Metabolic Conversion of Indicine N-Oxide to Indicine in Rabbits and Humans

Garth Powis, Matthew M. Ames, and John S. Kovach

Division of Developmental Oncology Research, Department of Oncology, Mayo Clinic, Rochester, Minnesota 55901

ABSTRACT

Indicine N-oxide, a pyrroloidizidine alkaloid N-oxide that exhibits antitumor activity without some of the toxic effects associated with other pyrrolizidizidine alkaloids, is metabolized to indicine in rabbits and humans. Indicine can be detected in the plasma and is excreted in the urine in a dose-dependent manner following the i.v. administration of indicine N-oxide. The p.o. administration of indicine N-oxide leads to an increased plasma concentration and an increased urinary excretion of indicine. The hepatic microsomal fraction and the gut flora both catalyze the anaerobic reduction of indicine N-oxide to indicine in vitro. Whole-animal studies suggest that the gut flora play a major role in the metabolic reduction of indicine N-oxide by the rabbit. Indicine N-oxide is not actively excreted in the bile, and it is probable that indicine N-oxide finds its way into the gut by passive diffusion following i.v. administration. Neomycin and erythromycin, which reduce the number of anaerobic bacteria in the gut, lead to decreased plasma levels and a decreased urinary excretion of indicine.

INTRODUCTION

Indicine N-oxide, a pyrrolizidine alkaloid, is an active antitumor agent derived from extracts of Heliotropium indicum Linn. (Boraginaceae) (20). It is currently undergoing clinical trial as an antitumor agent. The pyrrolizidine alkaloids are distributed widely in nature as the tertiary base of the N-oxide and are found typically in species of Senecio, Crotelaria, Heliotropiaceae, and Boraginaceae (8). The ability of some of these alkaloids to cause an unusual chronic liver disease characterized by centrilobular necrosis, enlarged cells, and enlarged nuclei in grazing animals (9) has prompted an extensive study of their chemical and toxicological properties (8). Demonstration of antimitotic properties, mutagenicity, and ability to cause chromosome breakage suggested that the alkaloids might possess antitumor activity (28). Several of the pyrrolizidine alkaloids were found to exhibit activity in a variety of experimental tumor models (10, 29, 30, 39), but the acute and chronic toxicities associated with all the active agents precluded their clinical use (10). Indicine and indicine N-oxide were not included in this study. The antitumor activity appeared to be associated with the allylic ester group of the molecule (10) which imparts alkylating ability and hepatotoxicity (12). The use of the N-oxides, which are generally less toxic than the tertiary base (32), failed to produce a more favorable ratio of desired activity to toxicity (10).

Indicine N-oxide exhibits antitumor activity in several systems including the murine leukemias without the acute toxicity associated with other pyrrolizidine alkaloids (28). This is perhaps surprising in view of the close structural similarity between indicine N-oxide and heliotrine N-oxide (Chart 1) which was rejected as a useful antitumor agent because of a low ratio of desired activity to toxicity (10). The major toxic effect of indicine N-oxide in experimental animals is bone marrow hypoplasia, and liver dysfunction is minimal and is not associated with abnormal histological findings (23).

The hepatotoxic effects of pyrrolizidine alkaloid N-oxide have been attributed to their reduction to the parent alkaloids (33). We considered it important to the understanding of the toxicity and the antitumor activity of indicine N-oxide to study the metabolic reduction of indicine N-oxide. Our studies have shown that in the rabbit the i.v. injection of indicine N-oxide produces a dose-dependent increase in the plasma levels and urinary excretion of the parent alkaloid, indicine. Both liver and gut flora are capable of reducing indicine N-oxide, although gut flora appear to play the major role in the formation of indicine.

MATERIALS AND METHODS

Male New Zealand White rabbits, weighing 2.5 to 3 kg, were administered indicine N-oxide in 0.9% NaCl solution, at doses between 25 and 118 mg/kg body weight, into an ear vein over a 30-sec period. Venous blood samples were collected from the other ear at various times. Urine was collected over short periods through a catheter (No. 8 French feeding tube; Sherwood Medical Industries, St. Louis, Mo.) using 5 ml sterile 0.9% NaCl solution to flush the bladder. Urine samples were collected over extended periods, such as overnight, by placing the animal in a metabolic cage. In one animal, the bile duct was cannulated under phenobarbital anesthesia. The animal was allowed to recover for 3 hr before being given indicine N-oxide, and bile and urine were collected from the exteriorized bile cannula and the bladder catheter for 24 hr.

