Cancer Chemother Pharmacol (1987) 20: 277–284

Cancer Chemotherapy and Pharmacology

Comparative effects of adriamycin and DNA-non-binding analogues on DNA, RNA, and protein synthesis in vitro

Mervyn Israel, John M. Idriss, Yoshihiro Koseki, and Vinod K. Khetarpal*

Departments of Pharmacology and Medicinal Chemistry, The University of Tennessee-Memphis, Memphis, TN 38163, USA

Summary. Drug-DNA binding is claimed to be the basis by which the antitumor antibiotic adriamycin (doxorubicin) inhibits DNA and RNA synthesis in vitro. However, in preliminary studies the DNA-non-binding adriamycin analogue *N*-trifluoroacetyladriamycin-14-valerate (AD 32) showed somewhat greater inhibition of DNA and RNA synthesis than adriamycin under identical conditions. The kinetics of macromolecule synthesis inhibition induced by adriamycin and AD 32, and the two principal DNA-non-binding metabolites of AD 32, *N*-trifluoroacetyladriamycin (AD 41) and *N*-trifluoroacetyladriamycinol (AD 92), have now been subjected to comparative study in cultured CEM (human leukemic lymphoblastic) cells. At equimolar concentrations (10 μ*M*), or at concentrations related to their 50% growth-inhibitory values vs CEM cells, AD 32 was consistently found to be more inhibitory than adriamycin of DNA and RNA synthesis, as measured by the incorporation of tritiated thymidine and uridine, respectively, into acid-precipitable fractions relative to untreated controls. Marked inhibitory activity was apparent with 10 μ*M* AD 32 even at the earliest sampling time (15 min); with adriamycin at the same concentration the maximal effect was not achieved until 3 h. AD 32 at 4.8 μ*M* concentration continued to show strong inhibition of nucleic acid synthesis, whereas adriamycin at 1.0 μ*M* was essentially inactive. Like AD 32, AD 41 and AD 92 showed greater inhibition than adriamycin of DNA and RNA synthesis at the early sampling times, although in all instances the effects of AD 32 were more profound. AD 32 at 10 μ*M* concentration produced a moderate but significant inhibition of the incorporation of tritiated methionine into protein compared with adriamycin, which at this concentration was not active. Parallel HPLC analytical studies with similar drug-treated cultures indicated that, while small amounts of adriamycin were found in cells treated with 10 μ*M* AD 32, the amount of adriamycin present at 15 min was only a small fraction (< 5%) of the amount of adriamycin achieved at 3 h in cultures treated with 1.0 μ*M* adriamycin, a concentration already shown to be only slightly inhibitory of nucleic acid synthesis under the culture conditions. The present study thus confirms the marked DNA and RNA synthesis-inhibitory effects of AD 32, and establishes that this inhibitory activity is not due to conversion of AD 32 into adriamycin. These findings accordingly call into question the validity of the drug-DNA binding mechanism as the explanation for the nucleic acid synthesis inhibitory effects seen with ADR.

Offprint requests to: M. Israel, Department of Pharmacology, University of Tennessee-Memphis College of medicine, 874 Union Avenue, Memphis, TN 38163, USA

* *Present address:* Drug Disposition Department, Rorer Central Research, Horsham, PA 19044

Abbreviations used: ADR, adriamycin (doxorubicin); AD 32, *N*-trifluoroacetyladriamycin-14-valerate; AD 41, *N*-trifluoroacetyladriamycin; AD 92, *N*-trifluoroacetyladriamycinol; AMNOL, adriamycinol; AD 48, adriamycin-14-valerate; AD 60, 13-dihydro-*N*-trifluoroacetyladriamycin-14-valerate; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; TCA, trichloroacetic acid; ID_{50}, 50% growth-inhibitory value relative to untreated controls

Introduction

The antitumor antibiotic ADR is known to bind avidly with double-helical DNA and to inhibit DNA and RNA synthesis [10]. Based upon several studies which showed no direct action of ADR on DNA polymerases [9, 12, 16], it has become commonly accepted that the inhibition of DNA synthesis by ADR is due to drug-DNA complexation, with resultant steric interference of DNA polymerase action. However, in preliminary studies with the DNA-non-binding ADR analogue AD 32, when cultured CEM (human lymphoblastic leukemic) cells were incubated for 2 h with AD 32 or ADR, then pulse-labeled with tritiated thymidine or uridine for 1 h, AD 32 was found to inhibit the incorporation of the radiolabeled precursor into DNA and RNA, respectively, to a somewhat greater extent than ADR [34]. Similar results with respect to thymidine incorporation into DNA were also seen in other studies in which AD 32 and ADR were compared [30, 33]. The present investigation was undertaken to evaluate the kinetics of macromolecule synthesis inhibition by AD 32 and its biologically active metabolites AD 41 and AD 92, compared with ADR, in an attempt to gain insight into the mechanistic properties of these agents. Cellular pharmacology studies with a sensitive high-performance liquid chromatography/fluorescence assay system were conducted in parallel to determine the metabolic fate of these agents under the culture conditions.

Materials and methods

Cells and culture conditions. Cultured CEM cells, initially obtained from Dr Herbert Lazarus, Dana-Farber Cancer Institute, were maintained in a humidified 5% CO_2–95%

air atmosphere at 37° C by serial dilution in Eagle's S-MEM medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum, 2 μ*M* glutamine, and 50 μg/ml gentamicin (Schering Corp., Kenilworth, NJ).

Drugs and radiochemicals. Bulk adriamycin hydrochloride was kindly provided by Farmitalia Carlo Erba, Milan, Italy, through the cooperation of Adria Laboratories, Inc., Columbus, Ohio. AD 32, AD 41, and AD 92 were prepared in these laboratories according to previously described procedures for these compounds [19, 21]. All drug substances were of at least 98% purity, as determined by HPLC. Drugs were dissolved in reagent grade DMSO (Aldrich Chemical Co., Milwaukee, Wisconsin), diluted with DMSO, and added to cultures so that the desired drug concentration was achieved in culture media containing 0.1% DMSO final concentration. Two concentrations of drugs were used for the radiolabeled precursor incorporation studies. In one set of experiments each agent was used at a high dose of 10 μ*M*. In another set of experiments, the amount of drug used was related to the 50% growth inhibitory value for the agent vs CEM cells after 48 h of continuous drug exposure; specifically, the concentrations employed were ADR, 1.0 μ*M*; and AD 32, AD 41, and AD 92, 4.8 μ*M*.

