**In Vivo Site Specificity and Human Isoenzyme Selectivity of Two Topoisomerase II-poisoning Anthracyclines**

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**ABSTRACT**

Anthracyclines exert antitumor activity by stimulating site-selective DNA cleavage by topoisomerase II (top2). DNA cleavage sites stimulated by two anthracycline analogues, dh-EPI and da-IDA, were investigated at the histone gene cluster of cultured Drosophila Kc cells. The two agents stimulated analogue-specific patterns of double-stranded DNA cleavage in Kc cell chromatin. Analyses of 47 base sequences of dh-EPI sites showed that the analogue largely followed the *in vitro* selectivity rule, the requirement of $5' TA$ at $3'$ ends of cleaved strands. da-IDA was more selective than dh-EPI, and thus fewer sites could be collected. Nevertheless, base sequences were consistent with its *in vitro* base preferences. DNA cleavage was then studied *in vitro* with Drosophila and human top2 isoforms. The tested drugs stimulated distinct *in vitro* patterns that corresponded to the *in vivo* patterns. Human top2a promoted cleavage patterns that were much more similar to those of Drosophila top2 (both *in vitro* and *in vivo*) than human top2β. Moreover, da-IDA showed a marked site-dependent preference for human top2β. Thus, DNA site selection *in vivo* is different for the two anthracyclines, and together with a degree of β-form specificity, may affect drug activity in human cells.

**INTRODUCTION**

Among the many drugs used in the chemotherapy of human cancers, anthracycline antibiotics have found one of the widest applications because of very broad and efficient activity against solid tumors and leukemias. A large body of evidence shows that doxorubicin and closely related anthracycline analogues transform top2 in a DNA-damaging agent by trapping an enzyme-DNA covalent complex wherein DNA strands are cut and covalently linked to the protein (1–4). The drug-trapped complex eventually triggers a cell death program (1, 5). Despite the wealth of knowledge in the field, the high cell-killing efficiency of anthracyclines relative to DNA cleaving activity has not found a sound basis at a molecular level. Promotion of apoptosis by anthracyclines has also been proposed to be caused by other molecular drug actions, such as free radicals generated by redox cycling or by covalent DNA-drug binding. To further establish the contribution of top2-dependent mechanisms to drug activity, here we have focused on molecular properties of top2-mediated DNA breaks in chromatin of living cells.

For chemically related top2 poisons, a good correlation exists between the extent and persistence of cellular DNA cleavage and the cytotoxic potency of drugs (1, 3, 4, 6). Interestingly, the cleavage: cytotoxicity ratio varies greatly among unrelated poisons and is lowest for anthracyclines (1, 3, 6, 7). Therefore, DNA breaks promoted by anthracyclines might be more lethal than those promoted by other top2 poisons; however, this remains to be proved fully. top2 poisons stimulate *in vitro* DNA breaks in a sequence-selective manner (1–4), and the site selectivity of anthracyclines has been studied extensively (3). In particular, doxorubicin requires $5' TA$ dinucleotides at the $3'$ terminus of the strand cut, corresponding to $-2$ and $-1$ positions, respectively (3, 8). In contrast, much less is known for base sequence preferences in the chromatin of living cells. We showed previously that dh-EPI, a potent anthracycline analogue, had a distinct locus and site specificity in the genome of *Drosophila* Kc cells (9, 10). In contrast to VM-26 and clerocidin, dh-EPI was completely unable to stimulate cleavage in the heterochromatic satellite III DNA, whereas it was very active at the histone gene locus (9, 10). However, drug nucleotide preferences remained to be established *in vivo*.

Thus, to determine the *in vivo* base preferences of anthracycline activity, we have investigated at nucleotide levels cleavage sites of dh-EPI and da-IDA (Fig. 1A) at the histone gene cluster of Kc cells and *in vitro* with Drosophila and human top2s. The two studied analogues are closely related to doxorubicin structure (11, 12) and mainly differ at the 3 and 4 position of the sugar (Fig. 1A). The histone gene cluster was selected for this study because top2-dependent DNA cleavage is much more easily detected *in vivo* in multicy copy DNA regions and is a locus of high activity of top2 poisons (9, 10). dh-EPI and da-IDA had different cleavage:cytotoxicity ratios in human HL60 leukemic cells; in particular, DNA lesions stimulated by da-IDA were more lethal than those of dh-EPI (6). Because the two analogues also stimulated distinct cleavage patterns with murine top2 (11, 12), genomic localization of cleavage may be a significant factor influencing drug activity. We show that the two analogues mainly maintain the *in vitro* base requirements in nuclear chromatin of *Drosophila* cells. Moreover, da-IDA showed a marked site-dependent preference for human top2β.

