Relationship between Lethal Effects and Topoisomerase II-Mediated Double-Stranded DNA Breaks Produced by Anthracyclines with Different Sequence Specificity

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SUMMARY

The role of the site selectivity of topoisomerase II poisoning in the cytotoxic activity of anthracyclines has not been established. In this article, we have thus studied the levels and persistence of double-stranded DNA breaks (DSB) along with the cytotoxic activity in human leukemic HL60 cells of seven anthracyclines, including doxorubicin, daunorubicin, and idarubicin, as well as sugar-modified analogues characterized by an altered sequence specificity. Epimerization at the 3′ position of the sugar moiety markedly affected the biological activity; indeed, a dramatic reduction of drug effects was evident for 3′-deamino-3′-epi-hydroxy-4′-deoxy-4′-amino-daunorubicin. The studied analogues could be gathered into three groups based on the DSB/cytotoxicity ratio. At equitoxic concentrations: (a) parent drugs and 3′-deamino-3′-epi-hydroxy-4′-deoxy-4′-amino-daunorubicin endowed with the same sequence specificity stimulated low DSB levels; (b) 3′-epi-daunorubicin and 3′-deamino-4′-deoxy-4′-epi-amino-idarubicin, which have a different sequence specificity, and teniposide (a structurally unrelated poison) stimulated higher amounts of DSB; and (c) 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-doxorubicin stimulated the highest DSB levels. For the last agent, a faster rate of cleavage resealing, which is consistent with a reduced DNA binding affinity, could account for the increased DSB/cytotoxicity ratio compared with parent drugs. However, for other analogues, the observed differences in DSB persistence/resealing could not completely explain the different DSB/cytotoxicity ratios. The results thus suggest that the cytotoxic potency of anthracyclines may be the result of an interplay of the level, the persistence, and the genomic localization of topoisomerase II-mediated DNA cleavage.

Antitumor agents such as anthracycline antibiotics, amsacrine, etoposide, and VM-26 are potent poisons of mammalian DNA topoisomerases II (1, 2). These enzymes are fundamental for the regulation of DNA topology in all living organisms (3, 4). Topoisomerase II, which is composed of two identical subunits, cleaves a double helix, then allows the passage of a second DNA segment through the break and eventually rejoins the strand breaks by acting as a two-gate molecular clamp (3). Anthracyclines and other antitumor poisons stabilize a transient DNA-topoisomerase II complex in which DNA strands are cut and covalently linked to the enzyme subunits (2, 5, 6). The complex can result in DNA damage upon detergent addition either in whole cells and with the purified enzyme (7).

In the past, the classical filter elution technique developed by Kohn (8) has allowed exhaustive investigations of drug effects at the cellular level. Early studies demonstrated that cellular DNA lesions occurred shortly after cell exposure to cytotoxic doses of poisons and rapidly reversed in the case of amsacrine and etoposide, VM-26, whereas they were more persistent in the case of anthracyclines (9–11). The high sensitivity of the method also allowed the demonstration that the drug-stimulated topoisomerase II DNA cleavage was strongly associated with the cytotoxic potency of topoisomerase II poisons (12–17). Nevertheless, accurate correlations between DSB levels and cell killing activity were not found when structurally unrelated compounds were compared (12, 18, 19).

Unrelated topoisomerase II poisons have been shown to stimulate drug-specific intensity patterns of DNA cleavage (2), whereas identical patterns were stimulated by closely related analogues of amsacrine (12), epipodophyllotoxins (20, 21), and anthracyclines (22, 23). Several groups have therefore addressed the question of whether drug-stimulated DNA cleavage patterns are also different in the genome of living cells. Pommier et al. (24) showed that amsacrine but not VM-26 selectively stimulated DNA cleavage at the c-myc P2 promoter. Genomic regions encompassing active gene coding sequences may be more prone to topoisomerase II-mediated cleavage by VM-26 than α-satellite repeats (25).

ABBREVIATIONS: VM-26, teniposide; DSB, double-strand break.

