Cancer Chemother Pharmacol (1999) 43: 419–426

ORIGINAL ARTICLE

Trevor W. Sweatman · Ramakrishnan Seshadri
Mervyn Israel

Pharmacology of N-benzyladriamycin-14-valerate in the rat

Received: 26 January 1998 / Accepted: 5 August 1998

Abstract *Purpose*: N-Benzyladriamycin-14-valerate (AD 198) is a semisynthetic anthracycline analogue superior to doxorubicin (DOX) both in vitro and in experimental rodent tumor models, and with differing mechanistic properties from those of the parental antibiotic agent. In the present study, we examined the metabolic fate and hematotoxicity of AD 198 in rats, with a view to determining whether some of the therapeutic properties observed for this drug might be due to a DOX prodrug effect. *Methods*: Samples of plasma, bile and urine were obtained at various times following intravenous (i.v.) [^{14}C]–AD 198 administration to rats and were analyzed by reversed-phase HPLC with flow–fluorescence detection and complementary liquid scintillography. In other animals, red blood cell and white blood cell (WBC) counts were determined for blood obtained by retrobulbar sampling on selected days from groups of animals receiving either AD 198 or DOX at several dose levels, as well as from vehicle controls. *Results*: Following a single iv dose of [^{14}C]-AD 198 (5 mg/kg; equivalent to the optimal murine antitumor dose) in anesthetized rats, a triphasic plasma decay pattern for parental drug was evident with extremely rapid α and β phases followed by a very long terminal elimination phase. Principal plasma products included N–benzyladriamycin (AD 288) and N–benzyladriamycinol (AD 298) together with very low levels of DOX and doxorubicinol (DOXOL). Analysis of bile from anesthetized and conscious animals receiving AD 198 revealed DOX to be the principal biliary fluorescent species together with low levels of AD 288, AD 298 and DOXOL; no parental drug was seen. By contrast, AD 288 was the principal urinary product, together with low levels of AD 298 and DOX; again, no parental drug was evident. Dose recovery (8 h) in the respective bile and urine of anesthetized rats was 12.4% and 13.2% based upon total fluorescence versus 1% and 15.3% of the administered radiolabel. In conscious animals, 13.4% of drug fluorescence was recovered in the bile (48 h), while in urine 16.6% and 77.1% of drug fluorescence and radiolabel, respectively, were eliminated over 72 h. The discrepancy between recovery of drug fluorescence and ^{14}C was due to the production of nonfluorescent hippuric acid (benzoylglycine) and N–benzyl daunosamine as a consequence of hepatic and renal drug metabolism. In the separate hematotoxicity studies, AD 198 (24.6 mg/kg i.v.; equivalent to the murine LD_{50} dose), produced a 45% reduction (nadir day 3–5) in WBC count, with recovery by day 10. By contrast, DOX (10 mg/kg i.v.; equivalent to the mouse highest nonlethal dose) produced an 80% decline in WBC with only partial recovery by day 17. *Conclusions*: By virtue of the low systemic DOX levels and low hematotoxicity observed in rats receiving AD 198, the in vivo therapeutic superiority of AD 198 cannot be attributed to substantial intracellular DOX generation. The conclusion that the therapeutic superiority of AD 198 compared to DOX results from the mechanistic differences between these two drugs is further supported by recent observations on their biochemical differences with regard to protein kinase C and topoisomerase II inhibition.

Key words Chemotherapy · Anthracycline · Animal pharmacology · Metabolism

Introduction

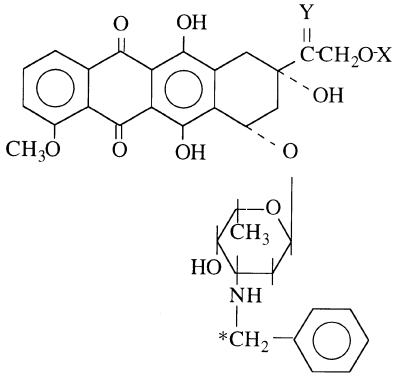
Doxorubicin (Adriamycin®, DOX), the most commonly prescribed antitumor antibiotic, possesses significant activity against a variety of human malignancies, including leukemias, lymphomas, sarcomas, and carcinomas such as those of the breast and lung [15]. However, the significant hematologic, gastrointestinal, and cardiac

This work was supported in part by NIH-NCI Research Grants CA44890 (TWS) and CA 19118 (MI).

T.W. Sweatman (✉) · R. Seshadri · M. Israel
Department of Pharmacology, University of Tennessee College of Medicine, 874 Union Avenue, Memphis, TN 38163, USA
Tel.: +1-901-448-4586; Fax: +1-901-448-4587
Email: TSWEATMAN@UTMEM1.UTMEM.EDU

toxicities of this agent have served to limit its full therapeutic potential. Moreover, DOX shows reduced effectiveness against tumor cell populations that exhibit the "classical" Pgp multidrug resistance phenotype, as well as those showing an altered topoisomerase II resistance [3]. These several considerations have led to various efforts to produce structural analogues that improve upon the spectrum of DOX antitumor activity, reduce its toxicities, or circumvent cellular drug resistance mechanisms. The present study concerned the metabolic profiling, in a laboratory animal model, of a novel anthracycline analogue that appropriately demonstrates all three of these advantages.