Radiochemicals used for the incorporation inhibition studies included [methyl-^{3}H]thymidine (sp. act. 92.1 mCi/mmol), [5,6-^{3}H]uridine (sp. act. 44.4 mCi/mmol), and L-[methyl-^{3}H]methionine (sp. act. 200 mCi/mmol); these were purchased from New England Nuclear Corp., Boston, Mass.

ID_{50} determination. CEM cells (5×10^5/ml) were incubated in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) for 48 h with the test drugs over a range of concentrations from 0.01–5.0 μ*M*; culture conditions were the same as described above. Experiments were done in duplicate and repeated at least twice, in each instance with untreated cultures serving as controls. Two aliquots of each flask were counted by means of a Coulter Counter, Model Z_F (Coulter Electronics Inc., Healiah, Fla). The ID_{50} value was determined by plotting cell counts vs drug concentration and extrapolating the concentration of drug producing 50% inhibition of growth relative to the untreated control cultures.

Cytotoxicity assays. CEM cells grown in 75-cm^2 flasks were collected by centrifugation at 600 *g* for 10 min and were then resuspended in fresh medium at a density of 5×10^5 cells/ml and incubated at 37° C for 30 min. Cells were then exposed to ADR, AD 32, AD 41, or AD 92 at concentrations of 1.0, 5.0, 10.0, and 20.0 μ*M* and allowed to incubate at 37° C for 1, 2, or 3 h. After drug exposure, the cells were washed twice with 1 ml S-MEM, then resuspended in fresh drug-free medium and incubated for 2, 4, 8, or 24 h. At the indicated sampling times, trypan blue was added and the number of viable cells was scored. Results were expressed as the percentages of values recorded in untreated controls. Values shown are the means of triplicate sampling, with an intersample variance of 5%.

Precursor incorporation inhibition assays. The synthesis of DNA, RNA, and protein was measured by the amount of radiolabeled precursor (thymidine, uridine, and methionine, respectively) incorporated into TCA-insoluble materials derived from treated cells, compared with untreated controls. Cells were grown to a density of $0.6–1.0 \times 10^6$ cells/ml, harvested by centrifugation at 600 *g* for 10 min at room temperature, and resuspended in fresh MEM medium, supplemented as described above; for these studies MEM containing methionine was used, except for the assays in which protein synthesis was to be measured. The cell density was adjusted to 1×10^6/ml, and the flasks were incubated, with gentle agitation, for 30 min at 37° C in a flow of 5% CO_2-95% air. Drugs were then added, followed after 5 min by the addition of the tritiated precursor. Cultures were allowed to incubate for a total of 195 min. Aliquots (0.5 ml) of cell suspension were withdrawn at predetermined times and added to an equal volume of cold 20% TCA solution. The mixtures were agitated vigorously on a vortex mixer and kept on ice for 15 min, then filtered through Whatman GF/C filters. The filters were washed several times with cold 10% TCA solution, then twice with ethanol, and solubilized in 0.5 ml Protosol tissue-solubilizing solution (New England Nuclear Corp.) prior to being counted in Econofluor liquid scintillation fluid (New England Nuclear Corp). At the end of each experiment, the remaining cells were checked for viability by the trypan blue exclusion method and were found to be about 95% viable.

Cellular pharmacology studies. CEM cells, grown as described previously, were harvested and washed, then resuspended in fresh S-MEM medium, supplemented with 10% fetal bovine serum, at a density of 2×10^6 cells/ml. Fresh drug was added and the cultures were incubated for 6 h; drug concentrations used for this experiment were ADR, 1.0 μ*M*, and AD 32, AD 41, and AD 92, 10 μ*M*. At selected times, aliquots of cell suspension were withdrawn, and cells, separated from medium, were disrupted by sonication with ice cooling. Cell sonicates and media were extracted separately with 2×3 ml ethylacetate:1-propanol (9:1, by volume), and the extracts were evaporated to dryness under a stream of dry nitrogen gas. Residues were reconstituted in 50 μl methanol prior to analysis. Aliquots (5–10 μl) of the methanolic extracts were analyzed by reversed-phase HPLC. The equipment used consisted of an automated Waters Associates liquid chromatograph equipped with their Model 710B automatic sample injector, dual M6000A solvent delivery pumps, Model M721 System Controller, and Model 730 Data Module (Waters Associates, Milford, Mass). Separation conditions were as previously described [24], except that a phenyl/RADIAL-PAK column mounted in a Waters Z-Module radial compression unit was used with an increased flowrate of 5.0 ml/min for the acetonitrile-pH 4.0 ammonium formate buffer eluting solvent. Signals were monitored by means of a Kratos Schoeffel Model FS-970 flow fluorescence detector (Kratos Instrument Co., Ramsey, NJ) set for excitation at 482 nm (deuterium lamp) and having a barrier filter for emission cut-off below 550 nm. Identification of signals was based upon retention time analysis relative to authentic reference standards, and quantitation was accomplished electronically by reference to standard curves derived from these authentic materials. Aglycone metabolites and unidentified anthracycline-fluorescent species were quantified as equivalents of the original test drug added to the culture. The values given in Table 2 for the amounts of

Table 1. Percentage recovery of parent drug, and known and putative metabolite reference standards from CEM cell sonicates and medium determined by means of the ethyl acetate: 1-propanol (9 : 1, by volume) extraction procedure described under Methods

Test compound	Cell sonicate (%)	Medium (%)
ADR	58	73
AMNOL	71	71
Adriamycinone	68	96
AD 32	81	100
AD 41	76	92
AD 92	98	100
AD 48	79	100
AD 60	73	84

