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Configurational Requirements of the Sugar Moiety for the Pharmacological Activity of Anthracycline Disaccharides

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ABSTRACT. The amino sugar is recognized to be a critical determinant of the activity of anthracycline monosaccharides related to doxorubicin and daunorubicin. In an attempt to improve the pharmacological properties of such agents, novel anthracycline disaccharides have been designed in which the amino sugar, daunosamine, is separated from the aglycone by another carbohydrate moiety. In the present study, we examined the influence of the orientation of the second sugar residue on drug biochemical and biological properties in a series of closely related analogs. This structure–activity relationship study showed that the substitution of the daunosamine for the disaccharide moiety dramatically reduced the cytotoxic potency of the drug in the 4-methoxy series (daunorubicin analogs). In contrast, in the 4-demethoxy series (idarubicin analogs), the C-4 axial, but not the equatorial, configuration conferred a cytotoxic potency and antitumor activity comparable to that of doxorubicin. The configuration also influenced the drug's ability to stimulate topoisomerase II α-mediated DNA cleavage. Indeed, the glycosides with the equatorial orientation were ineffective as topoisomerase II poisons, whereas the compounds with axial orientation were active, although the daunorubicin analog exhibited a lower activity than the idarubicin analog. It is conceivable that the axial orientation allows an optimal interaction of the drug with the DNA–enzyme complex only in the absence of the methoxy group. Our results are consistent with a critical role of the sugar moiety in drug interaction with the target enzyme in the ternary complex. BIOCHEM PHARMACOL **57**;10:1133–1139, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. anthracyclines; chemistry; structure–activity; topoisomerase

Anthracyclines are the first class of intercalating agents proven to have clinical efficacy in antitumor therapy [1]. Doxorubicin remains one of the most effective and widely used drugs in antitumor chemotherapy. The efficacy of doxorubicin has stimulated several structure–activity relationship studies aimed at identifying critical modifications which might improve therapeutic activity [1, 2]. The mechanism of the cytotoxic and antitumor activity of intercalating agents is ascribed to their ability to interfere with DNA topoisomerase II function [3]. DNA binding and intercalation are necessary but not sufficient conditions for optimal activity of anthracyclines [4]. Although drug intercalation may be implicated in the mechanism of topoisomerase II poisoning, the mode and site of drug binding appear more critical than DNA binding affinity [3]. The external (non-intercalating) moieties of the anthracycline molecule (i.e. the sugar residue and the cyclohexene ring) could play an important role in the formation and stabilization of the ternary complex (DNA–drug–topoisomerase II) [4, 5]. In particular, the sugar moiety, located in the minor groove, is a critical determinant of the activity of anthracyclines as topoisomerase II poisons [5].

Anthracycline glycosides, obtained from microbial cultures or by synthesis and containing a disaccharide or trisaccharide chain, have been described previously [2]. A structural feature common to such oligosaccharides is the presence of the amino sugar as the carbohydrate moiety directly attached to the anthracyclinone. In biosynthetic compounds, the amino sugar is either daunosamine, *N*-methyldaunosamine, or rhodosamine. The synthesis of novel disaccharide anthracycline analogs with a 2-deoxy-L-rhamnose or a 2-deoxy-L-fucose moiety, as the first sugar residue directly linked to the aglycone, and daunosamine, as second sugar residue, is expected to provide new insights into the configurational requirements of the sugar moiety for drug activity and further structural information on the determinants of sequence specificity of drug activity.

