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A comparative study of cellular and molecular pharmacology of doxorubicin and MEN 10755, a disaccharide analogue

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Abstract

MEN 10755 is a disaccharide anthracycline endowed with a broader spectrum of antitumour activity than doxorubicin (DOX). To investigate the cellular and molecular basis of its action, cytotoxic activity, drug uptake, subcellular localisation, induction of DNA damage, and apoptosis were assessed in the human A2780 ovarian carcinoma cell line. Experiments with radiolabelled anthracyclines indicated that MEN 10755 exhibited reduced cellular accumulation and a different subcellular distribution (higher cytoplasmic/nuclear ratio) than DOX. In spite of the lower nuclear concentration, MEN 10755 was as potent as DOX in eliciting DNA single- and double-strand breaks, G2/M cell arrest, and apoptosis. Sequencing of drug-induced topoisomerase II cleavage sites showed a common DNA cleavage pattern for MEN 10755 and DOX. Cleavage sites were always characterised by the presence of adenine in −1 position. However, the extent of DNA cleavage stimulation induced by MEN 10755 was greater than that produced by DOX. Reversibility studies showed that MEN 10755-stimulated DNA cleavage sites were more persistent than those induced by DOX, thus suggesting a more stable interaction of the drug in the ternary complex. As a whole, the study indicated that the cellular pharmacokinetics of MEN 10755 substantially differs from that of DOX, showing a lower uptake and a different subcellular disposition. In spite of the apparently unfavourable cellular pharmacokinetics, MEN 10755 was still as potent as DOX in inducing topoisomerase-mediated DNA damage. Although the extent and persistence of protein-associated DNA breaks may contribute to the cytotoxic effects, the drug's efficacy as apoptosis inducer and antitumour agent could not be adequately explained on the basis of DNA damage mediated by the known target (i.e. topoisomerase II), thus supporting additional cellular effects that may be relevant in cellular response.

Keywords: Doxorubicin; Anthracycline disaccharides; Drug uptake; Apoptosis; DNA breaks; DNA topoisomerase

1. Introduction

The anthracycline antibiotic doxorubicin (DOX) has been one of the most extensively used agents in the chemotherapy regimens of cancer patients for the past 30 years [1,2]. The identification of topoisomerase II as the main molecular target of DOX cytotoxicity has provided a biochemical basis for the identification of more effective anthracyclines [3]. DNA topoisomerase catalyses the change in DNA topology via a concerted mechanism of transient DNA strand cleavage and religation. Anthracyclines stabilise a transient DNA–topoisomerase II complex in which DNA strands are cut and covalently linked to the enzyme subunits [4]. The stabilised complex results in DNA damage that is related to the cytotoxic effect [5,6]. Treatment with topoisomerase II inhibitors results in arrest of cells in the G1 and G2 phases of the cell cycle [5,6]. G2 arrest was observed after treatment with topoisomerase-targeted drugs, as with agents inducing other types of DNA damage. The net result of the signalling steps after treatment with topoisomerase-directed agents could be the initiation of cell death with the characteristic feature of apoptosis [5,6].

A common structural feature of anthracyclines is the presence of an amino sugar as the carbohydrate moiety

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Abbreviations: DOX, doxorubicin; DNA-SSB, single-strand breaks; and DNA-DSB, double-strand breaks.

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directly linked to the chromophore [1]. The non-intercalating moiety of the anthracycline molecule (i.e. the sugar residue) is believed to play a role in the stabilisation of the ternary complex DNA–drug–topoisomerase II [7]. Interestingly, it has been shown that disaccharide anthracyclines have peculiar antitumour properties [8]. A disaccharide anthracycline analogue of the series, MEN 10755, has been shown to produce a broader antitumoural effect than DOX in a wide panel of human tumours xenografted in nude mice, and to be active against tumours naturally resistant to DOX [9,10]. Furthermore, MEN 10755 was found to induce earlier and greater *in vivo* apoptosis than DOX in a variety of tumour types [9].

The aim of the study was to investigate the cellular and molecular bases of the efficacy of MEN 10755 and DOX in the human A2780 ovarian cancer cell line, with particular reference to cellular drug uptake and localisation, DNA damage, and induction of apoptosis. The results support an increased ability of the disaccharide analogue to induce topoisomerase-mediated DNA damage in spite of a reduced concentration at the nuclear level.

2. Materials and methods

2.1. Chemicals

DOX was purchased from Sigma-Aldrich and stock solutions (5 mM) were prepared in distilled water, aliquoted, and stored at −20°. MEN 10755 (7-*O*-(2,6-dideoxy-4-*O*-(2,3,6-trideoxy-3-amino-alpha-L-lyxo-hexopyranosyl)-alpha-L-lyxo-hexopyranosyl-(4-demethoxy-14-hydroxy daunomycinone hydrochloride) was synthesised at the Chemistry Department of Menarini Ricerche as previously described [9], and stock solutions (5 mM) were prepared in distilled water, aliquoted, and stored at −20°. Both compounds were stable in culture for 24 hr at 37° (data not shown). [^{14}C]DOX and [^{14}C]MEN 10755 were purchased from Amersham (53 and 56 mCi/mmol, respectively).

2.2. Cell line

Human A2780 ovarian carcinoma cells were maintained in RPMI-1640 (GIBCO BRL) supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U penicillin, and 100 μg streptomycin at 37° in a 5% CO_2, 95% air humidified incubator.