N-Benzyladriamycin-14-valerate (AD 198; structure, Fig. 1) is a semisynthetic lipophilic anthracycline analogue which shows superior antitumor activity to DOX in various animal tumor models, together with significantly lower systemic toxicity [4, 5, 7]. In addition, in a well-regarded murine model of anthracycline cardiotoxicity [1], AD 198 shows negligible potential for cardiomyopathy compared to DOX [9]. At the cellular and biochemical levels, significant differences exist between AD 198 and DOX. These include: irreversible cell cycle G_2/M blockade [22]; a weaker binding with purified mammalian DNA [7, 11]; production of protein-associated DNA strand breaks on alkaline elution assays, despite an inability to inhibit isolated mammalian DNA topoisomerase II [2]; potent membrane lytic activity [7]; rapid and extensive drug accumulation and high retention in both sensitive and drug-resistant cell lines [4, 8, 18]; and lack of cross-resistance in Pgp transport-modified and altered topo II multidrug-resistant cell lines [4, 10, 13, 18]. The ability of AD 198 to circumvent multidrug resistance has been documented in vivo, as well, in models of DOX-resistant P388 and L1210 leukemia, and B16 melanoma [4, 5, 7]. Recent studies have shown further an enhanced potency of AD 198, compared to DOX, for inhibition of protein kinase C (PKC), thereby suggesting perturbation of PKC-regulated pathways as an additional mechanism of cytotoxicity for this novel drug [16].



Code No.	X	Y
AD 198	$CO(CH_2)_3CH_3$	O
AD 288	H	O
AD 298	H	H, OH

Fig. 1 Structure of [^{14}C]-AD 198 and principal metabolites; * denotes the site of the radiolabel

In cell culture systems with bovine serum supplementation, AD 198 is converted, to a greater or lesser extent, into N–benzyladriamycin (AD 288) through the action of nonspecific serum esterases [5, 7, 8, 18]. However, in biochemical and therapeutic properties, the behavior of AD 288 is more similar to that of DOX than it is to AD 198. Both AD 288 and DOX inhibit topoisomerase II, albeit through different mechanisms (Lothstein et al., unpublished data), making it extremely unlikely that an AD 288 prodrug mechanism can serve as the sole explanation for the superior therapeutic activity of AD 198. Yet, despite considerable knowledge of the cellular pharmacokinetics and in vitro effects of AD 198, little is known regarding the metabolic fate of this drug in vivo. Accordingly, the present work was undertaken to establish the pharmacology and metabolic profiling of AD 198 in a laboratory animal model. In connection with this work, a comparison of the hematotoxic potential of intravenous (i.v.) AD 198 with that of DOX was also performed, as these results have a bearing on the drug metabolism studies.

Methods

Drugs and drug formulations

Unlabeled and ^{14}C isotopically labeled AD 198 (see Fig. 1 for site of the label) were prepared as previously described [5, 8]. This labeling site was chosen to enable the fate of the nonfluorescent daunosamine sugar portion of the molecule to be determined. For animal use, AD 198 was dissolved in Cremaphor/ethanol (Diluent 12; Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md], then diluted 1:4 with saline prior to i.v. administration. Commercially available DOX in saline for injection (Adriamycin PFS®) was used as reference agent in the hematotoxicity studies.

Animal pharmacology studies

All animal experiments were conducted under protocols approved by the University of Tennessee, Memphis Animal Care and Use Committee. Male Harlan Sprague-Dawley rats (225–275 g, $n = 5$) were each anesthetized with intraperitoneal (i.p.) sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Ill.) and placed on isothermal pads (Braintree Scientific, Braintree, Mass.) for the duration of the study. AD 198 was administered as an i.v. bolus dose (5.0 mg/kg per ml; equivalent to the murine optimal antitumor dose of 10 mg/kg) via a catheter implanted into the jugular vein. At various times thereafter (2.5, 5, 10, 15, 20, 30, 40, 60, 75, 90 min and 2, 3, 4, 5, 6, 7 and 8 h), blood samples were obtained from a cannula placed in the ipsilateral femoral artery. Samples were immediately centrifuged and equal volumes of the resulting plasma (70–750 µl) were pooled in sterile red-topped vacutainers and frozen (−70 °C), pending extraction and analysis. Bilateral ureteral and common bile duct catheterization afforded simultaneous collection of urine and bile (30-min aliquots in preweighed vials) from the individual animals. The filled sample vials were

re-weighed and frozen (−70 °C), pending analysis. Adequate hydration of the animals was maintained by i.v. infusion of saline at a rate equal to the volume of combined biliary and urinary excretion.

In separate studies, otherwise conscious male rats ($n = 6$) under momentary methoxyflurane (Metofane, Pitman Moore, Mundelein, Ill.) anesthesia received [^{14}C]-AD 198 (5.0 mg/kg per ml) via injection into the tail vein. These animals were replaced into metabolism cages for the separate collection of urine and feces at various time-points (12, 24, 36, 48, 60 and 72 h). Animals were allowed food and water *ad libitum* for the duration of the study. At each time-point, the urine volume was measured and an aliquot, together with the total fecal production, were frozen (−70 °C) for subsequent analysis.

Additional conscious animals ($n = 6$) surgically fitted with biliary catheters each received radiolabeled AD 198 (5.0 mg/kg per ml) via an indwelling femoral vein catheter. The continuous biliary outflow was collected on dry ice in aliquots (14, 24, 38, 48 h) through 48 h. For each period, the individual bile volumes were recorded and representative samples frozen (−70 °C) pending analysis.

