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Feature Articles

DNA Topoisomerase-trapping Antitumour Drugs

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INTRODUCTION

THE ABILITY to modulate the topological state of DNA is essential for the survival of eukaryotic and prokaryotic cells. The enzymes responsible for this critical function are known as DNA topoisomerases [1–3]. These enzymes change DNA topology by catalysing one DNA strand passage through strand breaks followed by a DNA religation event [1–3]. Topoisomerases are defined according to the transient intermediate of the enzymatic reaction: double-strand and single-strand DNA breaks for topoisomerases II and I, respectively [1–3]. DNA cleavage results from a transesterification reaction in which tyrosine residues of the enzyme bind covalently to DNA break termini. Topoisomerase II is known to be the target of clinically effective antitumour drugs, including doxorubicin and etoposide [4–6]. These compounds hinder the DNA religation step and trap the transient intermediate of topoisomerase II strand passage reaction, thus stimulating DNA cleavage [7, 8]. Camptothecins, some of which are currently being evaluated in clinical trials, specifically trap the intermediate of topoisomerase I catalytic reaction [7, 9]. In this report, we will refer to these antitumour drugs as topoisomerase-trapping agents. These agents stimulate toposiomerase-mediated DNA cleavage in purified systems and in living cancer cells. It is generally recognised that this effect is most likely the basis of drug cytotoxic and antitumor activities [7, 8, 10]. New compounds of different chemical classes have been recently shown to stimulate DNA cleavage by mammalian topoisomerase II (Table 1).

The molecular mechanism of topoisomerase-trapping by drugs has long been debated. Since DNA intercalating and non-intercalating agents are known to trap topoisomerase II (Table 1), it was proposed that different compounds could have distinct mechanisms, i.e. diverse binding sites on the DNA and/or the enzyme [7, 8]. Several investigations have addressed this issue, and different molecular models have been proposed for drug action [11–15]. Recent studies on the local DNA sequence dependence of doxorubicin stimulation of topoisomerase II DNA cleavage have led to the proposal of a molecular model of the ternary complex DNA-drug-topoisomerase II [16, 17], which may be common to intercalating and non-intercalating agents [16, 18], and to topoisomerase I [19]. This model can be a useful working hypothesis for the interpretation of structure–activity relationships among drugs from the same or different chemical classes. This review summarises these new findings and discusses their importance for the antitumour activity of topoisomerase II-trapping drugs.

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DEPENDENCE ON LOCAL BASE SEQUENCE OF DRUG STIMULATION OF TOPOISOMERASE-MEDIATED DNA CLEAVAGE

Mammalian topoisomerase II is a homodimeric protein that produces DNA double-strand breaks with the two subunits covalently linked to the 5′-phosphoryl ends [2, 3, 7, 8]. The 3′-hydroxyl ends are recessed by four base pairs. The base at the 5′ end of the break, covalently linked to the protein, is being referred to as base (+1), while the base at the 3′ end of the break is being referred to as base (−1) (Fig 1). Topoismerase II promotes cleavage non-randomly in purified DNA in the absence and presence of drugs. Each drug stimulates DNA cleavage at specific sites, which results in distinct cleavage intensity patterns that are similar for compounds from the same chemical family. The base sequence specificity of drug-stimulated DNA cleavage by topoisomerase II is presumably determined by the sequence selectivity of the enzyme itself together with the drug sequence specificity. In an attempt to understand the sequence-dependent drug action, recent work focused on the base sequence requirements at the cleavage site in order for different drugs to stimulate topoisomerase II-mediated DNA cleavage.

The most revealing observations were made with doxorubicin [16]. Two sets of non-overlapping topoisomerase II cleavage sites were identified in SV40 DNA based on the differential effect of doxorubicin. The first set included cleavage sites by topoisomerase II without drugs, in which doxorubicin was not able to further stimulate DNA cleavage. The second set included doxorubicin-stimulated cleavage sites, which were not observed without drug. Base preference analysis at the cleavage site of these two sets disclosed a striking and unexpected difference: adenines were very often present at the two positions [−1] (and always at least at one of them) in the 'doxorubicin-stimulated sites', whereas they were absent in the 'doxorubicin-unstimulated sites'. This observation strongly suggests that doxorubicin has to interact with (−1) adenine to stimulate DNA cleavage. The sequence selectivities of other drugs are different and, consistently, VM-26 and etoposide require a cytosine at position (−1), and *m*-AMSA an adenine at position (+1) [18]. Thus, each drug may recognise specific nucleotides at the break site. In the case of VM-26, however, weak stimulation of DNA cleavage can be seen also at sites completely lacking cytosines at positions (−1) thus indicating that in some cases VM-26 may interact with other bases as well, although with reduced strength. This is in agreement with the common observation that VM-26 is the least sequence-selective drug in stimulating topoisomerase II DNA cleavage. Fosse' *et al.* [20] have reported that an ellipticine derivative requires a thymine at positions (−1) for strong topoisomerase II-mediated DNA cleavage. Recently, in our laboratory, an analysis of the effects of single nucleotide mutations in synthetic oligonucleotides showed that removing the specific nucleotide required by the drug markedly reduced,

