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Preclinical report

Differential *in vitro* interactions of a series of clinically useful topoisomerase-interacting compounds with the cleavage/religation activity of the human topoisomerase IIα and IIβ isoforms

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The topoisomerase II (TOP2)-associated DNA cleavage activity and the DNA sequence preference of 20 antitumor drugs, including 15 TOP2-interacting compounds, have been defined. Four major classes of drugs have been identified: (i) those which enhanced the stabilization of cleavable complexes at a single major site (e.g. amsacrine, doxorubicin), or (ii) at many sites (e.g. etoposide, azatoxin), with chemically related compounds having very similar, although not identical, cleavage patterns (e.g. etoposide, GL331 and Top-53); (iii) those which inhibited DNA breakage (e.g. aclarubicin, actinomycin D); and (iv) those which did not visibly interfere with TOP2-mediated cleavable complexes (e.g. ICRF-187, camptothecin). All drugs tested induced similar overall patterns of sites of preferred DNA cleavage, in the presence either of the two known isoforms, TOP2α or TOP2β, although relative intensities of signals at each position varied. It has been further shown that etoposide and its derivatives blocked the religation step downstream of the DNA cleavage step, whereas amsacrine, ellipticine, azatoxin and genistein acted upstream through enhancement of DNA cleavage. The information provided by this mechanistically based comparison can now be exploited in designing or synthesizing novel TOP2-interacting agents. [© 1999 Lippincott Williams & Wilkins.]

Key words: Catalytic cycle, DNA cleavage, inhibitors, religation, topoisomerase IIα, topoisomerase IIβ.

Introduction

The nuclear DNA topoisomerases II (TOP2) are essential enzymes involved in many vital aspects of nucleic acid metabolism, including DNA replication, transcription, chromosome structure, condensation and segregation, as well as in the organization of the nuclear matrix.[1,2] Central to its physiological function, TOP2 alters nucleic acid topology by passing an intact double helix of DNA through the transient double-stranded break made in a second DNA helix.[2,3] The catalytic cycle of TOP2 can be divided into several discrete steps: (i) binding of the enzyme to DNA followed by the formation of a cleavable complex (CC) at preferred sequences on DNA, (ii) cleavage of the DNA within the CC, (iii) double-strand DNA passage, (iv) religation of DNA, (v) ATP hydrolysis and (vi) enzyme turnover.[1,4] Two isoforms have so far been identified in human cells, i.e. the α (TOP2α) and β (TOP2β) isoforms.[5,6] Both isoforms seem to be functionally equivalent in complementation studies in yeast,[7] yet not in human cells,[8] and they differ in terms of their cellular localization and levels of expression during the cell cycle, i.e. the β isoform is expressed throughout the cell cycle, whereas the α isoform is strictly dependent on cellular proliferation.[9] Several major classes of antineoplasic agents act by interfering with the action of these TOP2 enzymes by converting them into cellular toxins.[4] Although some chemical agents exist which can interfere with TOP2 function without enhancing DNA cleavage like bis-dioxopiperazines,[10,11] most antitumor drugs form ternary DNA-drug-enzyme complexes that stabilize the otherwise transient TOP2-mediated cleavage (TMC) of DNA.[2,4,12] The equilibrium established between the CCs with cleaved DNA versus those with religated DNA is thereby displaced towards an accumulation of CCs with cleaved DNA. However, detailed studies have revealed that drugs that led to a similar displacement of equilibrium could be discriminated by their differential mechanisms of action. For

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example, etoposide acts through a block of the religation step, whereas genistein acts by increasing the overall level of DNA breakage.[4] Furthermore, it has also been shown that different drugs have affinities for TOP2-DNA duplexes through specific preferred DNA sequences.[13,14] These observations led to the hypothesis that these cleavage site differences might contribute to differential anticancer activity through selective gene damage by specific drugs.[14] However, to date, only limited studies aimed at comparing cleavage specificity and TOP2 isoform preference of clinically useful antitumor drugs have been reported.[15-17] Therefore, we have compared systematically the activity of a series of clinically useful antitumor compounds on the cleavage/religation step of the catalytic cycle of the human TOP2α and TOP2β enzymes. Such information providing evidence of their specificity or selectivity might then be exploited in designing or synthesizing novel topoisomerase-interacting drugs.

