ABSTRACT

N-Trifluoroacetyladriamycin-14-valerate (AD32) and N-trifluoroacetyladriamycin (AD41) have been compared with Adriamycin and daunorubicin in CCRF-CEM human lymphoblastic cells. All four agents produce DNA damage, as measured by alkaline unwinding methods. Thymidine incorporation, a measure of DNA synthesis, is inhibited by AD32 and AD41 and completely blocked by the other drugs. AD32 and AD41 inhibit growth of the cells after exposure for 2 hr but are considerably less potent than is either Adriamycin or daunorubicin. In drug uptake studies, cellular levels after 2-hr incubations are lower for AD32 than for Adriamycin, and both are several fold lower than those of daunorubicin. The results indicate that AD32 and AD41 (or their metabolites) have DNA-related effects similar to those of Adriamycin and daunorubicin. These common actions may be the proximal cytotoxic events for the four agents.

INTRODUCTION

AD413 and AD32 are analogs of Adriamycin with apparent antitumor efficacies and selectivities different from the parent compound. AD32, which has been tested more extensively than has AD41, is reported to be more active than is Adriamycin against a number of transplanted murine tumors, including L1210 leukemia, P388 leukemias (lines sensitive and resistant to Adriamycin), and RigidBody osteogenic sarcoma (6, 13). In cultured CCRF-CEM (and WI38) cells, AD32 enters intracellular compartments and localizes in the cytoplasm, the rate of uptake being apparently independent of temperature (8). In contrast, the less lipophilic Adriamycin accumulates in cells by mechanisms that are temperature dependent (11).

DNA strand scission and inhibition of DNA synthesis presently appear to be important parameters of cytotoxicity of Adriamycin and daunorubicin (2, 17-19), but are not necessarily the determinants of selectivity (12, 15). Inhibition of DNA synthesis seems to be primarily a function of intercalation by these agents, whereas strand scission may be due to activated metabolites (1, 5, 10, 17). Despite apparent differences in pharmacokinetics and affinity for DNA of the unmetabolized drugs (20), AD32 and AD41 might still have proximal biochemical actions similar to those of Adriamycin and daunorubicin. The present study was undertaken to compare drug uptake and retention, cytotoxicity, and some DNA effects of these agents in a sensitive human acute leukemia cell line.

MATERIALS AND METHODS

Chromatographically homogeneous samples of AD32 and AD41 were gifts from Dr. M. Israel, The Sidney Farber Cancer Institute, Boston, Mass. Bulk supplies of Adriamycin, daunorubicin, and N-acetyldaunorubicin were kindly donated by Professor F. Arcamone, Farmitalia, Milan, Italy. AD32, AD41, and N-acetyldaunorubicin were dissolved in ethanol and diluted with PBS. Adriamycin and daunorubicin were dissolved in PBS with ethanol then added. Ethanol (0.5% final concentration) does not affect parameters as measured in this study.

CCRF-CEM human lymphoblastic cells were maintained (37°C; 5% CO2) in RPMI medium 1640 with 10% dialyzed, heat-inactivated fetal calf serum, 1% penicillin (10,000 units/ml)-streptomycin (10 mg/ml) (Grand Island Biological Co., Grand Island, N. Y.). Relative cytotoxicities of AD32, AD41, Adriamycin, and daunorubicin were determined by growth inhibition studies. Briefly, the 4 compounds at various concentrations (0.0078 to 4.0 μg/ml) were incubated with CCRF-CEM cells (8 x 106 cells/ml; 10 ml) for 2 hr, centrifuged, and resuspended in drug-free media (1.3 x 106 cells/ml; 6 ml). Cells were counted electronically 5 hr later while control cultures were in logarithmic growth. For drug uptake and retention, CCRF-CEM cells (1 x 106/ml; 40 ml) were incubated with Adriamycin, daunorubicin, and AD32 (10 μg/ml) for 2 hr, collected by centrifugation, washed twice with PBS, and reincubated in drug-free media. Aliquots (3 ml) of the cell suspensions were taken at various times, centrifuged, washed twice with PBS, lysed in dodecyl sodium sulfate (0.05%; 1 ml), and stored frozen. Analysis of samples for presence of fluorescent drug was essentially as described previously (16). Excitation (480 nm)-emission (560 nm) coefficients and ranges of linearity were determined for each compound with an Aminco-Bowman spectrophotometer (Silver Spring, Md.).

