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Comparative uptake and retention of adriamycin and *N*-benzyladriamycin-14-valerate in human CEM leukemic lymphocyte cell cultures

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Summary. *N*-Benzyladriamycin-14-valerate (AD 198) is a new lipophilic adriamycin (ADR) analogue that shows marked therapeutic superiority to ADR in murine tumor model systems yet differs mechanistically from ADR in a number of ways. Among its other properties, AD 198 produces a delayed but profound effect on cell-cycle progression and a pattern of continuing DNA damage in cultured cells briefly exposed to the drug. Using radiolabeled drug forms and radioassays combined with HPLC separation and fluorimetric detection techniques, aspects of drug accumulation, biotransformation, and retention in cultured human CEM leukemic lymphocytes were studied, in part to determine a possible pharmacologic basis for the latent effects seen with this drug. In addition, the cellular pharmacology of AD 198 and ADR were comparatively examined under identical experimental conditions. When CEM cells were incubated with drug at equi-growth inhibitory/minimally cytotoxic concentrations (AD 198, 1.0 μ*M*; ADR, 0.1 μ*M*), a number of differences were apparent. Under conditions of continuous 24-h drug exposure, a slow cellular accumulation and equilibration was observed with ADR (cell: medium equilibrium, 1:11 after 4–6 h), whereas the uptake of AD 198 was rapid and extensive (cell: medium equilibrium, 3:1 within 30 min). In drug-retention studies, when cells were pretreated at the same drug concentrations as before (AD 198 for 1 h; ADR for 4 h) and then transferred to drug-free media, both compounds re-equilibrated their intracellular drug content with the fresh media, losing about 50% of their respective anthracycline levels. Liquid chromatographic analysis of ADR-treated cultures under both sets of conditions showed the parent drug to be the only intracellular anthracycline species, whereas analysis of AD 198-treated cultures revealed two fluorescent signals corresponding to the parent drug and its 14-de-esterified biotransformation product, *N*-benzyladriamycin (AD 288). Levels of AD 288 rose from 2% of the total intracellular anthracycline content immediately on drug admixture to 61% following 24 h continuous drug exposure and to 69% at 24 h in cells exposed to drug for 1 h and then continued in drug-free media for 24 h. At all times, the balance of the intracellular anthracycline fluorescence was attributable to the parent drug; no ADR was detectable in AD 198-treated cells by either fluorescence detection or radioassay. Thus, AD 198 is not a prodrug form of ADR, and the in vitro effects of this agent, including the latent effects on cell-cycle inhibition and DNA damage seen in cells following short-term drug exposure, can be explained on the basis of the high levels of active parent drug and biotransformation product that accumulate and persist in the cells.

Introduction

With its broad spectrum of clinical activity, the anthracycline antibiotic adriamycin (ADR) has been of major importance in cancer chemotherapy for over 15 years [4]. However, considerations of toxicity (including a still poorly-understood cumulative dose-related cardiotoxicity), lack of effectiveness against a number of common carcinomatous neoplasias, and development of ADR resistance by initially sensitive tumors with collateral resistance to other antitumor agents are among the reasons for a continuing search for anthracycline analogues with improved therapeutic efficacy. Previous studies in our laboratories with lipophilic *N*-acyl anthracycline analogues have resulted in products that have shown, relative to parental ADR, high therapeutic activity and low toxicity, including the absence of cardiac toxicity, in advanced preclinical and clinical studies [11]. Originally, ADR cytotoxicity was thought to be due to direct drug-DNA interaction, with resultant interference of DNA polymerase action [3]. More recently, the essential cellular enzyme DNA topoisomerase II has been shown to be a major target of ADR action [18, 20]. DNA damage caused by the *N*-acyl ADR analogues AD 32 and AD 143, which bind weakly or not at all with DNA, is now recognized as being due to their biotransformation to a common

