Hepatobiliary Metabolism and Excretion of Adriamycin and N-Trifluoroacetyladriamycin-14-valerate in the Rat

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ABSTRACT

In connection with mechanism of action studies with N-trifluoroacetyladriamycin-14-valerate (AD 32), a superior Adriamycin (ADR) analog under development in these laboratories, serial bile samples were collected from male Sprague-Dawley rats given a single i.v. dose of either ADR (4 mg/kg) or AD 32 (20 mg/kg) and were analyzed for anthracyclines by thin-layer chromatography-fluorometry and high-performance liquid chromatography. For ADR, 20% of the administered dose was accounted for at 24 hr, whereas 80% of the AD 32 dose was excreted into the bile by this time. ADR underwent little biotransformation; 80% of the 48-hr cumulative fluorescence excretion was attributable to unchanged drug, one-half the remainder was adriamycinol, and the balance was polar conjugates. In contrast, AD 32 underwent extensive metabolism to N-trifluoroacetyladriamycin, N-trifluoroacetyladriamycinol, and polar conjugates, mostly glucuronides of N-trifluoroacetyladriamycinol and N-trifluoroacetyladriamycinol. Based on direct and indirect evidence, ADR was not a metabolite of AD 32.

INTRODUCTION

ADR, given singly or in combination, is now used extensively in the treatment of a variety of tumors (7, 10). Its clinical value is limited, however, by acute myelotoxicity and by the development of irreversible cardiomyopathy, normally seen when the total accumulated dose exceeds 550 mg/sq m (18). ADR analogs in which therapeutic activity is preserved, but which are less toxic, would be of considerable practical importance. In this regard, in connection with a broad program on ADR analogs, we earlier prepared AD 32 and found it to be superior to ADR in experimental rodent tumor systems and, in general, to be less toxic than ADR (14, 16). Thus, AD 32 at 50 to 60 mg/kg administered once daily for 4 days was curative in the murine L1210 leukemia system, compared to only a 50% increase in life span of tumor-bearing animals treated at an optimal dose with ADR (4 mg/kg daily, for 4 days) (14). AD 32 was also significantly more effective than ADR against the murine P388 leukemia, Ridgway osteogenic sarcoma, and a P388 subline selected for ADR resistance (14, 19). In preliminary rabbit chronic cardiotoxicity studies, AD 32 at dose levels 15 times those of ADR on a weight basis has not produced the massive myocardial degeneration seen in some of the ADR-treated animals (I. C. Henderson, M. Israel, A. Krishan, and W. J. Pegg, unpublished results).

In connection with mechanism of action studies with AD 32, we have observed that AD 32 does not bind to isolated double-helical DNA (21), whereas the accepted mechanism of ADR action involves DNA complexation and inhibition of DNA, RNA, and protein synthesis (9). By cytofluorescence, AD 32 was observed to enter live cells in culture rapidly and to localize in the cytoplasm, in contrast to the time-dependent uptake of ADR and localization of its fluorescence exclusively in the nucleus (17).

On the basis of the structural relationship of AD 32 to ADR (Chart 1), it is conceivable that ADR could arise in vivo from AD 32 by enzyme-mediated processes, in which case AD 32 would be serving as an ADR prodrug. We recently described a mouse urinary metabolite study in which ADR was shown not to be produced in significant amounts from AD 32 (20). The present study was undertaken to define comparatively, by use of a rat model, the in vivo hepatobiliary metabolism and elimination of ADR and AD 32. Examination of bile was clearly indicated, inasmuch as the biliary route is the major excretory pathway for ADR and metabolites in a variety of species (3, 12). The rat was chosen since bile flow is continuous (13) because the animal has no gallbladder. In addition to the question of ADR from AD 32-treated animals, differences in the bio-transformation and elimination of ADR and AD 32 were sought as a possible explanation for the increased dose of AD 32, compared to ADR, required for optimal therapeutic activity.

