Protein-associated DNA Breaks and DNA-Protein Cross-Links Caused by DNA Nonbinding Derivatives of Adriamycin in L1210 Cells

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ABSTRACT

The effects of Adriamycin derivatives on L1210 mouse leukemia cells were studied with the DNA alkaline elution assay. The exposure of exponentially growing cells to approximately equitoxic concentrations of N-trifluoroacetyladriamycin-14-valerate (13.8 μM) and its metabolite, N-trifluoroacetyladriamycin (9.0 μM) and N-trifluoroacetyladriamycinol (43.7 μM), for 1 hr in vitro resulted in a high frequency of protein-associated DNA breaks and DNA-protein cross-links. These effects were comparable to those observed with Adriamycin (2.8 μM) and with adriamycinol (26.9 μM). In contrast to Adriamycin and its metabolite adriamycinol, N-trifluoroacetyladriamycin-14-valerate and its two major metabolites do not bind to DNA. Despite the absence of this direct interaction, N-trifluoroacetyladriamycin-14-valerate and its metabolites produce alterations in DNA comparable with the effects of intercalating agents. No evidence for conversion of N-trifluoroacetyladriamycin-14-valerate to Adriamycin or adriamycinol was found in L1210 cells. The similar effects on DNA macromolecules, observed between intercalating and non-DNA-binding anthracyclines, are consistent with the concept that mechanisms other than direct interaction with DNA play a role in the toxic effects of these compounds.

INTRODUCTION

The anthracycline derivative AD32 exhibits greater antitumor activity than does ADR (3, 9) against a spectrum of experimental rodent leukemias and solid tumors (17, 30, 34). Phase I clinical studies for this drug have been completed; clinical antitumor activity and low toxicity, relative to ADR, have been documented (4–6). AD41 and AD92 are the major metabolites detectable in plasma bile, and urine of various animal species and humans treated with AD32 (12, 13, 15, 19, 21, 22). In contrast to ADR, all 3 compounds (AD32, AD41, and AD92) do not bind to DNA (8, 33). AD32 exhibits cytoplasmic, rather than nuclear, fluorescence in live cultured cells (27), yet it produces an inhibitory effect on DNA and RNA synthesis (28) and/or cell cycle traverse similar to that of ADR. AD32, unlike ADR, enhances rather than inhibits propidium iodide nuclear fluorescence (26). In vitro and in vivo, ADR is not a significant metabolite of AD32 (12, 13, 15, 17, 19, 21, 22, 28). Considerable evidence indicates that various intercalating agents including ADR induce single-strand breaks in the DNA of mammalian cells (2, 29, 32). Recently, strand breaks associated with DNA-protein cross-links (24, 31) have been demonstrated by the DNA alkaline elution technique (23, 25). Nonintercalating DNA binders or other drugs which inhibit nucleic acid synthesis were not shown to produce such protein-associated breaks (31). The availability of DNA nonbinding and therefore, nonintercalating, anthracycline derivatives allowed us to ask the question as to whether exposure to these drugs would result in macromolecular alterations different from or similar to changes caused by intercalators.

In the present study, the effects of AD32 and its 2 metabolites AD41 and AD92 were investigated using the DNA alkaline elution technique. The results of the present studies show that AD32, as well as AD41 and AD92, despite their failure to bind, still produces protein-associated DNA breaks comparable to those found with ADR and its major metabolite AMNOL.

MATERIALS AND METHODS

Propagation and Radioactive Labeling of Cells. All studies were done on L1210 leukemia cells (Sloan-Kettering) during exponential growth, with a 22-hr doubling time. Suspension cultures in McCoy’s Medium 5A (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 16% fetal calf serum and 1% Pen-Strep, were labeled for 24 hr with either [methyl-3H]thymidine (40 to 60 mCi/mmol; 0.1 μCi/ml) or [2,14C]thymidine (>50 mCi/mmol; 0.025 μCi/ml). Both isotopes were obtained from Amersham-Searle Corp., Arlington Heights, Ill.

Drug Treatment. The structures of the compounds used in the experiments are shown in Table 1. AD32, AD41, and AD92, prepared as described previously (16, 17), were dissolved in Diluent 12 [polyethoxylated castor oil:ethanol (Division of Cancer Treatment, National Cancer Institute, NIH):0.15 M NaCl. ADR (Farmitalia S.p.A., Milan, Italy) and AMNOL (17) were dissolved in 0.15 M NaCl. The [methyl-3H]thymidine-labeled “experimental cells” were resuspended into fresh warm medium at 9 × 10⁶ cells/ml and incubated in the dark with equitoxic concentrations of drugs (Table 1) for 1 hr at 37°C. “Control cells,” also labeled with [methyl-3H]thymidine, were incubated under identical conditions in medium with appropriate amounts of Diluent 12:0.15 M NaCl or 0.15 M NaCl alone. At the end of incubation, the cells were washed 3 times with PBS before further manipulation.

* M. Israel and A. Krishan, unpublished observations.

