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Brief Communication

N-Trifluoroacetyladriamycin-14-valerate, an Analog with Greater Experimental Antitumor Activity and Less Toxicity than Adriamycin[1]

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SUMMARY

N-Trifluoroacetyladriamycin-14-valerate (AD 32), an analog of adriamycin, exhibits significantly greater antitumor activity than does adriamycin or daunorubicin in two experimental mouse tumor systems under similar assay conditions (C57BL × DBA/2 F_1 male mice, agents administered i.p. each day for Days 1 to 4). Against the P388 leukemia at optimal dosages, AD 32 gave a +429% increase in median life-span with 3 of 5 60-day survivors compared to +132% for adriamycin (no 30-day survivors). In the L1210 leukemia system, AD 32 at several dosages consistently and reproducibly effected an increase in life-span in excess of 445%, with a high percentage of 60^+-day survivors compared to adriamycin (+42 to +54% ILS; no 30-day survivors). The reduced toxicity of AD 32 was evidenced by its optimal dose range, which is significantly greater than the lethal dose for 100% of mice of adriamycin, and by its lack of delayed toxicity. *In vitro*, AD 32 was somewhat less effective than was adriamycin in inhibiting the growth of CCRF-CEM cells; enzymatic conversion of AD 32 by cell-free culture medium was not observed. The unique growth-inhibitory properties of this analog indicate that the therapeutic effectiveness of the anthracycline antitumor antibiotics can be retained or enhanced by substitution on the glycosidic amino group.

INTRODUCTION

The anthracycline antibiotics daunorubicin (daunomycin) and adriamycin (doxorubicin) (structures shown in Chart 1) are currently important in the clinical management of neoplastic disease. Daunorubicin has found use primarily in the treatment of acute myelogenous leukemia (7, 23). Adriamycin, with a broader spectrum of antitumor activity, is of value, either alone or in combination, in the treatment of lymphatic leukemia, lymphomas, breast cancer, genitourinary tumors, epidermoid carcinomas, soft tissue sarcomas, and bone sarcomas (2, 3, 5, 7, 12, 20). However, both agents produce a range of toxic reactions, the most serious of which, with respect to long-term or maintenance therapy, is a dose-dependent, irreversible, and fatal cardiac toxicity (4, 15, 20). Analogs of adriamycin that might show greater therapeutic efficacy or less toxicity than the parent antibiotic would be of considerable importance. Of the adriamycin analogs reported thus far (1, 6, 8, 16, 18, 21, 24, 25), none has shown a clear superiority over adriamycin in experimental tumor systems and most have less activity or are inactive. In contrast, AD 32[2] (Chart 1), which was synthesized by us (13) in connection with an ongoing program on adriamycin analogs, shows markedly greater antitumor activity than does adriamycin in 2 experimental rodent tumor assay systems.

MATERIALS AND METHODS

Chemical Agents. Daunorubicin hydrochloride and adriamycin hydrochloride, manufactured and formulated for clinical use by Farmitalia, Milan, Italy, and distributed by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., were used for all *in vivo* studies. In cell culture experiments, similar ID_{50} values were obtained with DMSO solutions of these formulated agents and DMSO solutions of the free bases of daunorubicin and adriamycin. The free base was prepared, in each instance, by dissolving the corresponding formulated antibiotic in 0.05 M potassium carbonate:potassium borate:potassium hydroxide buffer, pH 10 (Fisher Scientific Co., Pittsburgh, Pa.), extracting the free base into chloroform, and evaporating the sodium sulfate-dried chloroform extract to dryness.

AD 32 was prepared according to the following sequence: daunorubicin free base → *N*-trifluoroacetyldaunorubicin → 14-iodo-*N*-trifluoroacetyldaunorubicin → AD 32. The final conversion was accomplished by refluxing an acetone suspension of 14-iodo-*N*-trifluoroacetyldaunorubicin and excess sodium valerate. Analytically pure material was

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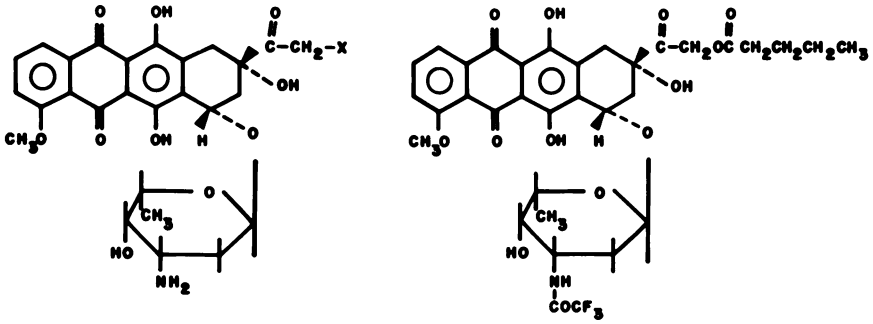
[2] The abbreviations used are: AD 32, *N*-trifluoroacetyladriamycin-14-valerate; ID_{50}, 50% inhibiting dose; DMSO, dimethyl sulfoxide; ILS, median increase in life-span.