2.3. Cytotoxicity assay

The cytotoxicity of DOX and MEN 10755 was determined using the sulforhodamine B (SRB) assay [11]. A2780 cells were plated in 96-well microtitre plates in 200 μL medium. After 24 hr, the cells were exposed to drugs at the appropriate dilutions and allowed to incubate for an additional 1 or 24 hr. After drug treatment, cells were washed with saline and incubated in drug-free medium for about 3 doubling times (72 hr), and the cellular viability was then measured by the SRB assay. The IC_{50} (the concentration achieving 50% cellular mortality compared to untreated control) was evaluated by a curve in which the surviving percentage of cells was reported as a function of the drug concentration.

2.4. Drug accumulation study

Exponentially growing A2780 cells were plated at 3×10^6 cells in 75-cm^2 flasks (Falcon). After 48 hr, [^{14}C]MEN 10755 or [^{14}C]DOX at 0.5 μM was added for varying times. At the end of incubation, cells were washed 3 times with cold PBS and 1 mL of lysis buffer was added. The cells were collected by a scraper in Falcon tubes with 4 mL of lysis buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% Nonidet P-40, and then incubated for 5 min on ice. One millilitre of cellular lysate was kept to evaluate the radiolabelled drug incorporated into the cells. To separate the nuclear from the cytoplasmic fraction, the cells were centrifuged at 3000 rpm at 4° for 10 min (Sorvall RC-28S). The recovery of nuclei was checked by phase-contrast microscopy. The nuclei pellet and the supernatant were collected, and the nuclei were resuspended in 1 mL of PBS. Total radioactivity associated with nuclei and cytoplasm was determined by scintillation counting (Ultima Gold XR, Packard) with a Packard β-counter. The drug level in each fraction was expressed as pmol for 1×10^6 cells.

2.5. Alkaline elution

The alkaline elution filter assays for DNA-SSB and DNA-DSB were performed essentially as described by Kohn [12]. Briefly, A2780 cells growing in the exponential phase were labelled for 24 hr with 0.01 μCi/mL of [methyl-^{14}C]thymidine (Amersham) at 37°. The labelled precursor was removed from the medium 24 hr before the drug treatments. Cells were then exposed to drug at different concentrations (0.1, 1, and 5 μM) for 1 hr at 37°. After treatment, the cells were washed twice with cold PBS, detached by gentle scraping, and deposited (5×10^5 cells) on a 2-μm pore size polycarbonate membrane filter (Millipore). For the measurement of DNA-SSB, cells on the filter were lysed with 2 mL of 2% SDS, 25 mM Na_2 EDTA, pH 9.6, and 0.5 mg/mL of proteinase K, followed by an eluting solution containing 0.1% SDS, 20 mM H_4 EDTA, and tetrapropylammonium hydroxide at pH 12.1. For the evaluation of DNA-DSB, the eluting solution had the pH adjusted to 9.6. Elution of DNA on the filters was at 0.03–0.04 mL/min for 12 hr. DNA-SSB and DNA-DSB were expressed as rad-equivalents using a calibration curve obtained by eluting A2780 cells irradiated in ice with various doses of γ-ray. The experiments for the evaluation of the time dependency of the reversal of DNA-SSB and DNA-DSB were per-

formed as follows: after a 1-hr exposure to a 1-μM drug concentration, cells were washed with PBS and then incubated for an additional 1, 2, or 4 hr in drug-free medium and processed as described above.

2.6. Determination of apoptotic cells and cell cycle analysis

A2780 cells were plated in 6-well plates and cultured for 16–18 hr before treatment with different drug concentrations. After various incubation times, cells were trypsinised and processed for fluorescence-activated cell sorter (FACS) analysis. A2780 cells were fixed in 70% cold ethanol at 4° for 60 min. The cells were then centrifuged, washed in PBS, and resuspended at 106 cells/mL in a 1-mL propidium iodide (PI) (Sigma Chemical Co.) solution (50 μg/mL in sodium citrate) containing 12.5 μL of RNase A (0.5 mg/mL) (Sigma) and 12.5 μL of Nonidet P-40 0.1% (Sigma). The cells were incubated in the dark at room temperature for 30 min and kept at 4° in the dark before being analysed. The PI fluorescence of cellular DNA was measured using a FACSort flow cytometer (Becton Dickinson). All data were analysed using the CELLFIT software (Becton Dickinson). In separate experiments, the TUNEL (TdT-mediated dUTP nick end labelling) method provided comparable results to those obtained with PI labelling.

2.7. Topoisomerase II cleavage assays

Recombinant human topoisomerase IIα was purified from a yeast strain as previously described [13]. The purified isozyme was stored at −80° in 500 mM Tris–HCl, pH 7.7, 200 mM KCl, 10 mM EDTA, 10 mM EGTA, and 10% glycerol. Oligonucleotide was synthesised by PRIMM s.r.l. and purified by gel electrophoresis. The upper DNA strand was ^{32}P-end labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6000 Ci/mmol). The labelled strand was annealed with a 1.5-fold amount of the cold complementary strand in 10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA at 70° for 5 min, and slowly chilled to 25°. Topoisomerase II DNA cleavage reactions were performed in a volume of 20 μL in 10 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiotreitol (DTT), 0.1 mM EDTA, 1 mM ATP, and 15 mg/mL of BSA for 20 min at 37°. Reactions were stopped by adding SDS and proteinase K (0.1% and 0.1 mg/mL, respectively, final concentration) and further incubated at 42° for 30 min. After ethanol precipitation, the samples were resuspended in 2.5 μL of 80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue, heated to 95° for 2 min, chilled in ice, and then loaded on to a 15% polyacrylamide sequencing gel (7 M urea, 89 mM Tris–HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA). Gels were run at 70 W for 2 hr.