Sample processing and analysis

Plasma samples were extracted from pH 8.5 Tris buffer (0.05 *M*) with 2 × 7 ml ethyl acetate:1-propanol (9:1, by volume), following the addition of N-trifluoroacetyladriamycin-14-octanoate (AD 28) as an internal standard. The combined extracts for each sample were evaporated to dryness under nitrogen at 37 °C and reconstituted in methanol (100 µl) for analysis. Bile samples from the anesthetized experiments, and urine samples from both anesthetized and conscious experiments, were diluted with demineralized water, as appropriate, and analyzed directly. Bile samples (1-ml aliquots) from the conscious animals were extracted, as for the plasma samples, prior to HPLC analysis.

Analysis was accomplished by reversed-phase HPLC, essentially as described previously [17], except for the use of a C_{18} column (4 µm particle size; 5 mm i.d. × 10 cm; Nova-Pak, Waters Associates, Milford, MA) and a flow rate of 1.0 ml/min. In addition to flow fluorescence detection (Schoeffel FS 970 flow fluorometer; 482 nm excitation, 550 nm barrier emission filter), the column eluate was collected at 30-s intervals in a Gilson FC-80K microfractionator (Gilson Instruments, Middleton, Wis.). Collected fractions were analyzed for radiolabel content by liquid scintillography (Biosafe II scintillant, RPI, Mount Prospect, Ill.) using a Beckman Model LS3801 counter (Beckman Instruments, Irvine, Calif.). In addition, aliquots of bile and urine were analyzed directly by scintillography for radiolabel content. Anthracycline levels in plasma samples and bile from conscious rats were quantitated, by reference to standard curves of parental drug and authentic samples of the principal biotransformation products added to blank rat plasma or bile and processed in a similar manner, and by reference to recovery of internal standard. Anthracycline levels in remaining bile and urine samples were quantitated by reference to standard curves produced by direct HPLC injection of standards. The assay was appropriate and specific for the major anthracycline components with linearity of detector response up to at least 4000 ng/injection for both directly injected and extracted component standards AD 198, AD 288 (N–benzyladriamycin), AD 298 (N-benzyladriamycinol), DOX and DOXOL (doxorubicinol). The limit of quantitation was 1 ng/injection for each of these components. Intra- and interday coefficients of variation (CV) values for component standards were < 5%.

Identification of nonfluorescent urinary metabolites

Urine from animals receiving [^{14}C]-AD 198 was concentrated by C_{18} Sep-Pak (Waters Associates, Milford, Mass.) extraction with elution in methanol and evaporation to dryness under nitrogen at 37 °C. The resulting material was purified by twice chromatographing on silica gel GF TLC plates (250 µm layer; Analtech, Newark, Del.) using chloroform:methanol:acetic acid (7:3:0.3, by volume) and the position of the radiolabeled materials determined with the aid of a Packard Model 7222 TLC chromatogram scanner. These areas were scraped and the radioactive material eluted from the silica with chloroform:methanol (7:3, by volume) and evaporated to dryness. The resulting two products at an approximate ratio of 7:3 were analyzed by reversed-phase HPLC, as above, with flow UV detection at 254 nm and liquid scintillography of the column eluate. The principal nonfluorescent radioactive urinary product was found to cochromatograph with a pure authentic sample of hippuric acid (Sigma, St. Louis, Mo; RT 19.5 min; 0–20% acetonitrile at 1 ml/min). Likewise, the secondary product, N-benzyldaunosamine, cochromatographed with authentic material derived in the laboratory by the acid hydrolysis of N–benzyldaunorubicin (RT 7.8 min; 0–40% acetonitrile at 2 ml/min). In both instances, analysis of the column eluate for ^{14}C by liquid scintillography revealed the elution of radiolabel to correspond exactly with the UV absorbing peak. Additionally, both hippuric acid (R_f 0.83; chloroform:methanol:water, 6:4:0.4 by volume) and N–benzyldaunosamine (R_f 0.68; butanol:acetic acid:water, 4:1:1 by volume) cochromatographed with authentic standards when analyzed on TLC (silica gel GF; 250 µm). Visualization was accomplished under short-wave UV light in a Chromatovue viewing chamber or by charring. Identification of one metabolite as hippuric acid was further confirmed by fast atom bombardment mass spectrometry (m/e 180).

Hematotoxicity studies

A total of 30 female Harlan Sprague-Dawley rats (215 ± 15 g body weight) were used for the study, with five animals randomized to each of six test groups. Test groups included: (A) vehicle control; (B) AD 198, 24.6 mg/kg; (C) AD 198, 15.0 mg/kg; (D) AD 198, 3.0 mg/kg; (E) DOX, 10 mg/kg; and (F) DOX, 5 mg/kg. Test agents were administered by slow-push tail vein injection to momentarily anesthetized animals (methoxyflurane inhalation anesthesia). Animal weights were recorded prior to retrobulbar blood sampling 1 day before the start of the experiment and on days 1, 2, 3, 5, 7, 10, 14, 17, and 21 after test agent administration. Upon collection, blood samples were immediately diluted with isotonic buffered saline, and red and white blood cell counts were determined in the usual manner using a Model ZM Coulter counter (Coulter Counter Electronics, Luton, UK). Except as noted, test animals were sacrificed on day 21 of the experiment after the last blood sampling.

