Published in final edited form as:

Toxicol Appl Pharmacol. 2014 December 15; 281(3): 303–309. doi:10.1016/j.taap.2014.10.015.

Characterization of Acute Biliary Hyperplasia in Fisher 344 Rats Administered the Indole-3-Carbinol Analog, NSC-743380

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Abstract

NSC-743380 (1-[(3-chlorophenyl)-methyl]-1H-indole-3-carbinol) is in early stages of development as an anticancer agent. Two metabolites reflect sequential conversion of the carbinol functionality to a carboxaldehyde and the major metabolite, 1-[(3-chlorophenyl)-methyl]-1H-indole-3-carboxylic acid. In an exploratory toxicity study in rats, NSC-743380 induced elevations in liver-associated serum enzymes and biliary hyperplasia. Biliary hyperplasia was observed 2 days after dosing orally for 2 consecutive days at 100 mg/kg/day. Notably, hepatotoxicity and biliary hyperplasia were observed after oral administration of the parent compound, but not when major metabolites were administered. The toxicities of a structurally similar but pharmacologically inactive molecule and a structurally diverse molecule with a similar efficacy profile in killing cancer cells *in vitro* were compared to NSC-743380 to explore scaffold versus target-mediated toxicity. Following two oral doses of 100 mg/kg/day given once daily on two consecutive days, the structurally unrelated active compound produced hepatic toxicity similar to NSC-743380. The structurally similar inactive compound did not, but, lower exposures were achieved. The weight of evidence implies that the hepatotoxicity associated with NSC-743380 is related to the anticancer activity of the parent molecule. Furthermore, because biliary hyperplasia represents an unmanageable and non-monitorable adverse effect in clinical settings, this model

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The authors declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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may provide an opportunity for investigators to use a short-duration study design to explore biomarkers of biliary hyperplasia.

Keywords

hepatotoxicity; biliary hyperplasia; rat liver; indole-3-carbinol

INTRODUCTION

NSC-743380 (1-[(3-chlorophenyl)-methyl]-1H-indole-3-carbinol) is an indole-3 carbinol analog that has shown growth inhibitory and cell-death inducing activities against cell lines derived from lung, colon, breast, ovary, and kidney cancers. A profile obtained using fifty-eight cancer cell lines showed the inhibitory activity of NSC-743380 to be unique and distinct demonstrating that there are both sensitive and resistant lines. The median 50% growth-inhibitory concentration (GI50) for NSC-743380 was 1.62 μ M. For eight of the most sensitive cell lines, the GI50 was 10^{-8} M (10 nM) (Liu *et al.*, 2012). NSC-743380 has also undergone testing for anticancer activity *in vivo* and produced complete regressions in A498 renal xenograft models when administered intraperitoneally (Guo *et al.*, 2011).

Mechanisms of action for NSC-743380-mediated induction of cancer cell death remain elusive, but there is some evidence that inhibition of STAT3 may be involved (Newton *et al.*, 2012, Guo *et al.*, 2011, Liu *et al.*, 2012). Treatment with NSC-743380 inhibited transcription factor STAT3 activation and induced ROS production in sensitive human lung cancer cell lines, but not in resistant cells (Liu *et al.*, 2012). Knockdown of STAT3 with shSTAT3 induced ROS generation and suppressed tumor cell growth. Moreover, scavenging ROS induced by STAT3 inhibition also diminished antitumor activity of STAT3 inhibition. *In vivo* administration of NSC-743380 suppressed tumor growth and p-STAT3 in lung tumors.

We characterized the toxicity of NSC-743380 after short-term administration to rats to obtain insight into the margin of safety for this agent and to identify major target organs for its toxicity. The results of these preclinical assessments identify the liver (characterized most notably by biliary hyperplasia) as the major target organ for toxicity of NSC-743380, and provide evidence that the mechanism of toxicity is directly related to the mechanism of antitumor activity.

MATERIALS AND METHODS

Test Articles and Formulation

The test articles were synthesized at the NCI. Oral formulations were prepared on the day of use by transferring the appropriate amount of test article into a mixing container, adding the appropriate volume of vehicle (Labrasol®, Cognis Corporation, Monheim am Rhein, Germany;), and stirring and/or sonicating until the test article was dissolved.