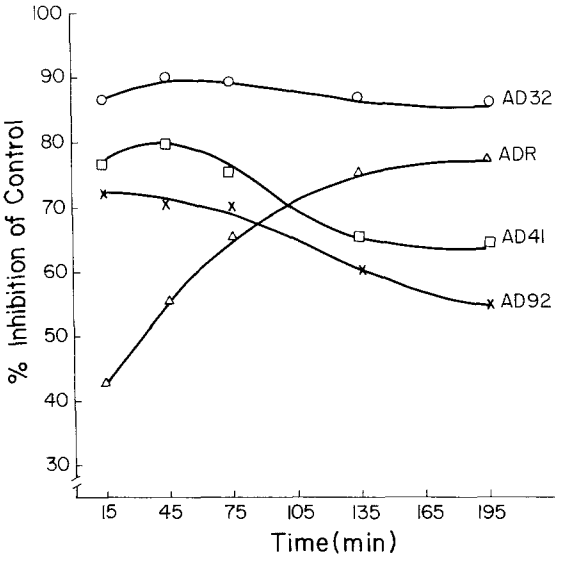

Fig. 1. Inhibitory effects of ADR and DNA-non-binding analogues AD 32, AD 41, and AD 92, each at 10 μ*M* concentration, on the incorporation of [methyl-^{3}H]thymidine into DNA in cultured CEM cells. Each *point* all figures represents the average value from at least three separate experiments, with intersample variance in the range of 5%

parent drug and metabolites present in the cells and in the media at the respective sampling times have been corrected for the efficiency of the extraction procedure; the percentage recoveries of pure drug reference standards, and known and putative metabolites, after addition to 1 ml of S-MEM media supplemented with 10% fetal bovine serum or to sonicates of 1×10^7 cells, are shown in Table 1. The limit of sensitivity of the assay for accurate quantitation is 0.002 nmol/10^6 cell sonicate and 0.01 μmol/ml medium.

Results

In the present studies comparing the effects of ADR and AD 32 on macromolecule synthesis in vitro, AD 41 and AD 92 were also included, as these substances are known biologically active metabolites of AD 32 [14, 21–23, 25–27, 28, 31, 34, 35, 40]. Initial experiments involved use of the four drugs at the same concentration (10 μ*M*). Figure 1 shows the effects of these equimolar amounts of the agents on the incorporation of tritiated thymidine into DNA, relative to untreated controls, over an observation period of 195 min. As seen here, a marked (85%) inhibitory

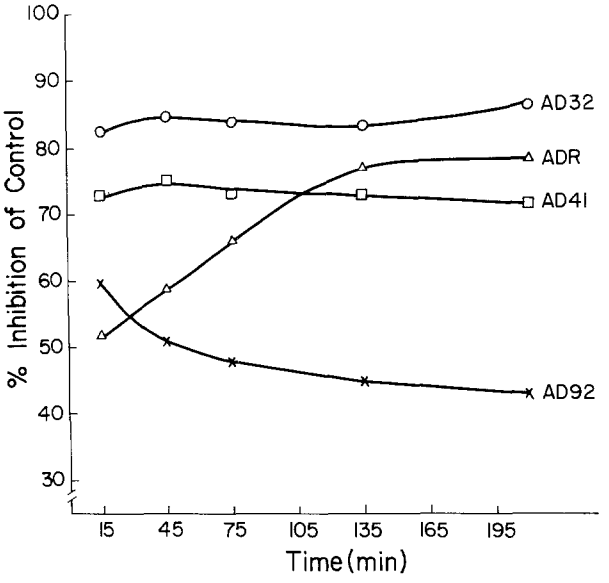

Fig. 2. Inhibitory effects of ADR, AD 32, AD 41, and AD 92, each at 10 μ*M* concentration, on the incorporation of [5,6-^{3}H]uridine into RNA in cultured CEM cells

effect of AD 32 was apparent at the earliest time sampled (15 min); inhibition of thymidine incorporation increased slightly over the next 30 min, then, following a slight decrease, remained relatively stable for the balance of the experiment. In contrast, by 15 min, ADR at the same concentration produced only 43% inhibition of thymidine incorporation into the DNA of treated cells, relative to controls; this inhibitory effect rose gradually but consistently, to reach 76% inhibition of DNA synthesis 3 h after the initial determination. While not quite as inhibitory of thymidine incorporation as AD 32, the AD 32 metabolites, AD 41 and AD 92, when assayed directly, were more inhibitory than ADR during the first 45 min of incubation; thereafter, their ability to inhibit DNA synthesis showed a measurable decline.

Figure 2 shows a similar behavior for the four drugs at equimolar concentrations with respect to their ability to inhibit the incorporation of tritiated uridine into RNA.[1] Compared with ADR, AD 32 again showed early high inhibitory activity; despite an increasing inhibitory effect for ADR, especially during the first 2 h of incubation, the activity of AD 32 was consistently greater than that of ADR throughout the entire experiment.

[1] The use of [5,6-^{3}H]uridine is fully appropriate for the studies described in this report. While some uridine may be converted to thymidine or cytosine and thence be incorporated into DNA, the amount of such incorporation is known to be of the order of only 5% or less [1, 13]. Of the two DNA precursor pathways, the salvage pathway by which uridine is converted into thymidine would make the greater contribution. The [5,6-^{3}H]uridine, as purchased, is labeled 64% at the 5 position and 36% at the 6 position. Uridine labeled at the 5 position which becomes incorporated into DNA as thymidylate will not be radioactive, as the label will be lost during its conversion to thymidine. Uridine labeled at the 6 position ending up as thymidylate in DNA will still show radioactivity, but the amount can be at most 1.8% (i.e., 36% of a maximum of 5%) of the total counts determined. Thus, theoretically no less than 95%, and in actuality more than 98%, of the radioactivity in the acid-precipitible fraction following incubation of cells with [5,6-^{3}H] uridine must be due to precursor incorporation into RNA

