Comparative Mammalian Metabolism of Adriamycin and Daunorubicin

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ABSTRACT

Both Adriamycin and daunorubicin undergo carbonyl reduction and glycosidic cleavage. We have investigated kinetic parameters of these reactions with several mammalian species including humans.

Adriamycin and daunorubicin showed similar Ks values for reduction in all organs studied except those from rabbits. Both rabbit liver and kidney have a much lower Ks for Adriamycin reduction. In contrast to the great similarity in Ks, the Vmaxs varied greatly according to drug, organ, and species. Daunorubicin showed a greater Vmax than did Adriamycin in all organs studied. The Vmax ratio of daunorubicin to Adriamycin ranged from 159 to 178 in mice and rats to 10 to 30 in humans. Liver and kidney always showed greater activity than did cardiac or skeletal muscle. These enzymatic data correlate with the quicker in vivo reduction of daunorubicin than of Adriamycin, with a greater percentage of daunorubicin than Adriamycin excreted in the reduced form, than and, inversely, with clinical efficacy in that Adriamycin has a wider spectrum of antitumor activity.

All organs showed high levels of glycosidic cleavage in the absence of oxygen. In vivo, relatively little of either Adriamycin or daunorubicin is in the aglycone form. Thus, tissues, especially liver, have a great potential for glycosidic activity that is normally not exhibited. However, in certain patients this may be an important mechanism of biotransformation.

Although the role of these activities in normal cellular physiology is unknown, their ubiquity points toward an essential function.

INTRODUCTION

Although the anticancer antibiotics, Adriamycin and daunorubicin, have similar structures (Chart 1), they differ in pharmacokinetic and pharmacodynamic characteristics (2, 7, 9, 16, 20, 32). For example, Adriamycin has greater antitumor activity as well as a higher therapeutic index than does daunorubicin (11, 25, 28). Whereas the in vivo metabolism of the antibiotics is qualitatively similar, quantitative differences are apparent (30).

The 2 major mammalian enzyme systems involved in the metabolism of both Adriamycin and daunorubicin are the cytoplasmic aldo-keto reductases (Chart 1A) and the microsomal glycosidases (Chart 1B) (3, 5, 6). The enzymatic products of the aldo-keto reductase, daunorubicinol or adriamycinol, retain inhibitory activity against both DNA and RNA synthesis (8, 24). Since the specific inhibitory activities of these metabolites are quite high, variations in the aldo-keto reductase responsible for the intracellular production of the metabolites affect the action of these drugs. The glycosidases presumably inactivate the anthracycline glycosides, although it is possible that the aglycone product of the glycosidases has biological activity when liberated intracellularly.

In this report, we have quantified and compared differences between Adriamycin and daunorubicin metabolism in human systems and several animal systems.

MATERIALS AND METHODS

Daunorubicin hydrochloride was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Adriamycin hydrochloride was provided by Farmitalia, Milan, Italy. Tissues were obtained from adult male DBA/2 mice, Sprague-Dawley rats, golden Syrian hamsters, and New Zealand White rabbits. Dog tissues were obtained from adult male purebred beagles. The human tissues were obtained on autopsy within 24 hr of death from adult males dying of accidental death that was not associated with drug usage. The animals were sacrificed in the midmorning hr, and all tissue extractions and preparations were at 0–8°C. The enzyme assays were done on extracts from individual animals.

Reductase activities were determined on 105,000 × g cytoplasmic extracts and ammonium sulfate fractions of these high-speed supernatant extracts (5). Tissues were homogenized in 0.1 m potassium phosphate buffer, pH 7.4 (1 g of tissue per 5 ml of buffer). The homogenates were clarified by centrifugation for 10 min at 12,000 × g in a Sorvall RC2B centrifuge followed by centrifugation for 60 min at 105,000 × g in a Beckman ultracentrifuge. Ammonium sulfate fractions (0.3 to 0.6 saturation) were prepared from rabbit liver, kidney, heart, and skeletal muscle high-speed supernatant extracts.

Microsomes for glycosidase activities were isolated from tissues homogenized in 0.05 m potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (1 g of tissue per 3 ml of buffer). The homogenate was centrifuged at 10,000 × g for 20 min, and the sediment was discarded. The 10,000 × g supernatant solution was centrifuged at 105,000 × g for 60 min to yield sedimented microsomes. The microsomal sediment was suspended in the phosphate buffer and centrifuged at 105,000 × g for 60 min. After the supernatant wash was discarded, the packed, washed microsomes were suspended in fresh potassium phosphate buffer and immediately assayed for the glycosidase enzymes.

