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Topoisomerase Poisoning Activity of Novel Disaccharide Anthracyclines

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ABSTRACT

Doxorubicin and idarubicin are very effective anticancer drugs in the treatment of human hematological malignancies and solid tumors. These agents are well known topoisomerase II poisons; however, some anthracycline analogs recently have been shown to poison topoisomerase I. In the present work, we assayed novel disaccharide analogs and the parent drug, idarubicin, for their poisoning effects of human topoisomerase I and topoisomerases IIα and IIβ. Drugs were evaluated with a DNA cleavage assay in vitro and with a yeast system to test whether the agents were able to poison the enzymes in vivo. We have found that the test agents are potent poisons of both topoisomerases IIα and IIβ. The axial orientation of the second sugar relative to the first one of the novel disaccharide analogs was shown to be required for poisoning activity and cytotoxicity. Interestingly, idarubicin and the new analogs stimulated topoisomerase I-mediated DNA cleavage at low levels in vitro. As expected, the cytotoxic level of the drug was highly affected by the content of topoisomerase II; nevertheless, the test agents had a yeast cell-killing activity that also was weakly dependent on cellular topoisomerase I content. The results are relevant for the full understanding of the molecular mechanism of topoisomerase poisoning by anticancer drugs, and they define structural determinants of anthracyclines that may help in the rational design of new compounds directed against topoisomerase I.

Human DNA topoisomerases I and II are the targets of several compounds with antitumor activity (Gupta et al., 1995; Capranico et al., 1997). These agents interfere with enzyme functions by stabilizing a reaction intermediate, in which DNA strands are cut and covalently linked to tyrosine residues of the protein. This action poisons the enzyme, transforming it into a DNA-damaging agent (Froelich-Ammon and Osheroff, 1995). The phenomenon of chemical poisoning of DNA topoisomerases has been conserved during evolution, probably because it is a very efficient mechanism of cell killing. Doxorubicin, an effective agent in the treatments of human cancers (Arcamone, 1981), is a potent poison of type II DNA topoisomerases (Binaschi et al., 1998).

The molecular action of topoisomerase poisons is peculiar. It is likely that these agents bind to a protein-DNA interface at the enzyme active site, thus hindering strand religation by topoisomerases (Capranico et al., 1997). This view is based on several lines of investigation, including sequence specificity of poison action (Capranico et al., 1997), photolabeling of DNA by cross-reactive poison analogs (Freudenreich and Kreuzer, 1994; Pommier et al., 1995), drug binding data (Shen et al., 1989; Hertzberg et al., 1989, 1990), and structural determinants of drug site selectivity (Capranico et al., 1994a, 1995, 1998). In the case of camptothecin, a topoisomerase I poison, drug receptor models have been suggested based on the crystal structures of enzyme-DNA complexes (Redinbo et al., 1998) and molecular modeling (Fan et al., 1998). In these models, camptothecin contacts specific amino acid residues as well as the GC base pair (bp) at the +1 position of the cleavage site that was shown to be required for camptothecin activity (Jaxel et al., 1991).

It is interesting that poisons such as actinomycin D, intoplicine, saintopin, and others (Trask and Muller, 1988; Wassermann et al., 1990; Poddevin et al., 1993; Leteurtre et al., 1994; Nabiev et al., 1994; Makhey et al., 1996) can act against both topoisomerases I and II, suggesting that some structural features of the drug receptor or the molecular mechanisms are shared by the two enzymes. Among anthracycline-related molecules, 3′-morpholinyl-doxorubicin, nogalamycin, and aclacinomycin A (Fig. 1) have been shown to be poisons of topoisomerase I but not of topoisomerase II (Wassermann et al., 1990; Nitiss et al., 1997; Sim et al., 1997). These findings point to the possibility of modifying the an-

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ABBREVIATIONS: SV40, simian virus 40; bp, base pair.

thracycline structure to increase the activity against the type I enzyme.

Precise structure-function relationships have been established in the case of antitumor anthracyclines. First, drug intercalation is necessary, but not sufficient, for topoisomerase II poisoning (Capranico et al., 1990). Second, the removal of specific groups, such as the 4-methoxy and 3′-amino substituents, greatly increases the drug activity (Capranico et al., 1990, 1994b). Third, the 3′ substituent of the sugar moiety markedly influences the sequence selectivity of anthracycline-stimulated DNA cleavage (Capranico et al., 1995). For anthracycline analogs that are able to poison topoisomerase I, it has been proposed that the saccharide moiety could confer this ability (Nitiss et al., 1997; Sim et al., 1997) because the moiety might confer a specific interaction of the drug with the DNA minor groove or the active site of the protein.

Recently, novel disaccharide anthracyclines with promising antitumor activity and a different spectrum of activity in animal models were developed (Arcamone et al., 1997a,b). The new analogs have the aglycone-bound sugar residue without the 3′-amino group and a second sugar, daunosamine, linked to the first sugar via α(1–4) linkage (Fig. 1). In the present work, we have examined the influence of the orientation of the α(1–4) linkage on the poisoning activity of the drug against recombinant human topoisomerase II isoforms, as well as against topoisomerase I. The studied analogs were compared to the parent drugs, doxorubicin and idarubicin. Our data provide strong evidence that topoisomerase II is the target of this series of anthracyclines, although low levels of activity against topoisomerase I have been detected from disaccharide anthracyclines as well as idarubicin.