For DNA unwinding, cells (1 to 2 x 104/ml; 9 ml) in logarithmic growth phase were prelabeled for 18 hr with [2-14C]thymidine (0.1 μCi/ml; 45 Ci/mol; Schwarz/Mann, Orangeburg, N. Y.). Drugs (1 vol) were added to cell suspensions (9 vol) and incubated at 37°C in closed flasks. To estimate DNA damage, a modified method of Rydberg (14) was used. Cells were collected by centrifugation, suspended in 1.0 ml PBS, lysed for 60 min with an equal volume of 0.1 N NaOH, and neutralized with 0.1 N HCl.
Effects of AD32 on DNA in Cells

(phenol red indicator). Sodium lauryl sarcosinate (0.5%) and disodium EDTA (0.01 N; pH 7.0) were added, and DNA in the lysates was sheared immediately by 6 rapid passages through a 22-gauge needle. Single-stranded and duplex DNA were separated by batch hydroxyapatite chromatography. Briefly, cell lysates made 10% with respect to formamide were incubated with 0.5 g hydroxyapatite for 15 min (60°) to bind DNA to the gel. After centrifugation (room temperature), gels were incubated at 60° and extracted sequentially with potassium phosphate buffers (5 ml; pH 7.0)—20% formamide: once with 0.01 N phosphate to wash, twice with 0.125 m phosphate to elute single-stranded DNA, and twice with 0.5 m phosphate to elute duplex DNA. Radioactivity in each fraction was determined by liquid scintillation counting; elution recoveries of chromatographed DNA exceeded 95%.

[methyl-3H]Thymidine (50 Ci/mmol; Moravek Biochemicals, City of Industry, Calif.) incorporation was carried out under culture conditions as above. After addition of drug to the cultures, [3H]thymidine was added (1.0 μCi/ml; final), and thereafter, aliquots (0.5 ml) were transferred to tubes containing cold 20% trichloroacetic acid (2 ml). The precipitates were collected and washed 2 times each with trichloroacetic acid (10%) and ethanol (95%) on fiber glass filters, dried, and counted by standard liquid scintillation methods.

The equation of Rydberg (14) describing time-dependent DNA denaturation in dilute alkali was used to express damage produced by agents in the present study:

$$M_n = \frac{-K}{\ln F_0} t$$  \hspace{1cm} (A)

$M_n$ is the number-average molecular weight between 2 breaks or unwinding points; $F$ is the fraction of double-stranded DNA remaining after alkaline unwinding for time $t$ (min). The unwinding constant $\beta$ was determined with CCRF-CEM cells, and the derived value, 0.62, was in good agreement with those values obtained by Rydberg (14). The kinetics of alkaline unwinding in 0.05 M NaOH are log-linear in the presence of the anthracyclines in CCRF-CEM cells and are comparable to the rates obtained after X-irradiation. The initial alkaline unwinding unit of DNA ($M_n$) was 4.2 × 10^4 daltons, estimated by applications of one-hit theory as described by Kohn and Grimek-Ewig (7).

The number of unwinding points ($p$) in cellular DNA was estimated from Equation A:

$$M_{n0} \ln F_0 = M_{n\alpha} \ln F_{\alpha}$$  \hspace{1cm} (B)

at constant unwinding times (60 min for CCRF-CEM cells). The subscripts 0 and $\alpha$ indicate control and experimental conditions, respectively. Values for $M_{n0}$ and $M_{n\alpha}$ obtained by the hydroxyapatite method have been confirmed in alkaline sucrose gradients (phage λ marker) with several anthracycline derivatives. The number of breaks ($n$) per unwinding unit is estimated as $(\ln F_0/\ln F_{\alpha}) - 1$.

RESULTS AND DISCUSSION

CCRF-CEM cells were incubated for 2 hr with AD32, AD41, Adriamycin, and daunorubicin. The cells were resuspended in fresh media, and growth inhibition was determined after 55 hr. As illustrated in Chart 1, the dose of Adriamycin inhibitory to 90% of the cells was 3-fold greater than that of daunorubicin but 6-fold less than those of either AD32 or AD41. The latter 2 compounds are nearly equipotent (after correction for differences in molecular weight) over the entire range of concentrations (0.0078 to 4.0 μg/ml) even though the incubations with drug were limited to 2 hr.

The relative growth inhibitory effects described above are consistent with the uptake of Adriamycin, daunorubicin, and AD32 at 2 hr (Chart 2). The prolonged retention of fluorescent compounds after washing and reincubation in drug-free media might reflect the strong macromolecular binding characteristics of Adriamycin and daunorubicin (4). Although AD32 is not known to have a strong affinity for DNA, it also shows a high degree of retention by the cells. However, the binding characteristics of metabolites of these agents are largely unexplored.

The analogs AD32 and AD41 were tested for production of DNA damage in CCRF-CEM cells and compared with
DNA damage in CCRF-CEM cells

CCRF-CEM cells (1 to 2 x 10⁶/ml) in log-phase growth and prelabeled with [2-'⁴C]thymidine were incubated with drug in 10 ml of media containing Roswell Park Memorial Institute Medium 1640 and 10% heat-inactivated fetal calf serum. DNA damage was assayed by the modified method of Rydberg (14) described in "Materials and Methods." Total radioactivity of DNA eluted from gels was 0.9 to 1.2 x 10⁵ cpm.

