at 37°C with shaking at 80 oscillations/min) for 20 min with DMSO (4 μl) or ANIT (final concentration, 5–100 μM), dissolved in 4 μl DMSO. Preliminary experiments and other studies [28–31] showed that these amounts of chemical or vehicle had no cytotoxic effects. After preincubation, 10 μl of radiolabelled substrate in physiological saline was added to each flask (final concentration 10 μM) and quickly mixed. The amount of radioactivity in each flask was ~80 nCi/ml. Sampling for uptake determination was carried out by the silicone oil centrifugation method [24] at appropriate times. After the pellet was dissolved in 3 M potassium hydroxide overnight, the samples containing [3H] were placed in scintillation vials containing 50 μl of 3 M hydrochloric acid to prevent chemiluminescence being produced by potassium hydroxide. After addition of 5 ml scintillation fluid to the vials and vortexing for 10 s, the amount of radioactivity in the supernatant and pellet was quantified using a Tri-Carb 4430 liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). Determination of the amount of radiolabel and protein concentration [32] allowed calculation of uptake per mg of protein. Initial rate of uptake (V₀) was determined from the slope of the lines in the linear range (20–80 s).
2.8. In vitro efflux of TC

For these studies, freshly isolated hepatocytes were preloaded with radiolabelled TC by incubation of 2 × 10^7 cells/2 ml for 20 min at 37°C with shaking at 80 oscillations/min. The final concentration of TC was 25 µM and the amount of radioactivity was 160 nCi/ml. After incubation, an aliquot (100 µl) of this concentrated cell suspension was added to 1.9 ml of fresh incubation medium which had been preincubated with DMSO (4 µl) or various concentrations (5–100 µM) of ANIT (dissolved in 4 µl of DMSO). Samples were then taken at appropriate intervals for determination of efflux of TC as described and referenced for the uptake studies. Initial rate of efflux ($V_0$) was determined from the slope of the lines in the linear portion (1–5 min).

2.9. Uptake and efflux of TC after in vivo exposure

All the procedures and protocols for these experiments were basically the same as for the in vitro exposure experiments, except that the hepatocytes isolated from ANIT- or corn oil-pretreated rats were incubated with various concentrations (2–100 µM) of radiolabelled substrate.

2.10. Data analysis and statistical procedures

The data were statistically evaluated using Student’s $t$-test, analysis of variance and Duncan’s test with a preset probability of $P < 0.05$. Each value is a mean which has been obtained from 3–8 experiments using hepatocytes from different rats.

![Fig. 1. In vitro effect of ANIT on initial rate of uptake ($V_0$) of taurocholic acid (10 µM). Each column represents the mean value and bars the S.E. ($n = 7$). None of the columns were significantly different to any other ($P < 0.05$).](image)
3. Results

3.1. Cell viability

Preincubation of the hepatocytes with various concentrations of ANIT for 20 min did not result in any decrease in intracellular potassium ion content or increase in leakage of cytosolic enzymes as compared with control. The mean levels (obtained from 6 separate experiments) of ALT, AST and LDH leakage for control cells when compared with their homogenate, were 11%, 5% and 11%, respectively. Relative values for the highest dose of ANIT (100 µM) were 10%, 6% and 11%, respectively. Furthermore, intracellular potassium ion content, as a sensitive index of cell viability, did not show any significant decrease in ANIT exposed cells as compared with the control cells. Mean levels of this parameter (obtained from 6 separate experiments) for control cells, 20 min after the incubation of the cells with DMSO (vehicle) and at the end of experiments were 77 and 73 µmol/g of cell, respectively, whereas, these values for the highest dose of ANIT (100 µM) were 76 and 68 µmol/g of cell, respectively.

Fig. 2. In vitro effect of ANIT on accumulation of taurocholic acid (10 µM) over time. Each column represents the mean value and bars the S.E. (n = 7). Columns with the same capital letters are not significantly different (P < 0.05).
3.2. Uptake of TC in vitro
Effects of various concentrations of ANIT on uptake of 10 μM TC were examined. As shown in Figs. 1 and 2, ANIT did not show any significant inhibitory effect on initial rate of uptake ($V_0$) or accumulation of bile acid.