Materials and Methods

Drugs, Enzymes, Yeast Strains, and Other Materials. Idarubicin was purchased from Pharmacia-Upjohn (Milan, Italy). The disaccharide anthracycline analogs used for testing were prepared in the chemistry department of Menarini Ricerche Sud (Pomezia, Italy), as described previously (manuscript submitted for publication). Anthracyclines were dissolved in deionized water. Camptothecin was provided by Drs. M. C. Wani and M. E. Wall (Research Triangle Institute, Research Triangle Park, NC), and was dissolved in dimethyl sulfoxide. Simian virus 40 (SV40) DNA, T4 polynucleotide kinase, agarose, and acrylamide were purchased from Bethesda Research Laboratories (Basel, Switzerland). [γ-^{32}P]ATP was obtained from Amersham Corp. (Milan, Italy). Calf intestinal alkaline phosphatase and restriction endonucleases were obtained from New England Biolabs (Taunus, Germany). Native murine topoisomerase II was purified from leukemic P388 cells as reported previously (De Isabella et al., 1990). Plasmids carrying human topoisomerase II cDNAs and *Saccharomyces cerevisiae* JN394*top2-4* and JEL1 strains were provided by C.A. Austin (Newcastle-upon-Tyne University, Newcastle-upon-Tyne, United Kingdom), and were described previously (Nitiss et al., 1992, 1993; Wasserman et al., 1993; Austin et al., 1995). Plasmid pEMBLyex, which contains a hybrid cyc-Gal1 promoter (Baldari et al., 1987), was obtained from E. Martegani (Milan University, Milan, Italy). P. Benedetti (Consiglio Nazionale delle Ricerche, Rome, Italy) provided plasmid ptac-hTop1 and the *S. cerevisiae* JN134*top1-1* strain (Nitiss and Wang, 1988; Bjornsti et al., 1989). The yeast JN394*top1*$^{-}$ strain was kindly provided by J. Nitiss (St. Jude Children's Hospital, Memphis, TN). Plasmid pEZ-hTOP1 was constructed by cloning a fused glutathione *S*-transferase-human topoisomerase 1 cDNA under the control of the cyc-Gal1 promoter of pEMBLyex (manuscript in preparation). Plasmid pEZ-hTOP1 expresses a topoisomerase I as detected by a DNA relaxation assay (see below) and a specific antibody (unpublished results) and was used in cell-killing experiments.

Purification of Recombinant Human DNA Topoisomerase Isoenzymes. Human topoisomerases IIα and IIβ were purified as described previously (Cornarotti et al., 1996; Binaschi et al., 1998). The plasmids YEpWOB6 and YEphTOP2β, which bear the human topoisomerase IIα and IIβ cDNAs, respectively (Wasserman et al., 1993; Austin et al., 1995), under the control of a galactose-inducible promoter, were transformed into the *S. cerevisiae* strain JEL1 (*MATα leu2 trp1 ura3-52 prb1-1122 pep4-3 Dhis3::PGAL10-GAL4*). The expression of the plasmid-borne human cDNA was achieved by the addition of galactose (2%) to the medium for 16 h. The purified isozymes were stored at −80°C in 50 mM Tris-HCl (pH 7.7), 0.8 to 1 M KCl, 10 mM EDTA, 10 mM EGTA, and 10% glycerol. The base sequences of the above plasmids showed that the recombinant topoisomerase IIα lacks the first 28 amino acid residues that are replaced by the first five amino acid residues of yeast topoisomerase 2 (Wasserman et al., 1993). The recombinant topoisomerase IIβ lacks the first 45 amino acids replaced by the first seven amino acid residues of yeast topoisomerase 2 and has a Ser165 to Arg substitution. A mutation of codon 165 was reported previously (Meczes et al., 1997). These amino acid changes do not affect the drug sensitivity of the β isozyme in vitro. For cleavage experiments, a human recombinant topoisomerase I was purified as detailed elsewhere (Pourquier et al., 1997).

SV40 DNA 5′-End Labeling and Topoisomerase II-Dependent DNA Cleavage. Labeled DNA fragments and cleavage reactions were performed as described previously (Capranico et al., 1994b, 1995). Briefly, SV40 DNA fragments were 5′-end ^{32}P-labeled with T4 kinase and [γ-^{32}P]ATP and were purified by agarose gel electrophoresis. DNA cleavage reactions (20,000 cpm/sample) were performed in 20 μl of 10 mM Tris-HCl (pH 6), 10 mM $MgCl_2$, 50 mM KCl, and 1 mM ATP, with or without drugs, at 37°C for 20 min. Reactions then were stopped by adding SDS and proteinase K (1% and 0.1 mg/ml, respectively) and incubation at 42°C for 45 min. Samples then were electrophoresed in a 1% agarose gel in 89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8; TBE buffer), and 0.1% SDS. Cleavage levels were determined by analyses of dried gels on a PhosphorImager model 425 (Molecular Dynamics, Sunnyvale, CA). To investigate anthracycline sequence specificity, DNA samples were analyzed with denaturing 8% polyacrylamide gels as described previously (Capranico et al., 1994b, 1995).

Topoisomerase I-Dependent DNA Cleavage Assay. The gel-purified, 177-bp *Pvu*II-*Hin*dIII fragment of pBluescript SK(−) plasmid (Stratagene, La Jolla, CA) was 3′-labeled on both ends with [α-^{32}P]dCTP (DuPont-New England Nuclear, Boston, MA). Approximately 200 ng of the pBluescript SK(−) plasmid fragment was incubated for 15 min at 25°C with 2 U of the Klenow fragment of DNA polymerase I in 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl containing dATP, dGTP, and dTTP (0.05 mM each). An 18-bp 3′-end-labeled fragment was excised by *Hin*dIII digestion, and a uniquely 3′-end-labeled, 159-bp fragment was purified with a Sephadex G50 column (Pharmacia, Milan, Italy). Topoisomerase I DNA cleavage reactions (50,000 cpm/sample) were performed in 10 μl of 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml BSA, 0.2 mM dithiothreitol, and the human recombinant enzyme for 15 min at 25°C. Reactions were stopped with 0.5% SDS (final concentration). Three volumes of denaturing loading buffer (80% formamide, 10 mM NaOH, 0.01 M EDTA, and 1 mg/ml dyes) were added. Samples were loaded on a denaturing 7% polyacrylamide gel in TBE buffer and run at 40 V/cm at 50°C for 2 to 3 h. Imaging and cleavage level determinations were performed with a PhosphorImager (Molecular Dynamics).