Protein estimations were determined by the method of Lowry et al. (22).

Reducase Assay. The reductases were assayed in dupli-
Glycosidase Assay. Water-saturated nitrogen gas was bubbled into tubes containing 50 μmoles Tris-HCl buffer (pH 7.8), 0.5 μmole NADPH, and 0.5 μmole daunorubicin or Adriamycin in 0.4 ml for 1 min/tube to displace oxygen (6). Microsome suspension (0.1 ml containing 0.1 to 0.3 mg protein) was added to each deoxygenated reaction mixture, and the tubes were again flushed with nitrogen before capping. The capped tubes were incubated at 37° for 15 min. The reactions were stopped by mixing 0.5 ml isopropyl alcohol into the reaction mixtures. The inactivated reaction mixtures were saturated with crystalline ammonium sulfate, mixed for 15 sec, and centrifuged at 100 × g for 10 min to separate the phases. Ten μl of the upper phase were applied to silica gel thin-layer plates for chromatographic separation, and the aglycones were quantified as previously described (7). All reactions were linear for the times and conditions used.

Glycosidase Assay. Water-saturated nitrogen gas was bubbled into tubes containing 50 μmoles Tris-HCl buffer (pH 7.8), 0.5 μmole NADPH, and 0.5 μmole daunorubicin or Adriamycin in 0.4 ml for 1 min/tube to displace oxygen (6). Microsome suspension (0.1 ml containing 0.1 to 0.3 mg protein) was added to each deoxygenated reaction mixture, and the tubes were again flushed with nitrogen before capping. The capped tubes were incubated at 37° for 30 min, and the reaction was terminated by the addition of 0.5 ml of n-butyl alcohol. The reaction mixtures were mixed with saturating amounts of NaCl crystals and were centrifuged at 100 × g for 10 min to separate the phases. Ten μl of the upper phase were applied to silica gel thin-layer plates for chromatographic separation, and the aglycones were quantified as previously described (7). The reactions were linear for at least 30 min.

Kinetic Constants. All kinetic determinations were carried out with 1 mM cofactor that was assumed to be saturating (17). Under these conditions, the enzyme reaction is described by the equation

\[ v = \frac{V_{\text{max}} S}{K_m + S} \]

where \( v \) is the rate of the reaction and \( S \) is the substrate concentration. Kinetic constants were determined by fitting the data to the above equation by the least-squares method and assuming equal variance for the velocities. Calculations were performed on the Tymshare computer network with FORTRAN programs that provided \( K_m \) and \( V_{\text{max}} \) and the S.D. of their estimate (14). Kinetic constants were calculated from the combined data of at least 3 fresh enzyme preparations from individual animals in the case of daunorubicin reduction and at least 2 enzyme preparations in the base of the Adriamycin reduction (only 1 enzyme preparation was used for humans and dogs).

Daunorubicin and Adriamycin concentrations were determined spectrally with a molar extinction coefficient of 11.4 × 10^3 at 485 nm.

RESULTS

All the mammalian tissue extracts catalyze the reduction of daunorubicin. Although all the extracts display similar \( K_m \) values, reductase activities differ among the various tissues (Table 1). This activity is highest in the kidney of the rat, mouse, and rabbit. Liver has activity equal to or greater than that of kidney in humans and dogs. Heart and skeletal muscle show low activity except for rat heart.

A similar survey of the enzymatic reduction of Adriamycin by high-speed supernatants was made. Because of the low Adriamycin reductase activity, kinetic constants were determined only for the enzymes from the most active tissues, liver and kidney (Table 2). For all species examined, daunorubicin activity was greater than that of Adriamycin in these tissues. \( V_{\text{max}} \) ratios varied from 178 to 10.6 (Table 3). In contrast, the \( K_m \) values for Adriamycin and daunorubicin were generally of the same order of magnitude. The only exceptions were in rabbit tissues.