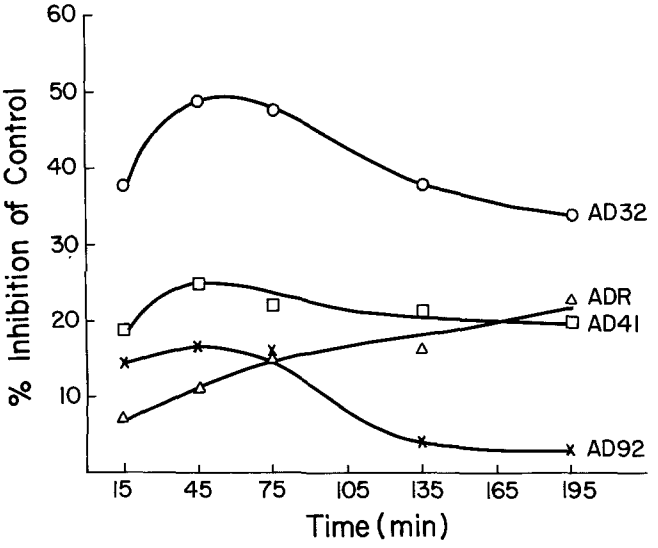

Fig. 3. Inhibitory effects of ADR, AD 32, AD 41, and AD 92, each at 10 μ*M* concentration, on the incorporation of L-[methyl-^{3}H]methionine into protein in cultured CEM cells

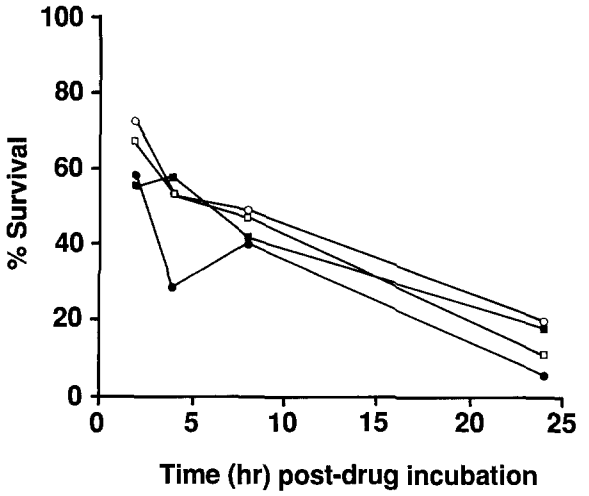

Fig. 4. Comparative cytotoxicity of ADR (1.0 μ*M*) and AD 32, AD 41, and AD 92 (5.0 μ*M* each). CEM cells were incubated with drug at 37° C for 3 h, then washed and resuspended in drug-free medium. Viable cell counts were determined at 2, 4, 8, and 24 h after drug incubation. – ○ –, ADR; – ● –, AD 32; – □ –, AD 41; – ■ –, AD 92

The effects of the four test agents on protein synthesis, as measured by the incorporation of tritiated-methionine, was also studied (Fig. 3). ADR has been reported [12] to have little effect on protein synthesis, even at concentrations as high as 20 μ*M*; our present results with 10 μ*M* ADR confirm these earlier observations. Nevertheless, AD 32 exhibited a moderate but significant inhibitory effect on protein synthesis, achieving a maximum of 48% inhibition relative to controls after 45 min of incubation. While over the course of the experiment AD 41 showed a very weak but stable inhibitory effect on methionine incorporation (equivalent in effect to the maximal activity of ADR at 3 h), the initial weak inhibition of protein synthesis produced by AD 92 disappeared after the first 75 min of incubation.

A second series of nucleic acid precursor incorporation inhibition experiments was conducted with the four test drugs, this time at concentrations related to their 50% growth-inhibitory values, as determined for 48 h incubations with continuous drug exposure. To establish the concentrations to be used in these studies, ID_{50} values were carefully redetermined, with the following results: ADR, 0.06 μ*M*; AD 32, AD 41, and AD 92, 0.30 μ*M*. These values are in close agreement with previously reported ID_{50} values for these compounds [20, 21, 34]. Since the precursor incorporation studies were to run only for about 3 h, the drug concentrations used were 16 times the 48-h ID_{50} values, i.e., ADR, 1.0 μ*M*, and AD 32, AD 41, and AD 92, each 4.8 μ*M*. Detailed cytotoxicity assays validated the similar toxicity of the four test agents at these concentrations (Fig. 4). The steep decline in cell counts between 2 and 4 h, followed by partial recovery, as seen here with AD 32, appears to be real; a similar effect was seen with 1.0 μ*M* AD 32 incubated with cells for 3 h and with 1.0 and 5.0 μ*M* AD 32 incubated for 2 h, whereas no similar effect was observed with the other compounds, regardless of the concentration or length of drug exposure used (data not shown).

The results of the tritiated thymidine and uridine incorporation studies with ADR at 1.0 μ*M*, and the other compounds at 4.8 μ*M*, are shown in Fig. 5 and 6, respectively. Once again, the marked inhibitory activity of AD 32 compared with ADR is apparent for both DNA and RNA synthesis. In fact, ADR at this concentration has relatively little effect on nucleic acid synthesis over the 195-min observation period. The other compounds, however, continue to exhibit a significant inhibitory effect even at half the concentration used earlier; the relative potency of the effect for AD 32, AD 41, and AD 92 was of the same order as seen earlier at the high concentrations, AD 32 being always the most inhibitory agent of both DNA and RNA synthesis at each timepoint.