Yeast Cell-Killing Tests. The JN394*top2-4* (*MATa ura3-52 leu2 trp1 his7 ade1-2 ISE2 rad52::LEU2 top2-4*) yeast strain was used to

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assess the role of topoisomerase II in cytotoxic activity of anthracyclines as described previously (Binaschi et al., 1998). Exponentially growing JN394*top2-4* yeast cells were diluted to 0.5 *A* in YPD (1% yeast extract, 2% peptone, and 2% glucose) medium and grown at a permissive or semipermissive temperature (25 and 30°C, respectively) for at least 2 h before the drug treatments. Then, cells were exposed to the indicated drug concentrations for 24 h at the same temperature. Appropriate dilutions of yeast cultures then were layered in triplicate onto agar plates in YPD medium, and colonies were counted after 3 days. To test the role of topoisomerase I in the cell-killing activity of anthracyclines, we used yeast JN134*top1-1 (MATα, rad52::LEU2, trp1, ade2-1, his7, ura3-52, ise1, top1-1)* or JN394*top1*$^-$ (*MATa ura3-52 leu2 trp1 his7 ade1-2 ISE2 rad52::LEU2 ÄTOP1*) cells bearing pEMBLyex or pEZ-hTOP1 plasmids. Exponentially growing cells were diluted to 0.5 *A* in selective ura$^-$ medium and treated with the indicated concentrations of camptothecin for 16 h at 30°C. Appropriate dilutions of yeast cells then were layered onto selective medium agar plates and colonies were counted after 3 days. Plates containing from 50 to 200 colonies were considered to determine cell survival rates.

DNA Relaxation Assay. The expression of human topoisomerase I in yeast cells from the pEZ-hTOP1 plasmid was determined by testing ATP-independent DNA relaxation activity in cell extracts. Exponentially growing yeast cells bearing pEZ-hTOP1 or pEMBLyex plasmids were incubated with galactose (2%) overnight to activate transcription of plasmid-borne topoisomerase I cDNA. Then, cells were centrifuged, washed, and resuspended in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM EGTA, 10% glycerol, and protease inhibitors. Glass beads then were added and the cells were disrupted by vortexing several times. Cell debris was centrifuged and the topoisomerase I activity was tested in the supernatants. Negatively supercoiled pBR322 DNA (0.25 μg) was incubated with serial dilutions of extracted proteins in 20 μl of 20 mM Tris-HCl (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol for 20 min at 37°C. DNA relaxation was followed by agarose gel electrophoresis and ethidium bromide staining.

Results

A novel disaccharide anthracycline, MEN10755, was shown to have a different spectrum of activity from the parent drug doxorubicin in animal models (Arcamone et al., 1997a) and is currently undergoing early clinical trials. The anthracyclines studied are related to MEN10755 because they have a second daunosamine moiety linked to the 4′-OH group of a 3′-deamino-daunosamine linked to the planar aglycone (Fig. 1). Two analogs, MEN10746 and MEN10733, have the second daunosamine linked in the axial orientation to the first sugar, whereas the other analogs, MEN10749 and MEN10732, have the linkage in the equatorial orientation. In addition, the 4-methoxy group was removed from the aglycone of MEN10746 and MEN10749 (Fig. 1).

First, we determined the stimulative effects of the drug on DNA cleavage produced by recombinant human topoisomerase II isoforms (Figs. 2 and 3). Overall levels of drug-stimulated DNA breakage were similar between the two human isoforms (Figs. 2 and 3, and data not shown), although some minor sites were more stimulated with one of the two isoenzymes (Fig. 2). Marked differences were observed among the analogs. MEN10746 and MEN10733 were more active than MEN10749 and MEN10732, respectively, showing that the axial orientation of the second sugar is a structural determinant of topoisomerase II poisoning. For both human isoenzymes, the analogs could be ranked in decreasing order of poisoning activity: idarubicin ≥ MEN10746 > doxorubicin > MEN10733 ≫ MEN10749 ≥ MEN10732 (Figs. 2–4). Thus, the removal of the 4-methoxy group increased the activity of disaccharide analogs against the isozymes, in agreement with previous results (Binaschi et al., 1998). A direct comparison with idarubicin (Figs. 2 and 3) showed that the novel disaccharide analogs were less potent than idarubicin. The latter stimulated maximal cleavage levels at 0.2 to 1 μM,