Animals

Animal husbandry and handling for all of the studies described conformed to the current AAALAC recommendations and current requirements stated in the “Guide for the Care and Use of Laboratory Animals” (National Research Council). Male F344 rats (Charles River Laboratories, Kingston, NY) between 9 and 14 weeks of age that weighed between 164 and 242 g at study initiation were used. Rats were held in quarantine for seven days. Animals were single-housed in polycarbonate cages equipped with automatic watering systems. Standard Harlan Certified Diet and municipal water were provided *ad libitum* in all studies. Animal room temperature and relative humidity values were recorded daily. Temperature ranged from 21 to 22°C and relative humidity ranged from 32 to 53% throughout the study. Fluorescent lighting was provided for 12 hours per day, followed by 12 hours of darkness. Prior to blood sample collections, rats were anesthetized with 70% carbon dioxide/oxygen mixture and blood was collected from the retro-orbital plexus.

Five-Day Exploratory Oral Toxicity Study

Twelve animals were randomly assigned, based on body weight, to 4 dose groups of 3 rats per group. On Days 1 through 5, each rat received a single daily dose of vehicle (0 mg/kg) or 100, 300 or 500 mg/kg NSC-743380 by gavage. Dose administration volume was 5 ml/kg. Animals were observed twice daily for clinical signs of toxicity. Body weights were recorded prior to dosing for calculation of individual dose administration volumes. Whole blood was collected from each animal following test article administration on Days 1, 2 and 5 for determination of plasma drug levels. Blood was also collected from all animals on Day 2 and from surviving animals on Day 5 for serum chemistry determinations (ALP, ALT, AST, GGT, creatinine, total protein and BUN). Only those animals administered 100 mg/kg/day survived the 5 days of treatment and were humanely terminated on Day 5. At necropsy, tissues were sampled and fixed in 10% neutral-buffered formalin, processed to 5 micron paraffin sections for H&E staining, and examined microscopically.

Comparative Toxicity Study of Parent Compound and its Major Metabolite

Twelve animals were randomly assigned, based on body weight, to 4 dose groups of 3 rats each. On Days 1 and 2, each rat received a single gavage dose of vehicle (0 mg/ml), NSC-743380 (parent compound), NSC-741908 (aldehyde metabolite) or NSC-751172 (carboxylic acid metabolite) at 150 mg/kg by oral gavage. Dose administration volume was 5 ml/kg. Clinical observations for evidence of toxicity were made twice daily throughout the study. Body weights were recorded pre-test and on Days 1 and 2 prior to dosing for calculation of individual dose administration volumes. Body weights were also recorded prior to termination. Whole blood specimens for determination of plasma drug and metabolite levels were collected at 0.25, 4 and 24 hours following test article administration on Day 1; the 24-hour specimen was collected prior to dosing on Day 2. An additional specimen was collected 4 hours post-administration on Day 2 for plasma drug and metabolite levels as well as serum chemistry (ALP, ALT, AST, GGT, creatinine, total protein and BUN). Animals were humanely terminated 4 hours after the final dose administration and the liver collected for histopathology examination. Liver tissues were

sampled and fixed in 10% neutral-buffered formalin, processed to approximately 5 micron paraffin sections for H&E staining, and examined microscopically.

Comparative Study Exploring Mechanism of Toxicity

Twelve male animals were randomly assigned, based on body weight, to 4 groups of 3 rats each. Prior to administration of test article or vehicle, rats in all groups received a single gavage dose of 5% (w/v) sodium bicarbonate in a dose volume of 1 ml/kg to enhance stability of the test article. (A comparison of the exposure between mice given NSC-743380 with bicarbonate with that of mice given NSC 743380 without bicarbonate showed that about 1/3 the amount of parent drug was measured in the plasma of the mice in which no bicarbonate was administered, *unpublished data*). Within one minute of administration of sodium bicarbonate, each rat in test article groups received an oral (gavage) dose (5 ml/kg) of a formulated test article (NSC-743380, NSC-754516 (structurally similar) or NSC-763131 (structurally unrelated, but with a similar in vitro anticancer activity profile in the NCI-60 panel) on Days 1 and 2; each test article was administered at 100 mg/kg/day. Rats in the control group received vehicle by gavage (5 ml/kg) on Days 1 and 2. Dose volumes were based on each animal's individual body weight recorded on each day of dose administration. Clinical observations were made twice daily. Body weights were recorded pre-test and prior to each dose for calculation of individual dose administration volumes, as well as prior to termination. Whole blood specimens were collected at 6 and 24 hours after dosing on Day 1 and 4 hours after dosing on Day 2 for plasma drug level determinations. Blood was also collected for serum chemistry (ALP, ALT, AST, GGT, creatinine, total protein and BUN) 4 hours after dosing on Day 2. All study rats received a gross necropsy and the liver was collected for histologic processing and microscopic examination.