Antibiotic treatment to reduce the gut flora of the rabbit consisted of the p.o. administration of 1 g erythromycin, 1 g neomycin, and 0.1 g nystatin, 36 and 12 hr before, and one-half of these amounts, 24 and 1 hr before the i.v. administration of indicine N-oxide. Aerobic and anaerobic gut flora were determined by quantitative plate cultures of suspensions of the gut contents using standard microbiological procedures. Blood samples were obtained over a 4-hr period, and urine was collected for 24 hr from 3 patients with advanced solid tumors receiving indicine N-oxide in Phase I clinical trial at a dose of 3000 mg/sq m, administered i.v. over a 10-min period.

Indicine N-oxide and indicine in the plasma and urine were assayed by a method previously described, based upon gas chromatography with electron capture detection of the pentafluoropropionyl derivative of indicine (2). For assay, indicine N-oxi

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2 To whom requests for reprints should be addressed.

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Indicine N-oxide was reduced to indicine with zinc and ammonium chloride. Heliotrine and heliotrine N-oxide were used as internal standards. Retronecine, the product of the side-chain hydrolysis of indicine, could also be detected by this method, eluting much earlier than indicine. Total conjugates were determined by hydrolysis in 0.5 n HCl at 100°C for 1 hr. Glucuronides were determined by hydrolysis with β-glucuronidase, 20 units/ml, at pH 4.5 for 12 hr at room temperature.

Hepatic microsomes were prepared by the method of Ernster et al. (15) and suspended in 0.15 M KCl. Protein was determined by the method of Lowry et al. (31). Metabolism of indicine N-oxide was determined at 37°C using an incubation mixture containing microsomal protein (4 mg/ml), Tris-HCl buffer, pH 7.4 (600 μmol), MgCl₂ (30 μmol), glucose-6-phosphate (75 μmol), glucose-6-phosphate:NADP⁺ oxidoreductase (EC 1.1.1.49; 6 units), NADP⁺ (6 μmol) or NADH (6 μmol), and indicine N-oxide (0.95 μmol), all in a final volume of 6 ml. Portions of the incubation medium were taken at 0, 5, 10, and 20 min for the determination of indicine. The formation of pyrrolic metabolites from indicine and retronecine was measured colorimetrically with Ehrlich’s reagent, as described by Mattocks and White (35), over a 30-min period at 37°C with the incubation medium described above and substrate concentrations of 0.3 to 3 mm. Metabolism of indicine N-oxide by the gut flora was determined at 37°C using a suspension of the total gut contents from a rabbit fasted for 24 hr, 24 mg protein per ml in 6 ml Dulbecco’s phosphate-buffered saline containing 20 mM glucose. Portions of the medium were taken for determination of indicine at 0, 5, 10, and 20 min. N₂ and CO used for gassing the incubation medium were passed through a deoxygenating solution (36).

Indicine N-oxide was obtained from the National Cancer Institute (Bethesda, Md.). Indicine was prepared by zinc and acetic acid reduction of indicine N-oxide as described by Kugelman et al. (28). Retronecine was also prepared from indicine N-oxide by the method of Adams and Rogers (1). NADH, NADPH, glucose-6-phosphate, glucose-6-phosphate: NADP⁺ oxidoreductase, and β-glucuronidase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Other reagents were the purest grades available.

RESULTS

Drug and Metabolite Levels in the Rabbit. Indicine N-oxide was administered by rapid i.v. injection to rabbits at 3 dose levels up to 111 mg/kg. Doses of anticancer agents are conventionally expressed in terms of body surface area (20), and for indicine N-oxide they were 0.45, 1, and 2 g/sq m.

Indicine N-oxide was removed rapidly from the plasma and exhibited a biphasic disappearance (Chart 2) with a mean (± S.E.: n = 3) t₁/₂ of 12.8 ± 1.7 min, t₁/₂ of 70.3 ± 5.8 min, and clearance of 16.1 ± 3.7 ml/min/kg body weight. The curves are superimposable, indicating that elimination processes are not saturable in this dose range. Indicine could be detected in the plasma at levels which appeared to be related to the dose of indicine N-oxide administered (Chart 2). At the lowest dose of indicine N-oxide, the indicine was below the limits of detection of the assay, i.e., less than 50 ng/ml. After an initial peak, possibly representing the small amount of indicine present in the indicine N-oxide preparation (5 different batches contained a mean ± S.E. of 0.039 ± 0.013% indicine), plasma levels of indicine fell to a relatively constant value after 3 hr. Indicine N-oxide and indicine were excreted in the urine, both free and in the form of conjugates (Table 1). Glucuronides and for indicine N-oxide they were 0.45, 1, and 2 g/sq m.