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3 The abbreviations used are: AD32, N-trifluoroacetyladriamycin-14-valerate; ADR, Adriamycin; AD41, N-trifluoroacetyladriamycin; AD92, N-trifluoroacetyladriamycinol; AMNOL, adriamycinol; PBS, phosphate-buffered saline (0.15 M NaCl: 0.71 mM KH₂PO₄, 4.28 mM K₂HPO₄, pH 7.4); HPLC, high-pressure liquid chromatography.

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Alkaline Elution Assay. The method used was that of Kohn and Ewig (24) and Kohn et al. (25) with only minor modifications. Experimental cells were assayed either directly or after exposure to 300 or 3000 R of X-irradiation (X-ray unit: 220 kV; 15 ma; half-value layer, 0.6 mm copper; target distance, 24.5 cm; 190 R/min) at a temperature of 0°. "Internal reference" lllC-labeled cells were exposed to 150 R at 0° and were lysed with 0.2% Sarkosyl (ICN Pharmaceuticals, Inc., Cleveland, Ohio). The filters were washed, and elution was carried out at pH 12.1 with tetrapropylammonium hydroxide. In order to decrease the nonspecific binding of protein to the filter, 0.1% sodium dodecyl sulfate (BDH Chemicals, Ltd., Poole, England):0.025 M trisodium EDTA solution (36) alone or containing 2% sodium dodecyl sulfate (BDH Chemicals, Ltd., Poole, England) was present only in cells treated with AMNOL (Table 2). The concentration range was 36% higher than that one calculated originally.

<table>
<thead>
<tr>
<th>Drug</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>ID50 (µM)</th>
<th>Drug concentration (µM)</th>
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</thead>
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<tr>
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<td>H</td>
<td>O</td>
<td>H</td>
<td>0.066</td>
<td>2.8</td>
</tr>
<tr>
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<td>26.9</td>
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<tr>
<td>AD32</td>
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<td>O</td>
<td>CO(CH2)3CH3</td>
<td>0.24</td>
<td>13.8</td>
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<td>H</td>
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</tr>
<tr>
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<td>COCF3</td>
<td>H</td>
<td>OH</td>
<td>1.03</td>
<td>43.7</td>
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</tbody>
</table>

* Calculation of drug concentrations is based on the ID 50 dose for each drug except AD32 (the used concentration is 36% higher than that one calculated originally).

RESULTS

DNA Alkaline Elution Assays. Typical plots of elution curves are shown in Charts 1 to 3. Compared to untreated controls, the following changes were observed with all the compounds: (a) increases in DNA elution rates of lysates of unirradiated drug-treated cells incubated with proteinase K (Charts 1 to 3, right); and (b) decreases in elution rates of lysates from drug-treated cells which had been irradiated with 300 or 3000 R and not incubated with the enzyme (left). This result indicates that the decreases in elution were prevented by pretreatment of lysates with proteinase K. Irradiation of experimental cells with 3000 R instead of 300 R did not increase the sensitivity of the assay, and Table 2 shows only the frequencies of DNA lesions in experiments performed with 300 R. The pattern of the elution curves (Charts 1 to 3) indicates an increased incidence of protein-associated DNA breaks and DNA-protein cross-links in cells treated with all the compounds, whereas a significant increase in DNA breaks over the controls was present only in cells treated with AMNOL (Table 2). The calculated DNA break frequency for ADR appears to be greater than that for AD32 and AD41 but still is within the limits established for control untreated samples. With approximately equitoxic drug concentrations being used in all experiments, the most striking alterations in DNA (protein-associated breaks and/or DNA-protein cross-links) were observed after the in-
cubation with AMNOL, AD41, or AD92. DNA alterations of this type caused by ADR or AD32 were lower in their frequency and comparable to each other (Table 2). The steep elution curve, indicating high frequencies of protein-associated breaks induced by AD41 (Chart 2, right), was observed consistently in 6 independent experiments. The tabulated frequencies of DNA breaks and cross-links obtained after drug treatments can be compared with values for untreated controls. The control values express the fluctuation in elution rates for lysates of untreated cells in various experiments.

The possibility that residual AD32 or its metabolites present in cell lysates could induce lesions of DNA after its release from the cell (23) was also tested using mixtures of untreated and drug-treated cells. Cells were mixed just before lysis, and aliquots of $10^8$ cells consisting of 30% $^3$H-labeled (no drug), 30% unlabeled (incubated with AD32) and 40% internal reference $^{14}$C-labeled cells were deposited on filters and treated as described in "Materials and Methods." In some aliquots, $^3$H-labeled cells were irradiated with 300 R, and all unlabeled cells were irradiated with 3000 R in order to minimize their interference with the elution rates of $^3$H- and $^{14}$C-labeled cells. The results of the experiment do not show DNA lesions induced by residual drug in the cell lysate.