Data Analysis/Statistics

Clinical chemistry data were analyzed for test article effects by analysis of variance. Data homogeneity was determined using Bartlett's test for homogeneity at the 0.05 level. When data were homogeneous, tests for differences between the vehicle and test article groups were made using Dunnett's test. For non-homogeneous data, tests for pair-wise differences between vehicle and each test article group were performed using Cochran and Cox's modified two-sample t-test. For the automated digital image analysis data, the Student's *t*-test was applied to compare each treatment group to the vehicle control group. Statistical significance for each comparison was reported at the 0.05 level.

Histopathologic Evaluations

Histopathologic evaluations of H&E stained tissues (two sections from the left lobe were included on each slide per animal) were conducted without prior knowledge of treatment group. Microscopic findings were graded semi-quantitatively based on severity from minimal (grade 1) to marked (grade 4). Minimal (grade 1) represented the least detectable lesion; mild (grade 2) represented an easily discernible lesion; moderate (grade 3) represented a change affecting a large area of the tissue represented on the slide; and marked (grade 4) represented the maximal lesion observed. Histopathologic evaluation of the liver included bile duct hyperplasia that was characterized by increased numbers of bile ducts in

hepatic portal areas. Proliferating bile ducts were often accompanied by subacute mononuclear inflammatory cell infiltration and adjacent hepatocellular necrosis.

Whole Slide Imaging and Analysis

H&E stained glass slides were scanned using the Aperio XT ScanScope whole slide imaging system with a 20X objective lens (Aperio, Vista, CA). One whole slide image was prepared from each animal that captured the entire tissue on the slide. Resulting SVS image files (a type of TIFF) were viewed using Aperio ImageScope. From each whole slide image, four separate areas of interest that contained similarly sized portal triads based on features of the portal vein, hepatic artery and bile duct were selected using the extract region feature to capture a high resolution portion of the image defined by dragging a box around the portal triad. The resulting TIFF images were imported to Definiens Developer software. Image analysis was performed using a customized rule set written with Developer XD version 2.0.4 (Definiens, Munich, Germany) that is based on Definiens Cognition Network Technology®. Individual bile ducts were identified as discrete units based on pixels with common morphologic features, including proximity to a portal vein and hepatic artery, and containing a lumen. The automated rule set applied to each captured image counted the number of bile ducts per portal triad. The average number of bile ducts for the four portal triads was determined for each animal and the mean $\pm$ standard deviation were calculated for each treatment group. Accurate capture of bile ducts using the automated software rule set was confirmed by the study pathologist.

RESULTS

Pharmacokinetic Profile of NSC-743380

Within 0.25 hours of administration, animals receiving NSC-743380 (parent compound), NSC-741908 (aldehyde metabolite) or NSC-751172 (carboxylic acid metabolite) at 150 mg/kg/day had notably high plasma concentrations (greater than 11,800 ng/ml) of the carboxylic acid metabolite that remained elevated up to 4 hours post-Day 2 dosing (Table 1). Although the aldehyde metabolite was detected within 0.25 hours of the initial administration of parent compound or aldehyde metabolite, by 24 hours post-Day 1 dosing, the aldehyde metabolite was less than 10.2 ng/ml in both groups, but was detected 4 hours post-Day 2 dosing. The aldehyde metabolite was less than 10.2 ng/ml at all time points in animals receiving the carboxylic acid metabolite. As expected, the plasma levels of parent compound were only detected in animals receiving the parent compound and ranged from a group average of 85.3 ng/ml at 0.25 hours post-Day 1 dose to 1,223 ng/ml 4 hours following Day 2 dosing.