MATERIALS AND METHODS

Chemical Agents

Bulk ADR hydrochloride, Lot 64498, was kindly provided by Farmitalia Co., Milan, Italy. AD 32 was prepared as previously described (14). ADR and AD 32 were formulated in 5% Emulphor:5% ethanol:90% NaCl solution (0.9% w/v) for i.v. injection at concentrations of 2 and 3.5 mg/ml, respectively. Emulphor was supplied as a 1:1 concentrate with ethanol by Paul Davignon, Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

The following anthracycline compounds (Chart 1) used as reference standards for the quantitation and identification of possible metabolites were synthesized in this labo-
Animals

Male Sprague-Dawley rats weighing 320 to 440 g were anesthetized with chloral hydrate (40 mg/100 g). A polythene cannula (PE 10) was placed in the common bile duct under direct vision, and the distal end was ligated; a similar catheter was placed in the left internal jugular vein. Both were withdrawn s.c. and brought to the exterior at the base of the tail. A coiled spring (length, 15 cm) was placed over the later stages of the study, absorption at 546 nm and fluorescence (see below) were monitored on a 2-channel spectrophotometer at an emission wavelength of 585 nm with excitation at 485 nm. For AD 32-treated animals, quantitation of acylated metabolites was achieved by reference to calibration curves constructed from AD 41 standards and the remainder extraction efficiency was 85 to 90%. This methanol extract was then subjected to each of the following analytical procedures.

Total Fluorescence. A 20-µl aliquot of the methanol extract was added to 1.5 ml of methanol in a disposable cuvet, and fluorescence was determined in an Hitachi Perkin-Elmer Model MFP-4 corrected-spectrum spectrophotometer at an emission wavelength of 585 nm with excitation at 485 nm. For AD 32-treated animals, quantitation of acylated metabolites was achieved by reference to calibration curves constructed from AD 41 standards added to control bile and extracted as described. ADR standards were used for ADR-treated animals.

TLC. Aliquots (20 µl) of both bile and methanol extract were applied to Silica Gel H TLC plates (5 × 20 cm; 250-μm layer) (Analtech, Inc., Newark, Del.). For reference purposes a mixture of standard compounds was prepared in control bile; a proportion of this was extracted as described. Three plates were prepared for each time point with reference standards, and 1 was developed in each of the following solvent systems: chloroform:methanol:water (80:30:3 by volume) (Solvent System 1); chloroform:methanol:acetic acid:water (80:30:3:3 by volume) (Solvent System 2); and chloroform:methanol:acetic acid:water (80:30:3:3 by volume) (Solvent System 3). The solvent front was developed in darkness, the plate was dried in air, and fluorescence was visualized under 254 nm light.

For AD 32-treated animals the area corresponding to ADR standard was scraped from the plate, taken up into methanol, and further analyzed by HPLC.

HPLC. Separation and quantitation of AD 32, AD 60, AD 41, and AD 92 were achieved with the use of a Waters Associates Model ALC/244 liquid chromatograph equipped with a phenyl/Corsil reverse-phase column (2 ft × 0.125 inch; Waters Associates, Milford, Mass.). The developing system was 27 to 40% acetonitrile in ammonium formate buffer, pH 4.0. Initially, absorption at 254 nm was monitored on a Hewlett-Packard Model 3380A recording integrator. In the later stages of the study, absorption at 546 nm and fluorescence (see below) were monitored on a 2-channel Texas Instruments recorder simultaneously with the 254 nm absorption signal. Samples of methanol extract (20 µl) were applied directly to the column. Concentrations were determined by estimation of peak areas and by reference to calibration curves constructed from AD 41 standards added to bile and extracted as described. For the purpose of this study, it was assumed that the quantum yield of the above compounds was similar. On an absolute mass basis, pure standard samples of the above acylated anthracyclines are detectable in this system at levels of 6 to 7 pmoles. In this study, for the methanol extracts of bile prepared as...
described, these agents were detectable in the presence of each other at a lower concentration limit of $1 \times 10^{-6} \text{ M}$, equivalent to 15 to 20 pmoles of each compound per injection aliquot.