Materials and methods

Chemicals and drugs

Aclarubicin, actinomycin D, amsacrine, cisplatin, daunorubicin, distamycin A, doxorubicin, ellipticine and genistein were purchased from Sigma (Saint-Quentin Fallavier, France), camptothecin from Cipla (Bombay, India), bleomycin from Roger Bellon (Neuilly-sur-Seine, France), and ICRF-187 hydrochloride (dexrazoxane) from Chiron (Suresnes, France). The other test compounds, i.e. azatoxin, etopophos, etoposide, GL331 (4′-demethyl-4β-*p*-nitro-aniline-4-desoxypodophyllotoxin), NK-611 (4′-demethyl-epipodophyllotoxin-9 (2-deoxy-2-dimethylamino-4,6-*O*-ethylene-β-D-glucopyranoside) hydrochloride, Top-53 (4′-demethyl-4 β-[2-[*N*-(2-(*N*′*N*′-dimethylamino) ethyl)-*N*-methylamino] ethyl]-4-desoxypodophyllotoxin) dihydrochloride, topotecan and vinorelbine were provided by Pierre Fabre Médicament (Castres, France). Test compounds were dissolved in 2.5% dimethylsulfoxide (DMSO) used as a vehicle and obtained from Sigma, except for cisplatin which was dissolved in 0.9% NaCl and, with bleomycin, a $Fe^{2+}(NH_4)_2$ mol/mol solution was used.

Enzyme preparation

Purified human TOP2α was purchased from Topogen (Colombus, OH). Human TOP2β was purified from recombinant yeast as detailed previously.[17,18]

Experimental procedure

The principles of the TMC and religation assays have essentially been described elsewhere.[2,13,14] Briefly, the total reaction volume of 20 μl contained: (i) 2 μl eukaryotic TOP2 cleavage buffer 10× (200 mM Tris-HCl, pH 7.5, 550 mM KCl, 65 mM $MgCl_2$, 20 mM ATP, 75 mM mercaptoethanol and 225 μg/ml bovine serum albumin); (ii) 4-8 ng of DNA (the nuclear matrix-associated region of the SV40 DNA, between positions 4100 and 4380 of the genome) radiolabeled (approximately 40 000 c.p.m.) with T4-polynucleotide kinase (Boehringer, Mannheim, Germany) with [γ-^{32}P]ATP. The radiolabeled 3′-end of the probe was cleavable by the endonuclease *Eco*RI; (iii) the vehicle with or without test compound; and (iv) 2 U either of human TOP2α or recombinant human TOP2β, which corresponds to the amount of enzyme decatenating 200 ng of kDNA in 10 min. The reaction mixture was incubated for 20 min at 37°C, then stopped by the sequential addition of 2 μl sodium dodecyl sulfate (SDS) 10% (w/v) for 15 min at 37°C and 2 μl proteinase K at 2 mg/ml in 30 mM EDTA for 60 min at 42°C. When evaluating the kinetics of religation, 2 μl 5 M NaCl was added to the reaction mixture initially incubated for 20 min at 37°C, and the reactions were stopped by the sequential addition of SDS and proteinase K. Samples were precipitated with 65 μl ethanol for 120 min at −20°C and pellets were resuspended in 6 μl loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The precipitation step could be controlled by the addition of a 404 bp radiolabeled DNA fragment. Samples were heat-denaturated and separated by electrophoresis on a 6% polyacrylamide urea-containing sequencing gel. Signals on dried gels, corresponding to overall TOP2-induced DNA cleavages, were autoradiographed as well as counted and quantitated using a BioRad molecular imager apparatus and its molecular analyst software (BioRad, Irvy-sur-Seine, France).

Data analysis

Following quantitation, the level of TMC at a specific DNA cleavage site is identified by the intensity of a single band. The signal observed is compared with the corresponding signal in the control reaction performed in the presence of vehicle only. Signals could therefore be considered as being enhanced, diminished or left unchanged (neutral). Alternatively, in the case of the kinetics of religation, the rate of disappearance of cleaved DNA products was ex-

pressed as a percentage of the signal observed at equilibrium, i.e. in the absence of high salt in the reaction. Furthermore, in order to take account of a loss of material during the precipitation step of the reaction, an additional 404 bp DNA fragment could be added so as to normalize signals. The kinetics of religation were considered as either 'fast' or 'slow', depending on whether the level of cleaved DNA products dramatically diminished in 60 s or was scarcely affected after 5 min.

Results

Comparative action of antitumor drugs on TOP2-induced DNA cleavage

The nuclear matrix-associated region of SV40 DNA, known to be preferentially cleaved by TOP2,[13,14] was mapped for DNA cleavage induced by either isoforms of the human TOP2 enzyme in the presence or absence of several classes of antitumor drugs. A 281 bp DNA probe was used for the assay. Upon its addition, TOP2α induced several TMC sites, although only weakly, indicative of transient CCs whose DNA has been cleaved, representing its baseline pattern of activity on DNA (Figure 1, lane 5) at preferred specific DNA sequences. The potential activities of a series of 20 known antitumor compounds, including 15 topoisomerase-interacting drugs, were evaluated. The overall results are summarized in Table 1. Overall five groups could be identified based on their effects on the stabilization of CCs under these experimental conditions.