In animals receiving 100 mg/kg/day NSC-743380 (active compound), NSC-754516 (structurally similar but inactive) or NSC-736131 (structurally diverse but active), detectable levels of each respective drug were found at 6 hours post dosing on Day 1 and at 4 hours after dosing on Day 2 (Table 2). Plasma levels of all three compounds were below the quantifiable limit of 2 ng/ml at 24 hours after the first dose. Mean group plasma drug levels of respective compounds were comparable in the NSC-743380 and NSC-763131 treated

animals compared to NSC-754516 which were considerably less at the 6 hour post-Day 1 dose and 4 hour post-Day 2 dose.

Toxicity Profile of NSC-743380 in Rats

Oral administration of 300 and 500 mg/kg/day NSC-743380 resulted in 100 percent mortality with deaths beginning as early as Day 3, whereas all animals receiving 100 mg/kg/day NSC-743380 survived to the scheduled termination on Day 5. Dose-related elevated liver specific enzymes, ALT and GGT were significantly greater than controls on Day 2 in all three dose groups (Table 3). ALP and AST were also significantly greater than controls on Day 2 in all three dose groups (Table 3). No other remarkable changes in serum chemistry related to liver toxicity were noted. The most notable histopathology findings attributed to NSC-743380 were hepatic biliary hyperplasia with local inflammation and hepatocellular necrosis, but not bile duct necrosis. Liver lesions increased in severity with increasing dose and were considered to be responsible for the elevated serum enzymes in all dose groups, as well as the moribund sacrifices and early deaths in animals administered 300 or 500 mg/kg/day NSC-743380. Additional characterization studies were conducted at 100 mg/kg of NSC-743380 because that dose was considered to be the MTD for a single dose administered once daily on two consecutive days.

Comparative Hepatotoxicity Profile of NSC-743380, its Major Metabolites and Structurally Similar or Diverse Compounds

Serum chemistry parameters indicative of liver toxicity measured after 2 days of oral administration of 150 mg/kg/day NSC-743380 were increased (58-fold and 53-fold for ALT and AST, respectively) compared with control animals administered equal dose volume of vehicle (Table 4). Also elevated was GGT, although the increase was not statistically significant compared to vehicle controls. In addition, ALP was increased approximately 5-fold above that of control animals. No other significant changes in serum chemistry were noted. In contrast to the parent compound, there were no significant changes in ALP, ALT, AST or GGT in animals treated with the same oral dose of the major metabolites [aldehyde (NSC-741908) or carboxylic acid (NSC-751172)] (Table 4).

Histopathological lesions consistent with serum chemistry alterations were noted in animals receiving the parent compound (NSC-743380). Specifically, liver findings included biliary hyperplasia with local inflammatory infiltration and hepatocellular necrosis (Table 5, Figure 1). There were no hepatic lesions observed in animals administered either major metabolite (NSC-741908 or NSC-741172) (Table 5, Figure 1). The results suggest that the liver toxicity observed was due to the parent compound, but not the major metabolites or metabolites generated from them.

To examine whether the hepatic toxicity induced by NSC-743380 was associated with its cytotoxic activity in tumor cells, we compared the liver toxicity profiles of this compound with that of NSC-763131, a structurally diverse compound with similar pharmacologic activity in the NCI-60 panel and NSC-754516, a structurally similar compound that was inactive in the NCI-60 panel. Clinical chemistry evaluations did provide evidence of hepatic toxicity of NSC-743380 and NSC-763131 (the structurally diverse compound with similar

potency) after 2 days oral administration at 100 mg/kg/day (Table 6). Although statistical significance was not attained due to variability in serum enzymes for this particular set of experiments, substantial elevations in ALT and AST were seen two of three rats per group receiving NSC-743380 (active compound) or NSC-763131 (different structure, but inactive). Severe hepatotoxicity, mainly characterized by biliary hyperplasia with or without concomitant inflammatory cell infiltration and hepatocellular necrosis was observed in all three of the rats receiving NSC-743380 and in two of the three rats receiving NSC-763131 (Table 6, Figure 2). Taken together, these findings provide clear biologically relevant changes associated with these two compounds. In contrast, plasma transaminases were not increased in rats receiving NSC-754516 (structurally similar, inactive compound) nor were histopathologic changes observed.

