Role of DNA Breakage in Cytotoxicity of Doxorubicin, 9-Deoxydoxorubicin, and 4-Demethyl-6-deoxydoxorubicin in Murine Leukemia P388 Cells

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ABSTRACT

Formation and persistence of DNA single- and double-strand breaks stimulated by doxorubicin, 9-deoxydoxorubicin, or 4-demethyl-6-deoxydoxorubicin in murine leukemia P388 cells were compared in relation to drug DNA affinity, cellular pharmacokinetics, and cytotoxicity. Although cellular uptake and retention and DNA affinity of the anthracycline derivatives were similar to those of the parent drug, cytotoxic potency was quite different, 9-deoxydoxorubicin being much less cytotoxic than doxorubicin, and 4-demethyl-6-deoxydoxorubicin the most effective. After 1-h exposure of cells to cytotoxic drug levels, the extent of DNA strand breaks produced by 4-demethyl-6-deoxydoxorubicin was greater than that produced by doxorubicin, whereas 9-deoxydoxorubicin induced very few DNA breaks. As for the parent drug, proteolytic treatment of cell lysates on the filter was needed to detect DNA cleavage produced by the analogues. A linear increase of DNA breaks was observed for 2 h following 4-demethyl-6-deoxydoxorubicin or doxorubicin addition; by contrast, DNA break levels reached a plateau after 45 min of exposure to 9-deoxydoxorubicin. DNA lesions produced by the derivatives persisted, and doxorubicin-induced DNA breaks even increased after drug removal, indicating an absence of DNA break resealing under our conditions. These observations indicate that modifications of the chromophore moiety of the anthracycline may enhance both drug cytotoxicity and specificity of drug-target interactions, and thus provide further strong evidence that the anthracycline effect on DNA integrity is a critical aspect of the mechanism of drug action.

INTRODUCTION

Doxorubicin is one of the most useful anticancer agents with a broad spectrum of activity (1). Its therapeutic interest has stimulated numerous investigations on the mechanism of action of anthracyclines in an attempt to find a molecular basis for rational synthesis of analogues (2). It is now well known that anthracycline antibiotics exert multiple actions at the cellular level (3). The cytotoxic activity of these drugs has been largely attributed to inhibition of DNA synthesis and functions caused by their binding to DNA by intercalation (4, 5). Although findings on structure-activity relations are compatible with intercalation as the most critical event for drug action, factors other than simple DNA intercalating ability are probably involved in determining cytotoxic potency and antitumor activity. Recently, it has been proposed that intercalating agent-mediated DNA strand breaks occur through the primary interaction of the drug with DNA topoisomerase II (6). This nuclear enzyme has been recognized as being a critical multidrug target. Indeed, intercalating agents and epipodophyllotoxins have been shown to interfere with DNA topoisomerase II (7–9), producing protein-associated DNA strand breaks in proliferating tumor cells and isolated nuclei (10–14), which have been related to cytotoxic effects (6–15).

Structure-activity studies of a large number of aminooacidines (16, 17) and of epipodophyllotoxin congeners (18) have demonstrated a high degree of correlation between cytotoxicity and ability to form protein-linked DNA breaks in intact tumor cells. In the case of anthracyclines, the role of drug-induced DNA cleavage is not yet completely established. No quantitative relation between DNA SSB (20) and cytotoxic effects has been found for a number of chromophore-modified anthracyclines, although increased cytotoxic potency is generally associated with an enhanced ability to produce protein-associated DNA breaks (14). There is general agreement that several factors, mainly cellular pharmacodynamics, are involved in modulation of the cytotoxic potential.

In a further attempt to throw light on the role of topoisomerase II-mediated DNA strand breaks produced by anthracyclines in the mechanism of drug action, this paper reports a detailed comparison of DNA breakage, DNA binding affinity, cellular pharmacokinetics, and cytotoxicity produced by doxorubicin, 9-deoxydoxorubicin, and 4-demethyl-6-deoxydoxorubicin (Fig. 1). These compounds are of particular interest because of their similar DNA affinities and superimposable cellular pharmacokinetic behavior. The results are evidence for a causal relation between drug-induced DNA lesions and cell-killing activity.