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addition, in a recent study from our laboratory, DNA cleavage sites stimulated by VM-26 and a potent doxorubicin analogue were shown to be distinct at a nucleotide level in two loci of Drosophila melanogaster Kc cell genome (26). These findings have documented, therefore, that the sequence-specific action of the poison also occurs in the nuclear chromatin of living cells. However, it remains to be established whether differences in DNA cleavage sites in the cell genome affect the cytotoxic potency of antitumor poisons.

Recently, we demonstrated that the anthracycline derivative VI has a sequence specificity distinct from that of II and I (27). This derivative requires a guanine at position 2 at the DNA cleavage site, whereas I excludes guanines and prefers thymines at the same position (22, 27). This finding has prompted us to further investigate the relations between cellular DSB levels and cytotoxicity by anthracyclines to evaluate the role of DNA cleavage localization in drug cytotoxic potency. In this study, we determined DNA DSB levels stimulated by several anthracycline analogues in human leukemic HL60 cells by the filter elution technique, together with cytotoxic effects. The quantitative relations between levels of DNA cleavage and of cytotoxicity were different analogue compounds that had different sequence specificities.

Materials and Methods

Chemicals. Anthracycline analogues were obtained from Pharmacia-Upjohn (Milan, Italy), and VM-26 was purchased from Bristol Italiana (Latina, Italy). Anthracyclines and VM-26 were dissolved in deionized water and ethanol, respectively, and diluted in the appropriate buffer immediately before use.

Filter elution assay. DNA DSBs stimulated by anthracycline analogues were determined in intact HL60 cells with the neutral filter elution procedure (8) described in detail elsewhere (28). Briefly, cellular DNA was labeled with 0.075 μCi/ml [2-14C]thymidine (Amersham International, Milan, Italy) for 24 hr at 37°. The labeled precursor was removed 16–18 hr before drug treatments. Levels of DSBs were expressed as rad-equivalents by using calibration curves obtained by irradiation of drug-untreated HL60 cells with γ-rays.

DNA binding parameters. The DNA binding affinity of anthracyclines was studied by means of fluorescence methods previously described (29–31). Calf thymus DNA was used in these experiments, and compounds were assayed at 0.1 mM NaCl at 25°C.

Cytotoxicity test. Human leukemia HL60 cells were cultured in RPMI-1640 medium plus 10% fetal calf serum (Flow, Irvine, UK) at 37°C in 5% CO2. Drug cytotoxicity was determined with a cell growth inhibition test. Cells (5 × 10^6 cells/ml) were treated for 1 hr with different drug concentrations. Cells were then centrifuged, washed, and resuspended at 10^6 cells/ml, and cultured in drug-free medium at 37°C for 3 days. Cells were counted with a Coulter counter, and cell growth was expressed as the fraction of surviving cells in treated samples compared with those in control samples.

Flow cytometry. Cellular DNA fluorescence was measured with a FACScan flow cytometry (Becton Dickinson, Mountain View, CA). Control and drug-treated cells were collected, washed with phosphate-buffered saline, and fixed in 70% cold ethanol. Fixed cells were centrifuged, washed in phosphate-buffered saline, and incubated with 5 mg/ml propidium iodide (Sigma Chemical, Milan, Italy) in the presence of 50 units/ml of RNase A (Sigma) for 1 hr at 4°C. For each sample, 10^6 cells were analyzed.

Results

Tumor-cell killing potency. The cellular effects of anthracycline analogues modified at 3′- and 4′- positions (compounds IV–VII) and parent drugs I, II, and III (Fig. 1) were investigated to examine the role of DNA cleaving activity and sequence specificity in the cytotoxic potency of topoisomerase II poisons. VI has been demonstrated to have a markedly different sequence selectivity compared with parent drugs (27), and V had a somewhat intermediate sequence selectivity between 3′-epi-DNR and parent drugs (27), whereas the other two analogues (IV and VII) stimulated cleavage intensity patterns similar to those of the parent drugs (27, 32).

Cytotoxic activity was determined on human HL60 leukemic cells using a cell growth inhibition assay after 1-hr drug exposures (Fig. 2 and Table 1). The most cytotoxic analogues were characterized by the lack of the methoxy group at the 4 position of the planar ring system (III, IV, and V) (Fig. 2). The most active compound on HL60 cells was III, which, in contrast to compounds IV and V, maintained the 3′-amino group in the natural configuration. The epimerization of NH2 was found to be unfavorable (Fig. 2) because VI was 6-fold less cytotoxic than the parent drug II (Table 1). VII was the least active agent (Table 1; Fig. 2). Thus, the epimerization of 3′-NH2 or the substitution with an epimerized OH caused the anthracycline molecule to be less active against cultured human cancer cells.