Automated Image Analysis and Comparison with Pathologist's Evaluation

To assess biliary hyperplasia in a more quantitative manner than is possible with standard histopathology, an automated image analysis system using Definiens Developer software was employed. The results based on automated detection and counting of bile ducts using the image analysis software were consistent with the pathologist's scores (Table 7). Furthermore, the quantitative results allowed for a more rigorous analysis of statistical comparison between treatment groups. Biliary hyperplasia based on number of bile ducts per portal triad was significantly greater in the NSC-743380 and NSC-763131 treated groups than in vehicle controls or in rats treated with the pharmacologically inactive NSC-754516. These findings were consistent with the composite scores based on histopathology findings of biliary hyperplasia that was accompanied by inflammation and hepatocellular necrosis in some animals.

DISCUSSION

The promising efficacy of NSC-743880 in tumor xenograft models warranted exploration of its toxicity in order to optimize decision-making during the early stages of its development as an anticancer agent. The most notable finding in an exploratory toxicology study was biliary hyperplasia in rats receiving only two daily oral doses. There was no observable necrosis of the biliary epithelium in treated animals; however, necrosis in the hepatocellular parenchyma was observed in some animals and was primarily minimal in severity and localized adjacent to regions of biliary hyperplasia and inflammatory cell infiltration. Consistent with these observations, increases in ALT and GGT were seen. Notably, GGT has been shown to be a specific marker of bile duct lesions in rat liver following bile duct obstruction and ANIT feeding (Alpini *et al.*, 1988, Leonard *et al.*, 1984). Rat hepatocytes have 1/30th of the GGT levels compared to human hepatocytes; thus, virtually all GGT is contained in bile duct epithelium in rats. This is in contrast to other species in which GGT is normally found in high concentrations in both the hepatocyte and bile duct epithelial cell. Therefore, elevation in circulating GGT in the rat is more likely to be an indicator of bile duct injury than is its elevation in other species (Cattley *et al.*, 2012). Since basal levels of serum GGT in rats are very low or undetectable, small increases in GGT activity have been reported to be biologically relevant following short-term ANIT treatment (Leonard *et al.*, 1984).

The categorization of toxicological effects as chemical-based, on-target, or off-target was recently discussed during a symposium at the annual meeting of the Society of Toxicologic Pathology (Rudmann, 2013). Chemical-based toxicity is defined as toxicity that is related to the physicochemical characteristics of a compound and its effects on cellular organelles, membranes, and/or metabolic pathways. On-target refers to exaggerated and adverse pharmacologic effects at the target of interest in the test system. Off-target refers to adverse effects as a result of modulation of other targets; these may be related biologically or totally unrelated to the target of interest. Generating data to the point at which one can reasonably categorize whether toxicity is chemical-based, on-target, or off-target is a key step towards understanding the mechanism of agent toxicity. Our experimental plan combined classic pathology with medicinal chemistry (comparisons with an active but structurally different compound) to support the conclusion that the toxicity associated with NSC-743380 appears to be related to the anticancer activity of the parent molecule. The difference in exposure for the inactive molecule makes it impossible to rule out the contribution of this difference in exposure to the resulting lack of toxicity, thus we cannot conclude from these data alone that the toxicity is mechanistically associated with efficacy. We do not propose that a single target is responsible for efficacy of toxicity at this point. This is especially important to note because typically, more than a single target and pathway may be required for therapeutic activity or all targets engaged by a molecule are not fully defined. Although, the approach we applied here can be broadly applied to define whether therapeutic activity is required for toxicity, we have also shown that there are caveats to using a medicinal chemistry approach such as the need to obtain equivalent exposure and similar PK behavior. This is especially important when more than a single target and pathway may be required for therapeutic activity or all targets engaged by a molecule are not fully defined.

The hepatoprotective function of STAT3 has been well-documented in many murine models of liver injury (Horiguchi *et al.*, 2010, Mair *et al.*, 2010). Thus, it is possible that inhibition of STAT 3 contributed to biliary hyperplasia. In fact, using a model of Sclerosing Cholangitis, it was shown that mice lacking the multidrug resistance gene 2 (*mdr2*^{-/-}), conditionally inactivated STAT3 in hepatocytes and cholangiocytes leads to strongly aggravated hepatocellular damage and fibrosis (Haga *et al.*, 2003). These are interesting possibilities worthy of exploration.