MATERIALS AND METHODS

Chemicals. Doxorubicin, 4-demethyl-6-deoxydoxorubicin, and 9-deoxydoxorubicin were obtained from Farmitalia-Carlo Erba (Milan, Italy) (Fig. 1). Drug solutions were prepared in distilled water immediately before use. [2-14C]Thymidine (50 mCi/mmole) in aqueous solution was purchased from Amersham International (Amersham, United Kingdom). All other chemicals of analytical reagent grade were from usual commercial sources.

DNA Binding Parameters. Fluorescence studies of binding of anthracyclines to calf thymus DNA were carried out using a Perkin-Elmer Model MPF 44A. The method for determining the DNA binding parameters from fluorescence quenching has been described (4). DNA binding parameters of drugs were determined under the same conditions of ionic strength (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.0, 0.5 mM EDTA).

Cell Lines and Drug Treatments. The murine leukemia P388 cell line was cultured as already described (14). Exponentially growing cells were exposed to drugs at 37°C for the indicated time periods. Cytotoxic activity of 1-h drug treatment was determined by the growth inhibition test (14) and by soft agar colony formation assay (19). In order to examine DNA break resealing and drug efflux, after 1-h drug treatment, cells were centrifuged, washed twice with cold phosphate saline solution, and then resuspended at the same cellular density in prewarmed culture medium for the indicated time periods.

Filter Elution Methods. Filter elution procedures were essentially as reported by Kohn et al. (20) and described elsewhere (14). Briefly, cells were labeled with 0.04 µCi/ml of [14C]thymidine for 24 h, followed by an overnight chase period. γ-Ray irradiation of cells was carried out on ice with a cesium-137 source (1020 rads/min). Cells were layered on 2-µm pore size polycarbonate membranes (Nucleopore, Pleasanton, CA)

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and lysed with a solution of 2% SDS, 0.1 M glycine, 25 mM disodium EDTA (pH 9.6), and 0.5 mg/ml of proteinase K (Merck, Darmstadt, Federal Republic of Germany). The DNAs on the filter were eluted with 0.1% SDS, 20 mM EDTA (acid form), and tetrapropylammonium hydroxide (Eastman Kodak, Rochester, NY), pH 12.15 or 9.6, for SSB and DSB, respectively. SSB and DSB frequencies were calculated as already reported and expressed in rad-equivalents (20, 21). DNA-protein covalent links were demonstrated by lysing cells with a solution of 0.2% Sarkosyl (Sigma Chemical Co., St. Louis, MO), 2 M NaCl, and 40 mM disodium EDTA (pH 10), without proteinase K, and eluting without detergent at pH 12.15.

Cellular Drug Content and Efflux. Cells were centrifuged at 0°C, washed with cold saline buffer, and lysed with distilled water and AgNO₃ (3.1% final concentration). The drug was extracted with 3 ml of water-saturated normal butyl alcohol, and the fluorescence intensity of extracts was determined with a fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT) at the following excitation and emission wavelengths, respectively: 500 and 590 nm for 9-deoxydoxorubicin and doxorubicin; and 445 and 580 nm for 4-demethyl-6-deoxydoxorubicin. The average yield of the extraction procedure was more than 95%.

High-pressure liquid chromatography analysis of the cellular extracts did not show the presence of metabolites of any anthracycline after 1-h exposure. The average yield of the extraction procedure was more than 95%.

**RESULTS**

Cytotoxicity Studies. Cytotoxic activity of the tested anthracyclines was examined by the growth inhibition test and by colony formation assay. The relative cytotoxic potency of these drugs was similar using different methods. Fig. 2 shows the effects of doxorubicin and derivatives on the survival of P388 cells after exposure to the drug for 1 h. Under the same experimental conditions, the relative order of effectiveness in producing 50% inhibition of colony-forming ability was: 4-demethyl-6-deoxydoxorubicin > doxorubicin > 9-deoxydoxorubicin (Table 1).