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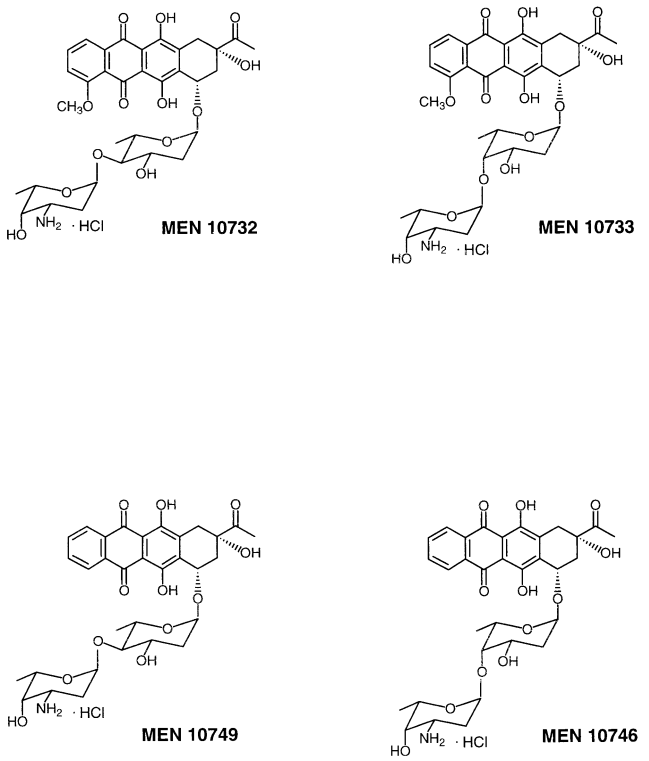

FIG. 1. Chemical structures of disaccharide analogs.

In an attempt to improve the pharmacological properties of anthracyclines, we have designed and synthesized novel anthracycline disaccharides without the amino group in the aglycone-bound sugar residue [6]. The second sugar residue, daunosamine, is bound to the first sugar via α(1–4) linkage (Fig. 1). In the present study, we examined the influence of the orientation of the second sugar residue on drug biochemical and biological properties in a series of closely related analogs. Antitumor efficacy studies performed with an idarubicin analog with the optimal configuration support the pharmacological interest of the new series of synthetic anthracyclines.

MATERIALS AND METHODS

Drugs

The four disaccharide anthracyclines (Fig. 1) were synthesized through a convergent synthetic strategy, involving the construction of the disaccharide moieties and their successive coupling with the aglycones, as previously reported [6]. In particular, we synthesized and evaluated the biological properties of the following novel compounds: MEN 10732 and MEN 10749, where the disaccharide moiety is the daunosaminyl α(1–4)-2-deoxy-L-rhamnosyl derivative and the aglycone daunomycinone and idarubicinone, respectively; MEN 10733 and MEN 10746, where the disaccharide moiety is daunosaminyl α(1–4)-deoxy-L-fucosyl derivative and the aglycone daunomycinone and idarubicinone, respectively.

DNA Binding

Calf thymus DNA was purchased from Sigma Chemical; the concentration in dilute solution was determined spectrophotometrically by using a molar extinction coefficient of 12.824 M^{-1} at 260 nm (base pairs). Fluorescence titration experiments were performed using a Perkin Elmer MPF 44 spectrofluorimeter. The excitation wavelength was 470 nm, and the emission ranged from 550 to 590 nm. Titrations were carried out by recording the decrease in intrinsic fluorescence of a drug solution (about 5 mM, 1.2 mL solution in 1-cm cuvette) by adding discrete aliquots of DNA solution (1 mM). All measurements were carried out for 1 hr at 20° in 0.05 M sodium phosphate, pH 7.0, 0.15 M NaCl, and 1 mM EDTA buffer. Binding data were cast into the form of a Scatchard plot or r/Cf versus r, where r is the number of moles of drug bound per mole of DNA base pairs and Cf is the concentration of free drug. The data were fitted to the neighboring exclusion model [7]:

$$r/Cf = K_i(1 - nr)[(1 - nr)/1 - (n - 1)r]n^{-1}$$

were K_i is the apparent binding constant to an isolated DNA binding site and n is the exclusion parameter [8].