Drug dosages used in this study relate to previously obtained toxicity data for the test materials in mice. Thus, for DOX, the 10 mg/kg rat dose is equivalent to a mouse dose of 20 mg/kg, a value that approximates the maximal sublethal dose of this agent in this species; the second DOX dose was then selected at 50% of the higher dose. Administration of DOX was accomplished using Adriamycin PFS, a clinical injectable preparation preformulated at a concentration of 2 mg/ml. For AD 198, the mouse i.v. single dose LD_{50} has been carefully titrated at 48–49 mg/kg, equivalent to 24.6 mg/kg in the rat; the other AD 198 doses represent levels approximately five-eighths and one-eighth of the top value. Based on considerations of drug solubility, the highest AD 198 dose level was prepared at a concentration of 8.2 mg/ml and administered in a volume of 0.3 ml/100 g animal body weight. The intermediate dose was formulated at a concentration of 7.5 mg/ml and administered in a volume of 0.2 ml/100 g body weight. The lowest AD 198 dose was prepared at a concentration of 3 mg/ml and administered in a volume of 0.1 ml/100 g body weight. For the vehicle control test group, animals received a volume of 20% NCI Diluent 12 equivalent to the largest volume of vehicle being used with any drug-treated group, i.e. 0.3 ml/100 mg body weight.

Results

Animal pharmacology

Recognizing the importance of dose selection in animal pharmacology studies, the therapeutically relevant dose

of AD 198 (5 mg/kg) for these pharmacology studies was selected as being nontoxic but equivalent to the optimal drug dose (10 mg/kg) in several murine antitumor studies [4]. Plasma anthracycline levels following [^{14}C]-AD 198 administration are shown in Fig. 2. Plasma anthracycline fluorescence was primarily accounted for in terms of four species (see Fig. 1 for structures of AD 288 and AD 298): parental AD 198, its 14-deacyl metabolite (AD 288), the corresponding 13-dihydro derivative of AD 288 (AD 298), and DOX. Trace levels of three unidentified plasma products were evident in samples prior to 1 h; these were omitted from Fig. 2 for the sake of clarity. At the earliest time-point (2.5 min), levels of parental AD 198 (550 ng/ml) were already exceeded by those of the principal biotransformation product, AD 288 (770 ng/ml). AD 298 (110 ng/ml) and DOX (30 ng/ml) were also evident. For DOX, these low circulating plasma concentrations represented the maximum observed during the course of this study.

Levels of AD 198, AD 298 and DOX declined rapidly (< 30 ng/ml each by 60 min) from this time-point, whereas the concentration of AD 288 fell more slowly to reach comparable levels only by 6.5 h. For AD 198 and AD 288 the plasma concentration-time profiles declined in a multiexponential manner and were thus computer-fitted to two- and three-compartment body models using nonlinear least squares regression (WinNonlin, SCI Software, Cary, NC). In both instances, a three-compartment body model best described the data, as determined by comparison of the Akaike Information Criterion [24]. Respective α, β and γ plasma half lives for AD 198 and AD 288 were as follows: 0.011 vs. 0.039 h, 0.117 vs. 0.297 h, and 357.2 vs. 4.7 h. The extremely long terminal half-life for AD 198 over the course of the 8-h study likely reflects enterohepatic recirculation of this lipophilic drug. Based upon anthracycline AUC (0–8-h) values, systemic exposure in plasma was highest for AD 288 (436 ng-h), intermediate for AD 198 (168 ng h) and lowest for DOX (23 ng-h). The result for AD 198 was to be anticipated given the previously observed rapid tissue sequestration of this lipophilic agent in vitro.

Further pharmacokinetic analysis of additional lipophilic metabolites was complicated by their involvement in the process of enterohepatic recirculation. Analysis of the HPLC column eluate for radiolabel showed excellent agreement with retention times for AD 198, AD 288 and AD 298. However, during the course of the study there was a marked increase both in the level of organic solvent-insoluble ^{14}C that remained in the aqueous phase following sample extraction and in the levels of nonfluorescent polar materials detectable in the column eluate. Thus, as seen in Fig. 2, when calculating the plasma anthracycline concentration based upon the specific activity of the labeled material and recovery of ^{14}C in plasma, there was an increasing discrepancy in values.

Figure 3 shows the cumulative biliary elimination of anthracycline fluorescence following administration of AD 198. Although no parental drug was found, a number of metabolic products were evident in the bile. The principal biliary product was DOX, with lower levels of AD 298, AD 288 and DOXOL. Low levels of a product previously identified as 4-O-desmethyl-AD 288 [6] were also seen. Trace levels of five other fluorescent products were also detected; these were omitted from Fig. 3 for clarity. By 8 h, 12.4 $\pm$ 3.8% of the fluorescent dose and 13.2 $\pm$ 2.3% of the radiolabel dose, respectively, were accounted for in the bile. In conscious animals receiving unlabeled AD 198, this metabolic pattern was essentially unchanged with recovery of 13.4 $\pm$ 2.2% of the dose ($n = 5$) by this route in 48 h. For each

