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Original Articles

Pharmacologic Studies with Radiolabeled N-Trifluoroacetyladriamycin-14-valerate (AD 32)

Comparison of Total Anthracycline Fluorescence and Radioactivity in Mouse Serum and Urine

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Summary. *In connection with pharmacologic studies with AD 32, isotopically-labeled drug prepared from 1-[^{14}C]-trifluoroacetic anhydride and adriamycin-14-valerate was used to determine murine serum and urine levels of radioactivity. Other studies, performed in parallel, measured serum and urinary total fluorescence. Serum fluorescence disappeared in a biphasic pattern, with an initial rapid rate of disappearance followed by a somewhat slower phase. For the first hour, serum radioactivity levels were not significantly different than those measured by fluorescence. After this, however, serum radioactivity decayed at a much slower rate than did fluorescence. Furthermore, a large fraction of the injected radioactivity was found excreted in the urine, whereas urine accounted for only a small fraction of the fluorescence. These results suggest the formation, in part, of a hitherto unrecognized nonfluorescent metabolite, most probably N-trifluoroacetyldaunosamine.*

Introduction

N-Trifluoroacetyladriamycin-14-valerate (AD 32) is a semisynthetic analog of adriamycin (ADR) first prepared at the Sidney Farber Cancer Institute [7, 8] and currently undergoing Phase II clinical evaluation. In murine test systems, AD 32 was found to be therapeutically superior to and less toxic than the parent antibiotic [8, 16, 21]. In rabbits AD 32 showed less toxicity in general, and much less cardiac toxicity in particular, than ADR when approximately equivalent myelosuppressive doses of drugs were given on a weekly schedule [5]. On the basis of its in vivo antitumor activity and low toxicity, clinical trials with AD 32 were initiated at the SidneyFarber Cancer Institute in October 1977. Clinical antitumor activity and low toxicity have been documented in connection with Phase I studies [2–4].

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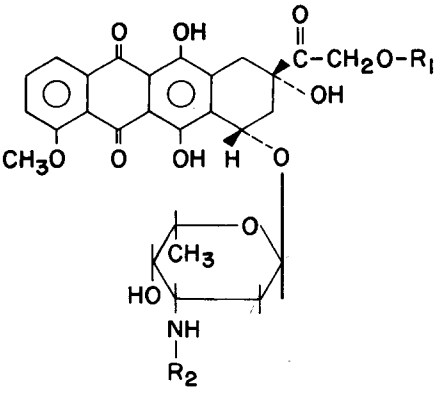
Structurally, AD 32 may be considered to be a disubstituted derivative of ADR (Fig. 1). In contrast to ADR, however, AD 32 essentially does not bind to isolated DNA preparations [20]. Furthermore, in live cultured cells AD 32 shows differences from ADR in transport and major site of fluorescent localization [15]. Previous pharmacological studies have shown that the administration of AD 32 does not result in significant levels of ADR in the urine of mice [10], bile of rats [13], plasma, bile, and urine of monkey [12], and plasma and urine of humans [9].

AD 32 undergoes extensive biotransformation in vivo, with *N*-trifluoroacetyladriamycin and *N*-trifluoroacetyladriamycinol and their conjugates as the major metabolites seen in biological fluids. As these metabolites



Adriamycin: R_1 = H
R_2 = H

AD 32: R_1 = $COCH_2CH_2CH_2CH_3$
R_2 = $^{*}COCF_3$

Fig. 1. Structural relationship of adriamycin and AD 32. The *asterisk* indicates the site of the radiolabel in the [^{14}C]-AD 32 preparation used in this work

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continue to possess the intact *N*-trifluoroacetylated glycosidic function, we decided to prepare a sample of [^{14}C]-labeled AD 32 bearing the radiolabel on the trifluoroacetamide carbonyl group and to use this material for studies on the serum and tissue levels of AD 32 and its metabolites. Comparison of the levels of metabolites as determined by radioactivity vs those seen by fluorescence assay then becomes a valuable methodology for detecting, quantifying, and isolating possible nonfluorescent metabolites that still contain the intact trifluoroacetyldaunosamine moiety.