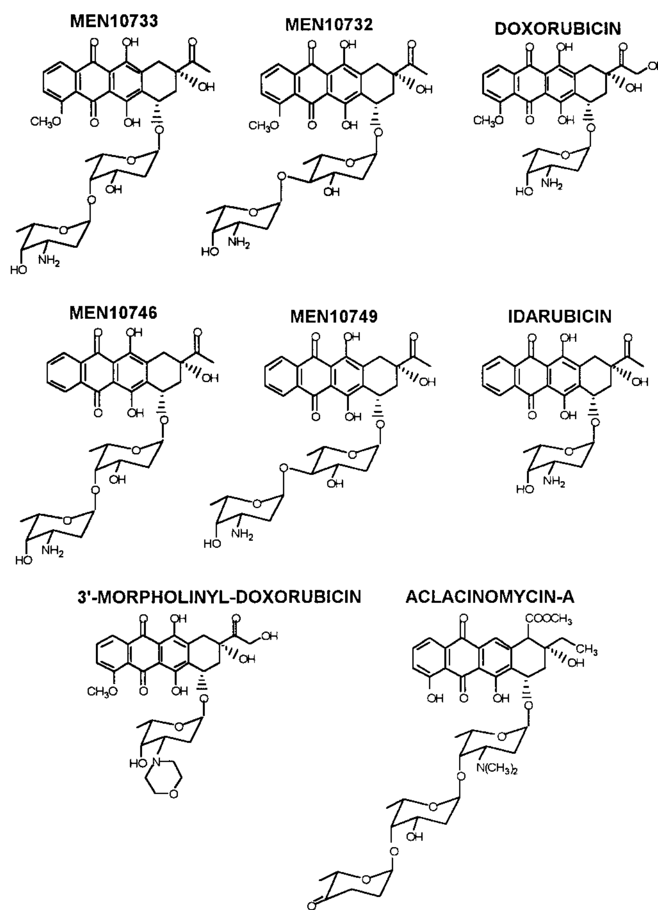

Fig. 1. Chemical structures of relevant anthracycline analogs.

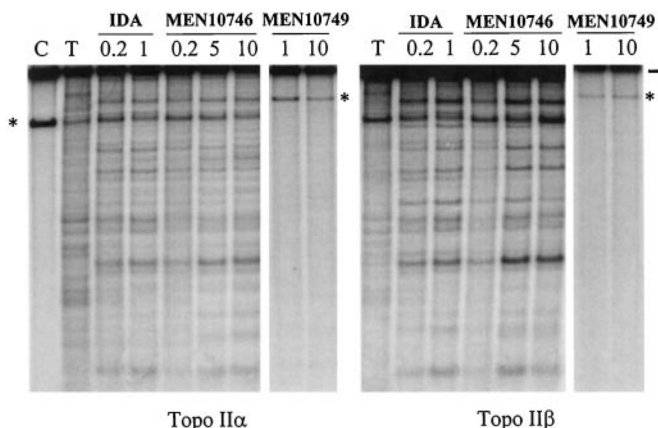

Fig. 2. DNA cleavage stimulation by the 4-demethoxy anthracycline analogs in the presence of human topoisomerase IIα and IIβ. SV40 DNA was reacted with the enzyme, with or without the indicated drug concentrations in 20 μL of 10 mM Tris-HCl (pH 6), 10 mM $MgCl_2$, 50 mM KCl, and 1 mM ATP at 37°C for 20 min. Cleavage reactions were stopped with SDS and proteinase K, and DNA samples were electrophoresed in a 1% agarose gel. *, contaminating band. Bar, full-length SV40 DNA.

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whereas MEN10746 and MEN10733 achieved the maximal effect at 5 to 10 μM (Figs. 2 and 3). At higher concentrations, all of the studied analogs suppressed cleavage (not shown).

The sequence specificity of the action of anthracycline disaccharides then was investigated in a plasmid DNA (Fig. 4). Drug-stimulated cleavage intensity patterns were identical among doxorubicin, idarubicin, and MEN10746 at nucleotide levels, showing that the sequence specificity of MEN10746 is identical with that of the parent drugs. Similar data were obtained with MEN10733 (not shown). Drug potencies evaluated at nucleotide levels were consistent with the results of agarose gels: maximal cleavage stimulation was detected with 0.1 to 1 μM and 1 to 10 μM with idarubicin and MEN10746, respectively (Fig. 4).

Next, we investigated the test anthracyclines with a topoisomerase I DNA cleavage assay in comparison with camptothecin (Fig. 5). MEN10733 and MEN10746, but not MEN10749 and MEN10732, stimulated topoisomerase I-dependent DNA cleavage, therefore indicating that the axial configuration of the second sugar was important for topoisomerase I poisoning, as was observed for topoisomerase II poisoning. Interestingly, idarubicin itself showed some activity at 10 μM (Fig. 5): site 4 was stimulated at consistent levels by the compound (Table 1). For topoisomerase I cleavage, the analogs ranked in the following order: idarubicin $>$ MEN10746 $>$ MEN10733 $\gg$ MEN10732 $\geq$ MEN10749. However, none of the anthracyclines was as active and potent as camptothecin. As expected (Capranico et al., 1997), a 100 μM concentration of the drugs tended to suppress topoisomerase I-DNA breakage (Fig. 5). The lowest active concentrations of idarubicin, MEN10733, and MEN10746 were 0.1, 1, and 0.1 μM, respectively, in the case of topoisomerases II (Fig. 4; Table 1), whereas the active concentrations were in the 10 to 100 μM range in the case of topoisomerase I (Fig. 5; Table 1). Thus, the comparison of cleavage stimulation levels demonstrated that the studied anthracyclines were much more potent and active against topoisomerases II than topoisomerase I.

Nevertheless, because a minimal activity of topoisomerase I poisoning was observed in vitro, we then asked the question whether the studied anthracyclines could poison the enzyme in vivo and whether this activity determined the drug cytotoxicity. Therefore, we measured the effects of cellular topoisomerase I or II contents on anthracycline cytotoxicity in yeast (Figs. 6-8). Yeast JN134*top1-1* cells lack a functional

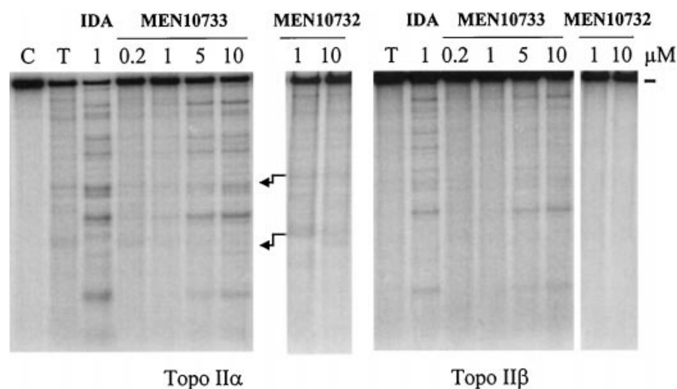

Fig. 3. DNA cleavage stimulation by the 4-methoxy anthracycline analogs in the presence of human topoisomerase IIα and IIβ. See legend to Fig. 2 for details.