**MATERIALS AND METHODS**

**Materials.** dh-EPI and da-IDA were provided by A. Suarato (Pharmacia-Upjohn, Milan, Italy), dissolved in DMSO, and diluted in water before use. T4 polynucleotide kinase, agrose, plasmid DNAs, and acrylamide were purchased from United States Biochemical Corp. (Cleveland, OH). Other enzymes were from New England Biolabs (Taunton, MA). YEpWOB6 (provided by C. Austin, Newcastle-upon-Tyne University, Newcastle-upon-Tyne, United Kingdom) or YEphTOP2 -165S plasmids that expressed top2α and top2β, respectively (13, 14). Enzymes were purified and stored as described already (14, 15). YeplTop2β-165S plasmids that expressed top2α and top2β, respectively (13, 14). Enzymes were purified and stored as described already (14, 15). YeplTop2β-165S plasmid, which encodes for a wild-type top2β, was obtained mutating the Arg$^{165}$ to Ser of YeplTop2β plasmid, which encodes for a mutant enzyme (16).

**Low-Resolution Mapping of Cleavage Sites.** Drosophila Kc cells were cultured as described (9). Exponentially growing cells were treated with 10 μM dh-EPI or da-IDA for 30 min at 25°C. Then, cells were centrifuged; lysed in 0.1% SDS, 20 mM Tris-HCl (pH 8), and 0.5 mg/ml proteinase K; and incubated overnight at 37°C. DNA was purified as described (9, 10) and digested with HindIII to generate the 5-kb major repeat of the histone gene cluster (Fig. 1C).
Fig. 1. A. structures of dh-EPI and da-IDA. B. DNA cleavage stimulated by dh-EPI and da-IDA at the histone gene repeat of *D. melanogaster* Kc cells. Cells were treated with 10 μM dh-EPI or da-IDA for 30 min at 25°C. DNA were purified and analyzed with the end-labeling technique. Lane C, untreated cells. Cleavage sites in the SAR and in the H2A-H2B gene region are shown on the left and right panels, respectively. Numbers and lines mark cleavage sites. Asterisks indicate histone gene repeat variants shorter than 3 kbp. C, diagram of the histone gene cluster (not drawn to scale). Cleavage sites are indicated by vertical bars and numbers. Bars without numbers correspond to sites described in a previous report (9). HP and HB probes were used in the left and right panels of B, respectively. Horizontal arrows show genes and direction of transcription. Open circles, uniquely positioned nucleosomes.

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Agarose gel electrophoresis and Southern blotting were performed by standard methods. 32P-labeled HP or HB probes (Fig. 1C) were used to map cleavage sites in the SAR or H2A-H2B region, respectively (9).