Purification of Recombinant Human DNA Topoisomerase IIα

Human topoisomerase IIα cDNA in plasmids YEpWob6 was expressed in *Saccharomyces cerevisiae* strain JEL1 (*MAT* α *leu trp1 ura3-52 prb1-1122 pep4-37* Δ*his3*:PGAL10-GAL4) under the control of a galactose-inducible promoter and purified as previously described [9, 10]. The expression of the plasmid-borne cDNA was achieved by the addition of galactose (2%) to the medium for 15 hr. After cell harvesting and lysis, DNA was precipitated with polymin P; then Celite (Fluka) was added to the protein mixture. The enzyme was eluted with 1 M KCl from Celite and precipitated with ammonium sulfate. Phosphocellulose column chromatography was then performed, and the human α isoform was eluted with a KCl gradient. Topoisomerase-containing fractions were identified by SDS-PAGE and DNA relaxation activity. The purified isozyme was stored at −80° in 50 mM Tris–HCl, pH 7.7, 200 mM KCl, 10 mM EDTA, 10 mM EGTA, and 10% glycerol.

End-labeling of SV40 DNA and DNA Cleavage Reactions

Uniquely 5′-end ^{32}P-labeled DNA fragments were prepared as previously reported [5]. Briefly, linearized SV40 DNA fragments were dephosphorylated and 5′-end-labeled with T4 kinase and ^{32}P-ATP (Amersham). After digestion with an appropriate restriction endonuclease, uniquely 5′-end-labeled fragments were separated by gel electrophoresis and purified by electroelution and ethanol precipitation. DNA cleavage reactions (20,000 cpm/sample) were performed in H buffer (10 mM Tris–HCl, pH 6, 10 mM $MgCl_2$, 50 mM

KCl, and 1 mM ATP) with or without drugs at 37° for 20 min. Idarubicin was used at a 1-μM dose, whereas the analogs were tested at 0.2, 1, 5, and 10 μM. Reactions were stopped by adding SDS and proteinase K (1% and 0.1 mg/mL, respectively) and incubated at 42° for 45 min. Samples were then electrophoresed in a 1% agarose gel in 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8, and 0.1% SDS.

The degree of drug stimulation of isozyme-mediated DNA cleavage was determined by volume integration using the Image Quant program on a Molecular Dynamics PhosphorImager 425 Model. A cleavage level of each sample was calculated as the ratio of the radioactivity of seven major cleavage sites to the total radioactivity in the lane and normalized relative to the control lane. Then, the drug stimulation factor was determined as the ratio of the cleavage level in the presence of the drug to the level without the drug.

Cell Lines and Cytotoxicity Studies

The human tumor cell lines used in the study included one ovarian carcinoma (A2780); one cervical squamous cell carcinoma (A431); one non-small cell lung cancer (NSCLC) (H460); and two SCLC* and their doxorubicin-resistant sublines (POGB and POGB/DX, POVD and POVD/DX). All cell lines were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum. POGB- and POVD-resistant cell lines were selected by continuous exposure to increasing drug concentrations and maintained in the absence of the drug [11, 12]. To evaluate the cytotoxic effect in A2780, A431, and H460 cell lines 24 hr after seeding, cells were treated with the drugs for 1 or 24 hr and then incubated in drug-free medium for a further 72 or 48 hr, respectively. At the end of the experiment, cells were trypsinized and counted by a Coulter Counter (ZBI Electronics). In experiments performed on SCLC (POGB, POVD) and on their resistant derived cell lines, cells (5×10^4) were seeded in 96-well tissue culture plates and treated 24 hr later with the drug. After 4 days, the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay was performed [13]. The plates were read at 550 nm with reference at 620 nm on a scanning multiwell spectrophotometer (Easy Reader 400 AT, SLT-Lab Instruments). The results were expressed as IC_{50}, calculated from dose–response curves, and defined as the drug concentration required for a 50% reduction of absorbance compared to control cells.