Incubation time

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (µg/ml)</th>
<th>2 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>n/Mn₀</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>0.81</td>
<td>0.80</td>
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<tr>
<td>AD32</td>
<td>1</td>
<td>0.75</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
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<td>1.4</td>
</tr>
<tr>
<td>AD41</td>
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<td>0.67</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.47</td>
<td>2.5</td>
</tr>
<tr>
<td>Adriamycin</td>
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<td>0.67</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.63</td>
<td>1.1</td>
</tr>
<tr>
<td>Daunorubicin</td>
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<td>0.72</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.63</td>
<td>1.1</td>
</tr>
</tbody>
</table>

a F, duplex DNA/total DNA; n/Mn₀, number of breaks per alkaline unwinding unit. See "Materials and Methods" for explanation of derived values. The cells (iced and in an air atmosphere) were calibrated for DNA damage with x-irradiation: 300 rads produces 1.67 strand scissions per alkaline unwinding unit (4.2 x 10⁶ daltons).

Prelabeled CCRF-CEM cells were incubated with AD32, Adriamycin, or N-acetyldaunorubicin for 2 hr, washed, and reincubated in drug-free media. Aliquots were removed at varying times, and cellular DNA damage was quantitated.

The N-acetyl derivative, which possesses only marginal biological activity, was included as a control: like AD32, it lacks the charged amino group and has a weak affinity for DNA (4, 20). Results shown in Chart 3 indicate progressive DNA damage produced by Adriamycin after cells were washed. AD32 again shows evidence of early strand scission, followed by partial recovery after washing, and then progressively increasing amounts of damage. The brief partial recovery (i.e., a decrease of n/Mn₀) after washing has been replicated in 2 other experiments.

Chart 3 shows results of a study of thymidine incorporation into CCRF-CEM cells. Incorporation is linear for about 2 hr in control cells. At this time, inhibition by each agent (10 µg/ml) was: Adriamycin, 45%; AD32, 72%; AD41, 80%; daunorubicin, 92%. In addition, another effect is also apparent: the incorporation of [³H]thymidine in cells treated with Adriamycin and daunorubicin is completely blocked from 1 hr until the end of the experiment (4 hr). In contrast, cells with AD32 and AD41 show almost constant rates of thymidine incorporation (but slower than initial control rates) during the entire incubation period. The complete inhibition by daunorubicin and Adriamycin can be interpreted as due both to intercalation and to DNA damage presumably by activated metabolites. The inhibition of DNA synthesis by AD32 and AD41 is difficult to explain as a function of their binding but may be due to the DNA damage shown above.

The studies described here indicate that AD32 and AD41 partially inhibit thymidine incorporation and introduce cuts or at least alkali-labile regions in DNA. Growth and cytotoxicity studies by others (6, 8, 13) raised the possibility that AD32 and AD41 might be acting differently in a qualitative sense from Adriamycin and daunorubicin. A basis for this distinction might be that AD32 and AD41 are different from the other 2 with respect to DNA binding. DiMarco (4) in previous studies with Adriamycin, daunorubicin, and a
AD41, and as a presumed consequence of the loss of the positive charge, the derivatives do not bind to DNA with the specificity or affinity of Adriamycin and daunorubicin (4, 20).

The present studies indicate that AD32 and AD41 (or their biotransformation products) have proximal DNA-damaging actions as do Adriamycin and daunorubicin (17). In this regard, it is important to note that Handa and Sato (5) and Bachur et al. (1) recently described evidence of free radical formation from Adriamycin, daunorubicin, and other polycyclic quinones in microsomal oxidation-reduction systems. Such activated metabolites might bind covalently to DNA, perhaps introducing labile regions within the DNA structure that appear as strand breaks under alkaline conditions. In this regard, Lown et al. (10) have demonstrated anthracycline-induced damage of isolated, covalently closed circular DNA in the presence of reducing agents. As another alternative, but without supporting evidence, Byfield et al. (3) and Lee and Byfield (9) have proposed that anthracycline-induced perturbations of DNA structure directly activate intracellular nucleases with resultant frank strand damage.

The inhibition of thymidine incorporation is probably a direct function of the amount of drug taken up and retained by intercalation. In addition, DNA damage may result from covalent binding of activated drugs. The extent of damage would presumably depend upon the amount of drug retained by the cells as well as the efficiency of activation and the affinity of activated drug for the DNA target. These parameters may also be interdependent in that template activity is altered by both intercalation and DNA damage (or adduct formation), while DNA repair may be impeded by the bound forms of each agent. Because AD32 and AD41 have low DNA affinities, it is reasonable to expect that activated forms might attack random intracellular targets with a higher frequency than would activated intercalating drugs with stronger DNA affinities. This would account for the relatively lower potencies of AD32 and AD41 if DNA damage and inhibition of DNA synthesis are determinant parameters.

REFERENCES

Effects of \textit{N}-Trifluoroacetyladriamycin-14-valerate and Related Agents on DNA Strand Damage and Thymidine Incorporation in CCRF-CEM Cells

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