Effect of Allyl Alcohol-induced Sublethal Hepatic Damage upon Doxorubicin Metabolism and Toxicity in the Rabbit

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ABSTRACT

A model of hepatic dysfunction in vivo has been developed in rabbits to determine the effects of sublethal hepatic necrosis upon doxorubicin pharmacology. Eight New Zealand white rabbits were given 3 mg/kg doxorubicin i.v. Plasma doxorubicin and metabolite pharmacokinetics were determined and toxicity assessed by nadir complete blood counts. Hepatic function was assessed by the pulmonary excretion rate of $^{14}$CO$_2$ from $[^{14}]$Caminopyrine. Hepatic necrosis was produced by i.v. injection of 1.35 mg/kg of a 2% allyl alcohol solution. Doxorubicin administration and pharmacokinetics were repeated.

Doxorubicin enhances the hepatotoxicity of allyl alcohol. Hepatocellular necrosis does not alter the plasma pharmacokinetics of doxorubicin but does increase the plasma exposure of doxorubicinol. Doxorubicin-induced myelosuppression is enhanced by allyl alcohol pretreatment. These data suggest that in circumstances of reduced hepatocellular volume or acute hepatocellular necrosis, a key plasma marker of doxorubicin-induced acute toxicity may be doxorubicinol.

INTRODUCTION

Cancer patients receive multiple drugs during their treatment, many of which are either detoxified or excreted by the liver. Dox, a major clinical antineoplastic agent, is reduced in cellular cytoplasm by aldehyde and ketone reductases (1–7) and detoxified in the hepatocyte most likely by NADPH cytochrome P-450 reductase catalyzed reduction of the oxygen linked glycoside to deoxyglycone forms (8, 9) (Fig. 1). Dox and doxorubicinol are excreted unchanged by the liver and to a small extent (approximately 6%) in the urine (1, 3–5, 10, 11). Small amounts of aglycones are found circulating in human and in rabbit plasma (12).

Clinical-pharmacokinetic data from 8 patients suggested that moderate changes in hepatic function caused increased Dox toxicity reflected in altered pharmacokinetics (13, 14). Other studies have suggested that Dox pharmacokinetics and metabolism correlated with deranged hepatic function as measured by bromsulphthalein retention time (13), bilirubin (13, 15), or indocyanine green clearance (16); yet other work has failed to correlate drug-induced toxicity to pharmacokinetics (17–21).

We hypothesized that correlations of Dox pharmacokinetics with acute toxicity in humans have not been possible because (a) parent compound may not be the sole or optimal marker for drug activity or toxicity, (b) assay techniques were not sensitive enough to detect important metabolites, (c) there was insufficient knowledge about the activity and toxicity of Dox metabolites and, (d) there were too many uncontrolled variables in studied clinical populations. These variables include the effects of coadministration of drugs that are hepatically metabolized, variations in the degree of illness among cancer patients, and undefined clinical or subclinical hepatic disease which may alter drug pharmacokinetics and pharmacodynamics.

In order to control possible variables, we have developed the rabbit as an economically feasible animal model of Dox metabolism. This model has the advantage of repetitive blood sampling and repetitive drug administration in the same animal. Compared to rodents, Dox metabolism in the rabbit is closer to the human (1, 22, 23). In order to simulate acute hepatic dysfunction, we induced sublethal hepatic lesions in rabbits with allyl alcohol. We then evaluated Dox pharmacokinetics, metabolism, hepatic function, and toxicity in paired studies with allyl alcohol-induced hepatic lesions. These studies were performed in the rabbit to determine, in a controlled model, whether pharmacokinetics affected the toxic ongoing events in the liver and whether they predicted pharmacodynamic changes as measured by myelosuppression. These lesions caused a small increase of Dox AUC and an increase of Doxol AUC. Myelotoxicity was enhanced by allyl alcohol pretreatment.