In reversibility experiments of the drug-induced DNA cleavage complex, the assay was performed with 1 μM of drugs. After the first incubation (37°, 20 min), samples were treated with NaCl (0.5 M, final concentration) for the indicated times (see legend to Fig. 6) before SDS/proteinase K treatment.

3. Results

3.1. Cellular drug accumulation and subcellular distribution

Fig. 1 shows the kinetics of total drug contents in whole cells (panel A) and in nuclear (panel B) and cytoplasmic (panel C) compartments in A2780 cells incubated for 30–240 min with 0.5 μM [^{14}C]MEN 10755 or [^{14}C]DOX, 0.5 μM being the lowest drug concentration necessary for drug detectability in the cytoplasmic compartment. During the observation time (2 hr), DOX was mainly localised inside the nuclei (10.6 $\pm$ 2.5 pmol/10^6 cells), and a lower level of radioactivity was detected in the cytoplasm (1.1 $\pm$ 0.1 pmol/10^6 cells). The nuclear accumulation of MEN 10755 (3.6 $\pm$ 1.3 pmol/10^6 cells) was 3-fold lower than that observed with DOX ($P < 0.05$), whereas the cytoplasmic accumulation (1.9 $\pm$ 0.3 pmol/10^6 cells) was greater than that observed with DOX ($P < 0.05$). After 1 hr of exposure, the cytotoxic effect of MEN 10755 was lower than that of DOX, but it increased following a long-term exposure (Table 1). The increase was more marked than that observed for DOX. Thus, the effects of short-term exposure were consistent with a reduced intracellular accumulation of MEN 10755.

3.2. Induction of DNA-SSB and DNA-DSB

The alkaline elution profile, after 1 hr of drug exposure, showed a linear increase in DNA-SSB induction by MEN 10755 and DOX at concentrations ranging from 0.1 to 5 μM (Fig. 2A). DNA-SSB induced by MEN 10755 appeared similar to those produced by DOX. Even in the experiments under neutral conditions (pH 9.6) (Fig. 2B), the DNA-DSB profile obtained with the two anthracyclines was comparable.

The persistence of DNA cleavage was investigated (under both elution conditions) after drug removal. Cells were exposed for 1 hr to an equimolar drug concentration (1 μM) and, after washing, incubated in drug-free medium for various times (Fig. 3A and B). The levels of DNA-SSB were similar for MEN 10755 and DOX up to 4 hr after drug removal, although an increase, not statistically significant, was observed with both compounds 2 hr after drug washing (Fig. 3A). A time-dependent increase in DNA-DSB was observed with both drugs following drug removal (Fig. 3B).

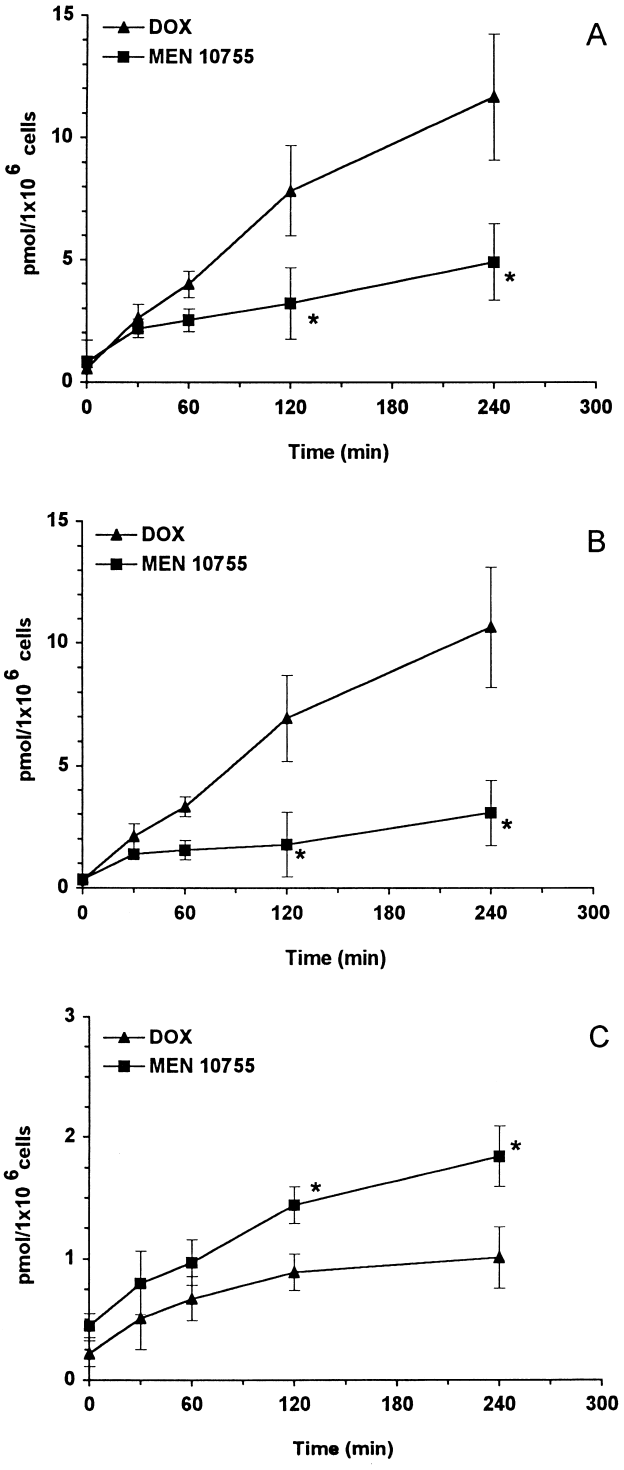

Fig. 1. Time-course of whole cell (A), nuclear (B), and cytoplasmic (C) uptake of anthracyclines in A2780 cells after incubation with [^{14}C]MEN 10755 (■) and [^{14}C]DOX (▲) at 0.5 μM of extracellular drug concentration. At the indicated times, cells were washed and analysed for drug content by scintillation counting as described in Materials and Methods. Each point is the mean of three independent determinations. Bars, SE. *$P < 0.05$ compared to DOX by Student's *t*-test.