Abbreviations: ADR, adriamycin (doxorubicin); AD 198, *N*-benzyladriamycin-14-valerate; AD 288, *N*-benzyladriamycin; AD 32, *N*-trifluoroacetyladriamycin-14-valerate; AD 143, *N*-trifluoroacetyladriamycin-14-0-hemiadipate; AD 41, *N*-trifluoroacetyladriamycin; [^{14}C]-AD 198, [benzyl-α-methylene-^{14}C]-*N*-benzyladriamycin-14-valerate; [^{14}C]-ADR, [14-^{14}C]-adriamycin; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; DMSO, dimethylsulfoxide; S-MEM, Eagle's minimum essential medium for suspension culture; PBS, phosphate-buffered saline (pH 7.0)

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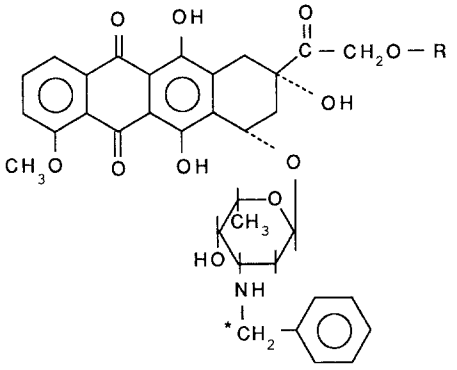

AD 198: R = $COCH_2CH_2CH_2CH_3$

AD 288: R = H

Fig. 1. Structure of AD 198 and its principal metabolite AD 288. The asterisk (*) denotes the site of the ^{14}C label in the radioactive AD 198 preparation used in the study

metabolite AD 41; unlike the parent drugs, this product is a strong inhibitor of DNA topoisomerase II [18].

The *N*-alkyl ADR analogue AD 198 (Fig. 1) is another lipophilic anthracycline compound of current developmental interest. This interest is based on the therapeutic superiority of AD 198 to ADR seen in murine tumor model systems [7, 8] and the differing mechanistic properties shown by the two drugs. In vitro and in vivo, AD 198 undergoes loss of the 14-valerate substituent by the action of nonspecific serum and tissue esterases to yield AD 288 as the initial and principal metabolite [11]. This metabolite is less active than AD 198 in vivo, showing activity more comparable with that of ADR [7, 8, 11]. Both AD 198 and AD 288 intercalate with DNA but in other respects show qualitative and quantitative differences with respect to DNA interactions [9, 21]. Although both AD 198 and AD 288 produce protein-associated DNA damage on alkaline elution filter assay [1, 17], neither compound inhibits purified mammalian DNA topoisomerase II [1, 2], a curious observation for a pair of DNA-interactive anthracycline antitumor drugs.

Other mechanistic differences between AD 198 and ADR have been noted. By precursor incorporation-inhibition studies, ADR has been found to exhibit preferential inhibition of DNA synthesis [9, 10]; in contrast, AD 198 selectively inhibits RNA synthesis, whereas AD 288 fails to show a preference [9]. AD 198 also exhibits a profound lytic effect on the plasma membranes of CEM cells after short-term drug exposure, an effect not seen with ADR or AD 288 under similar or more forcing culture conditions [11].

When the comparative effects of AD 198, AD 288, and ADR on cell-cycle progression were measured, AD 198 was found to produce an unusual pattern of delayed, profound, irreversible G_2/M blockade in murine L1210 cells following short-term drug exposure [21]. Consistent with this observation, alkaline elution studies have revealed that a brief cellular exposure to AD 198 results in an extended period of continuing DNA damage [17]. Based on these findings, the present study was undertaken to examine aspects of AD 198 cellular pharmacology in an attempt to discern a possible pharmacologic explanation for the latent cell-cycle inhibitory and DNA damage effects seen with this agent. Two additional objectives were intended: to evaluate AD 198 and ADR comparatively with respect to drug uptake, biotransformation, and retention, using the same experimental approach and conditions; and to determine whether the formation of ADR from AD 198 might account for some of the in vitro biological effects observed with the new analogue.