![Chart 1. Structure of indicine N-oxide and heliotrine N-oxide. The hydroxyl group of the pyrrolizidine ring (C-7) is in the a position for heliotrine N-oxide and in the β position for indicine N-oxide.](chart1.png)

<table>
<thead>
<tr>
<th>Indicine N-oxide</th>
<th>Human (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>123.2 ± 15.4⁵ (35.9)⁶</td>
</tr>
<tr>
<td>Conjugated</td>
<td>75.8 ± 23.3 (23.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Indicine</th>
<th>Rabbit (mg)</th>
<th>Human (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>20 ± 1.2 (0.6)</td>
<td>110.3 ± 7.2 (2.1)</td>
</tr>
<tr>
<td>Conjugated</td>
<td>6.8 ± 3.6 (1.9)</td>
<td>56.8 ± 18.3 (1.1)</td>
</tr>
</tbody>
</table>

*Mean ± S.E.  
Numbers in parentheses, percentage of the dose administered.

Table 1

Urinary excretion of indicine N-oxide and indicine

Values are the mean excretion of indicine N-oxide and its metabolites in the urine in 24 hr following the i.v. administration of indicine N-oxide at the doses shown. There were 3 individuals in each group. Conjugates refer to acid-labile conjugates. Dose: rabbit, 2 g/sq m; human, 3 g/sq m.
accounted for 29% of total conjugates in the rabbit (individual results not shown). Urinary excretion of indicine N-oxide over 24 hr is shown in Chart 3. Most of the indicine N-oxide was excreted within 2 hr of administration. The 24-hr urinary excretion was 83, 73, and 65% of the low, medium, and high doses, respectively. Indicine was excreted in the urine throughout the 24-hr collection period (Charts 3, 6, and 7).

**Drug and Metabolite Levels in Humans.** The removal of indicine N-oxide from the plasma was more variable and slower in humans than in rabbit (Chart 4). At the highest dose studied, 3000 mg/sq m, t1/2 (n = 3) was 40.3 ± 29.9 min, t1/2 was 85.6 ± 41.8 min, and clearance was 1.8 ± 0.2 ml/min/kg body weight. Indicine N-oxide and indicine were excreted in the urine in the free form and as conjugates (Table 1). Glucuronides accounted for 39% of the total acid-labile conjugates (individual results not shown).

**In Vitro Metabolism of Indicine N-Oxide.** Indicine N-oxide was reduced to indicine under anaerobic conditions by rabbit gut flora and by the hepatic microsomal fraction in the presence of NADPH. The rate of reduction was linear for 20 min under the assay conditions used. The Km for both pathways was similar, 0.51 mM for the gut flora and 0.43 mM for the hepatic microsomes (Chart 5). Some characteristics of metabolism are shown in Table 2. The formation of indicine was completely inhibited by oxygen in all cases. Carbon monoxide produced an almost complete inhibition of NADPH-dependent microsomal metabolism and a 71% inhibition of metabolism by the gut flora. Metabolism was almost completely confined to the microsomal fraction with only a little metabolism present in the hepatic supernatant fraction which showed a marked NADH dependence and no inhibition by carbon monoxide. The NADPH dependence and the inhibition by carbon monoxide of the reduction of indicine N-oxide to indicine suggests that microsomal metabolism is mediated by cytochrome P-450. This has been reported for other N-oxides (24). It was surprising to find, therefore, that NADH, which is much less efficient than NADPH in reducing cytochrome P-450 (21), was as effective as NADPH in reducing indicine N-oxide. NADH-dependent metabolism was inhibited over 80% by carbon monoxide showing it to be mediated by cytochrome P-450. The in vitro metabolic studies of indicine N-oxide shown in Table 2 were conducted at concentrations of indicine N-oxide found in the
plasma under therapeutic conditions, 0.16 mM, concentrations lower than the $K_m$. However, similar results were obtained with 1 mM indicine N-oxide.

Small amounts of retronecine were formed from indicine, although not from indicine N-oxide, by unfortified rabbit liver homogenates at 37°. No retronecine could be detected in the urine of rabbits receiving indicine N-oxide.

