Alterations in Adriamycin Efficacy by Phenobarbital

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SUMMARY

Adriamycin dosage should be reduced in patients with impaired liver function, since adriamycin disposition is influenced by liver metabolism and biliary excretion. It follows that drugs that increase the metabolism or excretory capacity of the liver may decrease adriamycin concentrations to suboptimal values. Adriamycin metabolism was therefore studied in mice pretreated with phenobarbital (75 mg/kg i.v.) by injection. After an i.v. dose of adriamycin (30 mg/kg i.v.), plasma fluorescence due to drug and metabolites was less and disappeared at a greater rate in phenobarbital-pretreated mice than control animals. When extracted with chloroform:isopropyl alcohol (1:1), the livers from the phenobarbital-pretreated group yielded a greater concentration of aglycones. Experiments with liver microsomes confirmed that aglycone production occurred at a more rapid initial rate in phenobarbital-induced livers. No increase in aldo-keto reductase (daunorubicin reductase) activity was noted. Phenobarbital-pretreated mice, inoculated i.p. with 1 million L1210 cells and then treated with adriamycin (6 mg/kg i.v.), had significantly lower survival than did controls (p < 0.01). These findings show that phenobarbital affects the disposition of adriamycin by microsomal enzyme induction and suggest that drugs that induce microsomal enzymes should not be used concurrently with adriamycin if optimal drug efficacy is desired.

INTRODUCTION

Pharmacokinetic studies of adriamycin show that metabolism of this anthracycline antibiotic is influenced by liver function. Patients with liver impairment develop toxicity with greater frequency than do patients with normal liver function. Plasma half-life of drug and metabolites is prolonged in patients with liver dysfunction. These findings have led to the recommendation that adriamycin dosage should be reduced according to the degree of liver dysfunction as assessed by sulfobromophthalein retention and serum bilirubin (8).

Adriamycin degradation pathways have been well elucidated (2). It is known that adriamycin is metabolized to adriamycinol by a cytoplasmic enzyme which is present in most mammalian tissues (1). Also, microsomal enzymes metabolize adriamycin extensively with aglycone formation from parent drug and metabolites as the major products. Adriamycin aglycone is less active than adriamycin as an antitumor agent, since the sugar moiety seems necessary for binding to DNA (11). Thus, it is of practical as well as theoretical importance to determine whether microsomal enzyme inducers increase the metabolism of adriamycin and whether this increase affects therapeutic efficacy. We studied the mouse as a biological model to demonstrate that microsomal enzymes play a critical role in the pharmacokinetics of adriamycin.

MATERIALS AND METHODS

Clinical grade adriamycin-HCl was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. Purified adriamycin was supplied by Farmitalia, Milan, Italy. NADPH was from P-L Biochemicals, Milwaukee, Wis., and phenobarbital was from Winthrop Laboratories, New York, N. Y. All other chemicals and solvents were reagent grade. Water was deionized and glass distilled.

Plasma Levels. NGP(S) mice weighing 32 ± 1 g were obtained from the NIH. These were divided into 2 groups: (a) control, which received i.p. injections of sterile, 0.9% NaCl solution, and (b) pretreated, which received i.p. injections of 2.4 mg phenobarbital (75 mg/kg) dissolved in 0.9% NaCl solution. Each group received 3 injections spaced 24 hr apart. Forty-five hr after the last injection, 1 mg clinical grade adriamycin (30 mg/kg) was given i.v. via a tail vein. After ether anesthesia, mice from each group were bled at timed intervals via the eye plexus into cold, heparin-coated glass tubes. After the blood was centrifuged, the plasma was removed, frozen in dry ice, and stored at −10°. When the sample collections were completed, 0.3 ml plasma from each mouse was mixed with 1.2 ml 95% ethanol:0.6 N HCl. The samples were then stored in a refrigerator for 24 hr, centrifuged, and read on a spectrophotometer at a fluorescence wavelength of 585 nm and an excitation wavelength of 470 nm (7). The total drug fluorescence was expressed as adriamycin equivalents.