Compounds which caused enhancement or stabilization of a large panel of TMC sites. This class is exemplified by etoposide (Figure 2, lane 3) and NK-611 (Figure 2, lanes 16–18) at 10^{-6} M or above. The position and intensity of TMC sites were similar, at equivalent concentrations, except for discrete, slight variations. This enhancement occurred at TMC sites that were already evident as part of the baseline activity of TOP2α, but were only really revealed by longer exposure times (data not shown) and generally were beyond detection under the standard exposure conditions (Figure 2, lane 2). Other etoposide-like compounds, i.e. Top53 and GL331, enhanced signals from a large number of CCs at 10^{-6} M or above, again like etoposide. Other unrelated compounds such as genistein and azatoxin also acted at a majority of preferred DNA sites. Patterns of TMC sites differed amongst these various compounds, although the chemically related drugs showed similar patterns, as it was the case for the cleavage patterns obtained with either etoposide and etoposide-like compounds, except for a few sites where the intensity of the signals differed. All the other compounds belonging to this class could be singled out according to their individual pattern of TMC sites, in terms of either the position of cleavage on DNA and/or the intensity of stabilization.

Compounds which caused enhancement or stabilization of one major TMC site. This class is exemplified by amsacrine at 10^{-6} M or above (Figure 2, lanes 10–12) and doxorubicin at 10^{-6} M (Figure 2, lane 15). Daunorubicin at 10^{-5} M and ellipticine at 10^{-4} M enhanced the stabilization of CCs at one major DNA site. Furthermore, the two chemically related compounds doxorubicin and daunorubicin stabilized the same single TMC site, although the optimally active concentrations differed (Table 1).

Compounds that inhibited the stabilization of CCs. Aclarubicin (Figure 2, lanes 4–6) as well as actinomycin D (Table 1) were found to inhibit the stabilization of CCs, as revealed by the disappearance of the baseline TOP2 activity, at 10^{-6} and 10^{-5} M or greater, respectively.

Compounds that did not interfere with the stabilization of CCs. Neither camptothecin (Figure 1, lane 4)

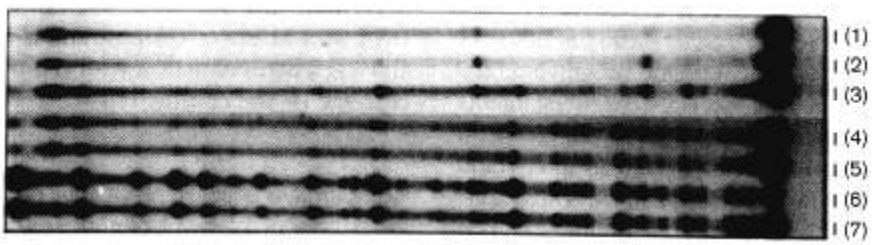

Figure 1. DNA cleavage patterns of TMC sites induced by topoisomerase inhibitors. Aliquots of 10^{-4}, 10^{-5} and 10^{-6} M distamycin (lanes 1–3), 10^{-5} M camptothecin (lane 4), and 10^{-4} and 10^{-5} M etoposide (lanes 6 and 7) were solubilized in vehicle (DMSO). Lane 5 contained vehicle only.

Table 1. Effects of 20 antitumor compounds, including known TOP2-interacting drugs, on the stabilization of CCs

Mechanism of action	Test compounds	Effects on CCs	Active concentration (M)
(A) TOP2 cleavable complex stabilizing agents			
(1) Non-intercalating agents			
	etoposide	enhances multiple sites	$\geqslant 10^{-6}$
	etopophos	no effect	–
	Top-53	enhances multiple sites	$\geqslant 10^{-6}$
	NK-611	enhances multiple sites	$\geqslant 10^{-5}$
	GL331	enhances multiple sites	$\geqslant 10^{-6}$
	azatoxin	enhances multiple sites	10^{-4}
	genistein	enhances multiple sites	10^{-4}
(2) Intercalating agents			
	amsacrine	enhances a single site	10^{-4}
	doxorubicin	enhances a single site	10^{-6}
	daunorubicin	enhances a single site	10^{-5}
	ellipticine	enhances multiple sites	$\geqslant 10^{-6}$
(B) TOP2 catalytic inhibitors only			
	aclarubicin	inhibition	$\geqslant 10^{-6}$
	ICRF-187	no effect	–
(C) Dual inhibitors of TOP1 and TOP2			
	actinomycin D	inhibition	$\geqslant 10^{-5}$
	distamycin A	enhances at single site/inhibition	$10^{-7}/10^{-6}$
(D) TOP1 inhibitors			
	camptothecin	no effect	–
	topotecan	no effect	–
(E) DNA-damaging agents			
	cisplatin	no effect	–
	bleomycin	no effect	–
(F) Tubulin-interacting agent			
	vinorelbine	no effect	–

Drugs are grouped according to their main proposed mechanism(s) of action, according to DeVita *et al.*[19] Compounds were shown either to enhance one or several cleavage sites or inhibit ('inhibition') the formation of CCs or they were judged as neutral ('no effect').