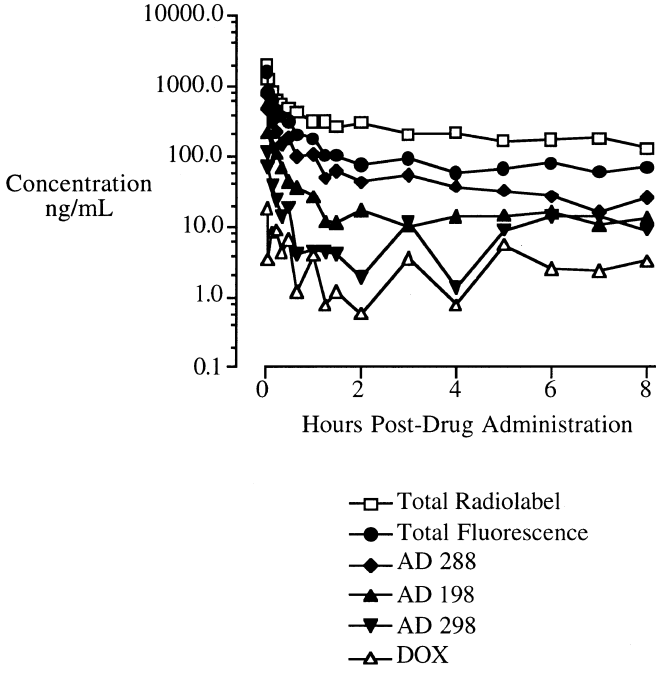


Fig. 2 Plasma anthracycline and radiolabel levels in rats following the administration of a single dose of [^{14}C]-AD 198 (5.0 mg/kg i.v. bolus)

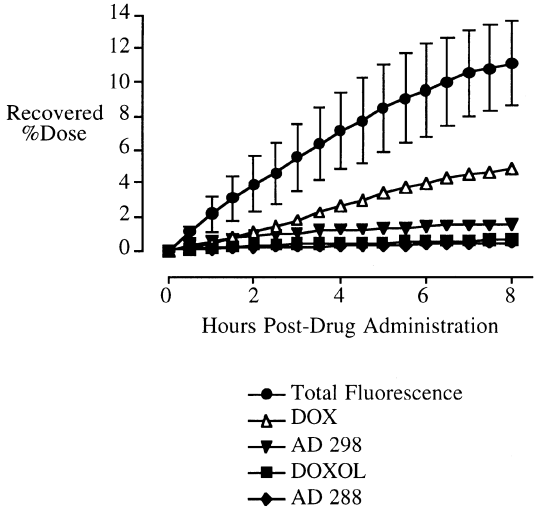


Fig. 3 Cumulative biliary elimination of anthracycline fluorescence following the administration of [^{14}C]-AD 198 (5.0 mg/kg i.v. bolus)

collection period (14, 24, 38, 48 h), DOX represented the principal biliary elimination product of AD 198 (53–65% total eliminated). Other products included AD 288 (5.9–10.9%), 4-O-desmethyl-AD 288 (8.4–12.2%), AD 298 (3–6.3%), DOXOL (3.4–9.3%) and a number of other minor unidentified fluorescent species.

Cumulative elimination of anthracycline fluorescence in Urine is shown in Fig. 4. In common with the biliary route, no parental AD 198 was detected through the urinary elimination route during the course of the study. Principal urinary biotransformation products included low levels of AD 288, AD 298, DOX and DOXOL. Over the course of the 8-h study, only 1.0 ± 0.3% ($n = 6$) of the administered drug fluorescence was recovered by this route. However, analysis of column eluate by liquid scintillography (Fig. 5) revealed the presence of a polar nonfluorescent species in urine, similar to that observed in plasma. Thus, recovery of ^{14}C in urine amounted to some 15.3 ± 8.5% of the applied dose. For conscious animals ($n = 5$) receiving [^{14}C]-AD 198, the pattern was similar, with recovery of 16.6 ± 2.1% of the fluorescent dose but 77.1 ± 5.3% of the applied radiolabel in 72 h following AD 198 administration.

Isolation and identification of the nonfluorescent materials was accomplished as described in Methods. In the pooled 0–24-h urine collection from these conscious animals, two radiolabeled products, hippuric acid (benzoylglycine) and N-benzyldaunosamine, were recovered at an approximate ratio of 7:3, respectively.

Hematotoxicity

Except for the high-dose DOX group, all test animals survived through the 21-day study period. One animal in the DOX 10 mg/kg dose group died accidentally on day

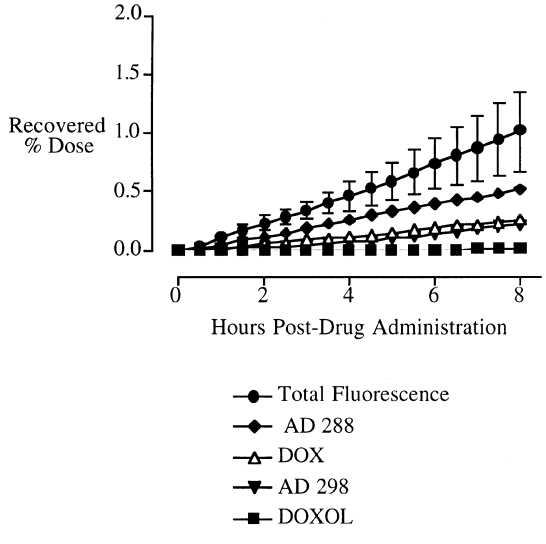


Fig. 4 Cumulative urinary elimination of anthracycline fluorescence following the administration of [^{14}C]-AD 198 (5.0 mg/kg i.v. bolus)

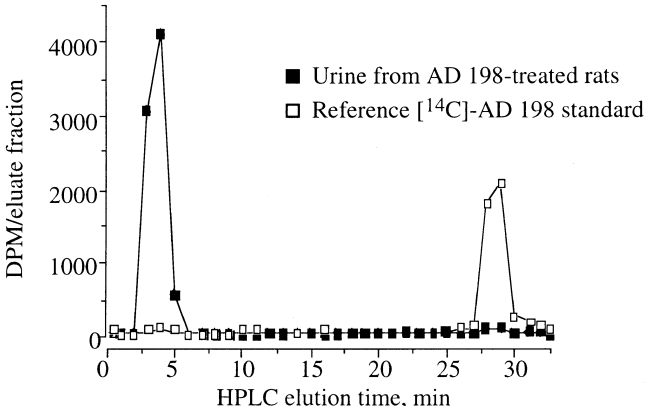


Fig. 5 Recovery of radiolabel in HPLC eluate following the injection of neat urine from rats receiving [^{14}C]-AD 198

14 prior to weighing and blood sampling while the four remaining test animals in this group, being in obvious distress, had to be sacrificed at various times before the end of the study. At sacrifice on day 21, all remaining animals looked normal and healthy.