High-Resolution Mapping of Cleavage Sites. Mapping of DNA breaks at nucleotide levels was based on premature termination of primer extension because of drug-dependent interruption of templates and on thermostable, nonproofreading Taq DNA polymerase (9, 10, 17). Genomic DNAs from control and drug-treated cells were digested with *MspI* and *Hae*III for the intergenic H2A-H2B and H2B coding regions, respectively. Four primers were used for the H2A-H2B intergenic region: upper strand, D10-1 (5'-CACCACCTTTTCCACG) and D10-3 (5'-GTCGCCAGACATTTTGCTTTGCG) from 160 to 180, and 179 to 200 positions, respectively; lower strand, D10-2 (5'-GTTTTCGGAGGCATTGTTCAC) and D10-4 (5'-CTTGGTGTTCTTCTGAGCC), from 429 to 409, and 481 to 460 positions, respectively (GenBankEMBL X14215). Primers for the H2B coding region were: D11-1 (upper strand, 5'-GTGAACAATGCCTCCGAAAACTAGTGG), from 409 to 435 positions, and D11-2 (lower strand, 5'-GCTTGGCCAAGCAATTTGCGT), from 639 to 619 positions. Primer extension was performed using 1 μg of genomic DNA and 5 x 105 cpm/sample of labeled primers. Taq DNA polymerase (1.25 units) was added in 50 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM MgCl2 supplemented with 10 μM of each deoxynucleotide triphosphate. Identical results were obtained when using other polymerases without proof-reading activity (9, 10). Samples were subjected to 30 cycles of 1/1/3 min at 94/58/72°C, followed by a final 15-min extension at 72°C. Then, samples were extracted with phenol/chloroform; precipitated in ethanol; resuspended in 2.5 μl of 80% formamide, 10 mM NaOH, 1 mM EDTA, and 0.1% dyes; heated at 90°C for 2 min; chilled on ice; and loaded onto 8% polyacrylamide gels. Sequence ladders were obtained as described already (9).

RESULTS

Analogue-specific, Double-stranded DNA Cleavage in Kc Cell Chromatin. We have determined DNA cleavage sites at the histone gene cluster of *Drosophila* Kc cells treated with two anthracyclines, dh-EPI or da-IDA (Fig. 1A), for 30 min at 25°C. The tested drugs stimulated different cleavage patterns in vitro, with murine top2 using SV40 DNA as substrate (11). Structure-activity relationships established that structural changes at the sugar 3' position were responsible for altered site selectivity (11, 12). dh-EPI, which has a 3'-OH group, has the same sequence specificity of doxorubicin, whereas da-IDA, which has no 3' substituent, stimulated distinct cleavage intensity patterns (11).

Double-stranded cleavage sites were mapped by the indirect end-labeling method (9, 10). Cleavage patterns were similar but not identical between the two analogues in the SAR and H2A-H2B intergenic region (Fig. 1B). Overall, dh-EPI promoted more cleavage sites than da-IDA, which was thus more selective. At the SAR, da-IDA stimulated cleavage at a subset of the sites detected with dh-EPI (Fig. 1, B, left panel, and C). In the H2A-H2B region, da-IDA stimulated sites 9, 10, and 11, whereas dh-EPI stimulated sites 9 and 10 and several others in the H3-H4 region (Fig. 1B and not shown; see also Refs. 9 and 10). It must be noted that site 11 was specific for da-IDA, because dh-EPI-dependent cleavage was very weak (Fig. 1B, right panel), and mapped in the middle of the H2B gene (Fig. 1C). Thus, the molecular actions of two closely related anthracyclines could be distinguished at the histone gene cluster of *Drosophila* Kc cells.

Analogue-specific Sites at Nucleotide Levels. To establish the in vivo sequence specificity of the studied drugs, we sequenced cleavage

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Cuts were mapped at nucleotide levels in both strands. Each strand was extended with two distinct and adjacent primers (see "Materials and Methods"), and extension stops specifically present in the "drug lanes" were detected with either primer at identical nucleotide positions (Fig. 2 and not shown). Thus, primer mispairing was unlikely to be the cause of the observed drug-dependent stops. Rather, they were likely attributable to the poison-dependent trapping of top2-DNA complexes that yielded interrupted templates (9, 10, 17).

At nucleotide levels, both dh-EPI and da-IDA stimulated high cleavage levels at the TATA box (site \( n \)) of the \( H2A \) histone gene (Fig. 2A). dh-EPI, but not da-IDA, stimulated other sites throughout the region. A rather high cleavage level was observed close to the CAP box of \( H2A \) (sites \( i \) and \( j \); Fig. 3A and not shown), and cleavage (site \( x \)) was weakly detected at the TATA box of \( H2B \) (Figs. 2A and 3A). A striking difference was observed in the \( H2B \) coding sequence (Fig. 2B). Site \( \theta \) was the strongest one for da-IDA, whereas site \( \mu \) was strongest for dh-EPI. Moreover, sites \( \theta \) stimulated by the two analogues were not at the same position but 1 bp far apart from each other (Figs. 2B and 3B). dh-EPI also stimulated weaker sites (\( \lambda \) and \( \eta \); Figs. 2B and 3B).