Materials and methods

Test drugs. The sample of [^{14}C]-AD 198 used in this study was prepared as follows. [α-methylene-^{14}C]-Benzyl chloride (sp. act., 5.05 mCi/mmol) was purchased from Pathfinder Laboratories (St. Louis, Mo). To this material (24 µl, 0.22 mmol) in anhydrous dimethyl formamide (6 ml) was added sodium iodide (600 mg, 4.0 mmol), and the mixture was stirred at room temperature for 1 h. Adriamycin-14-valerate (150 mg, 0.23 mmol), prepared as previously described [6], and sodium bicarbonate (75 mg, 0.89 mmol) were added sequentially and the reaction mixture was allowed to stir at room temperature for 48 h (monitored by HPLC). The reaction mixture was then filtered and the residue was washed with chloroform (3 × 20 ml). After the addition of more chloroform (200 ml), the filtrate was washed with ice-cold water (5 × 40 ml). The water washings were combined and back-extracted once with chloroform (50 ml). The combined chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure (water aspirator) on a rotary evaporator; residual dimethyl formamide was then removed with the aid of a high-vacuum pump.

The residue was chromatographed on Biosil A silicic acid (15 g; Biorad Laboratories, Richmond, Calif). Elution with chloroform yielded a reaction by-product, [benzyl-α-methylene-^{14}C]-*N,N*-dibenzyladriamycin-14-valerate (8 mg; 10% yield[1]; sp. act., 9 mCi/mmol; total activity, 89.1 µCi), chemically identical with an unlabeled reference sample. Elution next with chloroform containing 0.3%–0.5% methanol afforded the desired [^{14}C]-AD 198; center-cut fractions yielded 30 mg high-purity material, and an additional quantity of product was obtainable from early and late mixed chromatographic fractions by preparative TLC (silica gel plates, 1-mm-thick layer; Analtech, Newark, Del) with chloroform-methanol (98:2 vol/vol) as the irrigant. The total yield of [^{14}C]-AD 198 product was 40 mg (44%[1]), with a sp. act. of 3.8 mCi/mmol and a total activity of 223 µCi; it was chemically and radiochemically pure, homogeneous on TLC and HPLC [reverse-phase phenyl-RADIALPAK radial compression column (Waters Associates, Milford, Mass); gradient elution, 32%–65% acetonitrile vs ammonium formate buffer (pH 4.0) over 6 min; flow rate, 3.5 ml/min; fluorescence detection, 482-nm excitation wavelength, 550-nm emission

[1] Further elution of the column with chloroform containing 1% or more methanol afforded recovery of 70 mg unchanged adriamycin-14-valerate starting material. Product yields are correspondingly based on the unrecovered amount of the starting compound

cut-off filter; retention time, 8.60 min]; and identical in all aspects with an authentic unlabeled AD 198 reference sample. TLC radioscanning was run on an accessory Packard Model 722 TLC scanner connected to a Packard Model 7220/21 radiochromatogram scanner apparatus. Radioassays were carried out using Econofluor scintillation fluid (New England Nuclear, Boston, Mass) on a Beckman Model LS7500 liquid scintillation counter.

The hydrochloride salt of [^{14}C]-AD 198 was prepared in quantitative yield by treatment of pure free-base material with an equivalent amount of methanolic hydrogen chloride at 0° C, followed by evaporation of the solvent under reduced pressure and precipitation of the product from ethyl acetate by the addition of petroleum ether. The [^{14}C]-ADR material required for this investigation was prepared in our laboratories following a procedure described by Penco et al. [16]. The product showed >95% chemical and radiochemical purity (sp. act., 5.05 mCi/mmol). For use as HPLC reference standards, unlabeled ADR hydrochloride bulk material was kindly provided by Farmitalia Carlo Erba, Milan, Italy; unlabeled AD 198 and AD 288 were prepared according to our previously described procedures [7].