Cellular DNA breakage: relationship to cytotoxicity.

The cytotoxic potencies of the tested anthracyclines tended to be proportional to the in vitro stimulation activity of topoisomerase II DNA cleavage, whereas DNA binding parameters were poorly correlated to drug cytotoxic potency (Table 1). Therefore, the production of DSB in HL60 cells was next determined after 1-hr exposures to a range of drug concentrations (Fig. 3). DSB stimulated by all the tested analogues were protein-associated (data not shown), as expected for topoisomerase poisons (9, 19, 33). DNA cleavage increased with drug concentration; however, it decreased at concentrations equal to or higher than 1 μM II and III (Fig. 3). Suppression of DNA cleavage is expected at high doses of intercalating agents (33, 34) with high DNA binding affinity (33, 34).

Three groups of analogues can be observed with respect to the stimulation of DNA fragmentation (Fig. 3). III, IV, and V stimulated the largest amounts of breaks. Intermediate lev-
els of DNA breakage were detected when cells were treated with II or VI. Interestingly, a similar dose-response curve was observed for VM-26. Finally, I and VII stimulated very few strand breaks.

The data for VI were somewhat surprising: although the derivative was approximately 10-fold less cytotoxic than I (Table 1), it nevertheless stimulated a larger amount of protein-associated DSB than the parent drug (Fig. 3). The relationship between cytotoxic effects and DNA-DSB produced by the studied anthracyclines was examined in a more quantitative manner (Fig. 4). At equitoxic drug concentrations, IV stimulated the largest number of breaks, and V and VI stimulated intermediate amounts of DSB, whereas I, II, III, and VII stimulated only very low levels of DSB (Fig. 4). Surprisingly, the curves of VI and V closely overlapped that of VM-26 and were instead different from those of the parent drugs. Therefore, the latter analogues had a DSB/cytotoxicity ratio very similar to that of VM-26 and different from those of the parent drugs.

Persistence or resealing of DSB. Because cytotoxicity of topoisomerase II poisons has been shown to be influenced by both the level and the persistence of DNA cleavage (11, 17, 33), we evaluated the reversibility of cellular DSB stimulated by anthracyclines. The time courses of DSB were measured after 1-hr drug exposures; cells were exposed to drugs for 1 hr at 37°, lysed on the filter in the presence of proteinase K, and eluted at pH 9.6. DSB are expressed in rad-equivalents. Points, means of 2–4 independent experiments; bars, standard errors.

**TABLE 1**

Cytotoxic potency, DNA binding affinity constants, and in vitro topoisomerase II cleavage of the anthracycline derivatives studied

The compounds are listed in order of decreasing cytotoxic potency. IC₅₀ values were determined from dose-response curves from at least three independent experiments (see Fig. 2 for other details). K_app was determined in 0.1 M NaCl at 25°. n represents the apparent number of drug binding sites per nucleotide. Drug DNA cleavage activity is expressed in a semiquantitative manner as determined previously using an in vitro assay and murine topoisomerase II.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytotoxicity (μM)</th>
<th>DNA binding-affinity constants (K_app x 10ⁿ)</th>
<th>In vitro DNA cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n⁻¹</td>
<td>K</td>
</tr>
<tr>
<td>III</td>
<td>0.02</td>
<td>2.4</td>
<td>0.170</td>
</tr>
<tr>
<td>V</td>
<td>0.05</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>IV</td>
<td>0.17</td>
<td>1.0</td>
<td>0.072</td>
</tr>
<tr>
<td>II</td>
<td>0.20</td>
<td>4.8</td>
<td>0.160</td>
</tr>
<tr>
<td>I</td>
<td>0.80</td>
<td>6.5</td>
<td>0.179</td>
</tr>
<tr>
<td>VI</td>
<td>1.37</td>
<td>0.86</td>
<td>0.215</td>
</tr>
<tr>
<td>VII</td>
<td>2.60</td>
<td>0.32</td>
<td>0.149</td>
</tr>
</tbody>
</table>

n.d., not determined.