Separation and quantitation of ADR, AMNOL, and adriamycinone was achieved with the use of a Waters Associates Model ALC/202 liquid chromatograph equipped with a 25-cm Whatman Partisil PAC-10 modified normal-phase column and a developing system of chloroform:methanol:acetic acid:water (85:15:5:1.5 by volume). Signal detection was monitored with a Schoeffel Instrument Model SF-970 flow fluorescence detector, with an excitation wavelength of 482 nm, and with an emission filter (Schoeffel No. 2-73), with low wavelength cutoff near 550 nm. Samples of methanol extract (20 μl) were applied directly to the column. Concentrations were determined by estimation of peak areas and by reference to calibration curves constructed from ADR standards. The instrumental limit of detection of pure ADR standards by this methodology is 1 pmole or less; ADR added to bile and extracted as described could be detected in this system at a lower concentration limit of $1 \times 10^{-7} \text{ M}$, equivalent to 20 pmoles/injection aliquot, in the presence of the other anthracyclines used in this study.

In all instances where calibration curves were constructed for quantitation, the relationship between either the current (I) (fluorescence) or absorbance (a.u.f.s.) was linear. All samples were determined in duplicate and in subdued light.

Enzyme Assay. The presence of possible glucuronide conjugates was determined by incubation of bile samples in 0.05 M acetate buffer (pH 5.0; 37°) with serial concentrations of bovine β-glucuronidase (Sigma Chemical Co., St. Louis, Mo.). Samples were analyzed as described by TLC and HPLC and were compared to control samples incubated in 0.05 M acetate buffer.

Determination of Biliary Elimination Rate Constants and Half-Times

The rate of elimination of the individual anthracycline species present in bile was calculated from the equation

$$\text{Fraction at time } t = (1 - y/y') \times 100$$

where $y$ is the amount excreted at time $t$, and $y'$ is the total excreted. These values were plotted on a logarithmic scale versus time (Chart 4), and the individual slopes ($\beta$) were calculated by least-squares regression analysis. The half-times for biliary excretion were calculated from the equation

$$t_{1/2} = 0.693/\beta$$

RESULTS

The comparative cumulative anthracycline excretion for ADR and AD 32 is shown in Chart 2. At 3 hr 49% and at 24 hr 80% of the total dose of AD 32 was recovered in the bile, compared to 4.8 and 20% for ADR at the respective time points. In AD 32-treated animals, excretion plateaued at 24 hr; after 24 hr, fluorescent compounds could not be detected in 2 animals, and in the others fluorescence was just barely detectable. In the ADR-treated animals, fluorescent anthracycline compounds continued to be seen in the bile after 24 hr. In 1 ADR animal observed for 7 days, fluorescent compounds could still be detected; 45% of the administered dose had been recovered by this time.

In both groups the peak biliary concentration was at the time point of 0.5 to 1.0 hr; the peak biliary concentration for AD 32 was 2.23 $\times 10^{-3} \text{ M}$, and for ADR it was 8.45 $\times 10^{-4} \text{ M}$. For ADR-treated animals the biliary concentration at 24 to 48 hr (14.32 ± 3.5 $\times 10^{-4} \text{ M}$) was greater than that for AD 32-treated animals (1.48 ± 0.88 $\times 10^{-4} \text{ M}$).

TLC examination of bile samples at all time points from AD 32-treated animals revealed the presence of several metabolites. In Solvent System 2, the 0- to 0.5- and 0.5- to 1.0-hr samples contained both AD 32 and AD 60, just discernible by the naked eye under 254 nm light; these could not be detected, even by HPLC, in the remaining samples (1 to 48 hr), and together they accounted for less than 0.05% of the administered dose. AD 41 and AD 92 were clearly identifiable as major metabolites, with an area of faint fluorescence located in between. The latter could not be resolved by HPLC and may be artifactual. In Solvent System 1, 3 to 4 bands indicating polar metabolites were present, with $R_f$ values ranging from 0.15 to 0.3. No separate fluorescent spot corresponding to ADR standard ($R_f$ 0.32) was seen. In direct contrast, only a single spot corresponding to ADR standard was detected in the ADR-treated animals. No fluorescent spot indicating either an aglycone or AMNOL could be visually identified.