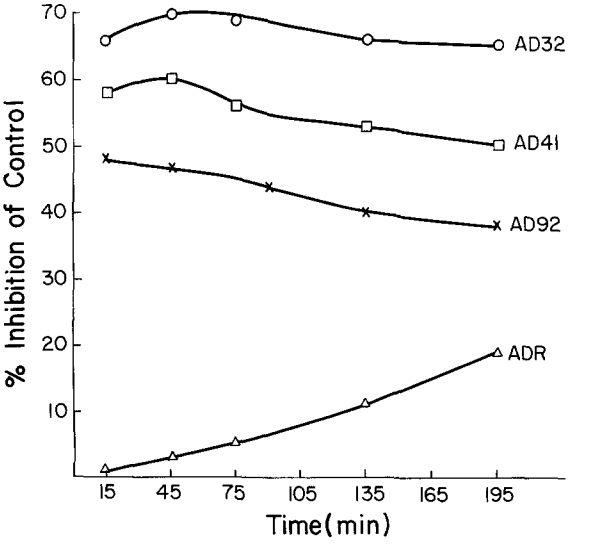

Fig. 5. Inhibitory effects of ADR, AD 32, AD 41, and AD 92 on the incorporation of [methyl-^{3}H]thymidine into DNA in cultured CEM cells. In this experiment the concentration of each drug was related to its ID_{50} value vs CEM cells for 48-h incubations (for ADR, 1.0 μ*M*; for AD 32, AD 41, and AD 92, 4.8 μ*M* each)

To determine the metabolic fate of the drugs during the precursor incorporation studies, parallel CEM cell cul-

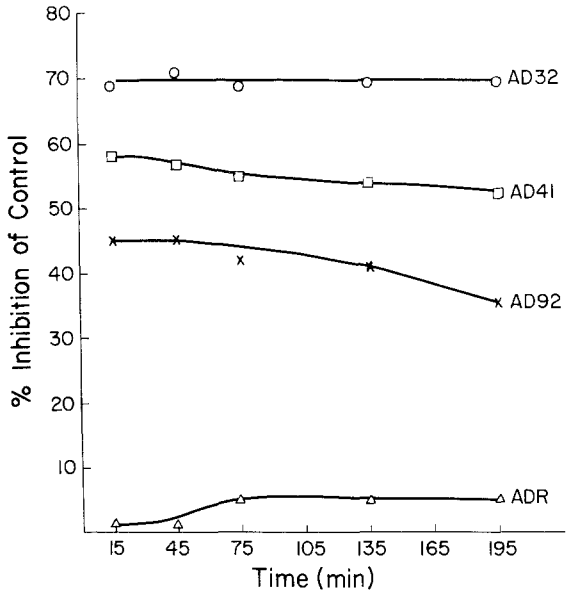

Fig. 6. Inhibitory effects of ADR, AD 32, AD 41, and AD 92 on the incorporation of [5,6-^{3}H]uridine into RNA in cultured CEM cells; drug concentrations were the same as those indicated for Fig. 5

tures were treated with the test agents and assayed by HPLC for 3 h and beyond. Drug concentrations used here were the high concentration (10 μ*M*) for AD 32, AD 41, and AD 92, and the low concentration (1.0 μ*M*) for ADR. These concentrations were selected with concern for detecting, quantifying, and interpreting the possible formation of ADR from AD 32 or AD 41. As a check of the extraction procedure, the percentage recoveries of parent drugs and of known and putative metabolites were determined for both culture media and cell sonicates. The efficiency of the extraction procedure for the reference standards from medium containing 10% fetal bovine serum was generally quite good, ranging from 71% for the most polar species (AMNOL) to 100% for the highly lipophilic AD 32 and AD 92 (Table 1). Recovery of materials from cell sonicates was less effective, but still more than adequate. The poorest recovery (58%) was seen with ADR; while this value may seem somewhat low, it should be noted that the extraction efficiency for ADR from cellular material according to the procedures used here is better than that reported in other studies [41, 42].

Results of the HPLC analysis of CEM cultures incubated for 6 h with 1.0 μ*M* ADR, or AD 32, AD 41, or AD 92, each at 10 μ*M* concentration, are shown in Table 2. For ADR, cells showed low initial drug levels, which increased consistently with time; the well-characterized, biologically less active principal ADR metabolite AMNOL began to be seen at 3 h incubation time; other signals detected included trace levels of ADR aglycones, which were not separately quantified in this study, and also chemically undefined anthracycline breakdown products. For AD 32, high intracellular parent drug levels were already evident at the earliest sampling time; these increased over the next 30 min, then began to decline between 3 and 6 h as increased enzymatic processing of parent drug, particularly conversion into AD 41, occurred. Low but increasing levels of ADR were found in the AD 32-treated cells as time progressed, but ADR was not detectable in the medium until 3 h into the experiment. Although AD 32 was used at 10 times the concentration of ADR, at each corresponding sampling time the amount of intracellular ADR derived from AD 32 was never more than 20%–25% of the intracellular ADR levels achieved in cultures treated with 1.0 μ*M* ADR. Intracellular parent drug levels in cultures treated with AD 41 showed a similar pattern to those of the AD 32-treated cultures, although the levels of AD 41 achieved in the AD 41-treated cells were considerably low-

Table 2. Concentrations of parent drug and metabolites found in cell sonicates and media at different times of incubation, as determined by HPLC analysis (for details see Methods). Drug concentrations used were: ADR, 1.0 μ*M*; AD 32, AD 41, and AD 92, each 10 μ*M*

Drug treatment	Signal detected	Cellular concentration (nmol/10^6 cells)				Media concentration (μmol/ml)			
		15 min	45 min	3 h	6 h	15 min	45 min	3 h	6 h
ADR	ADR	0.0121	0.0195	0.0712	0.1216	1.014	0.829	0.756	0.560
	AMNOL	ND	ND	0.0039	0.0089	ND	ND	T	T
	Others[a]	0.0131	0.0231	ND	0.0056				
AD 32	AD 32	0.8494	0.9569	0.9418	0.6328	8.08	6.386	4.09	1.80
	AD 41	0.1813	0.1315	0.2330	0.2608	0.40	0.694	3.387	3.851
	AD 92	0.0025	0.0047	0.0028	0.0031	ND	ND	ND	0.036
	ADR	0.0032	0.0040	0.0156	0.0381	ND	T	0.280	0.042
	AMNOL	ND	ND	ND	0.0025	ND	ND	ND	ND
	Others[a]	0.0343	0.0220	0.1228	0.0513				0.079
AD 41	AD 41	0.3029	0.3218	0.2434	0.2762	9.936	9.860	9.138	8.230
	AD 92	0.0035	0.0043	0.0018	0.0018	ND	ND	0.063	0.088
	ADR	0.0109	0.0100	0.0217	0.0713	ND	0.076	0.060	0.232
	AMNOL	ND	ND	ND	0.0060	ND	ND	ND	ND
	Others[a]	0.0542	0.1784	0.0353	0.0360	0.050	0.070	ND	0.059
AD 92	AD 92	0.1233	0.1376	0.1318	0.1434	10.232	10.920	10.360	9.753
	AMNOL	0.0049	0.0067	0.0104	0.0113	0.050	0.060	0.118	0.316
	Others[a]	0.0491	0.0805	0.0269	0.0149	0.302	0.395	0.038	0.215

ND, not detected; T, trace (see Methods)

[a] Expressed as equivalents of treatment drug

er than the amounts of AD 32 accumulated in the AD 32-treated cells. Relative to the intracellular AD 41 levels in the AD 41-treated cultures, however, the amounts of ADR derived from AD 41 were somewhat greater than those derived from AD 32.