Determination of Liver Drug and Metabolites. After being bled, each mouse was sacrificed by cervical dislocation, and the liver was immediately dissected free. Where possible, the gallbladder was removed. The liver was quick-frozen in dry ice and stored at −10° until all samples were obtained. Livers were extracted with 10 volumes (w/v) of ice-cold chloroform:isopropyl alcohol (1:1) in a Sorvall Omni-Mixer for 3 min. The homogenates were centrifuged at 4° at 27,000 x g for 15 min, and the clear supernatant solutions were concentrated under a nitrogen jet. The change in volume was recorded as a concentration factor (initial volume/final volume), and 50 μl were chromatographed on Quanta/Gram LQD (Quantum Industries, Fair-
field, N. J.) TLC	extsuperscript{1} plates. These were developed in an ascending fashion in CHCl	extsubscript{3}:CH	extsubscript{3}OH:CH	extsubscript{3}COOH:H	extsubscript{2}O (80:20:14:6). After the plates were dried, they were examined under 2537-Å light to locate the orange-red fluorescence of adriamycin and metabolites. The 2 major fluorescent areas were scraped from the plates and quantified fluorometrically as previously described (7). Fluorescence was then corrected with the concentration factor (observed fluorescence/concentration factor = corrected fluorescence).

**In Vitro Metabolism.** Mice from each group (control and pretreated) were sacrificed 45 hr after their last i.p. injection. Livers were immediately removed and homogenized in 5 volumes wet weight (w/v) of ice-cold, 0.1 M Tris-Cl, pH 7.8, in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 9,000 × g for 20 min at 4°, and the supernatant was used as a crude microsomal preparation. From similarly treated mice a supernatant fraction (109,000 × g for 60 min) was obtained for aldo-keto reductase activity. Protein concentrations were determined by the method of Lowry et al. (18).

Reaction mixtures (total volume, 0.3 ml each) containing the microsomal preparation (0.25 to 0.3 mg), purified 10⁻³ M adriamycin, 10⁻³ M NADPH, and 0.05 M Tris-Cl, pH 7.8 were incubated at 37° under nitrogen with agitation. NADPH was required in this reaction, and oxygen was inhibitory. Triplicate samples were removed at timed intervals. The reactions were stopped and extracted by shaking with 0.2 ml n-butyl alcohol and saturating quantities of solid NaCl according to the method of Bachur et al. (6). Ten μl portions of the organic phase were chromatographed on Silica Gel 60 (EM Laboratories, Elmsford, N. Y.) TLC plates and developed in CHCl	extsubscript{3}:CH	extsubscript{3}OH:CH	extsubscript{3}COOH:H	extsubscript{2}O (80:20:14:6). Areas corresponding to the aglycones of adriamycin and metabolites, adriamycinol, and adriamycinol with several unidentified metabolites were located with a 2537-Å UV light and scraped, and the fluorescence was measured.

Aldo-keto reductase activity was measured as previously reported (3).

**Identification of Compounds.** Adriamycin, adriamycinol, and the aglycone metabolites were identified by comparing R	extsubscript{f} values on TLC plates with those values obtained from pure compounds.

**Therapeutic Effect.** BALB/c mice weighing 18.5 ± 1.5 g were randomized to receive either phenobarbital (1.3 mg in 0.5 ml 0.9% NaCl solution) by i.p. injection or no pretreatment. Phenobarbital was given once a day for 3 days. The phenobarbital group was then divided into 3 sets. The 1st set received 1 × 10⁶ L1210 cells in 0.1 ml 0.9% NaCl solution by i.p. injection 8 hr after the last phenobarbital injection. The 2nd set was given adriamycin (0.1 mg in 0.2 ml sterile water) by tail vein injection 32 hr after the last phenobarbital injection. The 3rd set received the L1210 cells 8 hr after the last phenobarbital injection and adriamycin 24 hr after the cells. Matching sets of mice that received no pretreatment were used as controls.