Evaluation of Antitumor Activity

Female athymic Swiss nude mice, 8–10 weeks old, were used in the study (Charles River Italia). The experiments were approved by the Ethical Committee for Animal Experimentation of Istituto Nazionale Tumori. A431 cervical carcinoma, POVD SCLC, and A2780 ovarian carcinoma were established as s.c. tumor lines from cell lines (10^7 cells/0.2 mL/flank). For efficacy evaluation, mice were transplanted in both flanks with tumor fragments. Tumor-bearing mice were randomly divided into different groups before starting drug treatment. Each experimental group consisted of 8–10 tumors. Drugs were dissolved in water and delivered i.v. (10 mL/kg body weight). The s.c. tumors were measured in two diameters, and the TW was calculated by the formula: TW (mg) = tumor volume = length (mm) × width^2 (mm)/2. The following end points were used to assess antitumor efficacy: (i) optimal TWI % (opTWI%) in treated over control mice (mean TW of each group) evaluated as 100-(TW-treated/TW control × 100). The lowest value in the 30 days following the end of treatment is reported in Results; and (ii) LCK calculated by the formula: LCK = T − C/3.32 × Td, where T and C are the mean times (in days) required for the treated tumors and control tumors, respectively, to reach a predetermined size (usually 500 or 1000 mg) and Td is the mean doubling time in days measured from a best fit straight line of the control group tumors in exponential growth (range, 100–400 mg).

TABLE 1. ^{1}H NMR relevant data of disaccharide analogs (300 MHz, Me_2Sod_6, 298 K)

	Chemical shifts (ppm) and coupling constants (Hz)			
Proton	MEN 10732	MEN 10733	MEN 10749	MEN 10746
H-1′	5.45 (bs)	5.10 (bs)	5.20 (bs)	5.20 (bs)
H-1″	5.63 (bs)	4.90 (bs)	5.40 (bs)	4.90 (bs)
H-4′	3.32 (t, J = 9)	3.55 (bs)	3.10 (t, J = 9)	3.60 (bs)
H-4″	3.83 (bs)	3.55 (bs)	3.60 (bs)	3.60 (bs)

bs, broad singlet; t, triplet; J, coupling constant.

RESULTS

NMR Characterization of the Disaccharide Analogs

The configurations at positions 4′, 1′, and 1″ were confirmed by the characteristic values of proton–proton coupling constants and chemical shifts (Table 1). The rhamnosyl analogs MEN 10732 and MEN 10749 presented an H4′ resonance upfield in comparison with the fucosyl derivatives MEN 10733 and MEN 10746 and showed a triplet of J = 9 Hz, which is indicative of a transaxial arrangement between H4′ and H5′. However, the fucosyl analogs showed a broad signal for H4′, with coupling constants smaller than 2 Hz. All compounds presented a characteristic α configuration of the anomeric protons of both sugar moieties, as shown by the coupling constant pattern.

Drug Stimulation of DNA Cleavage Mediated by Human Recombinant Enzyme

To determine the drug activity in poisoning DNA topoisomerase II, DNA cleavage levels produced by human

* *Abbreviations:* SCLC, small cell lung cancer; TW, tumor weight; TWI, tumor weight inhibition; LCK, $\log_{10}$ cell kill; and SV40, simean virus No. 40.

TABLE 2. Drug stimulation of topoisomerase IIα-mediated DNA cleavage*

Drug	Stimulation factor†	
	1 μM	10 μM
Idarubicin	3.5	ND
MEN 10732	<1	<1
MEN 10749	<1	<1
MEN 10733	1.5	1.3
MEN 10746	3.2	4.2

*A cleavage level of each sample was calculated as the ratio of radioactivity of seven chosen cleaved fragments to the total radioactivity in the lane and normalized relative to the control lane.

†Stimulation factor, determined as the ratio of the cleavage level in the presence of drug compared to the level without drug. <1 indicates lack of stimulation. ND, not determined.

recombinant α isozyme with and without drugs were determined in the whole *SV40* genome with the use of agarose gels. Cleavage reactions were performed in H buffer, which increased enzyme binding to DNA. Compounds MEN 10732 and MEN 10749 did not stimulate topoisomerase IIα-mediated DNA cleavage (Table 2 and Fig. 2). In contrast, the disaccharide analogs with an axial orientation were effective in stimulating DNA cleavage. MEN 10746 was the most active agent, showing a stimulation factor similar to that of idarubicin. A comparison of the cleavage intensity pattern supported a similar sequence specificity of enzyme-mediated DNA cleavage stimulated by disaccharide analogs and idarubicin used as reference compound (Fig. 2).