nor topotecan (Figure 2, lanes 7–9) exerted any noticeable effect on the stabilization of CCs transiently induced by the enzyme TOP2α. These observations are consistent with their reported abilities to interfere specifically with the catalytic activity of the TOP1, but not with the TOP2 enzyme.[19] The prodrug etopophos[20] was inactive and ICRF-187, which acts at a late step of the catalytic cycle of TOP2,[21] also did not alter baseline TMC activity (Table 1). One tubulin-interacting agent, vinorelbine, and two DNA-damaging agents tested also fell into this categories although with differing patterns. Cisplatin, implicated in interstrand DNA adduct formation, did not stabilize TMC sites, whereas bleomycin, which induces double-stranded DNA breaks,[22] led to numerous cleavage sites on the DNA probe, but these were TOP2-independent (Table 1 and data not shown).

Compounds with 'dual' effects on stabilization of CCs. The drug distamycin A could be singled out in this respect, since it was observed to modulate the interaction between TOP2 and DNA through an increase in the formation of CCs at certain preferred DNA sites at 10^{-7} M (Figure 1, lane 3), whilst preventing the stabilization of CCs located at other DNA sites at lower concentrations (Figure 1, lanes 1 and 2), when compared with the baseline pattern of cleavage by the enzyme (Figure 1, lane 5).

Comparative action of antitumor drugs on the activity of the two isoforms TOP2α and TOP2β

A selection of these topoisomerase-interacting anti-

tumor agents was further studied to analyze their capacities to stabilize CCs formed by TOP2β. Reactions with either isoform of the enzyme were processed simultaneously (Figure 3). Starting from an equivalent amount of enzymatic activity, the baseline of TOP2β activity was hardly visible (Figure 3, lane 2), in contrast to that of TOP2α (Figure 3, lane 1). This difference might be due either to a lower frequency of TOP2β-containing CCs in a cleavable step than that of TOP2α-containing CCs in a similar step or could

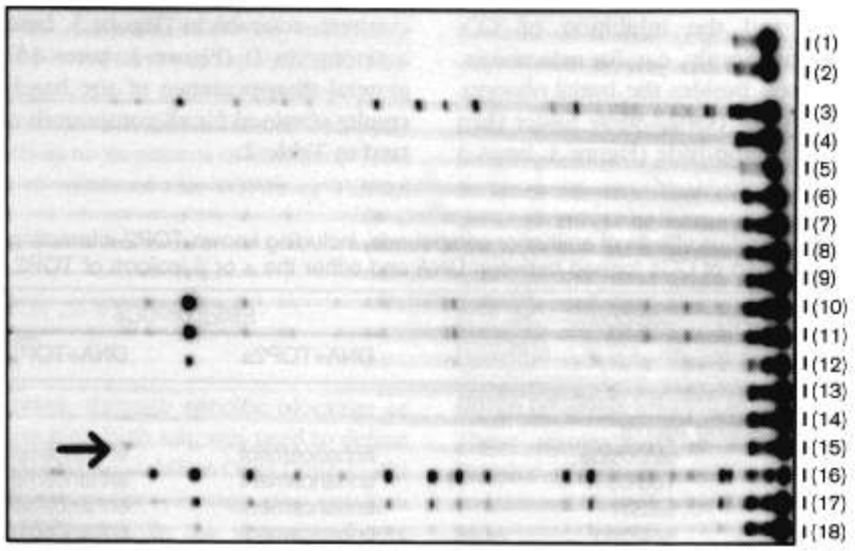

Figure 2. Comparison of DNA cleavage patterns of TMC sites induced by a series of known antitumor agents. Aliquots of 10^{-5} M etoposide (lane 3), 10^{-4}, 10^{-5} and 10^{-6} M aclarubicin (lanes 4–6),), 10^{-4}, 10^{-5} and 10^{-6} M topotecan (lanes 7–9), 10^{-4}, 10^{-5} and 10^{-6} M amsacrine (lanes 10–12), 10^{-4}, 10^{-5} and 10^{-6} M doxorubicin (lanes 13–15), and 10^{-4}, 10^{-5} and 10^{-6} M NK-611 (lanes 16–18) were solubilized in vehicle (DMSO). Lane 2 contained vehicle only and lane 1 DNA probe only.