**In Vitro Metabolism of Indicine.** The toxicity of the pyrrolizidine alkaloids is generally ascribed to their ability to be metabolized to highly reactive dihydropyrrolizines which can be detected with Ehrlich’s reagent (1, 42). Indicine and retronecine are metabolized by rabbit liver microsomes in the presence of O$_2$ and NADPH, to give products which will react with Ehrlich’s reagent (Table 3).

**Reduction of Indicine N-oxide In Vivo by Liver and Gut Flora.** Our *in vitro* studies showed that indicine N-oxide could be reduced to indicine with equal facility by liver microsomes or by the gut flora. Studies were designed to determine the relative importance of these 2 sites of metabolism in *vivo*. Indicine N-oxide, 56 mg/kg p.o., resulted in a 107% increase in the 24-hr urinary excretion of indicine compared to same dose of indicine N-oxide administered i.v. 5 days earlier to the same rabbit (Chart 6). The mean level of plasma indicine, between 2 and 5 hr, increased from 0.09 to 0.43 µg/ml following p.o. administration. Neomycin and erythromycin administered p.o. to the rabbit reduced the aerobic bacterial content of the gastrointestinal tract by 30% and the anaerobic bacteria by at least 98%. There was 76% decrease in the 24-hr urinary excretion of indicine after antibiotic treatment (Chart 7). The steady-state plasma levels of indicine were reduced from 0.17 to 0.05 µg/ml (results not shown). This dose of antibiotics had no apparent effect upon the ability of hepatic microsomes to reduce indicine N-oxide *in vitro*. Treatment with neomycin alone, which produced only a 30% decrease in the levels of anaerobic bacteria although still decreasing aerobic bacteria by 82%, resulted in a 47% decrease in the 24-hr urinary excretion of indicine (results not shown).

**Biliary Excretion of Indicine N-Oxide.** Having established that the gut flora could play a role in the metabolic reduction of indicine N-oxide, there remained the problem of how i.v. administered indicine N-oxide found its way into the gastrointestinal tract. Relatively little indicine N-oxide was excreted in the bile (Chart 8). The levels of indicine N-oxide in the bile were similar to those in the plasma at the midpoint of the bile collection period. Free indicine, however, was concentrated in the bile about 10-fold compared to plasma. Indicine conjugates accounted for only 18% of the total indicine excreted. Cannulating the bile duct failed to prevent the excretion of considerable amounts of indicine in the urine, 13.0 mg in 24 hr.

**Table 2**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hepatic microsomes</th>
<th>Hepatic supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADPH (ng/min/mg)</td>
<td>NADH (ng/min/mg)</td>
</tr>
<tr>
<td>O$_2$</td>
<td>0.0 ± 0.0$^a$</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>N$_2$</td>
<td>344.7 ± 14.7</td>
<td>337.0 ± 28.2</td>
</tr>
<tr>
<td>CO</td>
<td>1.4 ± 0.8</td>
<td>58.1 ± 24.7</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.E. of 3 observations.

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**Table 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indicine</td>
<td>0.71</td>
<td>0.12</td>
</tr>
<tr>
<td>Retronecine</td>
<td>1.16</td>
<td>0.13</td>
</tr>
</tbody>
</table>

The formation of pyrrolic metabolites was determined with Ehrlich’s reagent as described in the text at 37° under aerobic conditions over a 30-min period.
Administration of indicine N-oxide (i.v.) to the rabbit leads to a dose-dependent appearance of indicine in the plasma and to the excretion of indicine in the urine. Urinary excretion of indicine is relatively constant over 24 hr, unlike the excretion of indicine N-oxide which is almost complete within 2 hr of administration. Indicine was also found in the plasma and urine of patients receiving indicine N-oxide i.v. The rapid excretion of indicine N-oxide is not surprising. Pyrrolizidine alkaloid N-oxides have a high water solubility, and it has been suggested that they represent true detoxification products of the parent alkaloid (25, 34). It is likely that the pyrrolizidine N-oxides themselves, unlike the parent alkaloid, are not toxic (33). Alkaloid N-oxides are, in general, much less toxic than the parent alkaloid (5), and it has been suggested that the continuous reduction of N-oxides in the body leads to a slow release of active tertiary amine (38). At one time, it was hoped that administration of the N-oxide might provide a means of improving the therapeutic index of many alkaloids, although this expectation has not been borne out in most cases (5).