Discussion

ADR is perhaps the most active, broadest spectrum antitumor agent in current clinical use [5, 10]. However, the drug has only limited effectiveness against some of the more common carcinomatous solid tumors, and it manifests a number of serious toxic side-effects, including a total dose-dependent cardiotoxicity that can result in discontinuation of ADR treatment in responding patients.

AD 32 was discovered [19, 20] and developed in these laboratories in connection with a continuing search for improved ADR analogues. In animal model systems, AD 32 exhibited often highly significant therapeutic superiority compared to ADR, with very little toxicity [8, 17, 20, 31, 34, 36, 39, 43]. Activity against human disease, and the absence of clinical cardiotoxicity, were documented for AD 32 in connection with phase I/II trials [2, 3, 15]. Pharmacologic studies in laboratory animals and humans have demonstrated the more rapid and extensive metabolic processing of AD 32 than of ADR, with the formation of AD 41, AD 92, and biologically inactive aglycones as the principal metabolic products [14, 22, 23, 25–27, 28, 31, 35, 40]; AD 41 exhibits significant in vivo antitumor activity, but with more toxicity than AD 32, while AD 92 is much less active [21]. In vitro AD 32 and ADR exhibit similar biological effects with respect to DNA damage [4, 30, 35, 38], chromosomal breaks and translocations [30, 33], arrest of cell cycle traverse [33], and, as determined by previous methodologies, DNA and RNA synthesis inhibition [30, 33, 34]. These in vitro and in vivo properties of AD 32 are remarkable in light of this agent's failure to fit a commonly accepted basic tenent for anthracycline action, that requiring drug-DNA binding [11]. A number of studies, using various techniques, now support our original observations on the inability of this analogue to bind with double-helical DNA [6, 12, 30, 37, 40]. Consistent with this lack of DNA binding is the observation that cells exposed to AD 32 show drug-associated cytofluorescence in the cytoplasm, whereas cells exposed to ADR exhibit distinctly nuclear cytofluorescence [32]. Based on these properties, AD 32, in addition to its potential clinical value, is of continuing interest as a probe for exploring the mechanistic effects of anthracyclines in general.

In the precursor incorporation studies reported here drug and labeled precursor were added simultaneously to cells so as to obtain sequential data on cultures which otherwise required no further manipulation. The relative extent of DNA and RNA synthesis inhibition produced by the test agents used here, as measured by precursor incorporation methodology, is the same whether cells are simultaneously exposed to drug and precursor or are first incubated with drug for 2 or 3 h and then pulsed with labeled precursor. This is not true for all anthracyclines, however. For example, CEM cells incubated with the newer ADR analogue *N*-benzyladriamycin-14-valerate (AD 198), then pulse-labeled with tritiated thymidine or uridine, show inhibition of DNA and RNA synthesis, with a more profound inhibitory effect being seen on RNA compared to DNA synthesis, whereas, when drug and labeled precursor are added simultaneously to cultures, a marked stimulation of DNA synthesis, without commensurate effect on RNA synthesis, is noted [29].

With respect to the comparative nucleic acid synthesis-inhibitory effects of ADR and AD 32, the results of the present study clearly indicate that AD 32 inhibits the incorporation of tritiated thymidine and uridine into DNA and RNA, respectively, more rapidly and to a greater extent than does ADR. That AD 32 produces its inhibitory effects sooner than an equimolar amount of ADR is fully consistent with other observations on the rapid intracellular transport of the lipophilic analogue by passive diffusion, compared with the time- and temperature-dependent uptake of the more polar ADR [32]. Based on the time delay required for AD 32 to produce maximum inhibition of methionine incorporation into protein (Fig. 3), it is reasonable to conclude that protein synthesis inhibition is secondary to the effects of this drug on nucleic acid synthesis.

Interpretation of the DNA and RNA synthesis effects of AD 32 in cultured CEM cells must be done in context with the HPLC data on the metabolic disposition of this drug. The underlying question to be answered here is whether the nucleic acid synthesis-inhibitory activity of AD 32 can be explained in terms of the amounts of ADR derived metabolically from the DNA-non-binding analogue. Even after 3 h ADR at 1.0 μM concentration produces a barely marginal effect on thymidine incorporation (Fig. 5) and no effect on uridine incorporation (Fig. 6); at this time cells exposed to this concentration of ADR had accumulated a level of 0.0712 nmol parent drug/10^6 cells (Table 2). For ADR derived from AD 32 to be considered as the causative basis for the DNA and RNA synthesis-inhibitory activity seen with AD 32, especially at the early times when significantly high inhibitory activity is already being expressed, considerable conversion of AD 32 into ADR would have to have occurred. However, the HPLC data show that, even with a 10-fold higher concentration of AD 32 than ADR, the intracellular amounts of ADR derived from AD 32 after 15 min incubation are less than 5% of the maximal levels of ADR achieved at 3 h in cells incubated with 1.0 μM ADR, a concentration of the parent antibiotic which is essentially inactive with respect to nucleic acid synthesis inhibitory activity. Although AD 32 can be converted into AD 41 by the action of intracellular esterases, as well as by nonspecific esterases present in the fetal bovine serum used to supplement the media, AD 41 cannot be the causative agent for the nucleic acid synthesis inhibitory activity exhibited by AD 32, as parent AD 32 is consistently more active than AD 41 at either of the equimolar concentrations used in these studies.