Daunorubicin: X = H
Adriamycin: X = OH

AD 32

Chart 1. Structural relationship between daunorubicin, adriamycin, and AD 32.

obtained by column chromatography on Bio-Sil A silicic acid (100 to 200 mesh; Bio-Rad Laboratories, Richmond, Calif.) with chloroform containing 1% ethanol as the eluting solvent.

$C_{34}H_{36}F_3NO_{13}$

Calculated: C 56.43, H 5.01, F 7.89, N 1.94
Found: C 56.89, H 5.23, F 7.60, N 2.01

Complete details on the synthesis and structural characterization of this agent will be reported elsewhere. For the biological studies reported herein, material of 98% or higher purity was used.

Cell Culture Studies. The CCRF-CEM human lymphoblastic leukemic cell line (10) was used for *in vitro* assay. Actively growing CCRF-CEM cells were inoculated in triplicate into 13- x 100-mm round-bottom tubes in 1.0 ml of Eagle's minimal essential medium modified for suspension culture (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum (Microbiological Associates, Bethesda, Md.) at a density of 5×10^5 cells/ml. Daunorubicin, adriamycin, and AD 32 were dissolved and diluted in DMSO. Cultures were incubated with continuous exposure to the test agent for 48 hr at 37° in a humidified atmosphere of 95% air:5% CO_2. Cell counts were determined by means of a Model F Coulter Counter. The percentage of inhibition of growth was calculated using cultures to which DMSO had been added as controls. The assay procedure is a modification of a method previously described in detail (9).

In Vivo **Antitumor Assay.** Antitumor activity of test compounds was determined by measuring the median ILS of mice given i.p. inoculations of either 1×10^6 P388 or 1×10^5 L1210 lymphocytic leukemic cells as compared with untreated controls. The assay procedures are identical to the standard National Cancer Institute protocols (11) for these tumor systems, with treatment beginning on the 1st day after tumor inoculation but continuing once daily for 4 consecutive days rather than for 9 days. Agents were administered i.p., daunorubicin and adriamycin as 0.9% NaCl solutions of clinically formulated materials and AD 32 in 10% Tween 80. AD 32 was completely soluble in 10% Tween 80 up to and including the 70-mg/kg dosage (7.0 mg/ml); at 8.0 mg/ml, most of the sample was in solution, but there remained a trace of some finely suspended material. C57BL × DBA/2 F_1 male mice (The Jackson Laboratory, Bar Harbor, Maine) were used throughout this investigation. Percentage of ILS was calculated according to the equation:

$$\%ILS = 100 \times (t/c - 1)$$

where t is the median survival time in days of the treatment group, and c is median survival time in days of the 0 dose control group.

Table 1
In vivo antitumor activity of daunorubicin, adriamycin, and AD 32 versus murine P388 leukemia
Agent administered i.p. each day for Days 1 to 4.

Agent	Optimal dose (mg/kg)	% ILS	Survivors	
			30-day	60-day
Daunorubicin	2.0	+91	0/6	
Adriamycin	4.0	+132	0/6	
AD 32	40.0	+429	4/5	3/5

RESULTS

Against CCRF-CEM cells in culture adriamycin (ID_{50}, 0.066 μM) is somewhat less effective than is daunorubicin (ID_{50}, 0.035 μM). AD 32 gave an ID_{50} value of 0.24 μM. This lower order of activity *in vitro* is consistent with the observation of reduced toxicity *in vivo* for AD 32 compared to adriamycin but failed to correlate with the significantly greater therapeutic effectiveness *in vivo* of the adriamycin analog.

The antitumor activity of daunorubicin, adriamycin, and AD 32 against the murine P388 lymphocytic leukemia is shown in Table 1. In this system, daunorubicin exhibits a fairly reproducible ILS of +80 to +90%. With adriamycin the response is more variable, the ILS ranging from +122 to +175% at the optimal (maximally effective nontoxic) dose of 4.0 mg/kg each day for Days 1 to 4; on this schedule, 30-day survivors were infrequent and 60-day survivors were

Table 2

In vivo antitumor activity of adriamycin and AD 32 versus murine L1210 leukemia

Agent administered i.p. each day for Days 1 to 4.