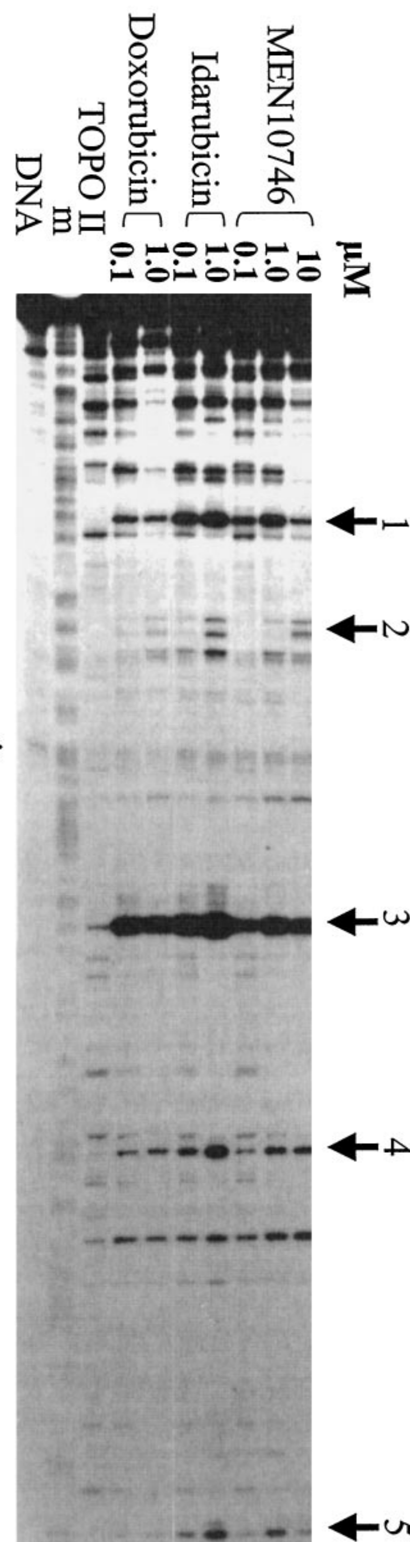

Fig. 4. Sequence specificity of topoisomerase II-mediated DNA cleavage stimulated by MEN10746. Cleavage reactions were performed as detailed in the legend to Fig. 1. DNA cleavage then was analyzed in a denaturing 8% polyacrylamide gel. Lanes: DNA, control sample; TOPO II, murine topoisomerase II without drugs; m, molecular weight markers. The numbers indicate drug concentrations. *, contaminating bands. Arrows and numbers indicate major sites of anthracycline-stimulated DNA breaks.

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topoisomerase I and the JN394*top1*⁻ strain carries a deletion of the yeast *TOP1* gene: these yeast strains have been used previously to demonstrate that camptothecin and other agents target topoisomerase I in vivo (Nitiss and Wang, 1988; Bjornsti et al., 1989; Nitiss, 1994; Nitiss et al., 1998). The yeast strain JN394*top2-4*, which is isogenic to JN394*top1*⁻, has a wild-type *TOP1* gene and a *ts* mutation of the *TOP2* gene (*top2-4* allele) that results in cell lethality at nonpermissive temperatures (35°C), and in a somewhat decreased enzyme activity at the permissive temperature (25°C; Nitiss et al., 1992, 1993, 1998; Nitiss, 1994). JN394*top2-4* cells can survive at semipermissive temperatures (30°C), although the cellular topoisomerase II content is even more reduced. Expression of the plasmid-borne human topoisomerase I in the JN134 and JN394 strains was determined by a DNA relaxation test (Fig. 7, bottom). Relaxation activity was absent in extracts of cells bearing the control pEMBLyex plasmid, whereas it was readily detected in cells bearing the pEZ-hTOP1 plasmid, showing that only the latter encoded for an active topoisomerase I.

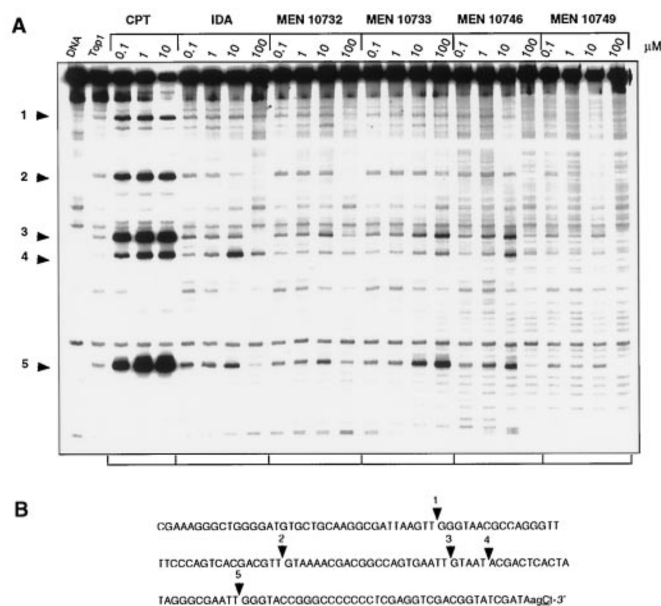

Fig. 5. Topoisomerase I-dependent stimulation of DNA cleavage by anthracycline disaccharides and the parent drug, idarubicin. DNA cleavage reactions were performed for 15 min at 25°C with a human recombinant topoisomerase I in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 µg/ml BSA, and 0.2 mM dithiothreitol. Reactions were stopped with 0.5% SDS and samples were analyzed by denaturing 7% polyacrylamide gels. A, arrowheads and numbers indicate sites of DNA cleavage stimulation by camptothecin. Anthracycline-stimulated DNA cleavage can be observed at sites 3, 4, and 5. B, mapping of drug-stimulated cleavage sites.