Table 1
Cytotoxic effect of DOX and MEN 10755 on A2780 cells following 1- and 24-hr drug exposure

Compound	IC_{50}[a] (nM)	
	1 hr	24 hr
DOX	56 ± 1*	9 ± 2
MEN 10755	268 ± 49	27 ± 11

[a] Drug concentration required to inhibit cell growth by 50%, determined from dose–response curves. Means ± SE of three independent determinations.

* $P < 0.05$ compared with MEN 10755, by Student's *t*-test.

3.3. Induction of apoptosis and perturbation of the cell cycle

Fig. 4 shows the level of apoptosis and the cell cycle distribution of A2780 cells following 24, 48, and 72 hr (Fig.

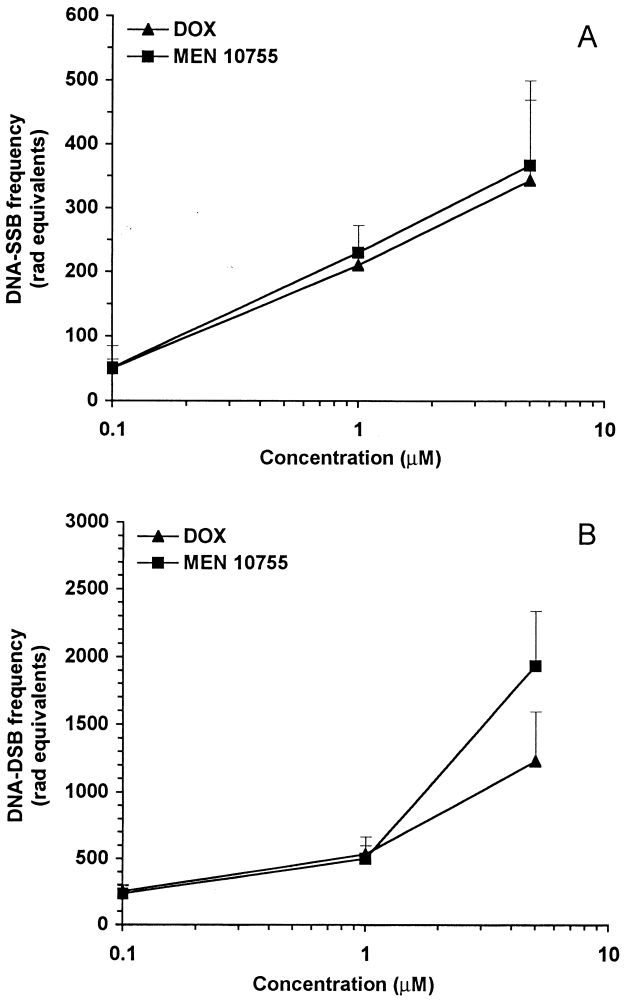

Fig. 2. DNA-SSB (A) and DNA-DSB (B) induced by DOX (▲) and MEN 10755 (■) in A2780 cells. Cells were exposed to drugs for 1 hr at 37°, lysed on the filter, and eluted at pH 12.1 (DNA-SSB) or pH 9.6 (DNA-DSB). Each point is the mean of three independent determinations. Bars, SE.

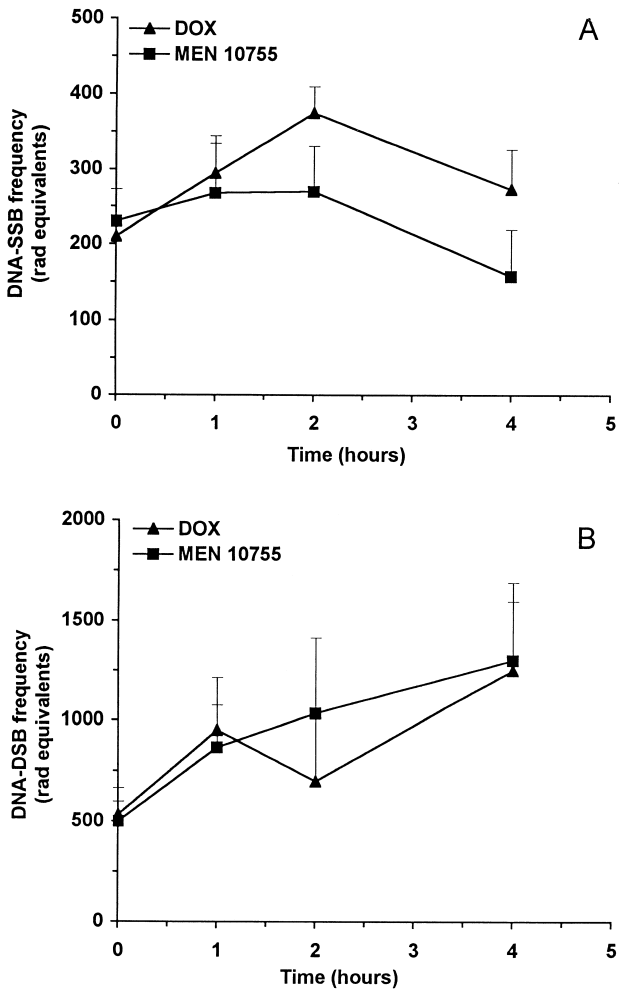

Fig. 3. Time-course of DNA-SSB (A) and DNA-DSB (B) persistence after incubation with DOX and MEN 10755. A2780 cells were preincubated with 1 μM of DOX (▲) or MEN 10755 (■) for 1 hr at 37° (time = 0) and then cultured in drug-free medium. At the indicated times, cells were lysed on the filter and eluted at pH 12.1 (DNA-SSB, panel A) or pH 9.6 (DNA-DSB, panel B). Each point is the mean of three independent determinations. Bars, SE.