Cells and culture conditions. CEM human leukemic lymphoblastic cells, originally provided by Dr. Herbert Lazarus, University of Miami School of Medicine, were maintained in the log-growth phase in a humidified 5% CO_2-95% air atmosphere at 37° C by serial dilution in S-MEM (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 m*M* glutamine, and 50 μg/ml gentamycin (Sigma, St. Louis, Mo). For drug treatment studies, drugs were dissolved in reagent-grade DMSO and added to cultures for a 1% final DMSO concentration in the cultures; previous studies in our laboratories have shown this DMSO concentration to have no effect on cell growth or viability.

Drug uptake studies. Cultures of CEM cells (10^6 cells/ml, in 75-cm^2 culture flasks) were incubated for up to 24 h in the presence of [^{14}C]-AD 198 (1.0 μ*M*) or [^{14}C]-ADR (0.1 μ*M*). Immediately after drug admixture and at other times (0.5, 1, 2, 4, 6, 8, and 24 h), duplicate cultures were harvested and aliquots of each were taken for determination of cell counts and viability and for analysis. Cell pellets were obtained by centrifugation (250 *g*, 4° C), washed with ice-cold saline (3 × 10 ml) and sonicated in cold saline (2 ml). Media and washings were analyzed for total radiolabel content by scintillation counting. One set of duplicate cell-pellet samples was analyzed for total radiolabel content by scintillation counting; the other was subjected to organic solvent extraction and analyzed by HPLC with flow fluorescence detection and liquid scintillation counting of the column eluate (see below).

Drug retention studies. As before, cultures of CEM cells were preincubated with [^{14}C]-AD 198 (1.0 μ*M*) or [^{14}C]-ADR (0.1 μ*M*) for 1 h or 4 h, respectively; based on results from drug uptake experiments, these incubation times were needed for cellular drug equilibration. The cells were harvested by centrifugation (250 *g*), washed at room temperature with S-MEM (10 ml, without serum), resuspended in drug-free serum-supplemented S-MEM, and incubated at 37° C for up to 24 h. At preselected times (0.5, 1, 2, 4, 6, 8, and 24 h), duplicate cultures were harvested, washed, and processed for analysis as described above.

Determination of total radioactivity. Aliquots of culture media (1.0 ml), saline wash (2.0 ml), and total cell sonicate for each time point were transferred to plastic scintillation vials (20-ml capacity; RPI Corporation, Mount Prospect, Ill) to which scintillant (Budget-solve, RPI Corporation) was added (10 ml), and the radiolabel content was determined in a Beckman Model LS7500 liquid scintillation counter.

HPLC analysis and quantitation. For the duplicate sample sets intended for HPLC analysis, cell pellets, processed and sonicated as above, were diluted with TRIS buffer (0.05 *M*, pH 8.5, 3 ml) and subjected to organic extraction (ethyl acetate: 1-propanol, 9:1 vol/vol, 2 × 8 ml). The two organic extracts for each cell pellet were combined and evaporated to dryness (37° C) under a stream of dry nitrogen gas. Dried samples were stored at −70° C pending analysis. The efficiency of extraction was monitored by comparison of the residual radioisotope content in the aqueous phase (postextraction) with that of the total radiolabel content.

Samples were reconstituted in methanol (60–100 μl) prior to analysis. HPLC separation conditions were as follows: reverse-phase 10-μm phenyl-RADIALPAK radial compression column (Waters Associates); initial mobile-phase conditions, 25% acetonitrile: 75% ammonium formate buffer, 0.05 *M* (pH 4.0); final conditions, 75% acetonitrile: 25% buffer; linear gradient, over 20 min; flow rate, 2.5 ml/min. Column eluate was monitored by flow fluorimetry (Model FS 970; Schoeffel Instruments, Ramsay, NJ), at a 482-nm excitation wavelength (550-nm emission filter). Fluorescent signals were identified and quantified by reference to standard curves constructed from authentic reference samples. Eluate from the fluorimeter was collected (1.25-ml aliquots) with the aid of a Gilson microfraction collector, and the radioactivity level of each fraction was determined by liquid scintillation counting as described above. Results of HPLC fluorimetric assays and radioassays were expressed on the basis of 10^6 viable cells.