MATERIALS AND METHODS

Materials. Doxorubicin hydrochloride was purchased as commercial material from Adria laboratories. Tetrahydrofuran was obtained from Fisher Laboratories of Allied Industries, Inc. and was HPLC grade. Ammonium formate (Fisher certified) buffer, 0.1% (w/v) (16 mM) was made daily with distilled water adjusted to pH 4.0 with formic acid (Fisher certified). The buffer was subsequently filtered and degassed using a 0.45-μm filter (Millipore, Milford, MA) before use. For chemical extraction, chloroform (Fisher certified), and ammonium sulfate (Fisher certified) were used. Allyl alcohol (J. T. Baker Chemical Co., Phillipsburg, NJ) was diluted in filtered 0.9% saline solution to a 2% concentration before use. [4$^{14}$Caminopyrine ([dimethyl-$^{14}$C aminojaminopyrine; specific activity, 90–100 mCi/mol) was obtained from Research Products International Corp., Mt. Prospect, IL. Aquasol was obtained from New England Nuclear, Boston, MA.

Synthesis of Dox Metabolites. The following metabolites were synthesized according to the published procedures of Takanashi and Bacheur (24): Doxol; doxorubicin aglycone; doxorubicinol aglycone; 7-deoxydoxorubicinol aglycone; and 7-deoxydoxorubicin aglycone. Identity of the standards was confirmed by negative ionization mass spectrometry using a Nermag R-10-10-B quadrupole mass spectrometer equipped with a Sidar 110 data system. Purity of all standards was confirmed by a single peak on HPLC at published retention times (12). The lack of other peaks in the HPLC trace at the sensitivity used suggested at least 95% purity.

Rabbits. Female, white, New Zealand rabbits, 3–4 kg were obtained from a local supplier of laboratory rabbits (Myrtles, Eagleville, TN) and maintained in clean, individual cages in the animal quarters at the Nashville Veterans Administration Medical Center. The animals were certified Pasteurella and disease free upon receipt and were kept in a separate clean room from other rabbits. The quarters are supervised by the local Animal Care Committee as required by United States Federal guidelines under the supervision of a veterinarian. Study procedures were reviewed by the veterinarian and the local Research and Development Committee prior to the study. Care was taken to minimize animal discomfort.
Liver Damage and Dox Metabolism

![Diagram of doxorubicin metabolism](image)

**Fig. 1. Proposed metabolic schema of doxorubicin metabolism (modified and reprinted by permission from Ref. 39).**

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Schema of study</th>
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<tbody>
<tr>
<td>Day</td>
<td>1 2 3 4 5 28 29 30 31 32 33</td>
</tr>
<tr>
<td>Doxorubicin (n = 8)</td>
<td>3 mg/kg 3 mg/kg</td>
</tr>
<tr>
<td>Allyl alcohol*</td>
<td>3 mg/kg</td>
</tr>
<tr>
<td>Pharmacokinetic study*</td>
<td>[14C]Aminopyrine breath test</td>
</tr>
<tr>
<td>[14C]Aminopyrine breath test</td>
<td>x x x x x x x x x</td>
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</table>

*Administration of 1.35 ml/kg of a 2% allyl alcohol solution i.v.
*Three-mi blood specimens were drawn after doxorubicin administration at 5 and 15 min and 1, 2, 4, 8, 12, 24, 36, 48, and 72 h. Complete blood counts were obtained on days 1, 5, 6-9, 28, and 33-37. Animals were sacrificed as described in the text. After the second WBC nadir, livers were removed fresh, fixed, and processed.

The rabbits were fed *ad libitum* and allowed unlimited water supply. The animals were sacrificed by intracardiac injection of 1 ml of T-61 euthanasia solution (Hoechst, Sommerville, NJ) which caused death within 15 s of injection.

Experimental Procedure. Eight rabbits were treated according to the schema in Table 1. The blood was immediately centrifuged at 4°C. The plasma supernatant was removed and frozen at −20°C until assay. Samples were assayed within 3 weeks of their collection. All studies were performed as paired studies in the same rabbits.