Because phenobarbital-pretreated mice averaged a 1.6-g weight loss prior to adriamycin treatment, 2 additional sets of mice were studied in a separate experiment. Neither set was phenobarbital pretreated. However, 1 set weighed an average of 16.8 g, and the other set weighed an average of 19.3 g. Both sets received L1210 cells and adriamycin as above.

The animals were inspected daily, and dead animals were counted and removed.

**RESULTS**

**Adriamycin Plasma Pharmacokinetics.** Phenobarbital pretreatment dramatically affects the plasma pharmacokinetics of adriamycin (Chart 1). Total adriamycin equivalents had a shorter half-life (16 hr) in phenobarbital-pretreated mice than in control mice (26 hr). Although plasma levels of total adriamycin and metabolites were very similar at 3 and 24 hr, a divergence began at 48 hr. Insufficient plasma was available to analyze for metabolite components.

**Liver Adriamycin and Metabolites.** In the phenobarbital-pretreated mice, adriamycin concentrations in the liver were low, initially, but attained the control concentrations by 48 hr (Chart 2A). Adriamycin aglycones were elevated in the phenobarbital-pretreated mice and remained so until at least 48 hr (Chart 2B). Early concentrations of aglycone were greater than those of the parent drug. Other metabolites were present only in much lower concentrations.

**In Vitro Studies with Microsomes.** Microsomes prepared from phenobarbital-pretreated mice metabolized adriamycin more rapidly than did control microsomes (Chart 3). The induced microsomes produced a significantly greater (p < 0.025) concentration of aglycones after 20 min. The requirements for NADPH and anaerobic conditions are similar to those necessary for the reductive glycosidase previously reported in rat liver microsomes (4). There was no difference between control and pretreated preparations for the production of the more polar metabolites migrating with the adriamycinol band on TLC plates.

**In Vitro Study with Aldo-Keto Reductase.** No difference was found in the rate of reaction or activity/mg protein

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	extsuperscript{1} The abbreviation used is: TLC, thin-layer chromatography.

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**Infographic: Chart 1.** Plasma clearance of adriamycin and fluorescent metabolites in control and phenobarbital-pretreated mice. Each point represents the average plasma fluorescence in adriamycin equivalents of 2 animals.
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Chart 2. Concentration of adriamycin (A) and aglycones (B) extracted from the livers of control and phenobarbital-pretreated mice. Each point represents the average value of 3 or 4 mice.

Chart 4. Mortality curves of control and phenobarbital-pretreated mice. Mortality is expressed as the probability of dying. Each point represents the death of an animal. A, mice that received adriamycin for L1210 cell tumor. Each group originally had 38 animals. B, mice that received either L1210 cells or adriamycin. Each group originally had 15 animals. PBARB, phenobarbital; ADRIA, adriamycin.

test for comparison of survivorship functions in samples subject to censorship (9). The survivorship functions were calculated according to the method of Kaplan and Meier (15). Phenobarbital pretreatment does not affect mortality, due to the natural history of i.p. L1210 cell tumor or due to the toxicity of adriamycin administered i.v. (Chart 4B). Similarly, there was no statistical difference in mortality when weight was the controlled variable. The probability of dying was significantly increased ($p < 0.01$) in the group pretreated with phenobarbital and given adriamycin as compared to the group that did not receive phenobarbital pretreatment.

DISCUSSION

Phenobarbital is a microsomal enzyme inducer as well as a stimulant to biliary excretion (16). The pharmacokinetics of a drug may be altered by phenobarbital if either or both of these mechanisms affects drug elimination. The pharmacokinetics of the cardiac glycosides, for example, is affected by phenobarbital pretreatment (19). Steady-state plasma concentrations of digitoxin were found to decrease approximately 50% in humans when phenobarbital was administered concurrently. Also, urinary excretion of the product of hydroxylation, digoxin, was increased more than 2-fold. Studies done in newborn rats show that biliary excretion of Strophanthin G is enhanced with a subsequent decrease in toxicity in phenobarbital-pretreated animals (17). Adriamycin is similar to digitoxin in that the metabolic pathway of adriamycin is known to involve both microsomal enzymes and biliary excretion (2). Altered adriamycin pharmacokinetics in phenobarbital-pretreated animals is thus predicted.