Results

Growth-inhibition assays [10] were used to establish the concentrations of AD 198 and ADR producing cytostasis with minimal cytotoxicity over 24 h continuous cellular exposure. These concentrations were determined to be 1.0 μ*M* for AD 198 and 0.1 μ*M* for ADR. At these concentrations, 24-h cell counts were essentially the same as those at time 0, with cell viability (trypan blue exclusion) of >95%.

The uptake of radiolabel by human leukemic CEM cells exposed continuously for 24 h to [^{14}C]-AD 198 or [^{14}C]-ADR at these concentrations is shown in Table 1. Results of the analysis of parallel [^{14}C]-AD 198-treated cultures by means of HPLC separation and fluorescence detection/liquid scintillation counting are given in Table 2.

As seen in Table 1, under conditions of continuous drug exposure, significant levels of AD 198 were rapidly attained; indeed, high intracellular drug levels were

Table 1. Distribution of radiolabel in cell sonicates, media, and washings at various times during the incubation of CEM cells with [^{14}C]-AD 198 (1.0 μ*M*) or [^{14}C]-ADR (0.1 μ*M*)[a]

Time (h)	AD 198-treated cells:				ADR-treated cells:			
	AD 198 equivalents[b] (ng/10^6 viable cells)	Mean percentage of recovered radioactivity[c]			ADR[d] (ng/10^6 viable cells)	Mean percentage of recovered radioactivity[e]		
		Cell sonicate	Media	Washings		Cell Sonicate	Media	Washings
0	125.0	23.4	73.3	3.3	0.8	1.3	97.0	1.7
0.5	339.5	72.9	23.7	3.4	1.6	3.0	92.1	4.9
1	359.2	71.9	23.0	5.1	1.4	2.6	94.7	2.7
2	377.4	74.2	21.5	4.3	2.1	3.9	91.6	4.5
4	388.1	77.6	17.4	5.0	3.5	6.1	89.1	4.8
6	375.1	78.2	16.6	5.2	4.1	7.2	87.2	5.6
8	363.5	75.0	18.7	6.3	4.1	7.8	87.0	5.2
24	359.1	77.1	17.7	5.2	5.0	7.8	85.0	5.7

[a] Continuous drug exposure for 24 h
[b] Calculated by reference to the specific activity of the [^{14}C]-AD 198 preparation
[c] Mean recovery of applied radiolabel from the combined totals (sonicate, media, and washings), 85.1% ±4.5%
[d] Calculated by reference to the specific activity of the [^{14}C]-ADR preparation. Parallel HPLC analysis, with flow fluorescence quantitation/liquid scintillation counting of column eluate, showed ADR as the only detectable fluorescence and radiolabel source
[e] Mean recovery of applied radiolabel from the combined totals (sonicates, media, and washings), 99.4%±3.3%

detectable immediately after drug admixture. A similar result was apparent even in cells maintained at 4° C for 30 min prior to drug addition (data not shown). At 37° C, partitioning of drug radioactivity between cells and media was essentially complete within 30 min, achieving a ratio of 3:1, with this distribution of drug radioactivity persisting for the remainder of the study. By contrast, and consistent with previous studies [10, 12], ADR accumulation was slow, temperature-dependent, and limited. Equilibration of ADR required a period of 4–6 h, at which time the cell : media ratio of radiolabel was 1:11.

HPLC analysis of cell sonicates from [^{14}C]-AD 198-treated cultures (Table 2) revealed the presence of only two fluorescent anthracycline signals. These were attributable to the parent drug and the AD 288 biotransformation product, which together accounted for all of the radioactivity contained in the cells; no additional fluorescence or radioactive signals were apparent. Levels of AD 288 increased throughout the course of the study, such that the biotransformation product was the predominant cellular anthracycline species at the end of the experiment (24 h after drug addition). At this time, parental AD 198 was still detectable in the cells. Similar analysis of [^{14}C]-ADR-treated cells by means of HPLC fluorescence detection/scintillation counting revealed unchanged drug to be the only detectable anthracycline fluorescence signal and