[14C]Aminopyrine Breath Tests in Rabbits. Rabbits were given 0.5 μCi of [14C]aminopyrine diluted in 1 ml of normal saline i.v. Their breaths were collected by a glass hood attached to the restrainer rack connected to suction. The exhaled 14CO2 was collected in 10 ml of a methanol:ethanolamine (2:1, v/v) solution in 20 ml scintillation vials. The exhaled breath was dehydrated in a sulfuric acid trap prior to collection in the methanol:ethanolamine. The 14CO2 was collected in 20-min fractions from injection to 180 min. After the addition of 5 ml of Aquasoil and 5 ml of methanol, the radioactivity in the collection vials was counted for 10 min. The Kt was obtained by calculating the slope from the logarithmic linear regression of cpm against time. Half-life was calculated from the equation 0.693/Kt.

Dox Assay. Specimens were assayed by HPLC after a chloroform:isopropanol (1:1, v/v) extraction (25) according to a recently described procedure (12). The technique was modified by the use of a 15-cm μBondapak phenyl column (Waters Associates, Millipore Corp., Milford, MA) in place of a 30-cm μBondapak phenyl column.

Complete Blood Count and Chemistry Assays. CBC including WBC, hemoglobin, hematocrit, and platelet count was performed using an ELT-800 cell counter (Ortho Diagnostics, Raritan, NJ). Serum assays for hepatic function tests including aspartate transferase, pyruvate transferase, total and direct bilirubin, lactic dehydrogenase, albumin, and alkaline phosphatase were assayed by Automated Stat/Routine Analyzer (Beckman Instruments, Inc., Brea, CA).

Pharmacokinetics. Doxorubicin plasma concentrations were fit by means of the nonlinear curve fitting program MLAB (26) to the equation

$$C_{0} = A e^{-\alpha t} + B e^{-\beta t}$$

where C0 is the plasma concentration at time t, A and B are constants, and α and β are linear elimination rate constants. A 1/(concentration)2 weighting function was used for curve fitting. The AUC was calculated for Dox by the log trapezoidal rule to infinity. Clearance was calculated by

$$\frac{D_{0}}{AUC}$$

where D0 is the administered dose. Volume of distribution was calculated by a noncompartmental method (27)

$$V_{dav} = D_{0} \frac{(AUMC)}{AUC^{2}}$$

where AUMC is the area under the moment curve. Areas under the curve for metabolites were calculated by the log-trapezoidal rule. A Student's t test for paired means was used to test for statistical significance.

Pathology. Liver from each rabbit was fixed by immersion in 10% neutral buffered formalin. Tissues were dehydrated routinely and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. Histological examination was made on the tissue from each animal without knowledge of its treatment by the examiner. Each slide was graded for the presence of degeneration and necrosis using the following grading schemes: (a) Degeneration: 1+, fine cytoplasmic vacuolation; 2+, vacuoles of a size occupying less than one-half the cytoplasm of the cells; 3+, vacuoles of a size occupying the entire cytoplasm of the cells; (b) Degree of Degeneration: 1+: 1-2 cell layers involved; 2+: 3-5 cell layers involved; 3+, 6-10 cell layers involved; 4+, diffusely involves the liver lobule; (c) Necrosis: 1+, 1-2 acidophil bodies/10 HPFs; 2+, 3-5 acidophil bodies/10 HPFs; 3+, 5-10 acidophil bodies/10 HPFs; 4+, >10 acidophil bodies/10 HPFs.

The extent of degeneration was determined by adding the grades of degeneration and degree of degeneration. Pathological data from the treated animals were compared to data on livers from animals which had been sacrificed without any intervention or had been treated with Dox only. The control animals had been cared for in the same environment over the same span of time as the treated animals but were not simultaneous controls. Student's t test for unpaired data was used to determine the statistical significance.