+++ doxorubicin activity; ++, less than doxorubicin activity; ++++, greater than doxorubicin activity.
up to 2 hr in drug-free medium; instead, they tended to increase for I and compound VII (Fig. 5B). In contrast, VM-26–stimulated DSB showed a rapid and almost complete reversal after 2 hr (Fig. 5A). However, protein-associated DSB dropped to 54%, 78%, and 80% of the initial cleavage level in cells treated with IV, V, and VI, respectively, after 60 min in drug-free medium (Fig. 5A). Therefore, for these analogues, partial reversal and no persistence of DNA cleavage were observed in HL60 cells.

DSBs were shown to be protein-associated in all cases (data not shown); however, in cells incubated with compounds IV and V, a large fraction of DSB (see below) was not protein-associated (data not shown) after 120 min in drug-free medium, which suggests that in these cases, DNA fragmentation was caused by factors other than DNA topoisomerase II. Therefore, for these two compounds, the levels of topoisomerase II-dependent DSB could not be determined at the 2-hr data point (Fig. 5A). Thus, the results showed that DNA cleavage of IV, V, and VI was less persistent than that of parent drugs, but it was also less reversible than that of VM-26.

Early apoptosis by high levels of anthracycline-stimulated DSB. We have further investigated the abrupt increase of DNA fragmentation after 2 hr from the removal of compounds IV and V from the medium by FACScan analysis under the same conditions as those used to measure DSB persistence (Fig. 6). Indeed, after 120 min in drug-free medium, a significant amount of fragmented DNA could be detected in HL60 cells incubated for 1 hr with IV and V at a drug level corresponding to IC50. We could calculate that 20% and 40% of HL60 cells were apoptotic after 2 hr from treatments with compounds IV and V. In contrast, for I and the other studied anthracycline analogues, DNA fragmentation was not detected under similar conditions by FACScan analyses (Fig. 6, B and C).

Discussion

Cellular DNA damage mediated by topoisomerase II is the primary lesion responsible for the antitumor activity of anthracyclines (23). Previous studies have documented that the extent and the persistence/resealing of cellular DNA cleavage correlate well with the cytotoxic potency of topoisomerase II poisons (17, 33, 34). In the present study, we have investigated seven anthracycline analogues, including VI and V, that showed an altered sequence specificity of topoisomerase II-mediated DNA cleavage in an in vitro assay (27). Our results suggest that in addition to the level and persistence, the site selectivity of drug-stimulated DSB may also contribute to the cytotoxic activities of the compounds studied.

The tested analogues were characterized by a wide range of cytotoxic potency and had structural modifications of the 3’- and 4’-sugar substituents and/or the removal of the methoxy group. A rough correlation was observed between the extent of DSB and cytotoxic levels because the most cytotoxic agents (the 4-demethoxy analogues III, IV, and V) were also the most effective in stimulating DSB (Table 1). This finding is in agreement with several published reports (32, 33, 35, 36). 3’-epi Derivatives, which are known to be less effective as topoisomerase II poisons in vitro (27) and in living cancer cells (Fig. 3), were also characterized by a reduced cytotoxic potency (Table 1). Nevertheless, I, which caused fewer DSBs with respect to VI (Fig. 3), was more cytotoxic. After drug removal, 20% of the DNA cleavage stimulated by VI was resealed, whereas in the case of I, DSB levels slightly increased. Differences in DNA cleavage persistence/resealing could partially explain the behavior of the different analogues, which stimulated different levels of DSB at equitoxic concentrations (Fig. 4). For example, IV stimulated 3000 rad-equivalents of DSB at the IC50, whereas I had the same rate of cell killing with 100 rad-equivalents. However, for IV, half of the DSB resealed after 60 min in drug-free medium, whereas for I, DNA cleavage persisted at the same level after 1 hr. The reversibility of the DSB stimulated by the analogue may be related to a reduced DNA binding affinity. Interestingly, VM-26, which stimulated a higher level of DSB than I at equitoxic levels, also stimulated DNA cleavage that was highly reversible. These observations emphasize the significance of the persistence of DNA lesions caused by topoisomerase poisons in the cell-killing mechanism. It is conceivable that long-lived covalent topoisomerase-DNA complexes have a higher probability of interfering with replication and
transcription machineries, thus triggering cell death processes (37).