Table 2. Drug uptake and biotransformation in CEM cells at various times following incubation with [^{14}C]-AD 198 (1.0 μ*M*)[a]

Time (h)	HPLC/fluorescence analysis, concentration (ng/10^6 viable cells)[b]:		Percentage of recovered radiolabel from column eluate[c]:		Total anthracycline content by radioassay (ng/10^6 viable cells)[d]
	AD 198	AD 288	AD 198	AD 288	
0	58.3	ND	97.2	2.8	69.4
0.5	132.4	2.8	97.9	2.1	160.6
1	179.8	5.4	96.8	3.2	198.5
2	163.5	13.5	93.0	7.0	185.1
4	198.8	32.9	83.3	16.7	231.0
6	256.9	56.6	78.6	21.4	303.0
8	168.8	42.0	77.4	22.5	214.1
24	79.7	114.1	36.0	64.0	189.3

[a] Continuous drug exposure for 24 h
[b] As determined by reference to standard curves for each species
[c] As determined by liquid scintillation counting (see Materials and methods). Figures represent the relative proportion of total radioactivity recovered in the fractions with retention times corresponding to AD 198 and AD 288. Mean recovery of injected radioactivity as AD 198 and AD 288 was 97.7%±3.9% for the 16 samples (8 time points, in duplicate)
[d] Calculated as AD 198 equivalents by reference to the specific activity of the [^{14}C]-AD 198 preparation
ND, not detected

Table 3. Retention of radiolabel in CEM cells pretreated with either [^{14}C]-ADR (0.1 μ*M*; 4 h) or [^{14}C]-AD 198 (1.0 μ*M*; 1 h) and subsequently cultured in drug-free media

Time (h)	Percentage of applied radiolabel[a] recovered in cell sonicate:		Drug concentration[b] (ng/10^6 viable cells):	
	ADR	AD 198	ADR[c]	Total (AD 198 + AD 288)[d]
0[e]	4.3	60.5	2.9	358.5
0.5	2.2	33.5	2.0	208.3
1	1.9	34.3	1.7	193.3
2	2.3	29.3	1.8	175.5
4	2.3	30.5	2.0	178.3
6	2.7	34.8	1.5	193.6
8		31.9		173.5
18	2.1		1.8	
24	1.7	35.9	1.7	181.9

[a] Expressed as the mean of duplicate cultures for each time point. Mean recovery of applied radiolabel from all sources (cell sonicates, culture media, saline washes), 98.3%±2.3% (ADR-treated); 100%±2.4% (AD 198-treated)
[b] As determined by liquid scintillation counting based on the specific activity of the respective agents
[c] As determined by HPLC fluorescence/liquid scintigraphy (see *Materials and methods*). ADR was the sole fluorescent and radiolabeled material in ADR-treated cells
[d] No other radiolabeled or fluorescent signals present
[e] Represents the respective drug concentrations attained by preincubation of cells for 1 h (AD 198) or 4 h (ADR) prior to replacement in drug-free media

the sole source of radioactivity is the cell sonicates (data not shown).

The retention of radioactivity in CEM cells pretreated with [^{14}C]-AD 198 for 1 h or [^{14}C]-ADR for 4 h, followed by their transfer to drug-free media, is shown in Table 3; the results of analyses by means of HPLC separation and quantitation of parallel [^{14}C]-AD 198-pretreated cell sonicates are provided in Table 4.

Following a 1-h exposure to [^{14}C]-AD 198, the levels of radioactivity in cell sonicates were similar to those seen in the uptake study. When these cells were placed in fresh drug-free media, repartitioning of drug radioactivity between the cells and the fresh media was evident at the earliest time point studied (0.5 h) and was essentially complete by this time (Table 3). Thereafter, there was little discernible change in the intracellular levels of radioactivity, which were approximately 50% of those achieved in cells left in continuous contact with the drug for 24 h.