The 10-hr difference in half-life of total plasma fluorescence after adriamycin administration between phenobarbila-
tal-pretreated mice and control animals can be accounted for if phenobarbital (a) increases the excretion of adriamycin, (b) increases the metabolism of adriamycin to nonfluorescent metabolites, (c) increases the clearance of fluorescent metabolites, or (d) changes the metabolic pathway toward metabolites which are more rapidly cleared. The experiments reported herein were performed to ascertain the probable mechanism.

Previous adriamycin studies have shown that the liver contains substantial amounts of aglycone metabolites including adriamycin aglycone and adriamycionol aglycone (2). The results of the extraction of the mouse livers in the present study confirm these findings and show that the amount of aglycones that are contained in the liver is increased, while the amount of adriamycin is decreased when the animals are pretreated. Since the primary products of microsomal enzymes acting on adriamycin are aglycones, it is postulated that the aglycones are formed in the liver and that the change in concentration is not due to a change in liver uptake. The induction of a reductive glycogenase is most likely responsible for this increase in aglycones (5).

When adriamycin undergoes carboxyl reduction, the metabolite, adriamycionol, is formed. This product did not appear to be a metabolite of consequence in the liver extractions or in the in vitro microsomal enzyme reaction. However, since adriamycionol is a pharmacologically active metabolite (1), attempts were made to induce it with phenobarbital. These attempts were unsuccessful. This is not surprising because the reductase appears in the cell cytosol and is not a microsomal enzyme (6).

The evidence suggests that an increased rate of metabolism to fluorescent products that are quickly cleared from the plasma is responsible for the decrease in total plasma fluorescence. However, these experiments do not rule out the possibility of increased biliary excretion as a further mechanism of elimination. Urinary excretion was not examined.

The experiments with the tumor-bearing animals show that this decrease in available adriamycin is detrimental. Animals with i.p. L1210 cells pretreated with phenobarbital and given adriamycin fared only slightly better than animals not given therapy. There was no significant difference in toxicity between control and pretreated animals as evidenced by the mortality curves of animals that received adriamycin but no L1210 cells. This finding is noteworthy because it suggests that therapeutic effect and toxicity are not dependent on the same mechanism. This relationship between efficacy and toxicity deserves further study.

Another cancer chemotherapeutic agent frequently used is cyclophosphamide. This drug is biotransformed into active alkylating metabolites by NADPH-dependent microsomal enzymes of the liver (12). In a study with a mouse model, it was found that there was only a slight initial increase in toxicity when cyclophosphamide was given to induced animals. There was no increase in therapeutic effect in mice with i.p. L1210 cell tumor. A pharmacokinetic study was done in humans, and the rate of formation of active metabolites was increased by phenobarbital induction, but the total amount of metabolites remained about the same (13).

Both adriamycin (10) and cyclophosphamide (14) are cell-cycle-nonspecific chemotherapeutic agents. The antitumor effect of these drugs is then expected to correlate with active agent exposure, that is, plasma concentration versus time area (C x T) (14). One would expect that the therapeutic effect of cyclophosphamide should remain relatively unchanged when microsomal enzymes are induced, because the total amount of alkylating agent is only slightly affected. However, in the case of adriamycin, the total amount of active agent is decreased, and a subsequent decrease in therapeutic effect is predicted. The results of the present study confirm that an increase in liver microsomal enzyme activity alters adriamycin disposition to a large enough extent that therapeutic effect is diminished. Although phenobarbital is not often used in cancer patients, other drugs which are inducers are prescribed. Although one cannot surmise that humans metabolize drugs like mice, or that all microsomal enzyme inducers act on the same enzyme systems, the clinician should always be alert to potential drug-drug interactions.

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