Materials and Methods

Chemical Agents

The unlabeled AD 32 used here was prepared for us by Farmitalia, Milan, Italy (lot no. 70440 E-396) under an arrangement involving the Sidney Farber Cancer Institute, Adria Laboratories Inc. (Wilmington, Delaware), and the National Cancer Institute. Drug was formulated in 5% Emulphor EL620 (polyethoxylated castor oil) – 5% ethanol – 90% aqueous NaCl solution (0.9% w/v) at a concentration of 3.5 mg/ml, and the final solution was filtered through a 0.45-μm membrane filter prior to IV injection. Emulphor was supplied as a 1 : 1 concentrate with ethanol by J. Paul Davignon, Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland.

The isotopically-labeled AD 32 was prepared by a modification of a synthetic route to AD 32 previously described by Israel and Modest [6]. [1-^{14}C]-Trifluoroacetic anhydride (143 mg, 0.681 mmol, in a breakseal vial) was purchased from New England Nuclear, Inc. (Boston, Massachusetts). The chilled vial was opened after the breakseal had been covered with 500 μl ethyl acetate. The resulting [1-^{14}C]-trifluoroacetic anhydride solution was added to a slurry of 51 mg (0.007 mmol) adriamycin-14-valerate [6] in 500 μl ethyl acetate. After 1 h at room temperature with occassional stirring only a small amout of unreacted solids remained. Several milliliters of water and chloroform were added, and the entire mixture was shaken together. The chloroform layer was separated, filtered through a plug of glass wool, and washed several times with pH 7.4 carbonate buffer. The organic solvents were evaporated under a nitrogen jet (vapors were trapped in a dry-ice trap for radiooactive waste disposal). The residue was dissolved in 2 ml methanol. The solution was allowed to stand in the dark overnight to decompose trifluoroacetate esters. The methanol was evaporated under nitrogen, as before. Precipitation of the crude [^{14}C]-AD 32 was accomplished by trituration from chloroform with petroleum ether. The crude product was purified by column chromatography on 100–200 mesh Bio-Sil A silicic acid (Bio Rad Laboratories, Richmond, California) (15 g) packed and washed with chloroform. Elution was with 10% methanol in chloroform. Trituration of a chloroform solution of the eluate with petroleum ether gave 39.4 mg AD 32 (71% chemical yield, based upon the starting adriamycin-14-valerate) of specific activity 2.9 mCi/mmol. The radiochemical purity, as determined by high-performance liquid chromatography (HPLC) (phenyl/Corasil column, acetonitrile-aqueous pH 4.00 ammonium formate, as previously described [11]) and liquid scintillation counting, was 98.3%. The chemical purity, as determined by HPLC with fluorescence detection, was 99.3%.

Formulation of radiolabeled compound for injection was done as follows: Small amounts (~175,000 cpm) of [^{14}C]-AD 32 were dissolved in the 5% Emulphor – 5% ethanol – 90% saline mixture described above. Unlabeled AD 32 was then added to dilute the label, and the radioactive solution was filtered through a 0.45-μm filter. The concentration of AD 32 in the radiolabeled preparation was determined by HPLC, by comparing the peak areas of a 1-μl injection of this solution with those of a 1-μl injection of a standard AD 32 solution. HPLC determinations were performed in triplicate. The concentration of AD 32 in the radiolabeled AD 32 preparations ranged from 2.6–3.1 mg/ml, with a standard error of the mean of approximately 5% of the determined concentration.

Determination of Radioactivity and Total Anthracycline Fluorescence in Mouse Serum Following Treatment with AD 32

Male A/JAX mice (Jackson Laboratories, Bar Harbor, Maine) weighing 16–29 g were injected via the tail vein with either unlabeled AD 32 (50 mg/kg) or radiolabeled AD 32 (50 mg/kg, ~175,000 cpm). The animals were killed by decapitation at various times after drug administration. Blood was collected on ice and centrifuged at 4° C, after which 100 μl serum was removed. When unlabeled AD 32 was injected into the mouse the resultant serum was transferred to a test tube and fluorescence was determined after extraction (see below). When radiolabeled AD 32 was injected, the serum was transferred to a scintillation vial, Aquasol liquid scintillation counting mixture (New England Nuclear, Inc., Boston, Massachusetts) was added, and the radioactivity counted on a Beckman LS-335 liquid scintillation counter.