Figure 6 shows the change in mean white blood cell counts following a single i.v. dose of AD 198 or DOX to female Sprague-Dawley rats. Results are expressed relative to the mean pretreatment white blood cell count for all the animals used in this study. No significant change in white cell counts was seen with the vehicle control group, further validating the innocuousness of the drug delivery formulation in its effect on the hematopoietic system. AD 198 was seen to produce a lowering of white blood cell counts (nadir by day 3–5), with recovery by day 10. The highest dose of AD 198 (24.6 mg/kg), equivalent to a drug dose resulting in 50% lethality in mice, produced a reduction by 45% from the starting mean total white cell count. By contrast, the higher DOX dose (10 mg/kg), equivalent to the highest nonlethal drug dose in the mouse, caused an 80% reduction in mean white cell counts, with only partial recovery of cell counts by day 17 and with animals exhibiting obvious clinical manifestations of toxicity. Indeed, in this experiment, DOX at only 5 mg/kg still resulted in leukopenia in excess of that seen with the highest dose of AD 198 (24.6 mg/kg), and even by day 21, recovery of white cell counts to normal had not been achieved.

No significant change was seen in red blood cell counts among the various test groups, except for the DOX 10 mg/kg test group; as previously mentioned, these animals were in a severely debilitated condition.

Figure 7 shows the change in mean body weights for the various test groups involved in the study. Except for the higher DOX test group, no significant differences were seen among the drug-treated groups relative to the vehicle control group. The effect on body weight of DOX at 10 mg/kg is fully consistent with the previously noted profound depression of bone marrow activity and clinical manifestations of toxicity seen in these animals.

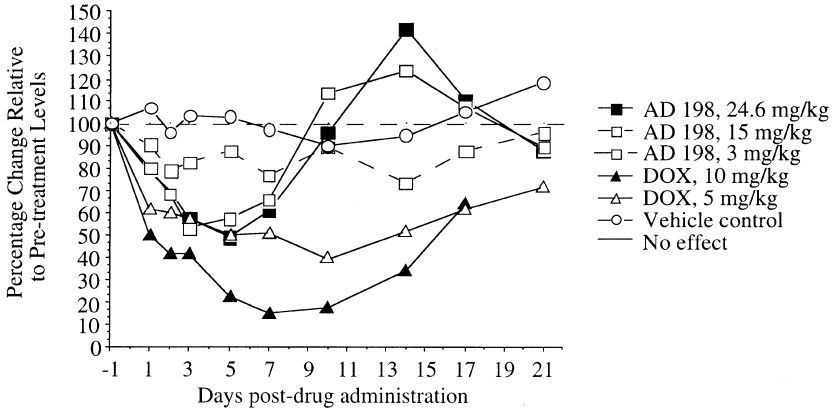


Fig. 6 Change in mean white blood cell count following a single i.v. dose of AD 198 or DOX

Discussion

As anticipated from the rapid and extensive cellular AD 198 uptake previously observed during comparative in vitro studies with DOX [4, 8, 18], AD 198 underwent extremely rapid distribution following i.v. administration of a therapeutically relevant drug dose. This rapid distribution was followed by a very prolonged elimination period, presumably due to enterohepatic drug recirculation. There was also evidence of a high degree of tissue sequestration in the bile, the principal excretory route, with the recovery of only 12.4% and 13.2% of fluorescent dose, in 8 and 48 h respectively, following drug administration. In comparison, previous studies have recovered 36% of a dose of DOX (10 mg/kg) by this route in 55 h [17]. In common with DOX (3.5% in 60 h) [17], little fluorescent material was recovered in the urine of anesthetized or conscious animals following AD 198 administration.

The pattern of excreted fluorescent anthracycline products was expected, based upon known biotransformation pathways. Thus, parental drug was rapidly and extensively metabolized, initially by the action of ubiquitous esterases, to yield AD 288. This product was then reduced, in part, to the 13-carbinol product, AD 298. Low levels of 4-O-desmethylated products, as found in the present bile samples, may represent a new biotransformation pathway resulting from the differential intracellular distribution of AD 198 relative to DOX. Alternatively, such products may have become apparent owing to improvements in chromatographic separation techniques. It is likely that some of the unknown products in bile may represent traces of 4-O-desmethyl metabolites of AD 298, DOX, etc. Low levels of DOX and DOXOL were evident in urine and plasma.

Since the liver and the kidney are the principal sites of N-debenzylation, it is not surprising that DOX was found as an elimination product in urine, and in significant levels in the bile. However, the levels of DOX seen here are meaningless with respect to cytotoxicity, since DOX itself does not undergo enterohepatic recirculation [21], or resorption from the urinary bladder [19]. The very low levels of DOX present in the plasma following AD 198 administration (AUC 0–8 h: 23 ng h) serve to reinforce the relative liver and kidney tissue specificity for the origin of this metabolite. In the context of therapeutic activity, these systemic DOX levels are 10- to 100-fold lower than the LD_{50} (8-h drug exposure; clonogenic assay) for this drug against human leukemic CEM cell lines in vitro [18]. Additionally, separate studies of AD 198 tissue distribution [20] have confirmed the absence of significant DOX levels in tissues other than liver or kidney.