As was evident from previously described uptake studies, the amount of radioactivity accumulated by CEM cells exposed for 4 h to [^{14}C]-ADR was limited; at this time cell sonicates contained only 4% of the applied radioactivity. As with [^{14}C]-AD 198, cells pretreated with [^{14}C]-ADR underwent a repartitioning of drug with fresh media.

Table 4. HPLC fluorescence and radioanalysis of CEM cell sonicates derived from cultures exposed to [^{14}C]-AD 198 (1.0 μ*M*) for 1 h and subsequently cultured in drug-free media

Time (h)	HPLC/fluorescence analysis, concentration (ng/10^6 viable cells)[a]:		Percentage of recovered radiolabel from column eluate[b]:	
	AD 198	AD 288	AD 198	AD 288
0[c]	313.3	13.7	93.6	6.4
0.5	166.7	15.7	89.1	10.9
1	178.9	25.6	85.8	14.2
2	152.9	31.7	85.0	15.0
4	154.1	40.1	76.4	23.6
6	158.9	64.0	63.7	36.3
8	159.9	67.1	65.0	35.0
24	63.5	140.1	26.4	73.6

[a] As determined by reference to standard curves for each species
[b] As determined by liquid scintillation counting (see *Materials and methods*). Figures represent the relative proportion of total radioactivity recovered in the fractions with retention times corresponding to AD 198 and AD 288. Mean recovery of injected radioactivity as AD 198 and AD 288, 91.3%±4.2% for the 16 samples (8 time points, in duplicate)
[c] Zero-time figures represent drug concentration in cells following 1 h preincubation of cultures with drug

This process was again evident at the earliest time point studied (0.5 h); thereafter, intracellular radioactivity remained essentially constant at approximately 50% of the original levels. As before, parental ADR was the only anthracycline species detectable in the cells, as determined by the HPLC separation and fluorescence/radiolabel detection technique.

Parallel quantitation of [^{14}C]-AD 198-treated cell sonicates by HPLC separation and fluorimetry/liquid scintillation counting (Table 4) again revealed the presence of only two fluorescent signals corresponding to AD 198 and AD 288; no ADR was found in these cells at any time. Together, AD 198 and AD 288 also accounted for essentially all of the cellular radiolabel content. Any variance between the amount of radiolabel injected onto the HPLC column and the total recovered in the column eluate can be attributed to experimental error rather than some undetected biotransformation product. Support for such a conclusion comes from the following observation: cleavage of the *N*-benzyl function, with the production of ADR or other aminoglycoside-unsubstituted products would have resulted in the liberation of the radiolabel from the anthracycline molecule. However, radioactivity was detectable in the column eluate from the cell extracts only at the retention times corresponding to AD 198 and AD 288; otherwise, the levels of radioactivity in the column eluate corresponded to background levels. Additionally, no fluorescent signals were detected at any times other than those corresponding to AD 198 and AD 288.

Following a 1-h drug exposure to [^{14}C]-AD 198, cellular levels of AD 288 were comparable in both the re-equilibration and continuous exposure experiments (13.7 ng/10^6 viable cells, Table 4, vs 5.4 ng/10^6 viable cells, Table 2). Although the overall levels of radioactivity and anthracycline fluorescence remained essentially constant between 0.5 and 24 h under both protocols, in each instance the relative intracellular distribution of AD 198 and AD 288 changed with time. Thus, the concentration of AD 288 rose, such that by 8 h it represented 25%–33% of the intracellular anthracycline content and by 24 h it was the major (66%–75%) anthracycline species found in the cells. At the latter time, AD 198 was still evident, representing the balance of the anthracycline content.

Discussion

By means of parallel studies using liquid scintillation counting for the direct determination of total radioactivity in media and cell sonicates, in addition to HPLC separation and quantitation of extracts of cell sonicates by tandem fluorescence detection and radioassay, detailed information concerning the accumulation, biotransformation, and retention of radiolabeled AD 198 and ADR drug forms in cultured cells was obtained. For the comparative purposes of this study, drug concentrations producing a similar biological endpoint were selected to produce static growth of cultures without the complication of excessive cell death or loss of cell viability. The results of the study provide a clear indication of a pharmacologic basis for the latent inhibition of cell-cycle progression and DNA damage effects seen with AD 198, namely, the persistence of markedly high intracellular anthracycline levels following short-term exposure of cells to this drug.