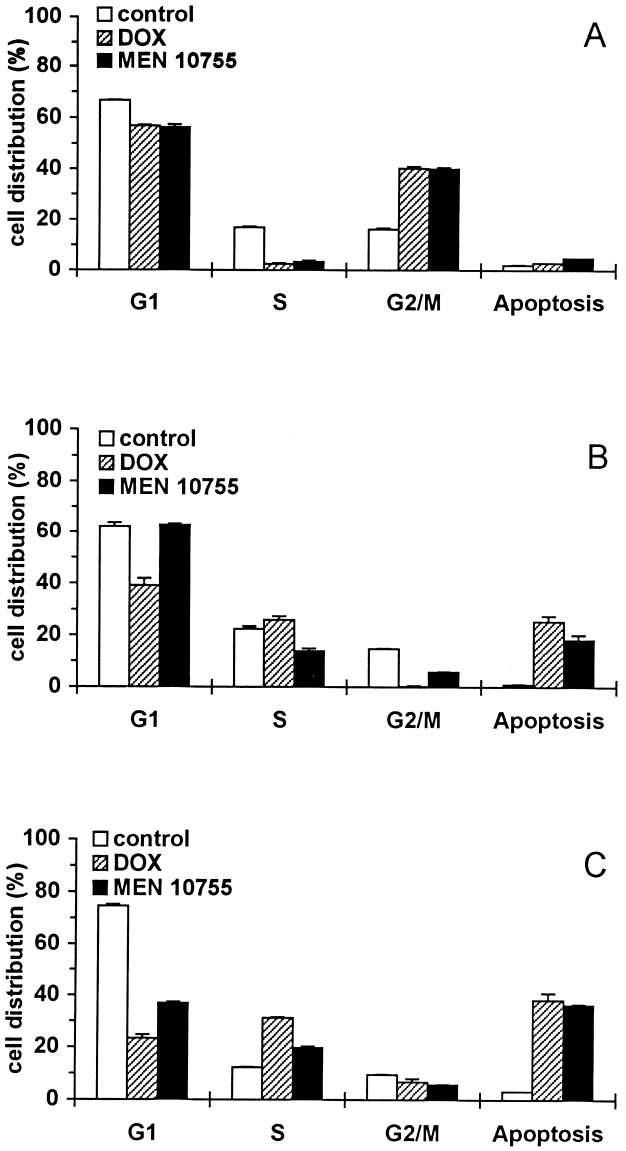

Fig. 4. Effect of DOX and MEN 10755 on the cell cycle phase distribution and apoptosis of A2780 cells, expressed as % of cell population after 24, 48, and 72 hr (panels A, B, and C, respectively) of drug exposure (0.1 μM). Each point represents the mean of three independent experiments. Bars, SE.

4A, B, and C, respectively) of exposure to 0.1 μM DOX or MEN 10755. Both compounds, after 24 hr of drug incubation, induced a cellular accumulation in the G2/M phase (Fig. 4A), a typical feature of the cellular response of topoisomerase II inhibitors [5]. Apoptosis was not evident at 24 hr of exposure. Between 24 and 48 hr (Fig. 4B) of drug incubation, a small number of MEN 10755- and DOX-treated cells started to undergo apoptosis. The phenomenon became more evident at 72 hr of exposure (Fig. 4C), with a concomitant reduction of cell distribution in the G1 phase.

3.4. Induction of the topoisomerase II-mediated cleavable complex

Induction of cleavage was analysed in a short DNA fragment corresponding to the 4230 to 4280 base position in the SV40 DNA genome sequence. Cleavage reaction was carried out by using the human recombinant topoisomerase IIα. MEN 10755 induced topoisomerase II cleavage sites with the same sequence specificity observed for DOX (Fig. 5). The high concentration (10 μM) of MEN 10755 resulted in a suppression of cleavage site stimulation similar to that shown under the same condition by DOX. It should be noted that some of the cleavage sites (4251 and 4243) were strongly stimulated by MEN 10755 and less so by DOX.

The cleavable complex induced by topoisomerase II inhibitors can be reverted by salt addition [14], and the time for reversibility is dependent on the stability of the drug–enzyme interaction [4]. NaCl was added to the reaction