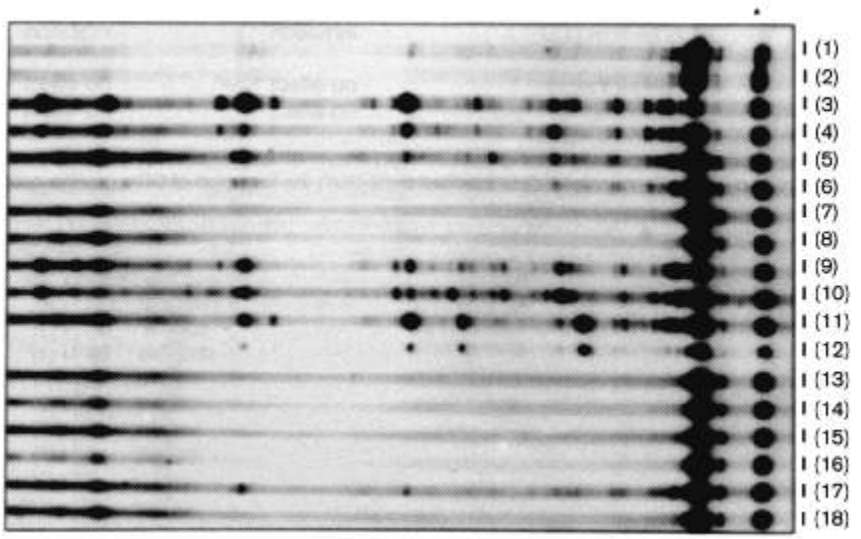

Figure 3. Comparison of DNA cleavage patterns induced by a series of TOP2-interacting agents in the presence of either TOP2α or TOP2β. Aliquots of etoposide (lanes 3 and 4), amsacrine (lanes 5 and 6), intoplicin (lanes 7 and 8), azatoxin (lanes 9 and 10), genistein (lanes 11 and 12), aclarubicin (lanes 13 and 14), actinomycin D (lanes 15 and 16) and ellipticine (lanes 17 and 18) were solubilized in vehicle (DMSO) at 10^{-4} M. Lanes 1 and 2 contained vehicle only. Reactions were performed in the presence of either TOP2α (odd lanes) or TOP2β (even lanes). A star indicates a 404 bp DNA fragment used to normalize the reaction after the precipitation step, as described in Materials and methods.

involve a technical bias. However, generally, the TOP2β-induced pattern of TMC sites appeared similar to that induced by TOP2α except for a uniformly lower intensity of all the bands, the faintest ones with TOP2α being below detection with TOP2β. These results imply that whilst our cleavage assay based on the TOP2β enzyme could appropriately analyze differential stabilization of CCs by drugs, it could not always distinguish between the absence of drug-mediated stabilization and the inhibition of CCs formation as observed previously, e.g. for aclarubicin, on TOP2α-DNA duplexes. Besides the initial observation that signals induced by TOP2β were lower than those induced by TOP2α, etoposide (Figure 3, lanes 3 and 4), amsacrine (Figure 3, lanes 5 and 6) and azatoxin (Figure 3, lanes 9 and 10), stabilized CCs at TMC sites that were similar in position but varied in intensity, depending on the TOP2 isoform used, TOP2α or TOP2β. In addition, genistein (Figure 3, lanes 11 and 12) and ellipticine (Figure 3, lanes 17 and 18) also induced patterns of TMC sites that were more similar in positions on the DNA and intensities in the presence of either enzyme, TOP2α or TOP2β. In contrast, aclarubicin (Figure 3, lanes 13 and 14) and actinomycin D (Figure 3, lanes 15 and 16) led to a general disappearance of the baseline activities. The results obtained for all compounds tested are summarized in Table 2.

Table 2. Comparative effects of antitumor compounds, including known TOP2-interacting drugs, on the stabilization of CCs formed between DNA and either the α or β isoform of TOP2

Mechanism of action	Compound	Effect on CCs	
		DNA+TOP2α	DNA+TOP2β
(A) TOP2 cleavable complex stabilizing agents			
(1) Non-intercalating agents			
	etoposide	enhancement	enhancement
	Top-53	enhancement	enhancement
	GL331	enhancement	enhancement
	azatoxin	enhancement	enhancement
	genistein	enhancement	enhancement
(2) Intercalating agents			
	amsacrine	enhancement	enhancement
	ellipticine	enhancement	enhancement
(B) TOP2 catalytic inhibitor only			
	aclarubicin	inhibition	inhibition
(C) Dual inhibitor of TOP1 and TOP2			
	actinomycin D	inhibition	inhibition
(D) TOP1 inhibitors			
	camptothecin	no effect	no effect
	topotecan	no effect	no effect

Drugs are grouped according to their main proposed mechanism of action, according to DeVita *et al.*[19] Compounds either enhanced ('enhancement') or inhibited ('inhibition') the formation of CCs, or were neutral ('no effect').