Generation of significant levels of DOX in the liver, with consequent loss of the N–benzyl substituent

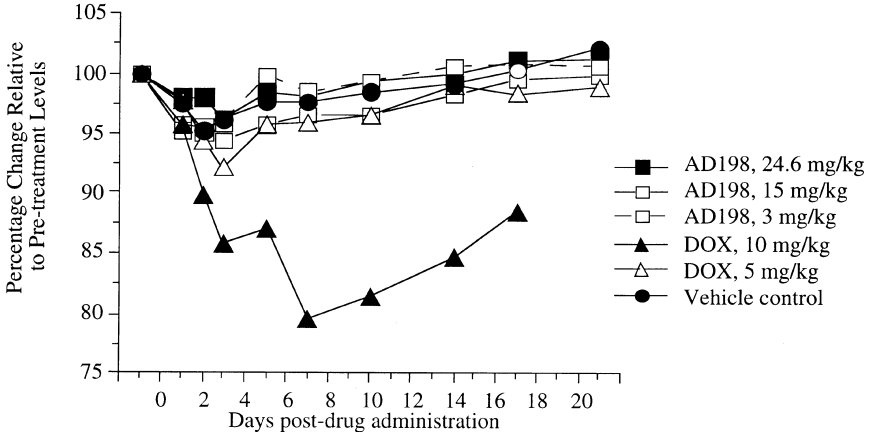


Fig. 7 Change in mean rat body weight following a single i.v. dose of AD 198 or DOX

containing the radiolabel, of necessity gave rise to nonfluorescent but radiolabeled metabolic products. Since the principal route of "detoxification" of aromatic carboxylic acids, such as benzoic acid, often involves conjugation with glycine [14, 23], hippuric acid (benzoylglycine) was a logical candidate as one of the two unknowns isolated from urine. This indeed proved to be the case, with confirmation by chromatography and mass-spectral analysis. Although N-benzyldaunosamine, the second nonfluorescent radioactive product, could arise intrahepatically from the hydrolytic or reductive loss of the sugar moiety and consequent generation of aglycones, specifically adriamycinone and 7-deoxyadriamycinone, no significant levels of either of these two products was evident in the bile. Alternatively, N-benzyldaunosamine might be generated by the intestinal microflora from anthracycline material eliminated in the bile, with subsequent reabsorption into the systemic circulation. Nevertheless, neither this biotransformation product nor the hippuric acid is of significance, relative to the biological activity of AD 198.

With regard to the hematotoxicity study, DOX was employed as a positive control at doses that were based upon the maximum sublethal single doze. Clearly, the top dose (10 mg/kg) was very close to lethal, with loss of weight, general condition and a severe reduction in white cell count. At half this concentration, the reduction in white cell count was almost identical to that seen in earlier studies [19]. In the present study, AD 198 required a five-fold increase in dose (24.6 mg/kg) to produce a comparable level of hematotoxicity to that seen with DOX (5 mg/kg). Even at the highest dose, AD 198 had no effect upon animal body weight. These data are strongly supportive of the metabolism studies, to the effect that systemic concentrations of DOX capable of producing leukopenia do not arise following the administration of AD 198. Such a contention is further supported by the absence of significant cardiotoxicity for AD 198, compared with DOX, when both were examined using the well-accepted Bertazzoli murine model [9].

In conclusion, the present studies demonstrate that AD 198 is very rapidly distributed into tissues following i.v. administration, with conversion to AD 288, the principal circulating metabolite occurring much more rapidly in vivo than in vitro. At the earliest time-point (2.5 min), plasma concentrations of AD 288 exceeded those of parental agent. Unlike AD 198 that appears to avoid P-gp both by rapid intracellularization [4, 8, 18] and through differential subcellular localization [12], AD 288 is a substrate for P-gp. Thus, significant conversion of AD 198 to AD 288 before access to the tumor cell population could result in reduced tumor exposure to the parental drug and diminished antitumor effect. At present, however, the effects of dose intensification on drug pharmacology remain unknown.

A variety of studies have clearly shown AD 198 and its initial and principal metabolite, AD 288, to be different from DOX relative to their cytoplasmic and nuclear mechanisms of action and with regard to experimental antitumor activity. Thus, since AD 288 is rapidly formed from AD 198 in vivo, it is reasonable to believe that the observed in vivo activity of AD 198 represents a blend of mechanistic contributions from parental drug and metabolite. A critical question addressed in the present study relates to whether AD 198 possibly serves further as a prodrug for DOX. Results of this and other studies clearly show that significant N–debenzylation of AD 198 occurs only in the liver and, to a limited extent, in the kidney. Thus, the DOX that is formed in this manner represents a true elimination product of no therapeutic consequence. Circulating levels of DOX are too low to produce either significant antitumor activity (based upon in vitro data) or leukopenia comparable with that seen with a direct therapeutic dose of DOX. Given the significant biochemical differences between AD 198, AD 288 and DOX, including the recently observed differences with respect to PKC and topoisomerase II inhibition, the explanation for the antitumor superiority of AD 198 almost certainly resides in mechanistic differences between these two drugs. These mechanistic properties continue to be the subject of study in our laboratories.