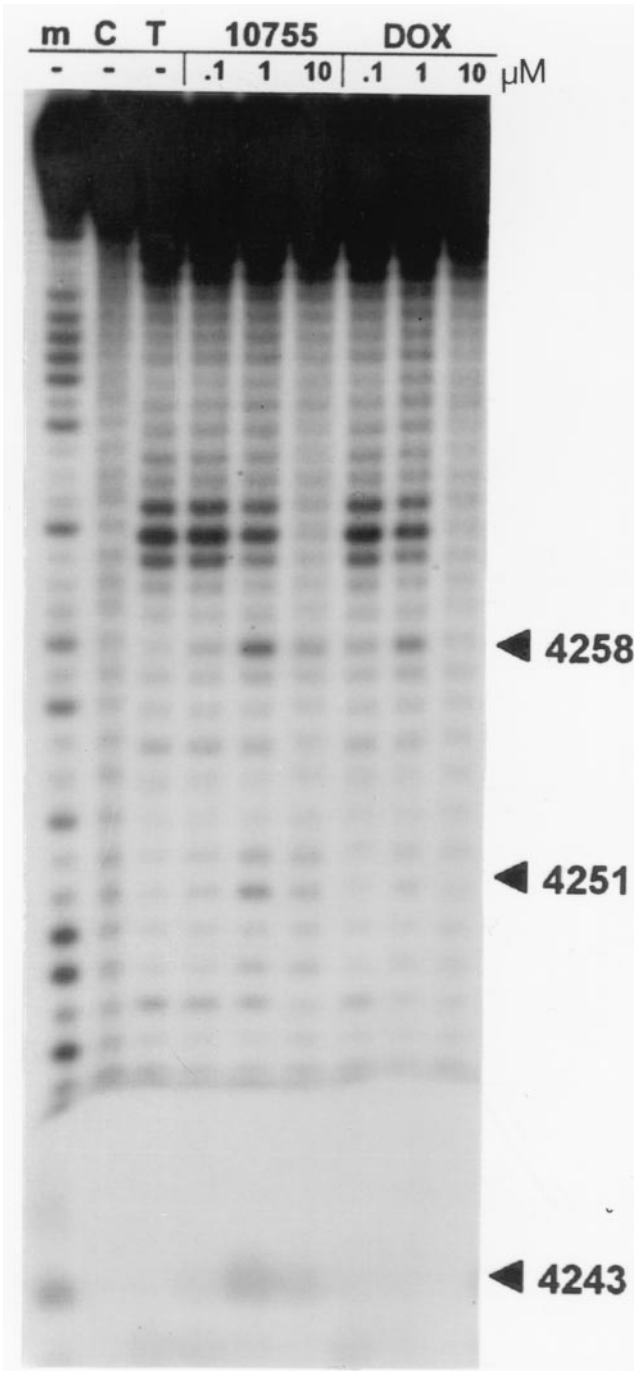

Fig. 5. Induction of topoisomerase IIα-mediated DNA cleavable complexes in SV40 oligonucleotide by MEN 10755 and DOX at the indicated concentrations (μM). Lane m, purine molecular markers; C, control DNA; T, DNA reacted with topoisomerase IIα alone. Arrows and numbers on the right indicate the cleavage sites.

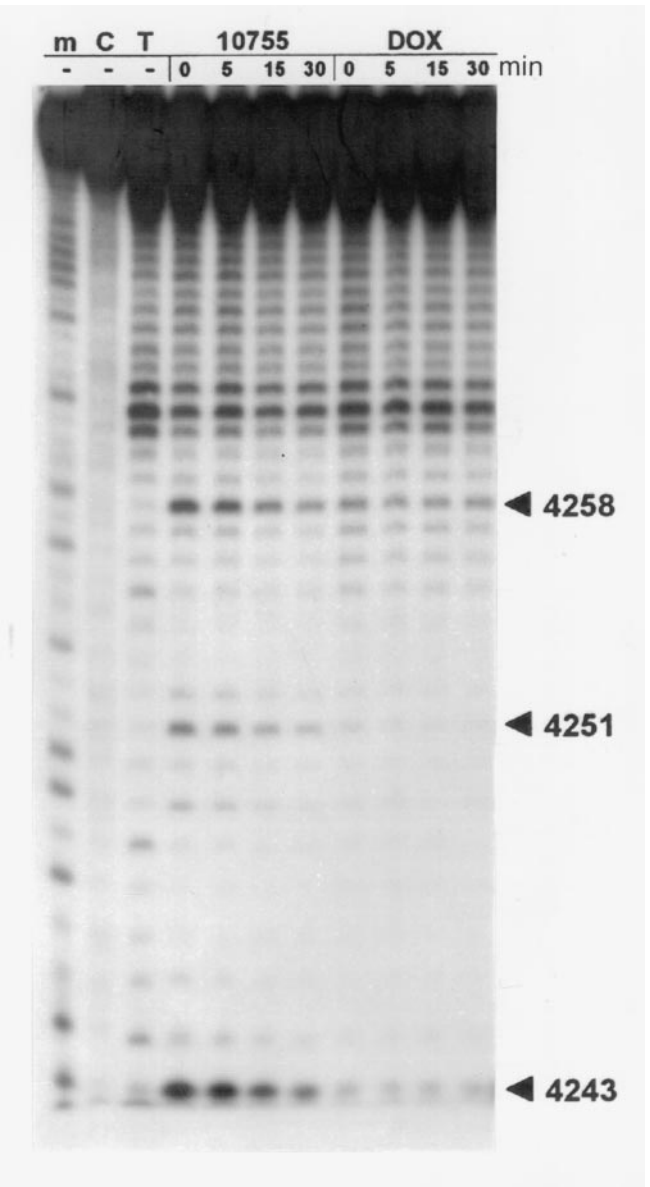

Fig. 6. Reversibility of anthracycline-induced DNA cleavable complexes following salt (NaCl, 0.5 M) treatment. The 5′-labelled SV40 DNA fragment was incubated with 1 μM MEN 10755 and DOX in the presence of topoisomerase IIα for 20 min at 37°. The reaction mixtures were then treated with NaCl (0.5 M, final concentration) at various times (0, 5, 15, and 30 min) before SDS/proteinase K treatment. Lane m, purine molecular markers; C, control DNA; T, DNA reacted with topoisomerase IIα alone. Arrows and numbers on the right indicate the cleavage sites.

mixture (final concentration, 0.5 M) which was then incubated for additional times, up to 30 min, before termination with SDS and proteinase K. As shown in Fig. 6, cleavage sites 4251 and 4243 of the SV40 DNA were stimulated by MEN 10755 (1 μM) and barely affected by DOX. MEN 10755-induced DNA cleavages were quite stable and slowly reversing. In fact, cleavage sites 4243 and 4251 were still present (although reduced) up to 30 min after salt treatment. Cleavage site 4258 was peculiar, being stimulated by both anthracyclines (intensity pattern: MEN 10755 > DOX) in an irreversible manner for up to 30 min.