**Metabolic Fate of ADR or AD32 in L1210 Cells.** Although the percentage of recoveries from culture media were good for most anthracycline compounds anticipated in this study (ADR, 95.4; AMNOL, 96.2; AD32, 96.0; AD41, 100.0; AD92, 84.0), the procedure when used for L1210 cells gave low recoveries of ADR and AMNOL (ADR, 17.9; AMNOL, 20.0; AD32, 90.0; AD41, 100.0; AD92, 100.0). The concentrations obtained for other anthracycline-fluorescent metabolites are presented as equivalents of AD32 or ADR, depending upon the drug used for the incubation.

The metabolic fate of AD32 or ADR when incubated with L1210 cells in culture media is shown in Table 3. It may be seen that parent AD32 tended to concentrate in cells under the incubation conditions. Of the total fluorescence in the media, 9% is due to AD32 metabolites while 25% of the total fluorescence in the cells could be accounted for as AD32 metabolites, a major portion as AD41. ADR was not detected as a metabolite of AD32 in either media or cells. Compared to AD32, ADR partitioned less to the cellular compartment under the given experimental conditions, a finding consistent with the known time-dependent intracellular transport of ADR (27). Other anthracycline-fluorescent signals, attributable to 7-hydroxy- and 7-deoxyaglycone products, were seen from both ADR and AD32. In all probability, some of these substances are listed twice in Table 3, having been detected at different retention times by reverse-phase and normal-phase HPLC. No attempt was made in this study to match these substances or to definitively characterize the aglycone metabolites, as no aglycone derivative of ADR or AD32 is known to bind with DNA or to have significant biological activity in vitro or in vivo. By retention time analysis relative to an authentic standard, the only signal common to both the ADR and AD32 incubations was observed at 5.85 min (reverse phase) and is due to 6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione. This compound is a known degradation product of anthracyclines (8) and is devoid of biological activity.

**Chart 1.** Effects of ADR and AMNOL on alkaline elution kinetics of DNA. Control (○, △) and drug-treated cells (●, ▲) were either left unirradiated (○, ●) or were irradiated with 300 R (△, ▲) and lysed (see "Material and Methods" for details). Cell lysates were subjected to alkaline elution without (pk−) or with (pk+) proteinase K treatment. The number of repeated experiments done with each compound is shown in Table 2.
The data presented above show the occurrence of protein-associated DNA breaks and DNA-protein cross-links not only following the treatment with ADR and its metabolite AMNOL but also with AD32 and its metabolites AD41 and AD92, anthracycline analogs which do not bind to DNA. AD32 is rapidly converted in vivo to AD41, especially in rodents, by the action of plasma and intracellular esterases, and AD41 is further metabolized to AD92 by aldo-ketoreductases in a manner analogous to the conversion of ADR to AMNOL (18). The finding that the frequency of protein-associated DNA breaks and DNA-protein cross-links is highest with AD41 is consistent with the report that AD41, like AD32, is therapeutically superior to ADR in the treatment of mouse tumors, but unlike AD32, its use is associated with a pattern of late deaths (18). The delayed toxicity of AD41 thus makes this drug inferior to AD32 as a therapeutic agent.

Any appreciable conversion of AD32 or its metabolites into ADR or AMNOL in the testing system would have to be considered in the interpretation of results. No evidence for such a conversion was detected in our L1210 cell line with a HPLC-fluorescence assay capable of measuring down to 5 pmol of ADR. In contrast, cells treated with an equitoxic dose of AD32 showed clearly detectable and quantifiable levels of the parent drug and its major, still biologically active, metabolite. The only anthracycline-fluorescent species found common to both ADR and AD32 is a known degradation product which does not bind with DNA and is otherwise biologically inactive. Also, DNA alkaline elutions with a mixture of untreated and AD32-treated cells do not indicate that residual AD32, or its metabolites present in cell lysates, could induce lesions of DNA after its release from assayed cells. Thus, induction of protein-associated DNA breaks and DNA-protein cross-links exhibited by AD32 in this system is not accountable in terms of an ADR-prodrug mechanism. Rather, AD32 and/or its metabolites must be capable of exerting the alterations of DNA macromolecules without the requirement of prior intercalation.

In conclusion, similar effect on DNA was produced by the non-DNA-binding AD32 and metabolites as to those caused by ADR and AMNOL in this system. It has been suggested that distortion of the DNA helix induced by intercalators leads to strand scission by a nuclease, possibly a DNA repair enzyme of the nick-closure type. The protein associated with the DNA break might be this enzyme, producing the break and remaining linked to the strand (31). While the biological significance of protein-associated DNA breaks and DNA-protein cross-links is not clear in the context of damage-repair processes (23), it could be expected that either (a) these lesions are caused by at least 2 independent mechanisms, one being operational for intercalators and the other for DNA nonbinding ADR derivatives; or (b) both groups of drugs affect DNA through a comparable mechanism which is in variance from the intercalation-induced distortion of the DNA helix. The latter possibility seems to be supported by observations that ADR and other quinone-containing drugs, such as AD32, might cause the damage in the cell either directly in a free-radical state or indirectly through oxygen-dependent free radicals, such as superoxide and hydroxyl radical (1).
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