Serum Extraction Procedure

To the serum sample 0.9 ml buffer [Tris-HCl, 0.05 *M*, pH 8.5, containing 3% w/v sodium dodecylsulfate (SDS)] was added. The resultant solution was extracted three times with 2-ml portions of ethyl acetate/1-propanol (9 : 1 by volume). The combined organic extract was evaporated to dryness in a vortex evaporator at 45°–50°. The residue was dissolved in 3 ml methanol, and 0.2 ml Tris-HCl buffer (0.1 *M*, pH 7.4) was added. Samples were then centrifuged to remove turbidity and fluorescence was read on a Perkin-Elmer Model MPF-4 corrected-spectrum spectrofluorometer. Native serum fluorescence was determined by injecting control mice with the Emulphor-ethanol-saline vehicle containing no anthracycline, decapitating the animals at comparable times, and proceeding with the analysis for total fluorescence as described. The average of at least three such experiments was used in the estimation of native fluorescence background.

Determination of Radioactivity and Anthracycline Fluorescence in the Urine of Mice Treated with Radiolabeled AD 32

Male A/JAX mice were injected IV with radiolabeled AD 32 (50 mg/kg, ~175,000 cpm). Each mouse was placed in an individual Lucite cage, the bottom of which was covered by a rubber-coated wire mesh screen. The animals were allowed free access to food and water during the course of the experiment. At the indicated times the animals were transferred from the cages. Fecal pellets remaining on the bottom of the cage or adhering to the mesh screen were removed. The cage together with the mesh screen were rinsed ten times with 10-ml portions of buffer (Tris-HCl, 0.05 *M*, pH 8.5, containing 3% w/v SDS) to dissolve the urine. Aliquots of the cage wash, in duplicate, were analyzed for total fluorescence and radioactivity.

Results

Serum levels of AD 32 as a function of time, determined either as total anthracycline fluorescence or as radioactivity, are shown in Fig. 2. Up to about 1 h after drug administration both total fluorescence and radioactivity decayed rapidly ($T_{1/2}\alpha \sim 2$ min) as drug distributed into extravascular spaces. At times longer than 1 h both fluorescence and radioactivity continued to decrease, but at a reduced rate; serum radioactivity decayed at a slower rate than did fluorescence. As determined from Fig. 2, β-phase serum half-lives for AD 32 were of the order of 1.1 h by total fluorescence and 10 h by radioactivity.

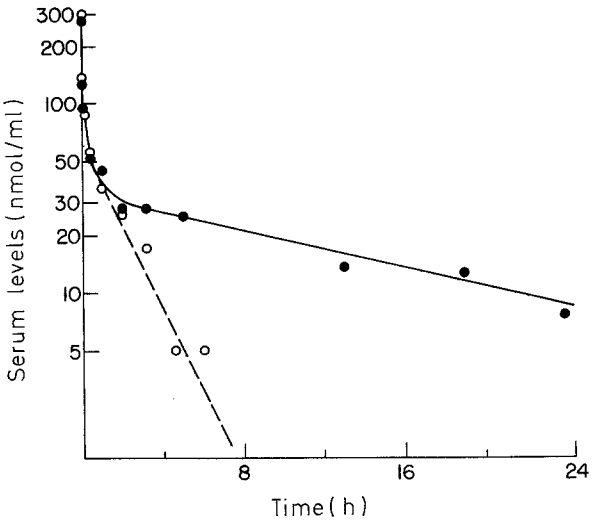

Fig. 2. Serum decay of AD 32 fluorescence (O—O) vs radioactivity (●—●) in mouse. Each animal (A/JAX males) received a single injection of AD 32, 50 mg/kg, via the tail vein; for the radioisotopic study the dose contained about 175,000 cpm [^{14}C]-AD 32. Each point represents the mean of four animals

Table 1. Urinary excretion of radioactive and fluorescent metabolites following a single IV administration of [^{14}C]-AD 32 (50 mg/kg, ~ 175,000 cpm) to A/JAX male mice

Experiment	Time after injection (h)	Radioactivity		Fluorescence	
		Nequiv[a]	% Total dose	Nequiv[b]	% Total dose
1	13	801	72	ND[c]	ND
2	13	881	65	ND	ND
3	13	798	46	41	2.4
4	13	1,166	67	63	3.6
5	15	918	72	117.4	9.2
6	15	918	52	91.2	5.2
7	24	1,139	69	ND	ND