At all of the tested concentrations, camptothecin was largely ineffective in JN134*top1-1* or JN394*top1*⁻ cells bearing a control pEMBLyex plasmid, whereas a dose-dependent cytotoxic activity could be documented when a human topoisomerase I was expressed at low levels (Figs. 6 and 7). Idarubicin and MEN10746 were, in general, the most effective analogs (Figs. 6–8). These analogs and MEN10749 showed a slight increase in cytotoxicity when human topoisomerase I was expressed in the JN134*top1-1* strain (Fig. 6, top and bottom): on average, a 3- to 4-fold increase was observed for these compounds (Fig. 6; Table 2). The topoisomerase 1-dependent increase of drug cytotoxicity was less significant in the JN394*top1*⁻ strain (Fig. 7). In concentrations ranging from 10 to 50 µM, MEN10733 and MEN10732 were ineffective in all of the strains (Figs. 6 and 7 and Table 2). In the JN394*top2-4* strain, 50 µM idarubicin, doxorubicin, and MEN10746 showed no activity at 30°C, whereas they reduced cell survival to 0.25, 1.5, and 3.5% at 25°C, respectively (Fig. 8). These values correspond to about 2-log increases in drug activity (see also Table 2), demonstrating a high dependence of anthracycline cell-killing activity on functional topoisomerase II contents. MEN10732, MEN10733, and MEN10749 were poorly active in the JN394*top2-4* strain at 25°C (not shown), in agreement with a much reduced activity in topoisomerase II poisoning. It must be noted that the active analogs were much less effective in the JN394*top2-4* than in the isogenic JN394*top1*⁻ strain (Table 2), although a wild-type topoisomerase I activity is present in the former and absent in the latter. Thus, the data showed a high dependence of anthracycline cytotoxicity on the cellular content of topoisomerase II, and a low dependence on topoisomerase I. The results appear to parallel the in vitro cleavage stimulation data (see above).

Discussion

The high clinical efficacy of doxorubicin and related anthracyclines has attracted considerable research attention to their mechanism of action. Topoisomerase II is generally considered to be the main target of these compounds (Capranico et al., 1997). Our current data show that idarubicin and novel disaccharide analogs can trap topoisomerase I-DNA complexes as well, although the studied drug analogs are much more potent and active against topoisomerase II. We also tested the role of drug poisoning of either enzyme for the biological activity of the drug by using a yeast system. The results show a high dependence of anthracycline cytotoxicity on the cellular content of topoisomerase II, and a lower dependence on topoisomerase I.

Therefore, classical anthracyclines may be considered dual

TABLE 1

DNA cleavage levels promoted by anthracyclines in the presence of type I or type II DNA topoisomerases[a]

Topoisomerase/Sites[b]	Camptothecin			Doxorubicin		Idarubicin			MEN10746				MEN10733			
	0.1	1.0	10	0.1	1.0	0.1	1.0	10	0.1	1.0	10	100	0.1	1.0	10	100
II/1–5	—	—	—	14	10	25	22	sup	28	25	13	sup	un	2.4	1.7	—
I/3	24	33	38	—	—	2.0	1.8	1.9	1.8	2.6	4.5	1.5	1.9	2.0	3.3	4.8
I/4	9.0	17	24	—	—	2.7	5.0	17	2.6	3.8	8.9	4.5	2.6	2.7	4.1	5.4
I/5	15	43	65	—	—	2.0	1.8	2.8	1.5	2.1	3.8	sup	1.8	1.7	4.4	6.5

[a] The values are fold increases of cleavage levels as compared with those without the drug. Dash, not tested; sup, cleavage suppression; un, undetectable increase. Doxorubicin and idarubicin at 10 and 100 µM, respectively, suppressed cleavage at all sites.

[b] The numbers indicate the sites shown in Figs. 4 and 5 for type II and type I topoisomerases, respectively. Cleavage at sites 1 and 2 of Fig. 5 was stimulated only weakly by anthracyclines.

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poisons of both type I and type II topoisomerases. It is tempting to raise the question of whether other known "specific" poisons of topoisomerase II may also trap topoisomerase I at a low level. Indeed, our data suggest that the dual poison family may be much larger than recognized previously (Capranico et al., 1997). A new distinction then could be made: 1) poisons with an equal potency against the two enzymes and 2) poisons with a lower (or much lower) potency against one of the two enzymes. Our conclusion also is strengthened by previous findings that, in contrast to present results, Δtop1 yeast mutants are hypersensitive to pure topoisomerase II poisons (such as etoposide and anasacrine) and top2ts mutants are hypersensitive to camptothecin at 30°C (Nitiss et al., 1993; Nitiss, 1994). The enhanced sensitivity in these cases is explained by the ability of the enzymes to substitute for each other in nuclear processes where a topoisomerase is essential.

In the case of the studied anthracycline analogs, topoisomerase II remains the main cellular target, in agreement with several other results (Capranico et al., 1997). This also is based on the observation that 1) yeast cell-killing levels depended more on the content of topoisomerase II than topoisomerase I, and 2) drug potency in vitro is lower for topoisomerase I than for topoisomerase II. Nevertheless, at drug concentrations achievable in patients, topoisomerase I might play a role, although a minor one, in the drug effects at cellular levels. Other studies will eventually establish the present findings in mammalian tumor cells.