Acknowledgement The authors are grateful to Dr. James T. Dalton, University of Tennessee College of Pharmacy, for the pharmacokinetic modeling data.

References

1. Bertazzoli C, Bellini O, Magrini U, Tosana MG (1979) Quantitative experimental evaluation of adriamycin cardiotoxicity in the mouse. Cancer Treat Rep 63: 1877–1883
2. Bodley A, Liu LF, Israel M, Seshadri R, Koseki Y, Giuliani FC, Silber R, Kirschenbaum S, Potmesil M (1989) DNA topoisomerase II interaction of doxorubicin and daunorubicin congeners with DNA. Cancer Res 49: 5969–5978
3. Danks MK, Yalowich JC, Beck WT (1987) Atypical multiple drug resistance in a human leukemic cell line selected for resistance to teniposide. Cancer Res 47: 1297–1301
4. Ganapathi R, Grabowski D, Sweatman TW, Seshadri R, Israel M (1989) N–Benzyladriamycin-14-valerate versus progressively doxorubicin-resistant murine tumours: cellular pharmacology and characterisation of cross-resistance *in vitro* and *in vivo*. Br J Cancer 60: 819–826
5. Israel M, Seshadri R (1986) N-Alkyl and N-benzyladriamycin derivatives. U.S. Patent 4 610 977, US Patent Office, Washington, DC
6. Israel M, Sweatman TW, Seshadri R, Koseki Y (1986) Metabolic studies in rats with N-benzyladriamycin-14-valerate (AD 198), a promising new anthracycline analog. Proc Am Assoc Cancer Res 27: 239
7. Israel M, Seshadri R, Koseki Y, Sweatman TW, Idriss JM (1987) Amelioration of adriamycin toxicity through modification of drug-DNA binding properties. Cancer Treat Rev 14: 163–167
8. Israel M, Sweatman TW, Seshadri R, Koseki Y (1989) Comparative uptake and retention of adriamycin and N-benzyladriamycin-14-valerate in human leukemic lymphocyte cell cultures. Cancer Chemother Pharmacol 25: 177–183
9. Israel M, Koseki Y, Jenkins JJ III (1991) Murine cardiotoxicity assay of the mechanistically novel adriamycin analog, N-benzyladriamycin-14-valerate (AD 198). Proc Am Assoc Cancer Res 32: 423

10. Krishan A, Sauerteig A, Gordon K, Swinkin C (1986) Flow cytometric monitoring of cellular anthracycline accumulation in murine leukemia cells. Cancer Res 46: 1768–1773
11. Lameh J, Chuang LF, Israel M, Chuang RY (1988) Mechanistic studies on N-benzyladriamycin-14-valerate (AD 198), a highly lipophilic alkyl adriamycin analogue. Anticancer Res 8: 689–694
12. Lothstein L, Sweatman TW, Dockter M, Israel M (1992) Resistance of N–benzyladriamycin-14-valerate in mouse J774.2 cells: P-glycoprotein expression without reduced N-benzyladriamycin-14-valerate accumulation. Cancer Res 52: 3409–3417
13. Maniar N, Krishan A, Israel M, Samy TSA (1988) Anthracycline-induced DNA breaks and resealing in doxorubicin-resistant murine leukemia P388 cells. Biochem Pharmacol 37: 1763–1772
14. Parke DV, Smith RL (eds) (1977) Drug metabolism from microbe to man. Taylor and Francis, London
15. Riggs CE (1992) Antitumor antibiotics and related compounds. In: Perry MC (ed) The chemotherapy source book, Williams and Wilkins, Baltimore, pp 318–358
16. Roaten JB, Bertics PJ, Wiepz G, Lothstein L, Israel M, Sweatman TW (1998) Protein kinase C inhibition by N-benzyladriamycin-14-valerate (AD 198). Proc Am Assoc Cancer Res 39: 306
17. Sweatman TW, Israel M (1987) Comparative metabolism and elimination of adriamycin and 4′epi-adriamycin in the rat. Cancer Chemother Pharmacol 19: 201–206
18. Sweatman TW, Israel M, Seshadri R, Koseki Y, Beck WT (1990) Cytotoxicity and cellular pharmacology of N-benzyladriamycin-14-valerate in mechanistically different multidrug-resistant human leukemic cells. J Cell Pharmacol 1: 96–102
19. Sweatman TW, Parker RF, Israel M (1991) Pharmacologic rationale for intravesical N–trifluoroacetyladriamycin-14-valerate (AD 32): a preclinical study. Cancer Chemother Pharmacol 28: 1–6
20. Sweatman TW, Seshadri R, Israel M (1992) Selective retention of N-benzyladriamycin-14-valerate (AD 198) in the lung following intravenous drug administration. Proc Am Assoc Cancer Res 33: 520
21. Tavolini N, Guarino AM (1982) Does adriamycin undergo an enterohepatic circulation. Proc Soc Exp Biol Med 169: 41–46
22. Traganos F, Israel M, Seshadri R, Kirschenbaum S, Potmesil M (1985) Effects of new N-alkyl analogues of adriamycin on *in vitro* survival and cell-cycle progression. Cancer Res 45: 6273–6279
23. Williams RT (1959) Detoxication mechanisms. Wiley, New York
24. Yamaoka K, Nakagawa T, Uno T (1978) Application of Akaike Information Criterion (AIC) in the evaluation of linear pharmacokinetic equations. J Pharmacokinet. Biopharm 6: 165–75