NSC-743380-induced biliary hyperplasia in the absence of observable biliary epithelial necrosis is an unusual finding. Bile duct ligation and chronic administration of ANIT are among the most widely used experimental models to study “typical” cholangiocyte proliferation. ANIT induces biliary hyperplasia coincident with biliary epithelial cell damage, bile duct obstruction and minimal local hepatocellular necrosis that has been described as single cell necrosis (Kossor *et al.*, 1995, Alpini *et al.*, 1998, Gaudio *et al.*, 2006), Lesage *et al.*, 2001). Our observations of mitotic figures in the bile duct epithelium without observable cell death suggest a *primary* proliferative response rather than a regenerative response secondary to cell death. However, we cannot rule out biliary obstruction in the pathogenesis of biliary hyperplasia induced by NSC-743380. Coincident necrosis of the biliary epithelium may be a confounding factor when exploring opportunities for specific biomarkers of biliary hyperplasia. Thus, NSC-743380-induced biliary

hyperplasia may provide a model with which biliary hyperplasia can be separated, at least temporally, from necrosis of the target cell population; this separation may be helpful for biomarker exploration. Moreover, having an accessible clinical biomarker for this adverse pathological effect could improve clinical monitoring and better inform the human relevance of this toxicity.

There is promise in translation of early mRNA responses from genomic profiling and biomarkers that will not require biopsy of liver tissue ((Cullen *et al.*, 2010). This genomic approach will benefit from short-term induction models that provide opportunities to measure performance characteristics and predictive properties.

Quantitative comparisons between various models of biliary hyperplasia will allow associations to be made between common molecular response pathways and disease pathogenesis that are distinguishable from discrete compound-related mechanisms of toxicity. This model system may be employed in the future to explore biomarkers of biliary hyperplasia and provide a means to further qualify biomarkers that hold promise.

Acknowledgments

This research was supported [in part] by the Developmental Therapeutics Program in the Division of Cancer Treatment and Diagnosis of the National Cancer Institute and with federal funds from the National Cancer Institute, National Institutes of Health, under Contracts No. HHSN261200800001E, N01-CM-201100019 and N01-CM-201100027.

Abbreviations

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANIT	α -naphthylisothiocyanate
AST	aspartate aminotransferase
BQL	below quantifiable limits
BUN	blood urea nitrogen
GGT	gamma glutamyl transferase
H&E	hematoxylin and eosin
IHC	immunohistochemistry
NA	not applicable
NCI	National Cancer Institute
PBS	phosphate-buffered saline
ROS	reactive oxygen species
SD	standard deviation

shSTAT3	short hairpin signal transducer and activator of transcription 3
SOP	standard operating procedure
SVS	stand visualization system; the baseline image file created using the Aperio whole slide imaging system
TIFF	tagged image file format

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Highlights

- 1-[(3-chlorophenyl)-methyl]-1H-indole-3-carbinol (NSC-743380) induced hepatotoxicity and biliary hyperplasia in rats when two doses of 100 mg/kg/day were administered orally, once daily for two consecutive days,
- The toxic effects of a structurally similar but pharmacologically inactive molecule were compared to NSC-743380 to explore scaffold versus target-mediated toxicity.
- The hepatotoxicity observed with NSC-743380 appears to be related to the anticancer activity of the parent molecule and is characterized as “on-target” toxicity.
- Biliary hyperplasia represents an unmanageable adverse effect in a clinical settings and this model may provide an opportunity for investigators to explore biomarkers of biliary hyperplasia.

FIGURE 1.

Representative images of H&E stained livers from vehicle control and treated groups; magnification 10X objective, bar = 100 µm. Compared with vehicle control (A), rats treated with NSC-743380 (B) had marked bile duct hyperplasia that was often accompanied by periportal necrosis. In contrast to the parent compound (NSC-743380), liver from rats treated with either the aldehyde metabolite (NSC-741908) or carboxylic acid metabolite (NSC-751172) appeared normal (C) and (D), respectively. Abbreviations: A, hepatic artery; D, bile duct; N, necrosis; V, hepatic vein.