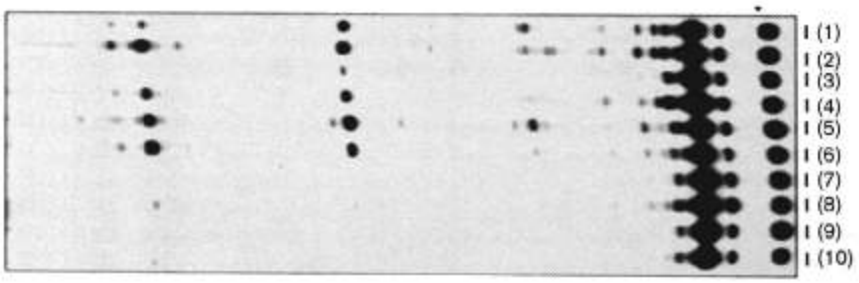

Figure 4. Comparison of DNA cleavage patterns induced by a series of TOP2-interacting agents in the presence of either TOP2α or TOP2β. Aliquots of 10^{-4} M etoposide (lanes 1 and 2), 10^{-4} M GL331 (lanes 3 and 4), 10^{-4} M Top-53 (lanes 5 and 6) and 10^{-5} M camptothecin (lanes 9 and 10) were solubilized in vehicle (DMSO). Lanes 7 and 8 contained vehicle only. Reactions were performed in the presence of either TOP2α (lanes 2, 4, 6, 8 and 10) or TOP2β (lanes 1, 3, 5, 7 and 9). A star indicates a 404 bp DNA fragment used to normalize the reaction after the precipitation step, as described in Materials and methods.

It is perhaps interesting to note that amongst the drugs tested, only the stabilization of TMCs induced by azatoxin and Top-53 seemed to be relatively more prominent with TOP2β, as compared with TOP2α. A comparison of etoposide and its two derivatives GL331 and Top-53 also revealed minor, but definite, variations in terms of the intensities of TMC sites at certain locations (Figure 4). Furthermore, clear differences could also be noted between CCs formed from DNA and TOP2α or TOP2β, with GL331 notably stabilizing many fewer TOP2β-DNA CCs than etoposide and Top-53 (Figure 4, lanes 3 versus 2 and 5). Therefore, it is apparent that each etoposide derivative can definitely be identified according to its pattern of stabilized TMC sites with respect to either of the TOP2α or TOP2β isoforms.

Action of compounds on the cleavage/religation equilibrium

The assay of religation, through specific blockage of the cleavage reaction with high salt, was used to define whether the drugs shown to stabilize CCs (Table 1) did so by: (i) a stimulation of the cleavage step or (ii) a block of the religation step. In the absence of test drug, TOP2α induced several weak TMC sites on DNA representing the baseline activity (Figure 5, lane 1). Upon the addition of high salt, these TMC sites disappeared rapidly with time (Figure 5, lanes 2 and 4). The inclusion of etoposide in the reaction resulted in a stabilization of TMCs at numerous sites on the DNA probe (Figure 5, lane 5). Following the addition of high salt, this pattern of TMCs hardly changed visibly with incubation times prolonged for up to 20 min (Figure 5, lanes 6–11). In contrast, whilst the addition of amsacrine also induced a characteristic pattern of TMCs (Figure 5, lane 12), the signals corresponding to cleavage dramatically decreased within 15–60 s after the addition of high salt (Figure 5, lanes 13–15), leaving only residual cleaved CCs after longer periods of incubation from 2 to 20 min (Figure 5, lanes 16–18). This differential effect of high salt concentration on drug-stabilized CCs was readily appreciated by quantifying, in the different lanes, one representative major site of cleavage for etoposide and amsacrine (arrowed signal in Figure 5). The resulting curves indicative of the kinetics of the disappearance of cleavage, i.e. of religation (Figure 6), were thus clearly different for these two drugs. These results can be summarized as follows: the kinetics of religation associated with amsacrine are considered 'fast', whilst those of etoposide are judged 'slow'.

Azatoxin, ellipticine, genistein, GL331, NK-611 and Top-53 previously found to enhance stabilization of