[a] Nequivalents of AD 32, as determined by comparison against a standard reference sample of [^{14}C]-AD 32

[b] Nequivalents of AD 32, as determined by comparison of fluorescence against a standard reference sample of AD 32

[c] ND, not determined

Table 1 shows the results of several experiments in which urinary levels of radioactivity and total anthracycline fluorescence were determined. Following a single IV dose of radiolabeled AD 32, a majority (60%—70%) of the radioactivity was excreted in the urine, whereas over the same time period only a small amount (2%—9%) of the administered dose was accounted for by urinary fluorescence. This latter observation is consistent with cited results in mice, monkey, and humans, in which little urinary excretion of anthracycline-fluorescent material was seen after AD 32 administration.

When cage washes were extracted with ethyl acetate/1-propanol, a predominant amount of the radioactivity partitioned into the organic phase. Cage washes from animals treated with unlabeled AD 32 but to which [^{14}C]-trifluoroacetate was added prior to extraction did not similarly partition radioactivity into the organic phase.

Discussion

Following administration of a single IV dose of AD 32 to mice, serum fluorescence due to AD 32 and its fluorescent metabolites drops rapidly with time for the first hour. After this rapid distribution phase, plasma fluorescence levels continue to decline at a reduced, but still rapid rate. This relatively rapid β-phase decline of AD 32 serum fluorescence is in contrast to the persistence of such fluorescence when ADR is administered to mice [19] and humans [1]. In addition, when ADR and AD 32 were given to cynomolgus monkeys, a similar more rapid β-phase decay of plasma fluorescence was seen with AD 32 [12].

Comparative studies with unlabeled and labeled AD 32 indicate disparities in observed serum drug levels, particularly at times greater than 1 h after drug administration. At these longer times, levels of radioactivity were several-fold greater than the levels of anthracycline fluorescence. Attempts were made to describe the serum decay data in terms of a standard two-compartment open pharmacokinetic model [17, 18]. While the data for both serum fluorescence and radioactivity could be described by this model, the fluorescence data were well accommodated, whereas the data for serum radioactivity did not fit as well, thus suggesting that the open two-compartment model was an oversimplification of the in vivo processes describing the handling of AD 32 radioactivity.

Taken together with the observations in urine of high radioactivity and low fluorescence, the results suggest that considerable cleavage of the radioactive label from the fluorescent chromophore has occurred. Based on several considerations, including the extractability of the label from aqueous alkaline media into organic solvents, the most probable nonfluorescent radioactive product

appears to be *N*-trifluoroacetyldaunosamine. A major site for hydrolytic, or possibly reductive, cleavage of the anthracycline to *N*-trifluoroacetyldaunosamine would be the small and perhaps also large intestine. The *N*-trifluoroacetyldaunosamine formed there could be reabsorbed into the circulation and then be eliminated via the kidney, thus accounting both for the persistence of radioactivity in serum long after fluorescence has decayed and for the high levels of radioactivity, despite little fluorescence, in urine, following a dose of radiolabeled AD 32.

The likelihood of intestinal cleavage of the anthracyclines is supported by several lines of evidence. Preliminary tissue distribution studies with labeled AD 32 in A/JAX mice indicate that at 1 h after drug administration a significant portion of the administered AD 32 dose can be accounted for in the small intestine [14]. Furthermore, levels of fluorescence in the small intestine appear to be somewhat higher than do those of radioactivity. Also, in an earlier study with rats bearing surgically placed indwelling biliary cannulae, 80% or more of an administered AD 32 dose could be accounted for by fluorescence in the bile [13]. The biliary products were identified principally as *N*-trifluoroacetyladriamycin, its carbonyl-reduced analog, *N*-trifluoroacetyladriamycinol, and polar conjugates of these two compounds, thus suggesting indirectly that the cleavage of the sugar must occur at a site distal to that of bile collection, i.e., the intestine.

The studies described here indicate the formation of a nonfluorescent metabolite, presumably *N*-trifluoroacetyldaunosamine, from AD 32. Present studies are concerned with the characterization of this metabolite, and with the isolation and identification of anthracycline aglycones, which must be concomitantly formed.

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