A major conclusion of our work is that the anthracycline

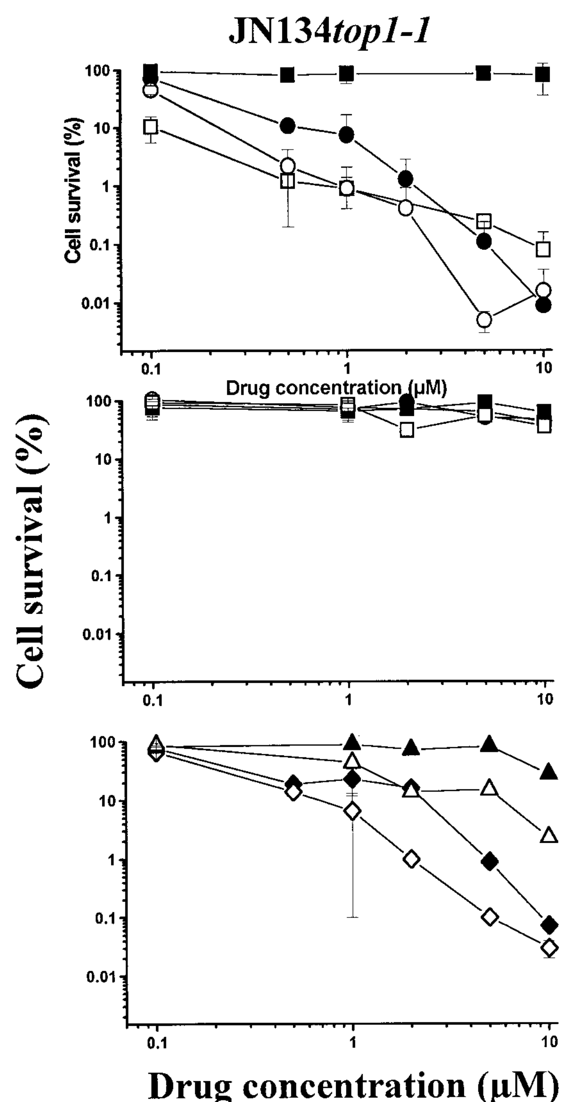

Fig. 6. Dependence of anthracycline cell-killing activity from the content of topoisomerase I in the JN134*top1-1* yeast strain. Cells were treated with the indicated drug concentrations for 16 h at 30°C. After drug removal, cells were plated and allowed to grow for 3 days in a selective medium. Colonies then were counted and cell survival was calculated relative to untreated cells. Open symbols represent yeast cells expressing a human topoisomerase I (pEZ-hTOP1); closed symbols represent yeast cells bearing a control pEMBLyex plasmid. Top: camptothecin, squares; idarubicin, circles. Middle: MEN10733, circles; MEN10732, squares. Bottom: MEN10749, triangles; MEN10746, diamonds. Values are means of two to four independent experiments. Bars, S.E., when larger than symbol size.

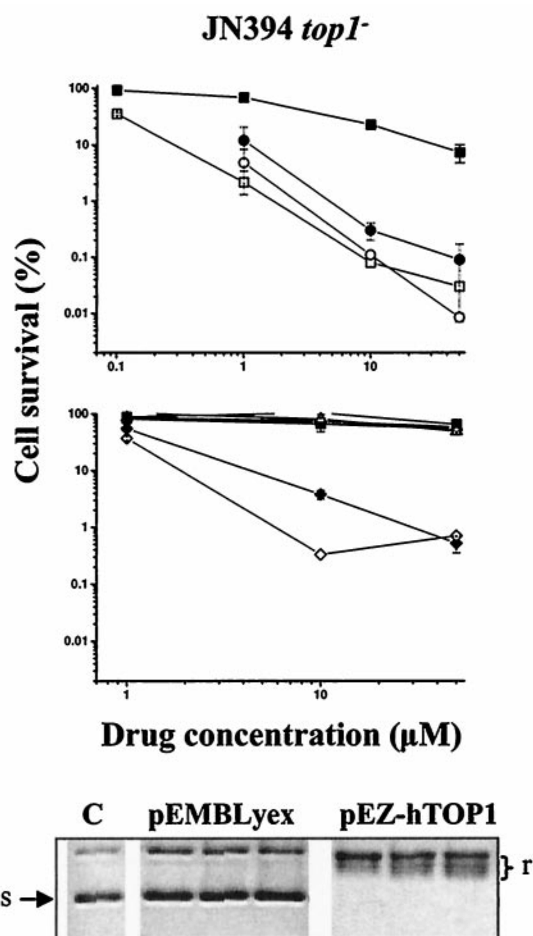

Fig. 7. Dependence of anthracycline cell-killing activity from the content of topoisomerase I in the JN394*top1*⁻ yeast strain. Top, see legend to Fig. 6 for experimental details. Open symbols are yeast cells expressing a human topoisomerase I (pEZ-hTOP1); closed symbols are yeast cells bearing a control pEMBLyex plasmid. Squares, camptothecin; circles, idarubicin; triangles, MEN10733; inverted triangles, MEN10732; diamonds, MEN10746. Values are means of two independent experiments. Bar, S.E., when larger than symbol size. Bottom, DNA relaxation activity in extracts of JN394*top1*⁻ and JN134*top1-1* cells bearing the control pEMBLyex or pEZ-hTOP1 plasmids. See *Materials and Methods* for experimental details. C, control DNA; s and r, supercoiled and relaxed DNA molecules, respectively. The amounts of proteins added to the samples were 8.8, 2.2, and 0.65 and 2.2, 1.1, and 0.65 μg (from left to right) for pEMBLyex and pEZ-hTOP1 lanes, respectively. A representative gel is shown.