Thus, the present study confirms earlier reports on the greater nucleic acid synthesis-inhibitory activity of AD 32 compared with ADR and further establishes that this inhibitory action is not due to the conversion of AD 32 into ADR or other DNA-binding biotransformation products. As noted earlier, DNA binding and resultant steric interference with DNA polymerase action are generally considered to be the mechanistic explanation for ADR-induced nucleic acid synthesis inhibition. This hypothesis obviously cannot serve as the explanation for AD 32, as this drug does not bind with DNA. Anthracyclines are also known to be membrane-active compounds. In this regard, we have previously reported that ADR and AD 32 alter plasma membrane permeability with respect to the intracellu-

lar transport of nucleoside precursors needed for nucleic acid synthesis [7, 18]. This nucleoside transport inhibition effect does not appear, however, to be responsible for the nucleic acid synthesis inhibitory effects of ADR and AD 32, as no correlation has emerged in attempts to relate nucleoside transport inhibition with the nucleic acid synthesis-inhibition and growth-inhibition properties of these and other anthracycline materials. Based on the properties exhibited by AD 32, it may well be that the nucleic acid synthesis-inhibitory effects seen with ADR may in fact not derive from drug-DNA complexation, as is commonly believed. Further studies are needed to identify the exact mechanistic bases for the nucleic acid synthesis-inhibitory effects of these agents.

Acknowledgments. The work described here was done under the support of research grants CA19118 (to the Dana-Farber Cancer Institute, Boston, Mass) and CA37209 (to the University of Tennessee-Memphis, Tenn) from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service. The authors are grateful to Mrs Lalita Seshadri for assistance with the preparation of the manuscript.

References

1. Baserga R (1962) A study of nucleic acid synthesis in ascites tumor cells by two-emulsion autoradiography. J Cell Biol 12: 633
2. Blum RH, Garnick MB, Israel M, Cannellos GP, Henderson IC, Frei E III (1979) An initial clinical evaluation of *N*-trifluoroacetyladriamycin-14-valerate (AD 32), an adriamycin analog. Cancer Treat Rep 63: 919
3. Blum RH, Garnick MB, Israel M, Canellos GP, Henderson IC, Frei E III (1981) Preclinical rationale and phase I clinical trial of the adriamycin analog, AD 32. In: Carter SK, Sakurai Y, Umezawa H (eds) New drugs in cancer chemotherapy. Springer, Berlin Heidelberg New York, p 7
4. Brox L, Gowans B, Belch A (1980) *N*-Trifluoroacetyladriamycin-14-valerate and adriamycin induce DNA damage in the RPMI-6410 human lymphoblastoid cell line. Can J Biochem 58: 720
5. Chabner BA, Myers CE (1982) Clinical pharmacology of cancer chemotherapy. In: DeVita VT, Hellman S, Rosenberg SA (eds) Cancer, principles and practice of oncology. Lippincott, Philadelphia, p 180
6. Chuang LF, Kawahata RT, Chuang RY (1980) Inhibition of chicken myeloblastosis RNA polymerase II activity in vitro by *N*-trifluoroacetyladriamycin-14-valerate. FEBS Lett 117: 247
7. Chuang RY, Chuang LF, Kawahata RT, Israel M (1983) Effects of *N*-trifluoroacetyladriamycin-14-valerate on [^{3}H]-thymidine uptake and DNA synthesis of human lymphoma cells. J Biol Chem 258: 1062
8. Dantchev D, Balercia G, Bourut C, Anjo J, Maral R, Mathe G (1984) Comparative microscopic study of cardiotoxicity and skin toxicity of anthracycline analogs. Biomed Pharmacother 38: 322
9. DiMarco A (1975) Adriamycin (NSC-123127): mode and mechanism of action. Cancer Chemother Rep [3] 6: 91
10. DiMarco A (1982) Anthracycline antibiotics. In: Holland JF, Frei E III (eds) Cancer medicine, 2nd edn. Lea and Febiger, Philadelphia, p 872
11. DiMarco A, Arcamone F (1975) DNA complexing antibiotics: daunomycin, adriamycin, and their derivatives. Arzneimittelforsch 25: 368; see also earlier references cited therein
12. Fachinetti T, Mantovani A, Cantoni L, Cantoni R, Salmona M (1978) Intercalation with DNA is a prerequisite for daunomycin, adriamycin, and its congeners in inhibiting DNase. I. Chem Biol Interact 20: 97
13. Feinendegen LE, Bond VP, Painter RB (1961) Studies on the interrelationship of RNA synthesis, DNA synthesis, and precursor pools in human tissue culture cells studied with tritiated pyrimidine nucleosides. Exp Cell Res 22: 381
14. Garnick MB, Israel M, Pegg WJ, Blum RH, Smith E, Frei E III (1979) Hepatobiliary pharmacokinetics of AD 32 in man. Proc AACR/ASCO 20: 206
15. Garnick MB, Griffin JD, Sack MJ, Blum RH, Israel M, Frei E III (1982) Phase II evaluation of *N*-trifluoroacetyladriamycin-14-valerate (AD 32). In: Muggia FM, Young CW, Carter SK (eds) Anthracycline antibiotics in cancer therapy. Nijhoff, The Hague, p 541
16. Goodman MF, Lee GM, Bachur NR (1977) Adriamycin interactions with T4 DNA polymerase. J Biol Chem 252: 2670
17. Henderson IC, Billingham M, Israel M, et al. (1978) Comparative cardiotoxicity studies with adriamycin (ADR) and AD 32 in rabbits. Proc AACR/ASCO 19: 158
18. Israel M, Idriss JM (1982) A possible mechanistic basis for the growth-inhibitory properties of *N*-trifluoroacetyladriamycin-14-valerate (AD 32), a non-DNA binding adriamycin (ADR) analog. Proc 13th Int Cancer Congress Seattle, WA, p 245
19. Israel M, Modest EJ (1977) *N*-Trifluoroacetyladriamycin-14-alkanoates and therapeutic compositions containing same. U. S. Patent No. 4,035,566
20. Israel M, Modest EJ, Frei E III (1975) *N*-Trifluoroacetyladriamycin-14-valerate, an analog with greater experimental antitumor activity and less toxicity than adriamycin. Cancer Res 35: 1365
21. Israel M, Pegg WJ, Seshadri R, Parker LM (1976) *N*-Trifluoroacetyladriamycin-14-valerate (AD 32): some in vivo structure-activity relationships. Abstracts, 5th International Symposium on Medicinal Chemistry, Paris, France, p 63
22. Israel M, Garnick MB, Pegg WJ, Blum RH, Frei E III (1978a) Preliminary pharmacology of AD 32 in man. Proc AACR/ASCO 19: 160
23. Israel M, Pegg WJ, Wilkinson PM (1978b) Urinary anthracycline metabolites from mice treated with adriamycin and *N*-trifluoroacetyladriamycin-14-valerate. J Pharmacol Exp Ther 204: 696
24. Israel M, Pegg WJ, Wilkinson PM, Garnick MB (1978c) Liquid chromatographic analysis of adriamycin and metabolites in biological fluids. J Liquid Chromatogr 1: 795
25. Israel M, Wilkinson PM, Pegg WJ, Frei E III (1978d) Hepatobiliary metabolism and excretion of adriamycin and *N*-trifluoroacetyladriamycin-14-valerate in the rat. Cancer Res 38: 365
26. Israel M, Khetarpal VK, Potti PGG, Seshadri R (1980a) Distribution and metabolism of *N*-trifluoroacetyladriamycin-14-valerate (AD 32) in tissues of A/JAX mice. Proc AACR/ASCO 21: 256
27. Israel M, Wilkinson PM, Osteen RT (1980b) Pharmacology studies with adriamycin and *N*-trifluoroacetyladriamycin-14-valerate (AD 32) in cynomolgus monkeys: additional evidence for the absence of an adriamycin-prodrug mechanism for AD 32. In: Crooke ST, Reich SD (eds) Anthracyclines: current status and new developments. Academic Press, New York, p 431
28. Israel M, Potti PG, Seshadri R (1985) Adriamycin analogues. Rationale, synthesis, and preliminary antitumor evaluation of highly-active DNA-nonbinding *N*-(trifluoroacetyl)adriamycin-14-*O*-hemiester derivatives. J Med Chem 28: 1223
29. Israel M, Idriss JM, Seshadri R (1986) In vitro studies with *N*-benzyladriamycin-14-valerate (AD 198), a new anthracycline analog. Abstracts 14th Int Cancer Congress Budapest, Hungary, vol 1, p 159
30. Kanter PM, Schwartz HS (1979) Effects of *N*-trifluoroacetyladriamycin-14-valerate and related agents on DNA strand damage and thymidine incorporation in CCRF-CEM cells. Cancer Res 39: 448