Cuts were mapped in both strands, and the complete results are reported in Fig. 3. Only in case of da-IDA, the \( \theta \) site on the top strand, had a 4-bp-staggered cut on the other strand (Fig. 3B), in agreement with the observed double-strand cut (site 11) in agarose gels (Fig. 1B). In the case of dh-EPI, the \( \theta \) sites on the two strands were not staggered by 4 bp. Thus, in contrast to da-IDA, dh-EPI stimulated cleavage at least at two distinct and alternative sites characterized by very weak cuts on one strand (Fig. 3B). dh-EPI action at nucleotide levels was consistent with an analogue-specific lack of double-stranded cleavage as observed in agarose gels (Fig. 1B). In the \( H2A-H2B \) intergenic region, sites \( n \) and \( u \) had a corresponding cut on the other strand with a 4-bp stagger for either analogue (Fig. 3A), consistent with double-stranded cleavage. It is likely that these two sites were mainly responsible for site 10 (Fig. 1B). Overall, 15 of 25 were "single" sites because they were not paired with a corresponding cut at the complementary strand. Because several of them were weak, the break on the other strand might have not been strong enough to be detected in our assay.

Base Preferences of in Vivo Anthracycline Action. Forty-seven dh-EPI-stimulated sites were collected, and base preferences could be determined. Nonrandom base distributions were observed at positions \(-1 \) and \(+1 \) by \( \chi^2 \) analyses, where adenines and guanines were strongly preferred, respectively (Table 1). Of the 47 sites, 23 (49%) had at least an adenine at position \(-1 \), and 3 (6%) had an adenine at the \(-1 \) position of the complementary strand only. Of the sites without adenosines at \(-1 \) positions of both strands (\([\sim 1]A\) sites), 14 (30%) were 1–2 bp close to sites with adenosines at \(-1 \) positions (\([\sim 1]A\) sites), and 7 (15%) were more distant from other sites (Fig. 3). Indeed, some breaks (20) constituted nine clusters of two to three adjacent cuts, 1–2 bp close to each other (Fig. 3), suggesting a reciprocal influence among very close sites (see "Discussion"). Because the majority of in vivo sites had adenosines at \(-1 \) positions, a further statistical analysis was then made by considering only the base sequences of \([\sim 1]A\) sites (Table 1, lower part). Although the sequences were limited, we could derive an in vivo consensus for dh-EPI action that completely agrees with the in vitro doxorubicin consensus (3, 8) showing a \( ^5 TA \) dinucleotide highly preferred at both \( 3' \)-termini of strand cuts (Table 1). Interestingly, a guanine was strongly preferred at position \(+1 \) (Table 1), and this was unexpected based on in vitro data (3). These data show that dh-EPI mainly maintained in nuclear chromatin the in vitro-established base requirements (3, 8).
Nevertheless, a significant fraction of sites appeared not to follow such a rule. The low number of sites stimulated by da-IDA did not allow an evaluation of its \textit{in vivo} local base preferences. However, all strong sites, n, u, and e, had the expected base requirement on both strands. Interestingly, site u, which is a strong double-stranded break site for da-IDA but not for dh-EPI (Fig. 1), has $5_{\text{9}}$GA$3_{\text{9}}$ at \(2_{\text{2}}\) and \(2_{\text{1}}\) positions at one of the two strand cuts. A guanine at position \(2_{\text{2}}\) has been shown \textit{in vitro} to be highly preferred by anthracycline analogues with modifications at the \(3_{\text{9}}\) sugar position (3, 11).

Analogue-specific DNA Cleavage Sites with Purified Enzymes. We then asked the question of whether the observed \textit{in vivo} sites were also cleaved by purified top2. DNA breaks were examined with the \textit{Drosophila} enzyme in a cloned intergenic H2A-H2B fragment (Fig. 4). Although the purified protein mediated strong cleavage with VM-26, it was weakly sensitive to both the tested analogues, and four sites only were detected in the whole region. Two of them (site n and e) were stimulated \textit{in vivo} as well (Fig. 2). Interestingly, da-IDA was more specific than dh-EPI because it stimulated only site n, whereas dh-EPI was active also at sites \(b\), \(g\), and \(e\) (Fig. 4). da-IDA was more selective than dh-EPI in an unrelated DNA fragment as well (not shown), showing that analogue specificity was not restricted to a particular substrate. Thus, drug interactions with \textit{Drosophila} top2 were analogue specific, supporting that they may occur \textit{in vivo} as well.