It was not possible to assay rabbit tissue high-speed cytoplasmic extracts for Adriamycin reduction because of nonlinear reaction rates. Therefore, rabbit tissue enzymes were processed by ammonium sulfate precipitation, and the resulting 3.0- to 0.6-saturated ammonium sulfate fractions were examined (Table 4). The ammonium sulfate fractions of rabbit liver and kidney gave first-order enzyme kinetics with time and yielded \( K_m \) values approximately 1 order of magnitude lower than those of daunorubicin and Adriamycin reductases in other species. The same rabbit liver and kidney ammonium sulfate fractions gave \( K_m \) values for daunorubicin similar to those obtained from the cytoplasmic extracts of these tissues (Tables 1 and 4). Since the \( K_m \) values for daunorubicin by rabbit high-speed cytoplasmic extracts (Table 4) and ammonium sulfate-purified fractions (Table 4) are experimentally indistinguishable, it is assumed that the \( K_m \) values for Adriamycin determined with ammonium sulfate-fractionated enzymes are directly comparable to the \( K_m \) values for Adriamycin determined on high-speed cytoplasmic extracts of other tissues. In such a
comparison, the rabbit liver and kidney enzymes have $K_m$ values for Adriamycin approximately an order of magnitude lower than the daunorubicin and Adriamycin reductases from all other species.

All the tissue microsomes possessed glycosidase activity (Table 5). Liver microsomes contained substantially more glycosidase activity than did kidney microsomes or heart sarcosomes in every species. Although glycosidase activity was remarkably high in all animal tissues, of the 3 organs examined, the hamster tissues contained the highest glycosidase activities. NADPH was a strict cofactor requirement for the glycosidase activity of all the microsomes (6).

Similar to the reductase, the glycosidase generally preferred daunorubicin over Adriamycin as a substrate. Liver microsomes preferentially cleaved daunorubicin at 1.5 to 1.7 times the rate of Adriamycin cleavage, which was similar to the substrate preference in most other tissues. However, a few tissues had higher activities with Adriamycin: i.e., mouse kidney and mouse, rat, and rabbit heart.

**DISCUSSION**

All of the tissues metabolized Adriamycin and daunorubicin via the cytoplasmic, aldo-keto reductase, and microsomal glycosidase pathways. However, the levels of enzymatic activities as well as the substrate specificities varied among the tissues. These data verify our previous findings that glycosidase activity is highest in liver. Aldo-keto reductase activities tend to be greater in liver and kidney than in heart or skeletal muscle, with the exception of rat heart.

Although the antibiotic reductases are ubiquitously distributed, for the most part they display similar kinetic properties. The daunorubicin reductase from all tissues and the Adriamycin reductase from all tissues except rabbit exhibited similar $K_m$ values that fall within a rather narrow range, suggesting similarities among the enzymes. The ratio of the $V_{max}$’s for daunorubicin over Adriamycin in liver and kidney tend to be similar for rat and mouse. Dog and human liver and kidney show differences that may suggest multiple reductases in these species. However, interspecies differences appear to be the major cause for the wide range of this ratio (Table 3).

The ubiquitous and constitutive distribution, as well as the functional conservation, as shown by the similarity in $K_m$’s, suggests a fundamental physiological role for the enzymes involved.

Of all the reductases, only rabbit tissue Adriamycin reductase had a unique $K_m$, but even in this animal the kinetics for daunorubicin reduction was not unusual or different from those of other animals. It is possible for a single rabbit enzyme to have a lower $K_m$ toward Adriamycin. Alternately, rabbit tissue may contain multiple reductases, one of which displays unique kinetics toward Adriamycin. The presence of numerous carbonyl reductases has in fact been observed in rabbit liver extracts (1).

Due to a high $K_m$ and substantial substrate inhibition (6), it is not possible to determine accurately the kinetic constants for crude microsomal glycosidase. For the purpose of this comparison, assays were therefore done at less than saturating substrate concentrations. The concentration of daunorubicin and Adriamycin used was selected

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (µmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.36 ± 0.05</td>
<td>74.8 ± 14.2</td>
</tr>
<tr>
<td>Heart</td>
<td>0.37 ± 0.05</td>
<td>40.8 ± 6.2</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.59 ± 0.02</td>
<td>18.9 ± 2.4</td>
</tr>
</tbody>
</table>

Table 1. Apparent aldo-keto reductase kinetic constants for daunorubicin.

$^a$ Mean ± S.D. of at least 3 observations.

$^b$ $10^{-10}$ mole/min/mg protein.

$^c$ $10^{-12}$ mole/min/mg protein.