Interaction with DNA. Table 1 summarizes the binding parameters for drug-DNA interaction. At neutral pH and in the presence of 0.1 M ionic strength, the doxorubicin derivatives exhibited similar binding ability with calf thymus DNA. Structural modifications in the chromophore produced an appreciable reduction in drug affinity for DNA. However, the association constant values of the derivatives were of the same order of magnitude as that of the parent compound and compatible with drug intercalation (4). The marginal changes in DNA binding ability did not account for marked differences in cytotoxic potency of these anthracyclines (Table 1).

Cellular Pharmacokinetics. Cellular drug content was measured after 1-h exposure to cytotoxic concentrations of each drug (Fig. 3). The drug accumulation by P388 cells was dose dependent, and no differences were observed among these anthracyclines. Drug uptake was also shown to be time dependent, as a linear increase of drug accumulation was observed in the presence of 1 μg/ml of drug over 2 h (Fig. 4A) without any appreciable difference among the studied anthracyclines. Moreover, cellular drug retention examined after 1-h treatment under the same experimental conditions was found to be virtually the same for the three compounds (Fig. 4B). Thus, in spite of the differential cytotoxicity (Fig. 2; Table 1), these anthracyclines showed similar cellular pharmacokinetic behavior.

Formation of DNA Strand Breaks. DNA single- and double-strand breaks produced by these anthracyclines after 1-h exposure to cytotoxic drug concentrations are shown in Figs. 5 and 6 as a function of cellular drug content. As already known for other anthracyclines (10, 14), the DNA breaks produced by
these derivatives were protein-associated, since proteolytic treatment of cell lysates on the filter was needed to detect DNA cleavage (results not shown). High levels of both SSB and DSB were produced by 4-demethyl-6-deoxydoxorubicin in a dose-dependent manner; in contrast, 9-deoxydoxorubicin produced very low DNA break frequencies, showing a biphasic dose-dependent curve. This biphasic dose-response curve has been reported already for other anthracycline derivatives and has been interpreted as inhibition of enzyme catalytic activity (14, 20). Obviously, this may complicate the role of drug interference with DNA topoisomerase on cellular death. Although doxorubicin induced DNA strand breaks at intermediate levels between the two derivatives, DNA breakage activity of the parent drug more closely resembled that of 9-deoxydoxorubicin. Therefore, the time courses of DNA SSB and DSB appearance were also studied over 2-h periods at drug concentrations which induced a comparable extent of DNA SSB after 1-h treatment (Fig. 7). It should be pointed out that these drug levels are within the cytotoxic range. The kinetics of DSB formation appear to be identical to those of SSB formation; however, DNA breaks produced by doxorubicin and 4-demethyl-6-deoxydoxorubicin increased linearly for 2 h, whereas DNA cleavage stimulated by 9-deoxydoxorubicin reached a plateau after 45 min of incubation. Thus, in addition to the slightly superior efficiency of doxorubicin in producing protein-associated DNA breakage as compared with 9-deoxydoxorubicin (Figs. 5 and 6), the saturation behavior exhibited by the derivative may be a kinetic difference relevant to cytotoxic potency.

Persistence of DNA Strand Breaks. Since resealing or persistence of DNA cleavage produced by anthracyclines may also be critical for cell killing activity (Refs. 11 and 22; Footnote 4), the time courses of DNA SSB and DSB after drug removal were also investigated (Fig. 8). Following 1-h exposure under conditions used in studies for DNA breakage formation, drug removal and continued incubation of cells in drug-free medium up to 2 h did not result in appreciable DNA break resealing. This binding was expected, since resealing of strand breaks is known to be a very slow process for anthracyclines (Refs. 11 and 22; Footnote 4). The extent of DNA strand breaks even increased following drug removal: this effect was marginal for the derivatives but marked for the parent compound (3-fold increase of DNA SSB after 2 h of incubation in absence of the drug). Since highly cytotoxic concentrations of 4-demethyl-6-deoxydoxorubicin induced high levels of DNA breaks (Figs. 5 and 6), persistence of DNA cleavage after 1-h exposure of cells to 1.8 µmol of this drug was also examined and showed a pattern similar to that found at lower concentrations (results not shown). Furthermore, since a biphasic curve was noted for DNA breakage produced by 9-deoxydoxorubicin (Figs. 5 and 6), the time courses of DNA SSB and DSB after removal of 9-