Liver microsomes and gut flora both reduce indicine N-oxide to indicine under anaerobic conditions. Reduction of pyrrolizidine alkaloid N-oxides by the hepatic microsomal fraction has not been reported previously. Carbon monoxide is a potent inhibitor of microsomal indicine N-oxide reduction suggesting the involvement of cytochrome P-450. Although, as has been pointed out (18), carbon monoxide inhibition is not proof of the involvement of cytochrome P-450, microsomal reduction of other N-oxides has been shown to be mediated by cytochrome P-450 (24, 27, 40). The possible involvement of cytochrome P-450 is particularly interesting in view of the finding that NADH is as effective as NADPH in supporting microsomal reduction of indicine N-oxide. NADH is only about one-tenth as effective as NADPH in reducing cytochrome P-450 (21, 26) and in supporting oxidative drug metabolism (21, 41). It is difficult, therefore, to explain the microsomal reduction of tertiary amine N-oxides in terms of the activity of cytochrome P-450 as a mixed-function oxidase with the N-oxide replacing oxygen as has been proposed by Sugiuara et al. (40).

The effectiveness of the liver in catalyzing the metabolic reduction of azo and nitro compounds in vivo, reactions which like the reduction of indicine N-oxide are inhibited by oxygen in vitro (16), has been questioned. Gillette (17) has pointed out that because of the high affinity of oxygen for cytochrome P-450 even low levels of oxygen would be expected to inhibit nitroreductase activity. The liver receives blood with a mean oxygen content around 3 mM (7) which is considerably higher than the level reported to inhibit microsomal reduction of tertiary amine N-oxides, 2 to 3 μM (40). On the other hand, there is evidence to suggest that the liver is not uniformly perfused in vivo and that at any moment some areas of the liver are poorly perfused (14, 19) and presumably hypoxic. The results of the present study suggest that in the rabbit gut flora are probably the major site for the formation of indicine from indicine N-oxide. The p.o. administration of indicine N-oxide produces an increase in the plasma levels and urinary excretion of indicine compared to i.v. administered indicine N-oxide. A short course of p.o. neomycin and erythromycin has been used to decrease the aerobic and anaerobic microflora in the gastrointestinal tract of patients before surgery (3, 37). Treatment of rabbits with neomycin and erythromycin leads to a decrease in the plasma levels and the urinary excretion of indicine. The gut flora have also been suggested to be the major site for the reduction of nicotine N-oxide in humans (4).

Cannulating the bile duct failed to prevent the excretion of considerable amounts of indicine in the urine following the i.v. administration of indicine N-oxide. It is probable that the unchanged indicine N-oxide molecule finds its way into the gut by passive diffusion. The liver and other tissues cannot, however, be ruled out as a site for the reduction of indicine N-oxide. A similar conclusion was reached by Dajani et al. (13) from their studies on the reduction of nicotine N-oxide in the rat.

It is not known whether indicine formed from indicine N-oxide contributes to the toxic or antitumor effects of indicine N-oxide. Work with other pyrrolizidine N-oxides has suggested that their toxic effects are due entirely to the formation of the parent alkaloid (33). Indicine is metabolized by the liver, as are other pyrrolizidine alkaloids (25) to dihydropyrrolizidine derivatives. These are thought to be the species responsible for the acute toxic effects of the alkaloids (34). They are highly reactive in vitro (11) and will react with protein (22) and cross-link strands of proteins.
of DNA (6, 43). A probable metabolic pathway for indicine N-oxide is shown in Chart 9. Indicine N-oxide is reduced by the gut flora to indicine which is then conjugated or oxidized by the hepatic microsomal fraction to pyrrolic metabolites. Small amounts of retrocine are also formed from indicine by the liver and this may be oxidized to its pyrrolic metabolite, dehydroretronecine. A suggested mechanism of alkylation by dehydroretronecine involves protonation and subsequent dehydration to form a stabilized carbonium ion which is thought to be the alkylating species (22). The ester group of dehydroretronecine would be expected to form an even more facile leaving group, due to resonance stabilization of the carboxylic anion which is removed. Reaction of the dehydroretronecine carbonium ion formed with water would yield dehydroretronecine. Culvenor (10) has concluded that the antitumor activity of the pyrrolizidine alkaloids is associated with the same functional region of the molecule as is hepatotoxicity. Indicine N-oxide appears to be unique among the pyrrolizidine alkaloids in having a favorable ratio of desired activity to toxicity, and it is possible that antitumor effects may not be mediated through the formation of indicine and dehydroindicine.

REFERENCES


36. Metabolism of Indicine N-Oxide

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