Sidney Farber Cancer Center protocol no.	Agent	Optimal dose (mg/kg)	% ILS	Survivors	
				30-day	60-day
202	Adriamycin	4.0	+45	0/5	
	AD 32	60.0	>+445[a]	4/5	4/5[b]
		70.0	>+445[a]	4/5	4/5[b]
233	Adriamycin	4.0	+54	1/5	0/5
	AD 32	50.0	>+445[a]	4/7	4/7[c]
		60.0	>+445[a]	3/6	3/6[c]
251	Adriamycin	4.0	+42	0/7	
	AD 32	50.0	>+400[a]	5/7	5/7[d]
		60.0	>+400[a]	6/7	6/7[d]

[a] Calculated as of Day 60.
[b] All 60-day survivors alive and well out past Day 245.
[c] All 60-day survivors alive and well out past Day 115.
[d] Experiment in progress; presently at Day 60.

not seen. In comparison to adriamycin, AD 32 was significantly more effective in prolonging survival of tumor-bearing animals. The median animal of the test group died of a nontumor-associated death (apparent pneumonia) on Day 74; the remaining 2 long-term survivors were alive and well on Day 150, when they were sacrificed.

The pronounced effectiveness of AD 32 against the L1210 leukemia is seen in Table 2. Adriamycin is only moderately effective against the usually refractory L1210 system but, as with P388, it is 1.5 to 2 times more effective than is daunorubicin in prolonging survival of mice bearing this tumor. In our studies with L1210, adriamycin at the optimal dose of 4.0 mg/kg each day for Days 1 to 4 does not give 60-day survivors. In contrast, AD 32 at several dosages consistently produces a high percentage of long-term survivors.

DISCUSSION

AD 32 is a semisynthetic analog of adriamycin, differing from the natural antibiotic in having a 5-carbon straight-chain ester function at the 14-carbinol position and trifluoroacetyl substitution on the glycosidic amino group. The compound inhibits the growth of cells in culture and produces a highly significant antitumor effect against 2 mouse leukemias *in vivo*. According to the prevailing explanation for the biological activity of the anthracycline antitumor antibiotics, which involves intercalation with DNA, the basic glycosidic amino group must be free for the expression of biological activity (8, 22, 25). In AD 32 the amino group is no longer basic, having been converted into an amide. In view of this, the superior experimental antitumor activity of AD 32 relative to adriamycin represents a unique observation.

The finding that daunorubicin is more effective *in vitro* with CCRF-CEM cells than is adriamycin is consistent with similar reports (6, 19) by other investigators with different cell lines. In a preliminary study, AD 32 was incubated at 37° with Eagle's minimal essential medium, fetal bovine serum, and pH 7.0 buffer. After 24 hr there was no indication by thin-layer chromatography that AD 32 had undergone enzymatic or chemical change. The activity of AD 32 *in vitro*, therefore, does not appear to require its prior modification before entering the target cell. In support of our preliminary observation, Lenaz *et al.* (16) recently reported that adriamycin 14-*O*-acyl derivatives similarly do not undergo extracellular metabolic conversion before being taken up by HeLa cells in culture. Intracellular conversion to an active metabolite cannot be precluded at this time.

With respect to toxicity, therapeutically effective dose levels of AD 32 are 10 to 15 times greater than the optimal dose of adriamycin on a weight basis and 8 to 12 times greater on a molar basis. In tumor-bearing mice, dosages of AD 32, 60 mg/kg, each day for 4 days are tolerated with little or no evidence of acute toxicity, whereas adriamycin, 8.0 mg/kg, each day for 4 days represents the lethal dose for 100% of the mice. In addition, the mouse shows myocardial cytopathological changes as a result of adriamycin administration (20 mg/kg i.p., single dose) (17). The very high percentage of long-term survivors with high doses of AD 32 each day for Days 1 to 4 suggests that this analog does not produce delayed toxicity in the mouse and raises the possibility that the myocardial degeneration produced by adriamycin is not effected by AD 32. There is therefore the hope that, in addition to having greater therapeutic efficacy, AD 32 will also show reduced cardiotoxicity. Evaluation of cardiotoxicity in the rabbit (14) will be scheduled as soon as sufficient material is available.

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