4. Discussion

The results presented in this study indicate that the disaccharide MEN 10755 has a lower cytotoxic potency than

DOX. The reduced cytotoxic potency of MEN 10755, more marked following short-term exposure, could be related to reduced intracellular accumulation. This finding is consistent with the pharmacokinetics and better tolerability of the analogue in *in vivo* studies [9,10,15].

Furthermore, the results indicate that MEN 10755 is characterised by: (i) a greater ability than DOX to induce DNA cleavage sites through a long-lasting interaction with topoisomerase II; (ii) a reduced cellular uptake as compared to DOX; (iii) a striking accumulation in the cytoplasmic compartment; and (iv) the ability to elicit DNA-SSB and DNA-DSB and to induce cell cycle arrest in G2/M and apoptosis, as would be expected on the basis of the primary mechanism of action. Indeed, when A2780 cells were exposed to the same extracellular concentration of DOX or MEN 10755, the apoptotic response achieved by MEN 10755 was comparable to that induced by DOX, but the effects were produced with intracellular concentrations of MEN 10755 about 50% lower than those of DOX and with a nuclear accumulation as low as one-third that of DOX. Although the cellular pharmacokinetics is consistent with a reduced cytotoxic potency, the present findings suggest that MEN 10755 could be more potent than DOX in inhibiting the nuclear molecular target (topoisomerase II) and that it may also act through some additional mechanism(s) involving the cytoplasmic compartment [16–18].

The finding that topoisomerase II is the main molecular target for the cytotoxic activity of MEN 10755 is substantiated by its alkaline and neutral elution profiles (Figs. 2 and 3), which indicate a strong, concentration-dependent, and slowly reversible induction of DNA-SSB and DNA-DSB. Furthermore, the experiments with isolated recombinant human topoisomerase IIα provided evidence for the ability of MEN 10755 to poison topoisomerase IIα and to stabilise the ternary complex that leads to DNA cleavage (Figs. 5 and 6). It is noteworthy that the sequence specificity observed with MEN 10755 is similar to that obtained with DOX, indicating that also for MEN 10755 the presence of adenine in position -1 is required for the induction of primary genotoxic lesions [19]. However, the intensity and persistence of MEN 10755-induced DNA cleavages were increased compared to those induced by DOX, suggesting a tighter and more effective interaction between MEN 10755, topoisomerase II, and certain DNA sequences [20]. The enhanced efficacy in producing topoisomerase II-mediated DNA cleavage may account for the observation that the two anthracyclines induced a comparable cellular response (Fig. 4), despite the fact that nuclear concentrations achieved with MEN 10755 were lower than those with DOX.

A reduced nuclear/cytoplasmic ratio in the subcellular distribution has also been described for idarubicin [21], an anthracycline analogue more potent than DOX in terms of cytotoxic activity and DNA cleavage stimulation by topoisomerase II [7]. Like idarubicin, MEN 10755 is chemically characterized by the lack of a methoxy group in position 4. The major chemical novelty of MEN 10755 lies in the presence of a disaccharide moiety that could result in reduced ability to cross the biological membranes in view of the steric hindrance and the different hydrophilicity. In fact, the cytotoxic effect of MEN 10755 was lower than that of DOX following a 1-hr drug exposure, but the difference became less marked when the exposure time was prolonged to 24 hr (Table 1). A relevant localisation of MEN 10755 in the cytoplasm of A2780 cells was confirmed by using confocal laser microscopy (data not shown). Recent papers have suggested that a cytoplasmic/mitochondrial topoisomerase and additional targets localised at the cytoplasmatic level could contribute to the activation of apoptotic pathways by anthracyclines [16–18,22]. Further studies are warranted to investigate whether MEN 10755 might have remarkable actions on cytoplasmic targets relevant for apoptosis.

In spite of the lower intracellular content of MEN 10755, the cell cycle perturbation and the induction of apoptosis caused by drug treatment were comparable with those induced by DOX. These findings are consistent with the pharmacokinetics data in nude mice bearing the human A2780 ovarian carcinoma xenograft, indicating an accumulation of MEN 10755 lower than DOX in tumour tissue and in organs such as heart, liver, and kidney [15]. In keeping with the cellular pharmacology results, despite the lower intratumoural accumulation, the antineoplastic effect exerted by MEN 10755 was strikingly superior to that of DOX in the A2780 as well as in other tumours [10,15].

The present results emphasize the observation common to other anthracyclines that the drug interaction with the known nuclear target does not adequately account for the marked difference in drug efficacy against solid tumours. For example, idarubicin, one of the most potent topoisomerase inhibitors, is inactive against solid tumours, including the A2780 ovarian carcinoma [8]. The finding that the introduction of a second sugar in the idarubicin molecule confers activity against solid tumours, despite a comparable activity as topoisomerase II poison, supports the hypothesis that additional (still unknown) cellular effects contribute to the improvement of activity in the disaccharide series [8]. Based on the observation that MEN 10755 is preferentially located in the cytoplasm and is more effective than DOX in inducing Bcl-2 phosphorylation [10,23], a tentative explanation for the change in activity could be a drug interaction with the mithocondria, thus favouring the apoptotic response triggered by DNA damage. Elucidation of such additional effects may offer new therapeutic opportunities.