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cyclines, drug was removed and cells were incubated in drug-free medium for the
deoxydoxorubicin (D). Points, mean of two to four independent determinations;
bars, SE, shown when larger than symbol size.

diately after drug removal. Drug concentrations were 1.72 μmol of doxorubicin
equivalents; [DSB], double-strand break frequency measured in double-strand
free medium. See legend to Fig. 8 for details.

was slight! lower for 4-demethyl-6-deoxydoxorubicin than for
among different classes of topoisomerase II inhibitors (22), the
ability to produce DNA SSB and DSB was reported to differ
with 4-demethoxydaunorubicin. This observation parallels a lower ability of 9-
deoxydoxorubicin. This observation parallels a lower ability of 9-
true ratio between DNA SSB and DSB introduced by ionizing radiation (21). A
[DSB] - 2, where s/d is the ratio between true DNA SSB and DSB, and K, is the
break rad equivalents. Values are means of several determinations. SDs are less
also shown by comparing DNA effects in Fig. 7. However, at
high concentrations of 4-demethyl-6-deoxydoxorubicin, the
[SSB]/[DSB] ratio increased (Table 2), suggesting that the
relative production of DNA SSB and DSB may be dose de-
pendent.

The relationship between cytotoxic effects and DNA strand
breaks is shown in Fig. 10. At equitoxic concentrations, doxo-
rubicin produced a lower level of SSB than derivatives. A lack of
precise correlation between SSB and cytotoxicity for anthra-
cyclines has already been noted (14-15). The effects produced
by 1-h exposure indicated a better correlation between DNA-
DSB and cytotoxicity.

**DISCUSSION**

The data presented in this study comparing doxorubicin and
two chromophore-modified derivatives provide some useful
insights into relations between DNA cleavage produced by these
intercalating agents and drug cytotoxicity. The studied deriva-
tives have been selected since structural modifications are not
expected to impair intercalation, in that all derivatives possess
the planar aromatic structure and no substituents are included.
Indeed, drug-DNA interaction studies indicated marginal
changes in binding properties. Clearly, these changes do not
account for more marked differences in cell killing activity
of these anthracyclines (Table 1). On the other hand, structural
modifications in the chromophore are expected to modify
the ability to induce DNA breakage (14). As already noted,
removal of the bulky methoxy or methyl group at position 4 increased
the ability to produce protein-associated DNA breaks. In addi-
tion, surprisingly, removal of the C-9 hydroxyl group decreased
the efficiency in inducing DNA breaks in spite of tight binding
to DNA.