FIGURE 2.

Representative images of H&E stained livers from vehicle control and treated groups; magnification 20X objective, bar = 100 μm. (A) Vehicle control; (B) NSC-743380 treated rat liver showing bile duct hyperplasia and mononuclear inflammatory infiltration; (C) Normal liver in NSC-754516 treated rat; (D) Bile duct hyperplasia in NSC-763131 treated rat. Abbreviations: A, hepatic artery; D, bile duct; I, inflammation; N, necrosis; V, hepatic vein.

TABLE 1

Group mean plasma drug levels of parent compound (NSC-743380), aldehyde metabolite (NSC-741908) and carboxylic acid metabolite (NSC-751172) after oral dosing of 150 mg/kg/day for 2 days.

Dose Group	Time Point (Post-dose)	NSC-743380 (parent) (ng/ml)	NSC-741908 (aldehyde) (ng/ml)	NSC-751172 (carboxylic acid) (ng/ml)
NSC-743380 (parent)	0.25 hour	85.3	31.6	14400
	4 hour	180.7	22.7	27900
	24 hour	494.3	< 10.2	15720
	4 hour (Day 2)	1223	21.1	68333
NSC-741908 (aldehyde)	0.25 hour	< 10	32.2	11800
	4 hour	< 10	44.4	46400
	24 hour	< 10	< 10.2	13567
	4 hour (Day 2)	< 10	22.5	43800
NSC-751172 (carboxylic acid)	0.25 hour	< 10	< 10.2	21833
	4 hour	< 10	< 10.2	15600
	24 hour	< 10	< 10.2	16333
	4 hour (Day 2)	< 10	< 10.2	20700

TABLE 2

Group mean plasma drug levels after 2 days oral administration of 100 mg/kg/day of NSC-743380, NSC-754516 (structurally similar to NSC-743380, but inactive in efficacy mouse xenograft model) or NSC-763131 (structurally different with same efficacy profile as NSC-743380).

Dose Group	Time Point (Post-dose)	Plasma Drug Level (ng/ml)
NSC-743380 (active)	6 hour	43.0
	24 hour	BQL ^a
	4 hour (Day 2)	63.9
NSC-754516 (same scaffold, inactive)	6 hour	5.8
	24 hour	BQL
	4 hour (Day 2)	7.5
NSC-763131 (different structure, active)	6 hour	53.3
	24 hour	BQL
	4 hour (Day 2)	179.7

^aBQL = below quantifiable limit of <2 ng/ml

TABLE 3
Group mean serum chemistry values measured after 2 days of daily oral administration of NSC-743380.

Dose Group	Values	Alkaline Phosphatase (units/liter)	Alanine Aminotransferase (units/liter)	Aspartate Aminotransferase (units/liter)	Gamma Glutamyl Transferase (units/liter)
Vehicle	Mean	311	54	75	0
	SD	17	4	8	0
NSC-743380 100 mg/kg/day	Mean	628*	1498*	1681*	2
	SD	84	486	678	1
NSC-743380 300 mg/kg/day	Mean	1547*	4215*	4461*	3
	SD	452	546	1279	0
NSC-743380 500 mg/kg/day	Mean	1966*	5186*	7618*	6
	SD	285	145	2733	2

* statistically significant difference from control (vehicle) group ($P = 0.05$)

TABLE 4

Group mean serum chemistry values measured after 2 days of daily oral administration of parent compound (NSC-743380), aldehyde metabolite (NSC-741908) or carboxylic acid metabolite (NSC-751172) at 150 mg/kg/day.

Dose Group	Values	Alkaline Phosphatase (units/liter)	Alanine Aminotransferase (units/liter)	Aspartate Aminotransferase (units/liter)	Gamma Glutamyl Transferase (units/liter)
Vehicle	Mean	204	68	84	0
	SD	3	10	11	0
NSC-743380 (parent)	Mean	956 *	3942 *	4454 *	3
	SD	122	393	428	1
NSC-741908 (aldehyde)	Mean	219	60	89	0
	SD	25	18	18	0
NSC-751172 (carboxylic acid)	Mean	248	69	84	0
	SD	20	15	12	1

* statistically significant difference from control (vehicle) group ($P = 0.05$)

TABLE 5

Summary of incidence and severity scores for histopathology findings in the liver by microscopic examination of H&E stained slides from animals administered 150 mg/kg/day of each test article for 2 days. Severity scores were graded as unremarkable (0), minimal (1), mild (2) moderate (3) or marked (4) for hepatotoxicity that was characterized by bile duct hyperplasia, mononuclear inflammatory cell infiltrate and necrosis.