**RESULTS**

[14C]Aminopyrine Breath Test. The [14C]aminopyrine breath test was adapted to rabbits from the technique used in the rat (28) to provide sensitive measurement of hepatic function. Using this technique we have found that the [14C]aminopyrine half-life is prolonged in the rabbits as they gained weight from 2.5-3.0 kg. We attributed this increase to the maturation from juvenile to adult hepatic function. Over a subsequent 4-month period the Kt and half-life stabilized with little variability within rabbits but substantial variability between rabbits. Additional work has documented increases in half-life in the presence of
LIVER DAMAGE AND Dox METABOLISM

Table 2 Doxorubicin pharmacokinetic parameters (n = 8)

<table>
<thead>
<tr>
<th></th>
<th>AUC (µmol x h)</th>
<th>Terminal half-life (h)</th>
<th>C₀ (µmol)</th>
<th>C₁τb (liter/min/kg)</th>
<th>Vm (liter/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment</td>
<td>2.03 ± 1.25*</td>
<td>23.1 ± 9.8</td>
<td>0.37 ± 0.27</td>
<td>0.058 ± 0.027</td>
<td>91.1 ± 49.9</td>
</tr>
<tr>
<td>Allyl alcohol pretreatment</td>
<td>3.06 ± 2.28</td>
<td>28.8 ± 12.4*</td>
<td>0.41 ± 0.22</td>
<td>0.060 ± 0.068</td>
<td>87.9 ± 46.9</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Fig. 2. Ratio of [14C]aminopyrine to each rabbit's individual control. Rabbits received a series of 3 daily, consecutive [14C]aminopyrine breath tests prior to allyl alcohol treatment. Animals were given allyl alcohol and a day later Dox, 3 mg/kg i.v. (C), Dox alone, 3 mg/kg i.v. (W), or allyl alcohol alone (Δ). [14C]-Aminopyrine half-life for each test day after treatment is divided by each individual rabbit's pretreatment control to obtain the [14C]-aminopyrine to control ratio. Allyl alcohol pretreatment plus Dox versus Dox alone: P = 0.04, day 2; P = 0.001, day 3. Allyl alcohol pretreatment alone versus allyl alcohol pretreatment plus Dox: P = 0.01, day 2; P = 0.05, day 3. Allyl alcohol alone versus Dox alone: P = 0.01, day 2; P = 0.06, day 3.

excretion over pretreatment control values. The addition of Dox to allyl alcohol caused the [14C]aminopyrine half-life ratio to increase in some but not all animals compared to allyl alcohol alone (Fig. 2). The changes lasted 2 days and returned to baseline by day 4.

Dox and Metabolite Pharmacokinetics. Dox pharmacokinetic data are listed in Table 2. No significant changes in Dox area under the curve, clearance, or volume of distribution were found in the paired studies. The terminal half-life was prolonged in the allyl alcohol pretreatment study. This difference is not large enough to have substantial effects on other important parameters of parent compound systemic exposure or elimination.

Evaluation of the metabolite data reveals increased exposure to the metabolite, Doxol (Table 3; Fig. 3). This increase was present in all but one of the rabbits treated. The degree of increase in the Doxol area under the curve varied markedly among the animals. This is reflected in the large S.D. The area under the curve of the other major metabolite detected, 7-deoxydoxorubicinol aglycone, was unchanged by the allyl alcohol pretreatment. Small amounts of doxorubicin aglycone were detected for short periods of time immediately after drug injection. 7-Deoxydoxorubicin aglycone was intermittently detected in some rabbits in the first h after injection but became undetectable after the first h. In the majority of rabbits, 7-deoxydoxorubicin aglycone was not detected in either Dox alone or allyl alcohol pretreatment studies. Two nonpolar, unidentified proposed metabolites, arbitrarily labeled “C” and “G” (12) were consistently detected in low concentrations in the rabbits when they received Dox alone or Dox after allyl alcohol pretreatment. Allyl alcohol pretreatment did not affect the areas under the curve of these proposed metabolites (Table 3).

Previous human Dox pharmacokinetic studies suggested that the ratio of Dox to Doxol AUC reversed from less than 1.0 in patients with normal hepatic function to greater than 1.0 in patients who had hepatic dysfunction (19). We saw the same phenomenon in the rabbit studies presented here.