Samples from each time point from both treatment groups were spotted side by side and developed in Solvent System 1. In bile from AD 32-treated animals, no fluorescent spot equivalent to that of ADR from ADR-treated animals was seen. Examination by normal-phase HPLC of scrapings from the area corresponding to and overlapping that of ADR revealed 2 peaks with retention times identical with those of AD 41 and AD 92; no peak was seen corresponding to ADR from ADR-treated animals or from ADR standards.

The cumulative biliary excretion of AD 41, AD 92, and polar metabolites from AD 32-treated rats is shown in Chart 3A, and for ADR and metabolites from ADR-treated animals it is shown in Chart 3B. Initially, AD 41 was the major metabolite, and elimination was complete in 6 to 12 hr. AD 92 was detected in the first 30 min after AD 32 administration and accounted for 10% of the administered dose at
that time point. AD 92 excretion in total exceeded that of AD 41, and its elimination was essentially complete by 12 hr. After an initial lag, perhaps due to enzyme saturation, the polar metabolites increased in proportion at each time point after 3 hr, and at 24 to 48 hr they accounted for the major fraction of the total fluorescence.

For ADR-treated animals ADR was the principal compound excreted in the bile. At 24 to 48 hr ADR accounted for 80% of the total drug fluorescence. Approximately one-half of the remainder was identified as AMNOL, and the difference must presumably have been due to conjugates and/or aglycones. The presence of aglycones may, however, have been artificial; examination of ADR standards, free of aglycones by HPLC analysis, revealed after TLC the appearance of adriamycinone at levels comparable to those seen in ADR bile samples.

The fraction of AD 41, AD 92, and polar metabolites from AD 32-treated rats, excreted with time and calculated as described in "Materials and Methods," is shown in Chart 4. A similar plot was made for ADR from ADR-treated animals. In each case a straight line was obtained with coefficients of determination >0.95. The values for the elimination rate constant and 1/2 for excretion calculated from the individual slopes are given in Table 1.

The concentrations of AD 41 and AD 92 increased by 700 and 90%, respectively, after incubation of the 3- to 6-hr AD 32 bile sample for 30 min with β-glucuronidase. Incubation for longer periods (up to 24 hr) resulted in a further small increase in the concentration of both AD 41 and AD 92, but about 20% of the original polar fluorescent material remained. Also seen were areas of faint fluorescence representing aglycones. Bile samples at other time intervals, upon incubation with β-glucuronidase, gave similar results. TLC analysis in Solvent System 1 again failed to demonstrate a fluorescent spot corresponding to ADR. The region on TLC plates corresponding to ADR standard accounted for less than 1.5% of total fluorescence at any time point, and no peak corresponding to ADR was seen by HPLC. The remaining polar fluorescence, unaffected by glucuronidase, was visualized on TLC as 2 separate areas with Rf values less than that of ADR. By normal-phase HPLC analysis, each revealed identical chromatograms, with 2 peaks that differed from ADR in retention time by 30 and 90 sec more rapid elution.