Dose Group	Liver Toxicity	
	Incidence	Mean Severity *
Vehicle	0/3	0
NSC-743380 (Parent compound)	3/3	4.0 (4.0)
NSC-741908 (Aldehyde metabolite)	0/3	0
NSC-751172 (Carboxylic acid metabolite)	0/3	0

* mean severity of affected rats in parentheses

TABLE 6

Individual animal serum chemistry values and severity scores for histopathology findings in the liver after 2 days of daily oral administration at 100 mg/kg/day of NSC-743380, NSC-754516 (structurally similar to NSC-743380), but inactive in efficacy mouse xenograft model) or NSC-763131 (structurally different with same efficacy profile as NSC-743380). Histopathology severity scores were graded as unremarkable (0), minimal (1), mild (2), moderate (3) or marked (4) for hepatotoxicity characterized by bile duct hyperplasia, mononuclear inflammatory infiltrate and hepatocellular necrosis.

Dose Group	Animal Number	Plasma Drug Levels (ng/ml)	ALT (units/liter)	AST (units/liter)	GGT (units/liter)	Histopathology Score
Vehicle	378	NA	55	121	2	0
	379	NA	64	98	2	0
	380	NA	58	119	0	0
	Mean	NA	59	113	1.3	NA
NSC-743380 (active compound)	SD	NA	4.6	13	1.2	NA
	381	87.9	264	330	0	4
	382	35.7	1631	1710	3	4
	383	68.0	67	107	0	4
	Mean	64	654	716	1.0	NA
	SD	26	852	868	1.7	NA
NSC-754516 (same scaffold, inactive)	384	8.3	71	117	2	0
	385	6.2	62	112	2	0
	386	8.0	79	110	0	0
	Mean	7.5	71	113	1.3	NA
	SD	1.2	8.5	3.6	1.2	NA
	387	109	64	109	0	0
	388	216	326	437	4	4
	389	214	846	1216	3	4
	Mean	180	412	587	2.3	NA
	SD	61	398	569	2.1	NA

NA, not applicable

TABLE 7

Quantitative Comparison of biliary hyperplasia induced by Indole-3-Carbinol-related compounds.

Comparison of Biliary Hyperplasia induced by NSC-643380 with its major metabolites			
Dose Group	Animal Number	Average Number of Bile Ducts per Portal Triad	Group Mean $\pm$ SD
Vehicle Control	101	1.8	1.3 $\pm$ 0.4
	102	1.3	
	103	1.0	
NSC-743380 (150 mg/kg/day) (Parent compound)	201	6.3	7.4 $\pm$ 3.2*
	202	11.0	
	203	5.0	
NSC-741908 (150 mg/kg/day) (Aldehyde metabolite)	301	1.0	1.6 $\pm$ 0.5
	302	1.8	
	303	2.0	
NSC-751172 (150 mg/kg/day) (Carboxylic acid metabolite)	401	1.0	1.2 $\pm$ 0.3
	402	1.0	
	403	1.5	

Comparison of mediated Biliary Hyperplasia induced by active versus structurally identical inactive molecule-			
Dose Group	Animal Numbers	Average Number of Bile Ducts per Portal Triad	Group Mean $\pm$ SD
Vehicle Control	378	1.3	1.8 $\pm$ 0.6
	379	2.5	
	380	1.8	
NSC-743380 (100 mg/kg/day) (active compound)	381	11.0	9.1 $\pm$ 1.8*
	382	8.8	
	383	7.5	
NSC-754516 (100 mg/kg/day) (same scaffold, inactive)	384	4.5	3.3 $\pm$ 1.6
	385	3.8	
	386	1.5	
NSC-763131 (100 mg/kg/day) (different structure, active)	387	8.3	7.8 $\pm$ 1.1*
	388	8.5	
	389	6.5	

* Statistically greater than vehicle controls; *P* 0.05