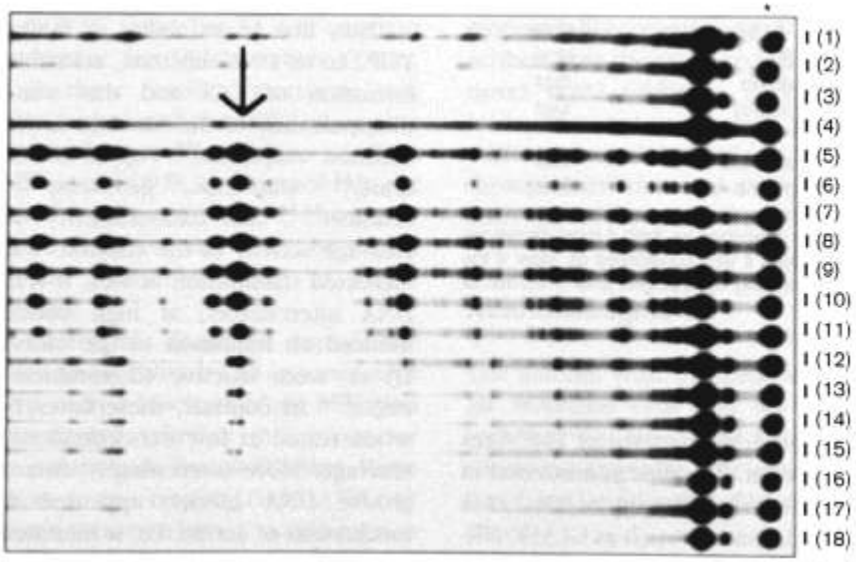

Figure 5. Assays of religation. The kinetics of DNA cleavage patterns induced by etoposide (lanes 5–11) or amsacrine (lanes 12–18) tested at 10^{-4} M in vehicle (DMSO). Lanes 1–4 contained vehicle only. Besides TMC control reactions (lanes 1, 5 and 12), end-points for the kinetics of incubation with high salt were 15 s (lanes 6 and 13), 30 s (lanes 2, 7 and 14), 60 s (8 and 15), 2 min (lanes 3, 9 and 16), 5 min (lanes 10 and 17) and 20 min (lanes 4, 11 and 18). The arrowed signal corresponding to one representative major TMC site for both etoposide and amsacrine was quantitated (see Figure 6). A star indicates a 404 bp DNA fragment used to normalize the reaction after the precipitation step, as described in Materials and methods.

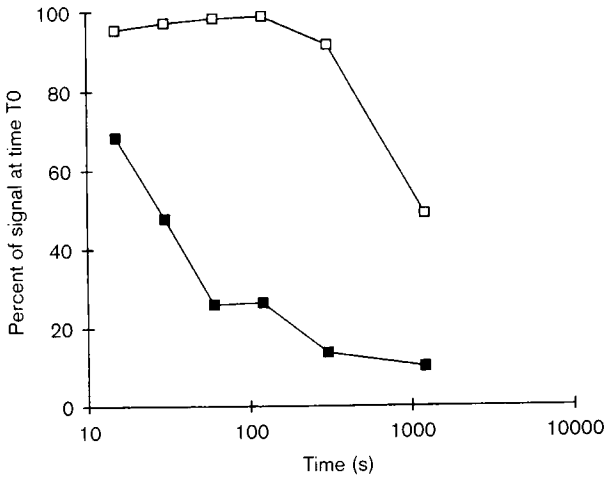

Figure 6. Kinetics of religation. Curves were obtained after quantitation of one representative cleavage site (arrow in Figure 5) induced by etoposide (□) or amsacrine (■). Results are expressed as a percentage of the signal at equilibrium, i.e. before the addition of high salt, at time (T0) zero.

Table 3. Effect of known TOP2-interacting antitumor compounds on the kinetics of religation

Mechanism of action[19]	Compound	Kinetics of religation
TOP2 cleavable complex stablizing agents		
(1) Non-intercalating agents		
	etoposide	slow
	Top-53	slow
	NK-611	slow
	GL331	slow
	azatoxin	fast
	genistein	fast
(2) Intercalating agents		
	amsacrine	fast
	ellipticine	fast

The kinetics of religation were described as 'fast' if most cleavages disappeared within 60 s, whereas it was considered as 'slow' if the intensity of TMC sites was only slightly changed after 5–20 min of incubation.

the cleavages with drug-specific patterns of TMC sites (Table 1) were assayed next. The data summarized in Table 3 show that, indeed, the intensity of TMC sites stabilized by etoposide derivatives such as GL331, NK-611 or Top-53 were hardly diminished after 5-20 min of incubation, i.e. with kinetics similar to those of etoposide. In contrast, most of the TMC sites stabilized by azatoxin, ellipticine or genistein were quickly religated, within 20-60 s after the blockage of the cleavage step by the addition of high salt in the reaction, being more like amsacrine (Figure 5), with only residual levels of cleaved DNA persisting after 5-20 min of incubation.

Discussion

It is of major importance, within the scope of identifying new inhibitors of the TOP2 enzyme, notably in the case of compounds derived from known leads, to determine whether they discriminate in terms of site of action within the catalytic cycle of the enzyme. Through a mechanistically based comparison of a wide range of TOP2-interacting antitumor compounds, this study focuses on the cleavage site selectivity and/or specific mode of action of compounds on the displacement of the cleavage/religation equilibrium for either α or β isoform of TOP2.

The methodology developed in this study appeared to provide a level of sensitivity sufficient to detect a baseline cleavage activity of human TOP2α and TOP2β on a DNA probe. The observed pattern of several weak sites of TMC reflects the cleavage/religation equilibrium of the enzyme. The ratio of cleaved fragment to uncleaved probe confirmed the fact that, in the absence of any drug, the equilibrium lies heavily towards religation.[2] Furthermore, this visible baseline cleavage activity of TOP2 allowed for drug-driven shifts of the equilibrium towards either an increased stabilization of CCs or an inhibition of the formation of CCs to be accurately evaluated.