Three classes of analogues could be distinguished on the basis of the relationship between DSB levels and cytotoxic activity (Fig. 4). One group, which stimulated very few DSB, gathered I, II, III, and VII; a second group, which stimulated an intermediate amount of DSB, included V and VI; and a third group, consisting only of IV, stimulated a larger amount of DSB (Fig. 4). Therefore, the studied analogues displayed different ratios of DSB to cytotoxic levels, which may be partially explained by the observed differences in persistence/resealing of DSB. Nevertheless, VM-26–stimulated DSBs were much more quickly resealed than DNA cleavage stimulated by any anthracycline analogue, although its DSB/cytotoxicity ratios fit with the curves of the second group (Fig. 4). Moreover, (a) III, which had DSB/cytotoxicity ratios similar to I, stimulated DSBs that were partially resealed after 2 hr in drug-free medium, as was the case with VI; (b) VII (VII) also had a DSB/cytotoxicity ratio similar to those of I and III, but DNA cleavage much increased after drug removal (Fig. 5B). Therefore, another factor may influence the lethal potential of DNA lesions caused by antitumor anthracyclines. Previous investigations of topoisomerase II-mediated DNA cleavage in an in vitro system have shown that V and VI analogues have an altered site specificity with respect to parent compounds (27). In particular, VI stimulated markedly different cleavage intensity patterns compared with I cleavage patterns. Moreover, recent studies demonstrated that the in vitro sequence specificity of VM-26 and compound IV was maintained in the nuclear chromatin of living cells (26). Thus, it is conceivable that VI and V may cause DBS at sites different from those of parent anthracyclines. Distinct genomic sites of topoisomerase II-mediated DBS may differ with potentially lethal consequences in human leukemic HL60 cells, and differences in DNA cleavage localization in the nuclear chromatin of living cells may thus critically influence drug activity. It has been reported that repair of ultra violet-induced pyrimidine dimers is heterogeneous in the mammalian genome. In particular, active genes are repaired faster than inactive genes (38), the transcribed strand is repaired preferentially over nontranscribed strands, and the promoter sequence is repaired more slowly than the region near the transcription initiation site (39). Therefore, DSB generated by DNA topoisomerase II at some genomic loci (i.e., active gene regions) may be more prone to be converted into irreversible lesions as a result of ongoing DNA-dependent process (i.e., transcription or replication), whereas at other loci (i.e., euterochromatin), DSB may be converted into irreversible lesions much less frequently because of the absence of these nuclear processes. Further
investigations with other poisons are needed to establish the role of the site selectivity in drug activity and the relevant molecular mechanisms.

Another conclusion can be drawn from our results: anthracyclines can induce an early apoptosis in HL60 cells. Early apoptosis has previously been described for other drugs (40); the authors suggested that for each drug, a threshold concentration (different for each drug) existed that could trigger an early DNA degradation process (40). In our experiments, we observed an early apoptosis for IV and V, which stimulated the largest extent of DNA DSBs after 1-hr incubation. The molecular basis of such an early event remains to be investigated; however, we suggest that a large amount of DNA cleavage may be necessary to reach the threshold value that triggers the early apoptotic process.

Finally, this structure-activity study emphasizes the critical role of the amino sugar in determining biological activity. In particular, these results support previous observations that the nature of the substituent at the 3′-position is critical for drug activity (35). Indeed, the amino group is not essential for drug activity because it could be removed or replaced by a hydroxyl group. However, epimerization of substituents at this position caused a marked reduction of drug efficacy. These observations provide further evidence that external interactions involving the sugar residue play a critical role in drug activity because it could be removed or replaced by a hydroxyl group. However, epimerization of substituents at this position caused a marked reduction of drug efficacy. These observations provide further evidence that external interactions involving the sugar residue play a critical role in determining a drug’s ability to stimulate enzyme-mediated DNA cleavage.

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