AD 198 is a highly lipophilic agent (octanol: PBS partition coefficient, >99), a potentially important factor in the tumor-penetrant and other membrane-interactive properties of anticancer agents. The present study demonstrates that, as in our previous experience with AD 32 and related lipophilic 14-acyl-substituted anthracycline analogues [12], the uptake of AD 198 into cultured cells is both rapid and extensive (Table 1) and, thus, indicative of intracellular transport by passive diffusion. By contrast, the cellular uptake of ADR is a slow process, requiring 4–6 h to achieve near equilibration. In this respect, the present results confirm earlier reports of a time- and temperature-dependent, active or facilitated cellular uptake of this drug [10, 12, 15].

Following the transfer of pretreated cells into drug-free media, intracellular AD 198 and ADR levels undergo re-equilibration with fresh media within 30 min (Table 3). During this repartitioning, a substantially higher proportion of the accumulated AD 198 dose ($\approx$33% of the original dose) than of the accumulated ADR dose ($\approx$2% of the original dose) is retained within cells.

The HPLC fluorescence detection/radioassay technique used in the present study revealed that ADR was the only detectable fluorescent and radioactive source in [^{14}C]-ADR-treated cells. As expected from our experience [10] and previous studies [11], analysis of [^{14}C]-AD 198-treated cells demonstrated the presence of parental drug and its 14-de-esterified product AD 288. Levels of the AD 288 biotransformation product increased over the course of both the continuous drug exposure and washout experiments (Tables 2, 4). Despite the ongoing conversion of AD 198 into AD 288, significant levels of parent drug still remained detectable at 24 h under both sets of experimental conditions. This is remarkable in comparison with AD 32, wherein similar 14-valerate de-esterification under corresponding conditions is complete within 8–10 h. Of further significance is the failure of the tandem high-sensitivity analytical techniques used in the present study to detect at any time the presence of ADR in [^{14}C]-AD 198-treated cells, either as a fluorescent signal or by radioassay. Thus, ADR cannot account mechanistically for the in vitro biological effects seen with AD 198.

The persistence of ADR in cells is generally explained in terms of its avid binding with DNA. In comparison with that of ADR, the interaction of AD 198 with DNA has been shown to be qualitatively and quantitatively different [1, 9, 11, 17, 21]. AD 288 shows a somewhat greater affinity for binding with DNA than does AD 198, and it is reasonable to believe that at least some of the AD 288 persisting in the cell is associated with DNA.

We currently believe that the in vivo and in vitro properties of AD 198, including its delayed, profound inhibitory effects on cell-cycle progression and its latent ability to produce DNA damage in cells long after their exposure to this drug, are attributable to the combined effects of the parent drug and its mechanistically somewhat different biotransformation product AD 288, which together accumulate and persist at markedly high levels in treated cells. It is unlikely that all of the intracellular drug exists in association with DNA; indeed, visual examination of cells by fluorescence microscopy shows the drug to be widely dispersed. Thus, the intracellular persistence of AD 198-associated fluorescence probably reflects the interaction of parent drug and metabolite with cellular com-

ponents other than or in addition to DNA. The precise nature and significance of these interactions remains to be defined.

An additional novel feature of AD 198 action not yet mentioned is the ability of this drug in vitro to circumvent mechanistically different types of cellular drug resistance [5, 13, 14, 19]. The high, persistent anthracycline levels achieved in cells following exposure to AD 198, together with the effects of this agent on membrane structure and function, may be important to our understanding how this agent can bypass biochemical mechanisms of cellular drug resistance. Appropriate studies to address this question are currently in progress. However, the lack of cross-resistance seen with AD 198 in cells made resistant to ADR or other DNA-interactive, natural-product antitumor drugs provides additional emphasis for the continued development of this anthracycline analogue for clinical trial.

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