Interesting results were obtained with human top2 isoforms.

### Table 1. Base frequency (%) and \(\chi^2\) values at dh-EPI-stimulated DNA cleavage sites in cellular chromatin

| Base | All sites (n = 47) | \([-5\) | \([-4\) | \([-3\) | \([-2\) | \([-1\) | \([+1\) | \([+2\) | \([+3\) | \([+4\) | \([+5\) | \([+6\) | \([+7\) | \([+8\) | \([+9\) |
|------|------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| A    | 28.3             | 28.3   | 28.3   | 23.9   | 47.8   | 17.4   | 19.6   | 28.2   | 28.3   | 21.8   | 32.6   | 30.4   | 21.7   | 8.7   |
| T    | 19.6             | 28.3   | 47.8   | 34.8   | 10.9   | 13.0   | 19.6   | 37.0   | 34.8   | 34.8   | 17.4   | 26.1   | 43.5   | 45.6   |
| C    | 23.9             | 21.7   | 10.9   | 15.2   | 8.7    | 23.9   | 23.9   | 17.4   | 13.0   | 21.7   | 30.4   | 28.3   | 8.7    | 26.1   |
| G    | 28.2             | 21.7   | 13.0   | 26.1   | 30.4   | 45.7   | 36.9   | 17.4   | 23.9   | 21.7   | 19.6   | 15.2   | 26.1   | 19.6   |
| \(\chi^2\) | 5.13              | \(<0.01\) | 21.6 | 4.96   | 35.2   | 40.3   | 16.9   | 4.10   | 5.02   | 2.97   | 8.77   | 4.33   | 18.1   | 25.2   |

| Base | \([-1]\) A sites (n = 23) | \([-5\) | \([-4\) | \([-3\) | \([-2\) | \([-1\) | \([+1\) | \([+2\) | \([+3\) | \([+4\) | \([+5\) | \([+6\) | \([+7\) | \([+8\) | \([+9\) |
|------|--------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| A    | 34.8                      | 17.4   | 21.7   | 8.7    | 100   | 8.7    | 26.1   | 30.4   | 39.1   | 17.4   | 43.5   | 26.1   | 30.4   | 4.3   |
| T    | 21.7                      | 21.7   | 43.5   | 43.5   | 13    | 17.4   | 30.4   | 17.4   | 56.5   | 17.4   | 26.1   | 34.8   | 43.5   | 34.8   |
| C    | 17.4                      | 34.5   | 13.1   | 30.4   | 17.4   | 26.1   | 30.4   | 26.1   | 8.7    | 21.7   | 30.4   | 8.7    | 26.1   | 26.1   |
| G    | 26.1                      | 26.1   | 21.7   | 17.4   | 60.9   | 30.4   | 17.4   | 17.4   | 17.4   | 26.1   | 26.1   | 17.4   | 17.4   | 26.1   |
| \(\chi^2\) | 4.8                       | 14.8   | 12.8   | 26.1   | 95.2   | 9.2    | 11.6   | 10    | 40.2   | 13    | 4.9    | 10.1   | 30.4   | 3773 |

In the statistical analyses, only one strand cut was considered for 4-bp staggered sites with \([-1]\) adenines at both strands.