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molecule has an inherent ability to poison both DNA topoisomerases I and II. Indeed, other anthracyclines were shown previously to poison specifically topoisomerase I (Wassermann et al., 1990; Nitiss et al., 1997; Sim et al., 1997). Therefore, the present results raise the question of whether structural modifications of the anthracycline molecule may increase the activity specifically against topoisomerase I. Structure-activity relationships have been determined in the case of topoisomerase II (Capranico et al., 1997; see also this introduction). The present results show that the activity against the type I enzyme was not affected by the number (one or two) of sugar residues linked to the planar aglycone. For the type II enzyme, we must first consider that the removal of both the 4-methoxy and 3′-amino groups increase topoisomerase II poisoning by the drugs (Capranico et al., 1994b). Indeed, an analog bearing both of the modifications showed enhanced cleavage stimulation levels as compared with analogs bearing only one modification (compare 3′-deamino-3′hydroxy-4′epiDOX, 4-demethoxy-3′-deamino-3′hydroxy-4′epiDOX, and idarubicin in Fig. 2, and 4′-*O*-daunosaminyl-daunorubicin and idarubicin in Fig. 3 of Capranico et al., 1994b). Because both the 4-methoxy and 3′-amino groups have been removed in the present disaccharide analogs, the similar activity of idarubicin and MEN10746 against topoisomerase II shows that the addition of a second daunosamine decreases the drug activity of cleavage stimulation with the type II enzyme. Idarubicin and MEN10746 also had a similar activity against type I topoisomerase; however, the specific effect of the 4-methoxy and 3′-amino groups cannot be established in this case and awaits further evaluation.

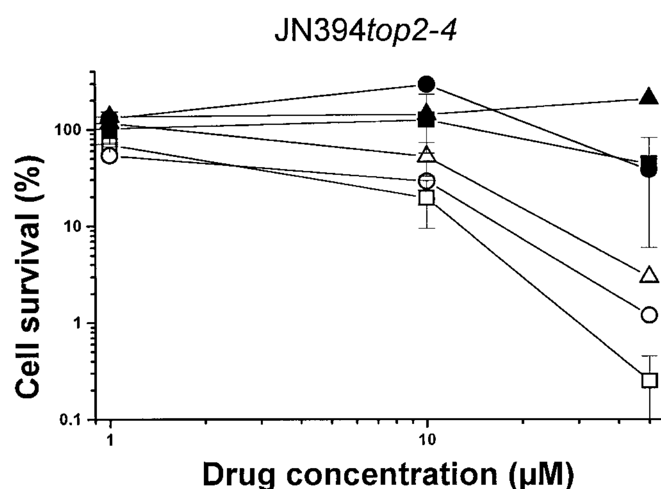

Fig. 8. Topoisomerase II expression determines yeast cell sensitivity to anthracyclines. JN394*top2-4* yeast cells were treated with the indicated drug concentrations for 24 h. After drug removal, cells were plated and allowed to grow for 3 days. Colonies then were counted, and cell survival was calculated relative to untreated cells. Open symbols, cells treated at 25°C. Closed symbols, cells treated at 30°C. MEN10746, doxorubicin, and idarubicin are triangles, circles, and squares, respectively. Values are means of two to three independent determinations. Bars, S.E., when larger than symbol size.

Although MEN10746 was as active as idarubicin in the cleavage and yeast assays, it was more potent than doxorubicin in the same experiments. Because doxorubicin, but not idarubicin, is effective in the treatment of human solid tumors, this finding is of interest, and the analog has been selected for further evaluation. In human cultured cells, MEN10746 also was more cytotoxic than doxorubicin (in agreement with the present findings), and it was equally effective in human tumor xenografts (Arcamone et al., 1999).

In summary, our data show the potential activity of classical anthracyclines against topoisomerase I and further extend some previous observations with a 3′-morpholinyl-doxorubicin (Wassermann et al., 1990). These findings support the idea that mechanistic aspects of chemical poisoning are shared by type I and II topoisomerases. DNA intercalation does not appear to play a major role for topoisomerase II poisoning of the studied analogs (unpublished data) and other intercalating agents (Capranico et al., 1997). Specific drug interactions with DNA and/or the proteins are likely to be determinant factors of enhanced drug effects against type I or II DNA topoisomerases. Our present findings are relevant for the full understanding of the mechanism of topoisomerase poisoning by anticancer agents, and they define important structural determinants of anthracyclines that may be of help in the rational design of new compounds directed against topoisomerase I.

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TABLE 2

Cytotoxic levels of the studied anthracycline analogs in yeast strains

Values are means of two to three independent determinations and indicate drug concentrations inhibiting 90% of cell colony formation. Note that drug treatments were for 16 h in the case of the JN134top1-1 and JN394top1⁻ strains, and for 24 h in the case of the JN394top2-4 strain.

Drug	JN134 top1-1		JN394 top1⁻		JN394 top2-4	
	pEMBLyex[a]	pEZ-hTOP1[a]	pEMBLyex[b]	pEZ-hTOP1[b]	25 °C[b]	30 °C[b]
MEN10746	2.30	0.70	4.2	1.9	25	>50
MEN10749	>10	5.90	>10	>10	>50	>50
MEN10733	>10	>10	>50	>50	>50	>50
MEN10732	>10	>10	>50	>50	N.D.[c]	N.D.
Idarubicin	0.50	0.22	1.15	0.7	14	>50
Doxorubicin	6.2	3.6	N.D.	N.D.	17	>50
Camptothecin	>10	0.11	32	0.28	N.D.	N.D.

[a] The highest drug concentration used was 10 μM.
[b] The highest concentration used was 50 μM.
[c] N.D., not determined.

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