Thus, due to its ability to inhibit the binding of TOP2 to its DNA substrate, aclarubicin prevented the formation of CCs and this was reflected by a disappearance of the baseline activity of TOP2.[23] In contrast, etoposide,[14] Top53, GL331, NK-611, amsacrine,[14,24] ellipticine,[25] genistein,[26,27] azatoxin,[24] doxorubicin[13,14] and daunorubicin[13] all stimulated the cleavage activity of the enzyme. With respect to this increased stabilization of CCs, it was also shown that DNA intercalators, at high concentrations, either induced an inhibition of the cleavage (actinomycin D) or were inactive (doxorubicin and daunorubicin).[12,13] In contrast, these latter DNA intercalators, when tested at low concentrations, could stimulate cleavage. More interestingly, distamycin A, a minor groove DNA binder, appeared to exert a dual mechanism of action, i.e. it inhibited the stabilization of CCs at a majority of TMC sites observed on the baseline pattern mediated by TOP2, at high concentrations, whereas it enhanced stabilization at a few different sites when tested at lower concentrations.[27] Finally, drugs that do not act as TOP2 inhibitors or act at a later step of the catalytic cycle (ICRF-187) were

found not to affect the baseline activity of TOP2, except for bleomycin known to induce a high rate of DNA breakage,[22] although in a TOP2-independent manner.

Overall, it has been shown that in the presence of either TOP2α or TOP2β, TOP2-interacting drugs from different mechanistic classes were characterized by diverse patterns of DNA cleavage. These patterns clearly differed amongst the compounds tested, although the patterns of closely chemically related drugs appeared similar, yet distinctive. For example, on the one hand, with etoposide-like compounds (etoposide, Top-53, GL331 and NK-611) and, on the other hand, with doxorubicin and daunorubicin. However, all other agents tested proved unique according either to their pattern and/or intensity of TMC sites generated (genistein, azatoxin, ellipticine).

It was further shown that the type of TOP2 isoform, either α or β, neither dramatically changed the ability of drugs to stabilize CCs nor altered the preferred TMC sites. These results thus indicate that TOP2 inhibitors tended to form similar ternary complexes with either isoform of TOP2 and the preferred sequences on DNA, as previously described for amsacrine.[16] However, clear differences in intensity at some locations and on a few sites were observed for most drugs, with a detailed study of the differential patterns induced by etoposide-like compounds demonstrating that they each could be individualized according to their own specific patterns of TMC obtained in the presence of either TOP2α or TOP2β. Only Top-53 and azatoxin exerted a net preference for TOP2β versus TOP2α. Therefore, although these results demonstrate that TOP2β is an *in vitro* target for all the TOP2-interacting compounds tested with site determinants on DNA mostly similar to those of TOP2α, one cannot rule out that the observed minor, although definite, changes at given preferred DNA sequences may in fact be critical determinants for the antitumor action of TOP2-interacting compounds.

Since it is well known that drugs that displace the CC equilibrium may do so either by an enhancement of the cleavage step or by a blockage of the religation step,[4] it was investigated whether this parameter could distinguish compounds by establishing these kinetics of religation. In this respect, GL331, NK-611 and Top-53 have been shown to have a mode of action similar to that of etoposide, since they were all found to efficiently block the religation step. In contrast, genistein, amsacrine, azatoxin and ellipticine did not; these interfered at the preceding step of the catalytic cycle of TOP2 (accelerate the forward cleavage reaction), since they did not decrease the rate of cleavage resealing in the presence of an overall increased stabilization of CCs. The result obtained with amsacrine, an inhibition of the religation step, is especially intriguing since it differs from a previous report,[10] but this might be explained by differences in terms of the precise experimental conditions used. In the present study, conditions aimed at mimicking the mode of action of TOP2 *in vivo*, i.e. relying on CCs that retain the cleaved DNA strands hybridized, instead of employing a suicide DNA stretch that is released and replaced by large excesses of another DNA fragment as described by Sorensen *et al.*[10]

Conclusion

In conclusion, this study has specifically identified the interference with TMC by certain clinically useful chemotherapeutic agents known to target TOP2. By comparing their patterns of TMC sites, compounds tested from different structural families may be uniquely idendified through (i) their mode of action relating to the steps of the catalytic cycle of TOP2 they target, i.e. formation of the CCs, increased cleavage or blockage of religation, (ii) the specific pattern of TMC sites observed in the case of stabilization of CCs, in terms of both the position of preferred DNA sites and the relative intensities at each site, and (iii) the preference for either the TOP2α or TOP2β isoform. Following these criteria, even various similar chemical derivatives can be distinguished, as demonstrated for etoposide-like compounds. The methodology described here therefore appears suitable for identifying novel TOP2 inhibitors or derivatives from known leads, and evaluating their mechanism(s) of action within the catalytic cycle of TOP2 enzymes, notably through their potential ability to stabilize CCs transiently induced by the human TOP2 enzymes.

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