Fig. 3. Map of \textit{in vivo} top2-promoted DNA cleavage sites. Cuts were mapped taking into account the addition of an untemplated base at 3' ends of extended primers by Taq polymerase (9). Vertical arrows and letters indicate cleavage sites (see also Fig. 2). Downward triangles, cleavage sites stimulated by dh-EPI with purified top2 (note that only the upper strand in A has been investigated \textit{in vitro}). ATG codons are in italics. The lowercase letters indicate regions that were not analyzed \textit{in vivo}.
two isozymes were more sensitive to anthracyclines than Drosophila top2, and the analogues stimulated cleavage at several sites (Fig. 5). da-IDA was again more selective (and potent) than dh-EPI. As expected (3), all of the observed cuts had an adenine at −1 positions (Fig. 3), and 6 of 16 in vitro cuts were also detected in nuclear chromatin (Figs. 3 and 5). The absence of sites in vivo is explained by chromatin proteins that restrict DNA accessibility to top2 (1, 3, 9, 21). Surprisingly, the site selectivity of human isozymes was in part, but significantly different, most notably with da-IDA (Fig. 5). The highest cleavage level was always found at site n with top2α. However, with top2β 0.5 μM of dh-EPI stimulated higher cleavage levels at site γ than n, and da-IDA at 0.5 μM stimulated comparable levels at sites γ, e, and site n (Fig. 5). Differences between the two human proteins were more striking with the more selective analogue, da-IDA. This compound exclusively stimulated cleavage at site n with top2α, whereas sites n, γ, δ, e, and e were detected with top2β (Fig. 5). Thus, da-IDA had a preference for top2β at a number of sites.

DISCUSSION

The present results demonstrate that two closely related compounds poison top2 at different preferred sites in the chromatin of Drosophila cells, and that the human top2β stimulated diverse cleavage patterns, particularly with da-IDA, as compared with top2α. Thus, DNA site selection in vivo, together with isoform selectivity of some anthracyclines, may affect drug cytotoxicity in human cancer cells.

Strand breaks were mapped at nucleotide levels in vivo by genomic primer extension with a thermostable DNA polymerase, and the observed cuts were largely promoted by the endogenous top2 based on the following considerations: (a) nucleotide-selective stops of primer extensions were dependent upon drug treatments of cells and were consistently observed in independent experiments (see also Refs. 9 and 10); (b) extension of different but adjacent primers was stopped at identical nucleotide positions when using the same genomic DNA as template; (c) major in vivo break sites were also in vitro sites of top2-dependent DNA cleavage; and (d) local base preferences of poisons, as established in vitro (3), were largely maintained in nuclear chromatin of Kc cells for anthracyclines (this work) and for VM-26 and clerocidin (9, 10, 17).

Here, we have reported 47 in vivo sites of DNA cleavage stimulated by dh-EPI and 9 sites stimulated by da-IDA, 3 of which were specific for this compound. Many of the dh-EPI sites (55%) had an adenine at −1 positions; however, a consistent number of sites (45%) did not. Interestingly, many [−1]noA sites (30%) were very close (1–2 bp) to [−1]A sites. The observation of site clusters in chromatin may indicate that the enzyme can adjust the precise cut site of one or two nucleotides. In fact, if drug stabilization of enzyme-DNA complexes at one site causes a local enrichment of enzyme molecules, this may lead to an increased top2 cleavage activity at nearby sites as well. Modulation of cleavage levels by close sites was indeed observed in SV40 DNA with murine top2 (22). Alternatively, the addition by Taq polymerase of two, instead of only one, untemplated nucleotides at 3′ termini of a fraction of extended strands might result in extra bands in the gel (9, 18).

However, 15% of [−1]noA sites do not apparently follow the in vitro rule and were not close to other sites, showing that anthracyclines can apparently stimulate in vivo DNA cleavage with a different sequence selectivity. The origin of these [−1]noA sites remains unclear, and some explanations can be proposed: (a) the lack of drug-preferred bases would result in a drug receptor for which the drug has a lower affinity (3). Then, it might be possible that strong chromatin-enzyme interactions may determine the formation of ternary DNA-enzyme-poison complexes in these cases; (b) recently, we showed that anthracyclines can poison human top1 at relatively high concentrations (20). Because these [−1]noA sites are apparently single-stranded cleavages, we cannot rule out the possibility that these breaks are generated by top1; (c) anthracyclines were shown to form a stable covalent adduct with DNA in the presence of formaldehyde or glyceraldehyde (23). We do not know whether the adduct may occur in cultured cells at 10 μM (our experimental condition); however, it might lead to topoisomerase-independent strand breaks that could be detected by our assay.