$^d$ Not determined.
because it gave optimal activity under the conditions of this assay. As a result, although individual enzymes were first order with protein and time, the comparison among the enzymes must be considered as an approximation. Despite the limitations of the assay, however, the results obtained predict what is observed in vivo, namely, that daunorubicin rather than Adriamycins is the preferred glycosidase substrate and that glycosidase activity is highest in the liver. In addition, this assay demonstrates the presence of glycosidase activity in all animal tissues that were examined.

Under our experimental conditions for testing enzyme activities, the microsomes contain very high glycosidase activities. This is misleading since the glycosidases probably do not normally function at these in vitro rates. From our studies on in vivo metabolism by rats, hamsters, rabbits, and humans (4, 7, 15, 20, 30) only low to moderate concentrations of aglycones, products of the glycosidases, are detected in tissues, bile, and urine compared to the products of the reductases. Therefore, tissues contain a great potential for glycosidic activity that normally is only partially expressed. As previously proposed (20), tissue glycosidic activity may be expressed if oxygen tension in the tissues decreases. This could happen in debilitated patients or in patients with compromised cardiovascular or respiratory functions.

The absolute activities of enzymes in human tissues must be assessed cautiously. Human tissues, by necessity, were obtained from autopsy material several hr postmortem. We do not know the postmortem effects on the enzyme activities in the human, but this may be related to the apparent low enzyme activities in the human tissues. Despite this unavoidable problem, the human enzymes display similar substrate specificity and Km values and about the same organ enzyme distribution as do enzymes from other fresh animal tissues.

At present, the rabbit is known to develop an anthracycline antibiotic-induced, dose-dependent cardiotoxicity that simulates the cardiotoxicity seen in humans (21, 23). However, cardiotoxic manifestations of these drugs have been reported in dogs (10), mice (27), hamsters (19), and rats (12). In vivo, both rabbits and humans show substantial (>50%) conversion of Adriamycin to adriamycinol and daunorubicin to daunorubicinol in urine and bile (7, 9, 20), whereas rats and dogs (15, 31) display a lesser degree of antibiotic reduction. A comparison of the toxicology and biochemistry of these agents seems an important future consideration.

We have previously shown that differences at C-9 affect the cellular uptake of the anthracyclines (8, 24). In this study and in other in vitro (7) and in vivo (7, 9, 20, 30) studies it is apparent that differences in the C-9 side chain profoundly affect metabolism on the same side chain as well as the metabolism at the distal glycosidic linkage. Since Adriamycin and daunorubicin exhibit only minor differences as agents affecting nucleic acid metabolism (13, 18), it is pertinent to suggest that other factors contribute to the determination of therapeutic index and spectrum of activity of the 2 agents.

One of the pertinent factors appears to be the greater hydrophilic nature of the reduced metabolites. It has been previously shown that cellular uptake of anthracyclines by L1210 cells is linearly related to their oil-water partition coefficient (8). The more hydrophilic the drug, the harder it is to cross hydrophobic membranes. This could also affect the ability of membrane-bound enzymes to metabolize these drugs and their metabolites.

The fact that the Km's for Adriamycin and daunorubicin reduction are similar but that Vmax vary up to 178-fold suggests that the C-9 side chain affects the ability of the reductase to reduce the moiety as opposed to its ability to bind the moiety. The data from the rabbit suggest that multiple reductases may have greater activity for one than

### Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Km (mM)</th>
<th>Vmax (mole/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.126 ± 0.032</td>
<td>3.0 ± 2.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.190 ± 0.032</td>
<td>7.7 ± 0.46</td>
</tr>
</tbody>
</table>

*10^-5 mole/min/mg protein.

### Table 3

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Vmax for daunorubicin/Adriamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>159</td>
</tr>
<tr>
<td>Mouse</td>
<td>155</td>
</tr>
<tr>
<td>Dog</td>
<td>35.6</td>
</tr>
<tr>
<td>Human</td>
<td>30.4</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Km (mM)</th>
<th>Vmax (mole/min/mg protein)</th>
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</thead>
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<tr>
<td>Liver</td>
<td>0.24 ± 0.032</td>
<td>0.020 ± 0.0032</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.33 ± 0.049</td>
<td>0.0078 ± 0.0014</td>
</tr>
</tbody>
</table>