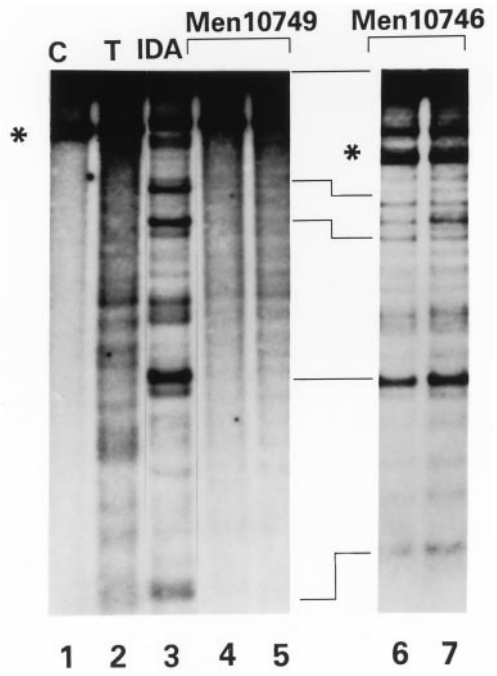

FIG. 2. Topoisomerase II-mediated DNA cleavage stimulated by disaccharide analogs. C: control DNA; T, topoisomerase II alone; 3, idarubicin (1 μM); 4, 5 and 6, 7: two drug concentrations (5 and 10 μM) of the tested analogs. Asterisks indicate a contaminating DNA fragment.

Cytotoxicity

The cytotoxic activities of the disaccharide analogs were studied on a large panel of human tumor cell lines of different histotypes. The pattern of cellular response to the tested anthracycline disaccharide analogs is shown in Table 3 and compared to the cytotoxic effects of doxorubicin, daunorubicin, and idarubicin. A common feature of the two disaccharide analogs of the daunorubicin series (MEN 10732 and MEN 10733) was a marked reduction in cytotoxic potency. Indeed, IC_{50} values were at least one order of magnitude greater than those of daunorubicin and doxorubicin, with no appreciable difference between the activity of the analogs with 2-deoxyl-L-rhamnose (MEN 10732) or with 2-deoxy-L-fucose (MEN 10733) as the first sugar residue directly linked to the aglycone. In contrast, in the 4-demethoxy series, the analog containing the fucose residue (MEN 10746) exhibited a cytotoxic potency comparable to that of doxorubicin in most cell systems and superior to that of doxorubicin in the ovarian carcinoma A2780 cells. The effects of MEN 10746 in the A2780 cell line were similar to those of idarubicin, which was the most potent anthracycline used in the study. Surprisingly, the increase in drug potency following removal of the methoxy group was not observed with the epimer containing the rhamnose residue (MEN 10749), which exhibited a cytotoxic activity comparable to that of the daunorubicin series.

The cytotoxic activities of anthracycline disaccharides were also investigated in the multidrug-resistant sublines of SCLC cell lines selected for resistance to doxorubicin (Table 4). POVD/DX and POGB/DX are characterized by overexpression of the multidrug-resistant *mdr-1* and *MRP*

TABLE 3. Cytotoxic activity of anthracycline disaccharides on human tumor cell lines

Cell line	Exposure time (hr)	IC_{50} (ng/mL)*						
		Doxorubicin	Daunorubicin	MEN10732	MEN10733	Idarubicin	MEN10749	MEN10746
A431	1	300 ± 140	150	4000	>10000	14 ± 11	>10000	200
	24	30 ± 10		400	500	4 ± 0	300	20
A2780	1	240 ± 110	70 ± 50	2500	4500	4 ± 3	4000	10
	24	23 ± 6	10	350	600	1.3 ± 0.9	70	1
H460	1	130 ± 120		400	1500	2 ± 1	4000	40
	24	25 ± 7		500	400	2.7 ± 0.6	100	30

*IC_{50} values were calculated by cell counting. See Materials and Methods for details.