3.3. Efflux of TC in vitro
There were no effects of ANIT on TC efflux from cells preloaded with 25 μM [3H]TC for either $V_0$ (Fig. 3) or continuous efflux of TC in the presence of any dose of ANIT (Fig. 4).

3.4. Total serum bile acids and the other liver function tests
In vivo treatment with ANIT (50 μmol/kg i.p. on each of 3 consecutive days) resulted in a highly significant rise in total serum bile acids. In addition, there was a slight increase in the activity of serum ALP and a very mild hyperbilirubinemia. None of the other enzyme liver tests was affected by ANIT (Table 1).

3.5. Uptake of TC after in vivo administration of ANIT
Uptake of various concentrations of TC (2–100 μM), using hepatocytes isolated from ANIT- and corn oil-pretreated rats, was not significantly different for either $V_0$ (Fig. 5) or accumulation of TC over time (Fig. 6).

3.6. Kinetic parameters of TC uptake after in vivo administration of ANIT
$K_m$ and $V_{max}$ for uptake after in vivo treatment of rats with ANIT or vehicle were calculated from Eadie-Hofstee plots. These values were no different for ANIT or ve-
Fig. 4. In vitro effect of ANIT on continuous efflux of taurocholic acid (25 μM) from hepatocytes, over time. Symbols represent the mean value and bars the S.E. (n = 5). Data for the other concentrations of ANIT were the same and, for the sake of clarity, they were excluded.

Vehicle treated animals. Mean values (obtained from 3–4 separate experiments) of $K_m$ for control and treated cells were 17.7 and 16.9 μM, respectively, whereas mean values for $V_{max}$ in control and treated cells were 2.53 and 2.49 nmol/min/mg protein, respectively.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total bile acids (μmol/l)</th>
<th>Total bilirubin</th>
<th>ALP</th>
<th>ALT</th>
<th>AST</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*a</td>
<td>14.7 ± 2.1 b (6)</td>
<td>1.5 ± 0.1 (4)</td>
<td>130 ± 14 (4)</td>
<td>40 ± 3 (4)</td>
<td>42 ± 2 (4)</td>
<td>79 ± 8 (4)</td>
</tr>
<tr>
<td>ANITd</td>
<td>74.1 ± 9.1* (7)</td>
<td>2.1 ± 0.1* (7)</td>
<td>173 ± 6* (7)</td>
<td>44 ± 6 (6)</td>
<td>37 ± 5 (6)</td>
<td>95 ± 18 (6)</td>
</tr>
</tbody>
</table>

*aCorn oil-treated rats (1 ml/kg i.p. for 3 consecutive days).

bValues are means ± S.E.

*cNumber of rats.

dDose (50 μmol/kg i.p. for 3 consecutive days).

*Significantly different from the respective controls (Student’s t-test, $P < 0.05$).
Fig. 5. Initial rate of uptake ($V_0$) of various concentrations of taurocholic acid by hepatocytes from ANIT-pretreated rats (50 μmol/kg i.p. on each of 3 consecutive days). Each column represents the mean value and bars the S.E. ($n = 3$ or 4). Columns with the same capital letters are not significantly different ($P < 0.05$). $V_0$ for the other concentrations of taurocholic acid showed the same pattern and the data were not included for the sake of clarity.

3.7. Efflux of TC after in vivo administration of ANIT

After in vivo administration of ANIT and subsequent isolation of hepatocytes from pretreated animals, efflux of TC from the cells which were preloaded with 25 μM $[^3H]$TC was studied. Initial velocity of efflux ($V_0$) of TC was not significantly different between ANIT- and vehicle-treated rats ($0.12 \pm 0.01$ vs. $0.11 \pm 0.01$, respectively). Continuous efflux of TC was not significantly different either (Fig. 7).