31. Khetarpal VK, Israel M (1982) Metabolism and disposition of *N*-trifluoroacetyladriamycin-14-valerate in mice bearing Lewis lung carcinoma. Fed Proc 41: 1572
32. Krishan A, Israel M, Modest EJ, Frei E III (1976) Differences in cellular uptake and cytofluorescence of adriamycin and *N*-trifluoroacetyladriamycin-14-valerate. Cancer Res 36: 2114
33. Krishan A, Dutt K, Israel M, Ganapathi R (1981) Comparative effects of adriamycin and AD 32 on cell cycle traverse, chromosomal damage, and macromolecule synthesis in vitro. Cancer Res 41: 2745
34. Lazarus H, Yuan G, Tan E, Israel M (1978) Comparative inhibitory effects of adriamycin, AD 32, and related compounds on in vivo cell growth and macromolecular synthesis. Proc AACR/ASCO 19: 159
35. Levin M, Silber R, Israel M, Goldfeder A, Khetarpal VK, Potmesil M (1981) Protein-associated DNA breaks and DNA-protein crosslinks caused by DNA non-binding derivatives of adriamycin in L1210 cells. Cancer Res 41: 1006
36. Parker LM, Hirst M, Israel M (1978) *N*-Trifluoroacetyladriamycin-14-valerate (AD 32): additional mouse antitumor and toxicity studies. Cancer Treat Rep 62: 119
37. Pearlman LF, Chuang RY, Israel M, Simpkins H (1986) The interaction of three second generation anthracyclines with polynucleotides, RNA, DNA, and nucleosomes. Cancer Res 46: 341
38. Potmesil M, Levin M, Israel M, Silber R (1982) Protein-associated DNA breaks caused by adriamycin or *N*-trifluoroacetyladriamycin-14-valerate in vivo: relationship to cell clonogenicity. In: Periti P, Grassi G (eds) Current chemotherapy and immunotherapy. American Society for Microbiology, Washington, DC, p 1439
39. Potmesil M, Levin M, Traganos F, Israel M, Darzynkiewicz Z, Khetarpal VK, Silber R (1983) In vivo effects of adriamycin or *N*-trifluoroacetyladriamycin-14-valerate on a mouse lymphoma. Eur J Cancer Clin Oncol 19: 109
40. Sengupta SK, Seshadri R, Modest EJ, Israel M (1976) Comparative DNA-binding studies with adriamycin (ADR), *N*-trifluoroacetyladriamycin-14-valerate (AD 32), and related compounds. Proc Am Assoc Cancer Res 17: 109
41. Sinha BK, Sik RH (1980) Binding of [^{14}C]-adriamycin to cellular macromolecules in vivo. Biochem Pharmacol 29: 1867
42. Tatsumi K, Nakamura T, Wakisaka G (1974) Comparative effect of daunomycin and adriamycin on nucleic acid metabolism in leukemic cells in vitro. Gann 65: 237
43. Vecchi A, Cairo M, Mantovani A, Sironi M, Spreafico F (1978) Comparative antineoplastic activity of adriamycin and *N*-trifluoroacetyladriamycin-14-valerate. Cancer Treat Rep 62: 111

Received May 12, 1987/Accepted July 15, 1987