TABLE 4. Pattern of cross-resistance to anthracycline disaccharides of resistant SCLC cell lines

Cell line	IC_{50} (ng/mL)*						
	Doxorubicin	Daunorubicin	MEN10732	MEN10733	Idarubicin	MEN10749	MEN10746
POVD	30 ± 10	30	320 ± 240	375 ± 30	1 ± 0	47 ± 3	9 ± 1
POVD/DX	850 (28)	200 (7)	>10000	>10000	80 (80)	ND	230 (25)
POGB	15 ± 7	21 ± 13	1000 ± 0	285 ± 49	1.5 ± 0.7	1950 ± 1300	20 ± 10
POGB/DX	385 ± 90 (26)	270 ± 190 (13)	>10000	>10000	24 ± 8 (16)	4750 ± 1700 (2.4)	1650 ± 1900 (82)

*IC_{50} values were determined by MTT assay, following a 96 hr-exposure. In parentheses: resistance index calculated by dividing IC_{50} values in resistant and parental cells. ND, not determined.

gene, respectively [11, 12]. Parental SCLC cell lines exhibited a pattern of cellular response to anthracycline disaccharides comparable to that of cell lines of different origin. Again, only MEN 10746 exhibited a cytotoxic activity similar to or higher than that of doxorubicin. However, a marked cross-resistance was found in both cell lines for all tested compounds, including MEN 10746.

Antitumor Activity Studies

A study of antitumor efficacy was performed only with MEN 10746, the most cytotoxic analog of the disaccharide series. Table 5 shows the antitumor effects achieved by the analog compared to doxorubicin and idarubicin. The optimal dose of each tested drug according to a schedule of every 7 days (repeated 3 times) is reported. As expected from cytotoxicity studies, the analog was less potent than reference compounds. In all tested tumors, the antitumor activity of MEN 10746 was comparable to that of doxorubicin in terms of tumor growth inhibition (TWI > 60%) and LCK values. Idarubicin was found to be ineffective, as was expected from previous studies on the treatment of solid tumors [2], in spite of its high cytotoxic potency, which is reflected by the maximum tolerated dose.

DISCUSSION

The results of this study provide new insights into the critical role of the sugar moiety in the biological activity of anthracyclines. Comparative studies of the disaccharide and monosaccharide anthracyclines indicated that only the 4-demethoxy derivative with an axial configuration (MEN 10746) achieved a cytotoxic potency comparable to that of doxorubicin. In contrast, a marked reduced potency was shown by the other 3 disaccharide analogs. No analog of the series overcame cellular resistance mediated by overexpression of membrane transport systems (MDR-1, MRP). Therefore, from a pharmacological point of view, the most relevant information arising from the present study is that the optimal (axial) configuration peculiar to the fucose residue of MEN 10746 conferred a significant *in vivo* activity against human solid tumors, unlike the known daunorubicin-related monosaccharide anthracyclines, including 4-demethoxydaunorubicin [2].

The results of the present study are consistent with a role of the sugar moiety in the biochemical and biological activity of anthracyclines and provide new information concerning relevant modifications leading to optimal drug activity. The influence of the configurational properties of the anthracycline disaccharides on cytotoxic potency reflected the drug's ability to stabilize topoisomerase II-mediated DNA cleavage. Indeed, the glycosides with the equatorial disposition of the disaccharide moiety (MEN 10732 and 10749) did not stimulate DNA cleavage, whereas stimulation was found with analogs with the axial orientation in the linkage between the two sugars. A role of aminosugar conformation is well known for monosaccharides analogs, as clearly documented by the lack of activity of the β anomers [14]. However, the dramatic change in pharmacological activity of the β isomers is associated with a different interaction with DNA [14]. Relevant to this point is the observation that, in this study, no correlation could be found between topoisomerase II inhibition and DNA binding affinity, since all tested compounds exhibited similar binding parameters (Table 6). This observation is consistent with a lack of influence of the second sugar on daunorubicin interaction with DNA [15].