*Mean ± S.D."
Table 5

Glycosidase activities

<table>
<thead>
<tr>
<th>Animal</th>
<th>Substrate</th>
<th>Liver</th>
<th>Kidney</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice (DBA/2) A b</td>
<td>135.7 ± 51.8 c</td>
<td>111.4 ± 7.7</td>
<td>14.5 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>208.1 ± 3.4</td>
<td>93.3 ± 1.6</td>
<td>11.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>A</td>
<td>128.9 ± 54.6</td>
<td>44.3 ± 27.8</td>
<td>49.1 ± 25.0</td>
</tr>
<tr>
<td>D</td>
<td>214.3 ± 101.8</td>
<td>84.0 ± 53.9</td>
<td>23.0 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>Hamster</td>
<td>A</td>
<td>315.0 ± 67.0</td>
<td>109.0 ± 28.5</td>
<td>50.6 ± 10.0</td>
</tr>
<tr>
<td>D</td>
<td>496.0 ± 110.0</td>
<td>239.3 ± 30.8</td>
<td>68.9 ± 24.2</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>A</td>
<td>225.6 ± 4.0</td>
<td>65.8 ± 11.0</td>
<td>36.0 ± 7.7</td>
</tr>
<tr>
<td>D</td>
<td>334.9 ± 168.3</td>
<td>97.8 ± 9.2</td>
<td>20.1 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>A</td>
<td>178.9 ± 28.3</td>
<td>29.2 ± 15.3</td>
<td>33.2 ± 20.0</td>
</tr>
<tr>
<td>D</td>
<td>250.6 ± 58.5</td>
<td>75.6 ± 17.9</td>
<td>36.3 ± 14.6</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>A</td>
<td>165.7 ± 68.2</td>
<td>8.0 ± 0.9</td>
<td>12.5 ± 4.1</td>
</tr>
<tr>
<td>D</td>
<td>250.7 ± 63.2</td>
<td>26.5 ± 14.9</td>
<td>21.8 ± 13.2</td>
<td></td>
</tr>
</tbody>
</table>

a Reactions were run for 30 min.
b Adriamycin; d, daunorubicin.
 c S.E. of from 3 to 5 different enzyme preparations.

for the other. Thus the stereospecificity of the C-9 side chain and the electron distribution about the carbonyl group, as distinct from a change in the hydrophilic nature of the molecule as a whole, affects the ability of the drug to be reduced.

Takanashi and Bachur (29, 30) examined the percentages of Adriamycin, daunorubicin, and their metabolites in human urine. Daunorubicin and daunorubicinol comprised 39 and 28%, respectively. Adriamycin and adriamycinol comprised 23 and 45%, respectively. Adriamycin and adriamycinol comprised 39 and 28% respectively.

Benjamin et al. (9) showed that urine excretion of daunorubicin and metabolites in humans was only 5.7% of the total dose. The greater ability of the aldo-keto reductase in the kidney and elsewhere to reduce daunorubicin rather than Adriamycin (Table 3) could account for the greater excretion of daunorubicinol. The greater ability of the kidney to convert daunorubicin to a relatively more water-soluble form than that for Adriamycin could account for the greater urinary metabolism of daunorubicin and its metabolites than of Adriamycin and its metabolites.

Bachur et al. (7) examined the tissue disposition of Adriamycin, daunorubicin, and reduced metabolites in the rabbit. Rabbit tissues showed a greater disposition of daunorubicinol than did adriamycinol. We suggest that this is due to the greater activity of the aldo-keto reductase for daunorubicin than for Adriamycin. Adriamycin thus seems able to stay in the parent form longer than does daunorubicin. This may account in part for their differences in therapeutic activity. If this is a true "cause and effect" relationship, then simultaneous administration of a pharmacologically acceptable reductase inhibitor could increase the therapeutic efficacy of these drugs.

Riggs et al. (26) have recently described bile from a patient treated with Adriamycin that contained at least 11 metabolites of Adriamycin. Takanashi et al. (29, 30) have described extensive metabolism of both drugs. The ability of enzymes to sulfate, conjugate, demethylate, etc., these drugs could depend on whether the parent drug was Adriamycin or daunorubicin and whether the drug was in the reduced and/or aglycone form.

However, a better correlation of in vitro and in vivo data will have to await a greater understanding of the various metabolic pathways that these drugs take as well as a better understanding of the in vivo effect of their various metabolites.

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