**DISCUSSION**

The metabolic fate of ADR, at least in terms of fluorescent species, has been previously studied. Anthracyclines are excreted primarily via the biliary route in the rabbit, dog, rat, and humans (4-6, 12, 24). A small amount of ADR and metabolites is excreted in the urine, but this accounts for only a minor proportion of the administered dose (5). Intracellular cytoplasmic aldo-keto reductases is the major catalytic factor converting the parent compound to AMNOL (11). Other enzymes can further biotransform both ADR and AMNOL, particularly a reductive glycosidase and a hydrolytic glycosidase found in most tissues, including

![Chart 3. a, cumulative biliary excretion of AD 32 metabolites in rats treated with AD 32, 20 mg/kg i.v. Values represent mean ± S.E., n = 4. b, cumulative biliary excretion of ADR and metabolites in rats treated with ADR, 4 mg/kg, i.v.](chart3.png)

![Chart 4. Excretion of AD 32 metabolites following single i.v. administration of AD 32 (20 mg/kg). The results represent the log percentage of AD 41, AD 92, and polar metabolites excreted with time, each expressed as a percentage of its total excretion; values are the mean ± S.E., n = 4.](chart4.png)

**Table 1**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Keq (hr⁻¹) ± S.E.</th>
<th>t½ (hr) ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADR</td>
<td>0.017 ± 0.0013</td>
<td>42.25 ± 3.78</td>
</tr>
<tr>
<td>AD 32 (total)</td>
<td>0.30 ± 0.04</td>
<td>2.42 ± 0.31</td>
</tr>
<tr>
<td>AD 41</td>
<td>1.13 ± 0.31</td>
<td>0.76 ± 0.19</td>
</tr>
<tr>
<td>AD 92</td>
<td>0.466 ± 0.08</td>
<td>1.64 ± 0.31</td>
</tr>
<tr>
<td>Polar metabolites</td>
<td>0.256 ± 0.04</td>
<td>2.89 ± 0.43</td>
</tr>
</tbody>
</table>

* Keq, biliary rate constant for elimination.
* Mean ± S.E.; n = 4.
* Based on total fluorescence.
liver (2). Once the parent drug and metabolites are in the intestine, further biotransformation takes place with the production of predominantly water-insoluble aglycones (8). While the biliary excretion of ADR has been quantitated in the rabbit (4), there are no published data pertaining to the rat.

Of interest in this study is the relative resistance of the ADR molecule to biotransformation. At 24 hr, 92% of the total recoverable fluorescence in the bile of ADR-treated rats is unchanged drug. This low rate of biotransformation probably accounts for the inability of earlier workers to detect any metabolites of ADR and confirms the conclusion of Felsted et al. (11) that ADR is a poor substrate for rat aldo-keto reductase. While adriamycinone was visualized by HPLC, the concentrations were quantitatively similar to those observed in control bile samples to which ADR standard had been added. This infers that either a hydrolytic glycosidase was present in the bile capable of producing the aglycone or that the aglycone is an artifact produced by the analytical procedure. The latter is a more plausible explanation, because adriamycinone or 13-dihydroadriamycinone is too insoluble to be excreted directly and would require conjugation in order to undergo elimination. Our results suggest that aglycone production and subsequent conjugation are not a significant metabolic pathway in the rat.

In direct contrast to ADR, AD 32 undergoes extensive biotransformation. The finding of very low concentrations of both AD 32 and AD 60 results from the rapid conversion of AD 32 to AD 41 by nonspecific esterases present in rat serum (15). Therefore, AD 41 appears to be the major metabolite, which then undergoes aldo-keto reduction to AD 92. AD 41 is superior to ADR as a substrate for enzymatic reduction; 1 hr after drug administration the amount of AD 92 exceeded that of AD 41. Both of these compounds are readily conjugated, which again is in contrast to ADR, for which small amounts of conjugated products were assumed but were not proved. ADR conjugation, however, does occur, as has been demonstrated both in the rabbit and in humans (5, 6, 12).

The kinetics of the 2 drugs are different (Table 1), as evidenced by the rate constants for biliary elimination and half-times for excretion. The former value reflects the β (or slow) phase of elimination from plasma, and the value for ADR (0.017 hr⁻¹) is comparable to that (0.025 hr⁻¹) reported for AD 32 as derived from plasma concentration-time curves (23). The long t₁/₂ for excretion (42 hr) is explained by retention in the tissues and represents binding to tissue components, including DNA.