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References

[1] Arcamone F. Doxorubicin. Anticancer antibiotics. New York: Academic Press, 1981.
[2] Weiss RB. The anthracyclines: will we ever find a better doxorubicin? Semin Oncol 1992;19:670–86.
[3] Zunino F, Capranico G. DNA topoisomerase II as the primary target of anti-tumour anthracyclines. Anticancer Drug Des 1990;5:307–17.
[4] Burden DA, Osheroff N. Mechanism of action of topoisomerase II and drugs targeted to the enzyme. Biochim Biophys Acta 1998;1400: 139–54.
[5] Kaufmann SH. Cell death induced by topoisomerase-targeted drugs: more questions than answers. Biochim Biophys Acta 1998;1400:195–211.
[6] Brown JM, Wouters BG. Apoptosis, p53, and tumour cell sensitivity to anticancer agents. Cancer Res 1999;59:1391–9.
[7] Zunino F, Capranico G. Sequence-selective groove binders. In: Teicher B, editor. Cancer therapeutics: experimental and clinical agents. Totowa NJ, Humana Press Inc., 1997. p. 195–216.
[8] Arcamone F, Animati F, Bigioni M, Capranico G, Caserini C, Cipollone A, De Cesare M, Ettorre A, Guano F. Manzini S, Monteagudo E, Pratesi G, Salvatore C, Supino R, Zunino F. Configurational requirements of the sugar moiety for the pharmacological activity of anthracycline disaccharides. Biochem Pharmacol 1999;57:1133–9.
[9] Arcamone F, Animati F, Berettoni M, Bigioni M, Capranico G, Casazza AM, Caserini C, Cipollone A, De Cesare M, Franciotti M, Lombardi P, Madami A, Manzini S, Monteagudo E, Polizzi D, Pratesi G, Righetti SC, Salvatore C, Supino R, Zunino F. Doxorubicin disaccharide analogue: apoptosis-related improvement of efficacy *in vivo*. J Natl Cancer Inst 1997;89:1217–23.
[10] Pratesi G, De Cesare M, Caserini C, Perego P, Dal Bo L, Polizzi D, Supino R, Bigioni M, Manzini S, Iafrate E, Salvatore C, Casazza A, Arcamone F, Zunino F. Improved efficacy and enlarged spectrum of activity of a novel anthracycline disaccharide analogue of doxorubicin against human tumour xenografts. Clin Cancer Res 1998;4: 2833–9.
[11] Skehan P, Storeng R, Scudiero DA, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. New colorimetric assay for anticancer-drug screening. J Natl Cancer Inst 1990;82:1107–12.
[12] Kohn KW. Measurement of strand breaks and cross-links by alkaline elution. In: DNA repair: a laboratory manual of research procedures. New York: Marcel Dekker, 1981. p. 379–401.
[13] Cornarotti MG, Tinelli S, Willmore E, Zunino F, Fisher LM, Austin CA, Capranico G. Drug sensitivity and sequence specificity of human recombinant DNA topoisomerase IIα (p170) and IIβ (p180). Mol Pharmacol 1996;50:1463–71.
[14] Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 1984;226:466–8.
[15] Gonzalez-Paz O, Polizzi D, De Cesare M, Zunino F, Bigioni M, Maggi CA, Manzini S, Pratesi G. Tissue distribution, antitumour activity and *in vivo* apoptosis induction by MEN 10755 in nude mice. Eur J Cancer 2001;37:431–7.
[16] Serafino A, Sinimbaldi-Vallebona P, Piermarchi P, Bernard P, Gaudiano G, Massa C, Rasi G, Ranagnan G. Induction of apoptosis in neoplastic cells by anthracycline antitumour drugs: nuclear and cytoplasmic triggering? Anticancer Res 1999;19:1909–18.
[17] Lin JH, Castora FJ. DNA topoisomerase II from mammalian mitochondria is inhibited by the antitumour drugs, m-AMSA and VM-26. Biochem Biophys Res Commun 1991;2:690–7.
[18] Block RB, Thorburn DR, Thompson GN, Dahl HH. A topoisomerase II cleavage site is associated with a novel mithocondrial DNA deletion. Hum Genet 1995;95:75–81.
[19] Capranico G, Binaschi M, Borgnetto ME, Zunino F, Palumbo M. A protein-mediated mechanism for the DNA sequence-specific action of topoisomerase II poisons. Trends Pharmacol Sci 1997;18:323–9.
[20] Capranico G, Kohn KW, Pommier Y. Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin. Nucleic Acids Res 1990;18:6611–9.
[21] Duffy PM, Hayes MC, Cooper A, Smart CJ. Confocal microscopy of idarubicin localization in sensitive and multidrug-resistant bladder cancer cell lines. Br J Cancer 1996;74:906–9.
[22] Bellarosa D, Ciucci A, Bullo A, Nardelli F, Manzini S, Maggi CA, Goso C. Apoptotic events in a human ovarian cancer cell line exposed to anthracyclines. J Pharmacol Exp Ther 2001;296:276–83.
[23] Pratesi G, Polizzi D, Perego P, Dal Bo L, Zunino F. Bcl-2 phosphorylation in a human breast carcinoma xenograft: a common event in response to effective DNA-damaging drugs. Biochem Pharmacol 2000;60:77–82.