The finding that the structural modifications did not appreci-
ably alter the cellular pharmacokinetics of these anthracy-
clines (drug uptake and retention) provides the interesting
opportunity of more easily correlating DNA alterations and
cytotoxic effects. The present results showed clearly that the
most important factor accounting for the cytotoxic potency of
these anthracyclines is the relative ability to stimulate DNA
breakage. However, a simple correlation between cell killing
and the extent of DNA cleavage immediately following 1-h
exposure did not adequately reflect the cytotoxic consequences
of DNA alterations produced by anthracyclines, since the ki-
biology of formation and disappearance of DNA lesions is expected to be relevant to the cytotoxic events. For example, DNA breakage induced by the less potent derivative, 9-deoxydoxorubicin, appeared saturable in contrast to the linear formation of DNA lesions caused by doxorubicin over 2 h of exposure (Fig. 7). In addition, in contrast to the two analogues, doxorubicin-produced DNA breaks continued to increase even following drug removal (Fig. 8). Thus, the extent of DNA breaks, coupled with the time course of formation and with the persistence of DNA breaks, contributes to a better understanding of the cytotoxic role of drug-induced DNA break formation. The persistence (i.e., lack of resealing and, even, increase) of DNA lesions following drug removal is a peculiar aspect of the interaction of anthracyclines with DNA topoisomerase II (11, 22) which might explain the already noted lack of precise correlation between cell killing and frequency of DNA breaks produced by different classes of topoisomerase II inhibitors (22, 23). Indeed the formation of DNA breaks in cultured cells has been reported to reach a plateau rapidly after exposure to epipodophyllotoxins (18) and to aminoacridines (11); a rapid disappearance of drug-induced breaks has been noted as well (11, 18). In contrast to persistence of DNA breakage observed with the drugs used in this study, a reversal of DNA SSB produced by other anthracyclines [4-demethoxydaunorubicin and 5-imino daunorubicin (22)] was found following cell exposure under similar conditions. This difference may reflect different drug retention and may explain the already noted absence of a quantitative correlation between cell killing and DNA breaks also for anthracyclines (11, 14, 22). However, additional cytotoxic events at the DNA level and/or sequence specificity of topoisomerase II-mediated effects may contribute to the cytotoxic action (and selectivity) of the intercalating antitumor agents. A decrease in cell-associated drug (Fig. 4B) following drug removal is accompanied by a persistence of DNA breakage (Fig. 8). This apparent discrepancy may be rationalized in terms of a faster drug efflux of only non-tightly bound drug inside the cells (i.e., cytoplasmic). DNA-bound drug is expected to be slowly removed, producing persistence of DNA breaks. The increased level of DNA lesions post-drug removal, observed only in doxorubicin-treated cells, was striking and could reflect some peculiar features in drug-protein interaction inside the chromatin.

The cellular effects of the anthracyclines studied may be relevant to the known difficulty in establishing clear structure-activity relations among anthracycline antibiotics (14). The reduced cytotoxic potency and marginal antitumor activity of the 9-deoxy derivative, which retains a similar DNA binding strength, support our hypothesis that a specific mode of interaction, rather than strength of binding, is important in determining biological activity (14). This observation is consistent with the belief that DNA intercalation is a necessary but not sufficient condition for antitumor activity (24–27). Although in vivo antitumor selectivity, and therefore efficacy, is expected also to depend on factors other than biochemical action at the cellular level, for example, pharmacological factors, the evidence that position 9 plays a critical role in cytotoxic activity and in DNA cleavage and, thus, the possibility of modulating specific effects on DNA topoisomerase II through chromophore modifications, may provide a basis for rational design of effective anthracyclines. The importance of the position 9 in the drug biological activity has been already emphasized (25, 28–31). Both substituents at C-9 have been suggested to play a key role in interacting with the bases on either side of the intercalated molecule in the minor groove of the double helix (32–34).

In particular, the role of the C-9 hydroxyl group has been rationalized in terms of the hydrogen bond between O-9 and two nitrogen atoms, N-3 and N-2, of an adjacent guanine base (32–34). From our findings, this specific interaction is also expected to influence the drug interference with topoisomerase II. One could speculate that removal of the crucial hydroxyl group in position 9 may change the sequence specificity of drug-DNA interaction and, therefore, the DNA localization of topoisomerase II-mediated DNA cleavage.

In summary, these experiments provide the first detailed comparison of protein-associated DNA strand breaks produced by chromophore-modified anthracyclines characterized by cellular pharmacokinetic behavior identical to doxorubicin and similar DNA binding strength. Interestingly, the time course of formation and of persistence of DNA lesions produced by anthracyclines was shown to be important as well as the extent of strand breaks (measured immediately following exposure). Our data indicating differences among these anthracyclines in their ability to cause cellular DNA cleavage further support a crucial role of drug interference with DNA topoisomerase II, a primary target for these agents.

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REFERENCES

DNA BREAKS AND CELL KILL PRODUCED BY ANTHRACYCLINES


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