TABLE 5. Antitumor activity of the anthracycline disaccharide analog (MEN 10746) in a panel of human tumor xenografts

Tumor line	1st day of treatment	Doxorubicin			Idarubicin			MEN 10746		
		mg/kg*	TWI%†	LCK‡	mg/kg*	TWI%†	LCK‡	mg/kg*	TWI%†	LCK‡
A431	4	7	79	0.84	1.1	44	0.21	8.5	66	0.54
POVD	15	7	71	0.41	1.2	41	0.24	8.5	80	0.65
A2780	11	7	70	1.75	1.2	35	0.27	10	68	1.48

*Optimal dose, according to a schedule of every 7 days (repeated 3 times). Drugs were delivered i.v.
†Optimal tumor weight inhibition after the end of drug treatments.
‡Log_{10} cell kill. See Materials and Methods for calculation.

TABLE 6. Binding parameters for interaction of anthracycline disaccharides with calf thymus DNA

Drug	$K \times 10^{-5}$ (M^{-1})	n
Daunorubicin	8.2 ± 1.4	3.0 ± 0.3
Idarubicin	4.5 ± 1.0	2.7 ± 0.2
MEN 10746	2.4 ± 0.4	1.5 ± 0.1
MEN 10749	3.5 ± 0.5	2.0 ± 0.5

K is the apparent binding constant. n is the exclusion parameter (for base pair).

The marked influence of the orientation of the second sugar residue was reflected in a substantial cytotoxic activity only for the 4-demethoxy analog MEN 10746. It is conceivable that the addition of the second sugar may reduce the cellular uptake of the drug as a result of an increased hydrophilicity. This drawback could be at least in part compensated for by an increased hydrophobicity of the chromophore following removal of the methoxy group. Thus, an increased cytotoxic potency of the 4-demethoxy analog MEN 10746 over that of the corresponding daunorubicin analog (MEN 10733) is likely the combined result of an increased efficacy at target (topoisomerase II) level and an enhanced drug accumulation. Both effects have been reported following removal of the methoxy group in monoglycosylated natural compounds [3, 16]. Indeed, 4-demethoxy derivatives of daunorubicin and doxorubicin are characterized by a high cytotoxic potency [16]. The increased efficacy as topoisomerase II poison is likely related to the mode of DNA intercalation. Removal of the bulky methoxy group at position 4 could allow a more flexible stacking of the planar chromophore between base pairs, thus favoring an optimal accommodation of the sugar moiety in the minor groove and stabilization of the ternary complex (drug–enzyme–DNA). Such an interpretation is consistent with a marked influence of the second sugar only in the demethoxy analogs of the disaccharide series. A comparison of the stimulation of topoisomerase II DNA cleavage by the four disaccharide analogs suggests that removal of the methoxy group is not a sufficient condition for an efficient inhibition of topoisomerase II. However, modification in the intercalating moiety could influence external interactions of the sugar and the cyclohexene ring in the ternary complex. In agreement with a previous study, no steric constraints are present at the 4′-C of anthracyclines in the ternary complex, whereas no bulky substituents are tolerated at the 3′-C in order to retain activity against the target enzyme [5]. However, the present results document that substituents at the 4′-position may dramatically modulate drug activity.

Such evidence has made it possible to optimize the therapeutic properties of the disaccharide analogs of the new series. Indeed, the effort led to the synthesis of a doxorubicin analog of MEN 10746. This novel compound (MEN 10755), selected for clinical development, exhibited a promising profile of preclinical activity with unexpected efficacy in the treatment of tumor models characterized by intrinsic resistance to doxorubicin [17].

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