Our results parallel previous observations with mammalian top1. Cleavage sites stimulated by camptothecin, a top1 poison, have been reported in the coding strand of the human 18S rRNA gene (24). Four of seven sites matched the poison-preferred nucleotides (5′ TG) at the cut site (25), and the authors also showed that in vivo and in vitro sites were generally consistent with each other, although relative intensities were somewhat different (24). An earlier study (26) investigated at nucleotide levels the in vivo sites stimulated by camptothecin in the SV40 genome during replication. Again, the large majority of the cuts had the drug-preferred nucleotides at the cut site. Thus, although some in vivo breaks cannot be predicted by in vitro-established, site-selective rules, altogether the results show that poisons of top1 and top2 mainly maintain in vivo the sequence selectivity shown in vitro (3, 9, 10, 24, 26).

How the differences in base specificity of distinct poisons may project on different lesion lethality is a matter of further investigation. Nevertheless, it may affect the site selection of cleavage genome wide (9, 10). This is best seen in the case of VM-26 and dh-EPI, two in vivo equally potent poisons. dh-EPI does not stimulate cleavage in the satellite III DNA, whereas VM-26 is weakly active in the histone gene region studied in this report (9, 10). Site selection in nuclear chroma-
tin is likely the result of a combination of several factors, including chromatin structure, enzyme site selectivity as well as drug sequence specificity (10).

A second major finding of the present study is the isoform specificity of da-IDA that shows a site-dependent preference for top2β (Fig. 5). This may add a further level of specificity to poison action in mammalian cells; an altered balance of the two isozymes in tumor cells may influence cleavage sites and cytotoxicity. Recently, it was reported that a specific point mutation in yeast top2 (Ser740 to Trp) confers resistance to quinolone and hypersensitivity to etoposide, markedly altering the enzyme sequence selectivity with and without poisons (27, 28). The mutation may change enzyme-DNA interactions, thus influencing the poison binding site at the protein-DNA interface (28). At the equivalent positions, a serine residue (Ser763) is present in top2α, whereas an alanine residue (Ala784) is present in top2β. It must be noted that Ser740 to Ala mutation has been shown to confer a low degree of quinolone resistance to yeast top2 (27). Therefore, we hypothesize that the amino acid difference, Ser763 versus Ala784, is responsible for the different site-dependent sensitivity to da-IDA of human top2 forms. Interestingly, da-IDA-stimulated patterns with Drosophila top2 were identical to those with top2α, consistent with the presence of a Ser in the insect enzyme at the equivalent position of the yeast Ser740. The observations thus suggest that anthracycline interactions may be subtly altered in the ternary complex with top2β.

With the filter elution technique, we observed that the two analogues were equipotent in stimulating double-strand breaks in human HL60 cells genome wide (6). Nevertheless, different cleavage:cytotoxicity ratios were found for the two analogues; in particular, da-IDA-stimulated breaks were more lethal than those of dh-EPI (6).

Because the more selective analogue, da-IDA, generally stimulate cleavage at a subset of dh-EPI sites, sites stimulated by both analogues may be more lethal than those stimulated by dh-EPI only. Diverse antitumor drugs, including nitrogen mustards, alkylating compounds, cisplatin, and topoisomerase poisons, kill cells by producing sequence-selective DNA lesions (29–33). Moreover, new pyrrole-imidazole polyamides have been designed that can specifically bind to genomic sites interfering with the biological activity of transcription factors (34, 35). Among clinically used agents, mitomycin C is a highly specific alkylating agent of CpG sites (36), and drug cross-linking activity has been shown to be greatly affected by local DNA structure (37). This might result in heterogeneous genomic localization of the damage with consequences on the drug biological activity. Specific interactions of cisplatin-DNA adducts with nuclear proteins can also occur that might influence drug activity (38, 39). A specific interaction of cisplatin-DNA adducts with a testis-specific protein (SRY) has been proposed to explain the high efficacy of cisplatin in the treatment of testicular tumors (29, 40). Thus, the sequence selectivity of several nucleic acid-targeted drugs or their specific molecular interactions can be important determinants of the biological activity of clinically useful or promising compounds.

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