Chemical Analysis of Hepatic Function. Acute elevation in transaminase assays were evident by 1 h after allyl alcohol infusion. Four h following allyl alcohol administration, alkaline phosphatase and γ-glutamyltransferase were 9 times baseline, aspartate aminotransferase was 34 times baseline, and alanine aminotransferase was 244 times baseline. Total bilirubin, total

Table 3 Dox metabolite areas under the curve (n = 8)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>No pretreatment (µmol/h)</th>
<th>Pretreatment with allyl alcohol (µmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>1.29 ± 0.92*</td>
<td>5.27 ± 2.88*</td>
</tr>
<tr>
<td>7-Deoxydoxorubicin aglycone</td>
<td>1.10 ± 1.47*</td>
<td>1.69 ± 1.41*</td>
</tr>
<tr>
<td>“C”</td>
<td>0.30 ± 0.12</td>
<td>0.21 ± 0.17*</td>
</tr>
<tr>
<td>“G”</td>
<td>0.34 ± 0.35</td>
<td>0.45 ± 0.25*</td>
</tr>
</tbody>
</table>

* Mean ± SD.

* Differs from no pretreatment controls, at P < 0.01.
protein, and serum albumin were unchanged. By 24 h, enzyme concentrations were decreasing.

Toxicity. CBC data shown in Table 4 reveal significant decreases in nadir hematocrit and hemoglobin determinations, WBC, and platelet counts between the no pretreatment and allyl alcohol pretreatment groups.

Pathology. The liver pathology data from rabbits treated with allyl alcohol and Dox was compared to the pathology of 2 separate control animals (nonsimultaneous). A group of 8 rabbits sacrificed over a year's time had received no treatment of any kind but was cared for in the same location as the treated animals. These 8 rabbits are referred to as control animals. A second group of 4 rabbits served as allyl alcohol only controls and are described above. After 10 days, the animals receiving allyl alcohol alone were sacrificed and their livers examined for pathology. These animals are referred to as controls plus allyl alcohol. Histological examination of the livers from the control rabbits showed predominately normal hepatic parenchyma. The liver parenchyma of the control rabbits maintained the normal hepatic architecture with portal tracts, central veins, and liver plates composed of one layer of hepatocytes (Fig. 4, top). The majority of these animals had no evidence of degeneration. The liver tissue from 3 of the 8 control rabbits had a diffuse, fine vacuolization of the cytoplasm, defined as 1+ degeneration, but no control animal had large cytoplasmic vacuoles. Necrosis was seen in the liver tissue of only one of the control rabbits. This was characterized by single acidophil bodies located nonspecifically within the lobule. Degeneration was seen in the liver tissue of 6 of the 8 treated rabbits (Fig. 4, middle). Degeneration in these animals was characterized by cloudy swelling, which appeared as a diffuse, fine vacuolization of the cytoplasm, or by the presence of large vacuoles in the cytoplasm which did not displace the nucleus. In 4 of the 8 treated rabbits the degeneration was located around the central vein, and in 2 animals it involved the entire lobule in a diffuse pattern. Evidence of spotty necrosis (Fig. 4, bottom) was seen in all treated rabbits. No massive necrosis was seen in any of the animals.

Comparison of the degree of degeneration and necrosis in the liver of the control and treated rabbits is listed in Table 5. The treated animals had a statistically significant increase in the extent of degeneration and necrosis when compared with Student's t test.