4. Discussion

The data presented here show that isolated rat hepatocytes do not lose their ability to transport bile acid in the presence of different concentrations of ANIT up to 100 μM. Lack of any inhibitory effects on the uptake and efflux of bile acid by ANIT indicates that raised concentrations of serum bile acids after exposure to this hepatotoxicant are not mediated by alterations in these functions.
These results are consistent with previous studies, where no change in initial uptake rate of TC by isolated perfused rat liver was observed [13,33], and the release of bile acids from hepatocytes exposed to doses of ANIT up to 1000 μM in vitro was normal [30].

Fig. 6. Accumulation of taurocholic acid by hepatocytes isolated from ANIT-pretreated rats (50 μmol/kg i.p. on each of 3 consecutive days). Symbols represent the mean value and bars the S.E. (n = 3 or 4).
As it is possible that the effects do not necessarily occur directly in this in vitro exposure situation, the transport of bile acid by hepatocytes isolated from ANIT-pretreated rats was further studied. Initial rate of uptake of various concentrations of model substrate (TC, 2–100 μM) and accumulation of TC by hepatocytes isolated from ANIT-pretreated rats did not show any significant difference when compared with the vehicle-treated rats. Additionally $K_m$ and $V_{max}$ were no different between ANIT- and vehicle-exposed animals. Similarly, in vivo treatment with ANIT had no effect on the process of bile acid efflux. These results are again in accord with the observation of Lavigne et al. [30] that functions of bile acid transport were preserved in hepatocytes isolated from ANIT-pretreated rats.

Theoretically, an increased SBA after ANIT treatment may be as a result of decreased hepatocellular uptake of bile acids, reduced canalicular transport of bile acids, regurgitation of bile acids across tight junctions and/or bile duct obstruction [13,20,33]. Bile duct obstruction is perhaps unlikely to be the cause of ANIT-induced elevation of serum bile acids observed here, given that the dose of ANIT used in our experiments did not cause any histological abnormalities in the liver sections as examined under light microscopy [2,34] and this dose is less than those (40–200 mg/kg) [12,20,35] causing profound cholestasis associated with evidence of degeneration and exfoliation of biliary epithelial cells, formation of bile plugs and bile duct occlusion. Kossor et al. [35] recently reported no changes in serum total bile acids.
after a single oral dose of 25 mg/kg of ANIT, which would seem inconsistent with our data and data of other studies [2,34]. However, it is difficult to make a direct comparison because of the different protocols adopted (our treatment regime used 3 i.p. doses of 9.26 mg/kg on each of 3 consecutive days). Furthermore, it has been demonstrated that increased SBA and onset of cholestasis at 16 h after ANIT treatment precede bile duct obstruction which occurs 48–72 h after ANIT administration [20,35]. Our data would suggest that either decreased hepatocellular uptake or hepatocanalicular transport of TC is also an unlikely reason for ANIT-induced elevation of SBA observed here, given the lack of any in vitro or in vivo effect on transport of bile acids by ANIT. Therefore, increased permeability of hepatic tight junctions which has been documented as an early event in ANIT-induced cholestasis [13–19] is perhaps more likely to be causally linked to an elevation of SBA observed under our experimental conditions.

The raised ALP after ANIT treatment in this study deserves comment. Since ALP is found in bile [36,37], the increased plasma activity of this enzyme may result from regurgitation into serum of hepatic ALP from damaged and disrupted tight junctions [38]. However, the extent of increase in serum activity of ALP after ANIT treatment is less than for bile acids presumably due to the greater molecular size (M_r 130 000) and hence a lower diffusion rate across tight junctions.

In conclusion, the data indicate that, unlike the case with solvents, ANIT-induced elevation of serum bile acids is not a consequence of its interference with bile acid transport. This is not inconsistent with the postulated mechanism of action of an increased permeability of hepatic tight junctions. Neither would it be inconsistent with bile duct obstruction as a mechanism at higher doses. Furthermore, the increased SBA after ANIT treatment in the absence of any alteration in bile acid transport (as evidenced by normal uptake function by cells) or hepatocyte integrity (as indicated by normal values for serum enzymes) may further substantiate the importance of these sensitive indicators of hepatobiliary dysfunction in response to hepatotoxicants.

References


[7] V. Kukongviriyapan, U. Kukongviriyapan and N.H. Stacey, Interference with hepatocellular sub-