The elimination rate constants for AD 32 (total) (0.3 hr⁻¹) and AD 41 (1.13 hr⁻¹) indicate a rapid plasma clearance. The value for AD 92 is comparable to that of AD 32 (total), but it cannot be determined from this study whether this is produced as a result of reductase activity in most organs and tissues or whether it is produced entirely by the liver. The former is more likely, inasmuch as the enzyme responsible has a ubiquitous distribution in mammalian cells (11).

The polar metabolites of AD 32 consisted principally of glucuronide conjugates of AD 41 and AD 92. Some faint areas of fluorescence visualized under 254 nm light are consistent with very low concentrations of aglycones. This may represent a small amount of true aglycone production with subsequent conjugation in vivo or may have resulted from the analytical procedure. The nature of the remaining metabolites, as yet unidentified and accounting for 20% of the total polar metabolites, is at present not resolved, but they are undoubtedly conjugates, possibly O-sulfates.

There was no evidence, either direct or indirect, that ADR was a significant metabolite of AD 32. This observation is consistent with a similar conclusion that emerged from a comparative study of anthracycline excretion in mouse urine (20). If ADR were formed in part from AD 32 by the action of intracellular amidases, we believe that at least a portion of this material would follow kinetics of elimination similar to those of ADR in ADR-treated animals. The differences in kinetics of ADR and of AD 32 and its metabolites, including the significant difference in anthracycline concentration in the 24- to 48-hr bile samples, therefore suggest that it is unlikely that ADR is the active principle of AD 32.

Looking at the data in another way, one should recall that the lower limit of detection of ADR under the experimental conditions used is 1 x 10⁻⁸ M (1 nmole/ml). Assuming a total bile production in the rat of 40 ml in 48 hr, we deduced that the total mass of ADR that may have been produced from AD 32 but may have remained undetected would be less than 40 nmoles. At 48 hr, 27% of the total dose of ADR was recovered in the bile of ADR-treated animals (Chart 2). Based on this consideration, the total conversion of AD 32 to ADR beyond detection would have been 150 nmoles (87 µg, equivalent to a dose of 300 µg/kg). This amount appears trivial when one considers the therapeutic dose requirements for ADR in experimental rodent tumor systems (14).

The mechanism of action of AD 32 continues to be speculative. AD 32, AD 41, and AD 92 all have about the same growth-inhibitory activity against cells in culture. However, in vivo, AD 32 and AD 41 produce significant prolongations of survival of mice bearing the L1210 leukemia, whereas AD 92 is only marginally active (15). The antitumor activity of AD 32 has now been explored in a wide range of mouse leukemia and solid tumor systems and in 1 rat system; AD 32 is almost always clearly superior to AD 92, whereas AD 92 is only marginally active (15). The antitumor activity of AD 32 has now been explored in a wide range of mouse leukemia and solid tumor systems and in 1 rat system; AD 32 is almost always clearly superior to ADR and is never inferior. The rapid conversion by both mouse and rat plasma esterases of AD 32 to AD 41, which itself is active but more toxic than AD 32 on parenteral administration, infers either that a fraction of the original AD 32 dose gains entry into the cell with resultant effect or that AD 41 may be the active principle. If the former is correct, then this would explain the reason for the increased dose of AD 32 compared to that for ADR. On the other hand, it is still possible that a minute amount of intracellular AD 32 or AD 41 undergoes conversion to ADR, which then becomes persistently bound to cellular components. After all, no published series has succeeded in accounting for more than 50 to 60% of an administered ADR dose as fluorescent compounds; the remainder must be either tightly bound or eliminated as nonfluorescent species currently incapable of detection. If the latter possibility is so, then similar metabolism must be considered for AD 32, with resultant implications for therapeutic activity and toxicity. These and other aspects of ADR and AD 32...
pharmacology are under active investigation in these laboratories.

REFERENCES


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