**DISCUSSION**

The rabbit model reported here provides important information that is unobtainable in human studies. Controlled,
Fig. 4. Upper, low power photomicrograph of the liver from a control rabbit. Note the liver plates (arrow) are one cell layer thick and the cytoplasm of the cells is homogeneous. H & E; bar, 300 μm. Middle, low power photomicrograph of the liver from a rabbit treated with allyl alcohol and Dox. Note vacuolization of the hepatocytes (arrow) which extends from the central vein (C) to the portal tract (P). The normal hepatic architecture is disrupted and the liver plates are expanded. H & E; bar, 300 μm. Lower, higher power detail of hepatocytes from the same rabbit liver pictured in middle section. Note the fine vacuolization in some hepatocytes (short arrow) and larger vacuoles in other liver cells (long arrow). Acidophil bodies (arrowhead) were located nonspecifically within the lobule. H & E; bar, 100 μm.
The influence of ranitidine on the pharmacokinetics and toxicity of doxorubicin.

CBCs, no change in any of the parameters from control were given the animals in this study. After 10 days of consecutive treatment with allyl alcohol, no differences in CBCs compared to control values. Allyl alcohol pretreatment induced increased myelotoxicity compared to the paired control. Doxol concentrations were consistently elevated in the animals after allyl alcohol pretreatment study when compared to the paired control. Doxol concentrations were not statistically changed after pretreatment with allyl alcohol compared to control values. Allyl alcohol pretreatment induced increased myelotoxicity compared to the paired control. Doxol concentrations were consistently elevated in the animals after allyl alcohol pretreatment. These elevations were reflected in the significant increase in the Doxol AUC compared to control values. Other aglycone metabolites were not affected by hepatic dysfunction as were Doxol concentrations. The enhanced Dox acute myelotoxicity observed in the allyl alcohol pretreatment study when compared to the paired controls is not artifactual: (a) the same volume of blood was obtained from the animals for each of the paired studies; (b) the pretreatment baseline CBC for the allyl alcohol study was the same as that for the Dox baseline study; (c) in a similarly designed paired study of a separate group of animals receiving ranitidine as a microsomal inhibitor, no differences in CBCs were found in the animals after a second course of Dox separated by a 4-week interval; (d) 4 animals as described previously were treated with allyl alcohol alone at the same dose given the animals in this study. After 10 days of consecutive CBCs, no change in any of the parameters from control were found (data not shown).

These data suggest that Doxol concentrations may play an important role in the definition of drug-induced myelotoxicity and may serve as an important predictive marker of drug-induced toxicity. This postulate is supported by other data. Chan et al. (19) after studying doxorubicin pharmacokinetics in hepatoma patients, found that noncirrhotic patients had Dox concentrations below those of Dox and had minimal drug-induced toxicity, whereas those patients with cirrhosis had elevated Doxol concentrations and increased drug-induced toxicity. Doxol is less cytotoxic in vitro to human myeloid progenitors (33-35) and pancreatic cancer cell lines (36) than Dox, yet it is functionally more cardiotoxic than Dox (37).

Based upon the metabolism of Dox, Doxol should be an important marker of drug toxicity. The aldehyde and ketone reductases that reduce doxorubicin to doxorubicinol are ubiquitous and probably not affected by reduction in hepatocellular volume or function (6, 7). Hepatic NADPH cytochrome P-450 reductase may be rate limiting with Dox detoxification (38). We hypothesize that in the setting of hepatic cellular necrosis, the detoxification of Dox to 7-deoxydoxorubicinol aglycone is more likely to be inhibited than the reduction of Dox to Doxol. If Doxol at physiological concentrations is more toxic than previously understood, as some of our preliminary pharmacological and toxicological data support, or if hepatic NADPH cytochrome P-450 reductase is rate limiting for drug detoxification, then in circumstances of reduced hepatocellular volume or acute necrosis, a key plasma marker of Dox-induced acute toxicity may be Doxol.

The data produced in a tightly controlled animal model of Dox metabolism may be applicable to human studies but would require verification in a clinical trial. Assay of Dox and metabolite pharmacokinetics following an initial dose in humans may provide important guidelines for upward or downward adjustment of dose in an individual. It would be ideal to have an inert marker that clinically predicts doxorubicin pharmacodynamics. Evidence that such markers (such as aminopyrine, indocyanine green) are of clinical value is lacking. The rabbit model may provide such evidence that will allow the design of rational clinical trials to answer these important questions.

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REFERENCES

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