*Table 1. Antitumour drugs that stimulate DNA cleavage by eukaryotic topoisomerases**

Chemical class	Specific agent	Intercalation	Reference
Topoisomerase II			
Anthracyclines	Doxorubicin; daunorubicin (DNR); 4-demethoxy-DNR (idarubicin)	yes	[4, 23]
Demethylepipodophyllotoxins	VP-16 (etoposide); VM-26 (teniposide)	no	[5]
Acridines			
9-Anilinoacridines	*m*AMSA	yes	[2]
Acridinecarboxamides	*N*-[2(Dimethylamino)ethyl]-acridine-4-carboxamide	yes	[68]
Actinomycins	Actinomycin D†	yes	[4, 81]
Anthracene derivatives			
Anthracene-9, 10-diiones	Mitoxantrone	yes	[4]
Anthracene bishydrazones	Bisantrene	yes	[4]
Anthrapyrazoles	Piroxantrone	yes	[69]
Ellipticines	2-Methyl-9-hydroxyellipticine	yes	[86]
Quinolones‡	CP-115,953;CP-67,804	no§	[72]
Flavonoids			
Isoflavones‡	Genistein; orobol	no	[73, 74]
Flavones	Quercetin; fisetin	yes	[74]
Terpenoides	Terpentecin; clerocidin	no	[77]
Benzisoquinolinediones	Amonafide; nafidimide	yes	[24, 79]
2-Nitroimidazoles‡	RO-15-0216	no	[78]
Naphtacene-5, 12-diones	Saintopin†	yes	[80]
Quinoline-5, 8-diones	Streptonigrin	no	[84]
Indoloquinolinediones†	AzalQD	no	[82]
Topoisomerase I			
Camptothecins	9-Aminocamptothecin, SN-38‖, topotecan	no	[9]

*The listed drugs trap nuclear topoisomerases. Antitrypanosomal drugs, pentamidine, berenil, samorin and ethidium bromide, were shown to stimulate DNA cleavage by mitochondrial, but not nuclear, topoisomerase II of trypanosomes [87].

†It has also been shown to stimulate DNA cleavage by topoisomerase I.

‡Evidence for antitumour activity has still not been provided.

§In the presenceof Mg^{2+} quinolones have been shown to unwind the DNA [88].

‖SN-38 is a metabolic derivative of CPT-11.

or even abolished, drug stimulation of DNA cleavage (unpublished results). These data support the idea that drug molecules likely form a ternary complex, DNA-drug-topoisomerase II, by interacting at the interface between the enzyme and DNA with the two cleaved nucleotides. Each topoisomerase II-trapping drug appeared to have distinct local base preferences, thus suggesting that each of them may specifically interact with certain bases at the cleavage site. Analogous observations have been made with camptothecin [19, 21]. This compound stimulates topoisomerase I DNA cleavage at sites with a guanine at position (+1), which in the case of topoisomerase I, is the nucleotide at the 5′ terminus non-covalently linked to the protein. Therefore, a common model may explain the action of both topoisomerase I- and II-trapping antitumour drugs [16, 19]. Consistently, affinity labelling experiments showed that camptothecin binds much more to the binary complex DNA-topoisomerase I than to either of the two isolated macromolecules [22]. The authors suggested that a binding site for camptothecin is created as the enzyme–DNA complex is formed.

DNA intercalation is necessary but not sufficient for antitumour activity [10] and for topoisomerase II-mediated DNA cleaving activity of anthracyclines [17, 23]. The same holds for antitumour acridines and other drugs [14, 24]. Nearly all of the known topoisomerase II-trapping drugs are DNA intercalators or have a planar ring system, which could intercalate between base pairs when additional stabilisation is conferred in the ternary complex. It has to be mentioned that a weak DNA binding of etoposide and camptothecin has been detected under particular conditions [25, 26]. Thus, it has been hypothesised that the drug molecule is intercalated between DNA bases in the ternary complex at the cleavage site [16]. An intercalation-like site might be formed by the two bases (−1) and (+1) and by aminoacid residues of the enzme [17]. This could prevent DNA religation by direct steric interference with the 3′-hydroxyl and 5′-phosphate groups [16, 17] or by causing a misalignment of rejoining strands (Fig. 1), as previously suggested in a different model [15].

The hypothesis that the drug molecule is placed at the cleavage site, at the interface between topoisomerase II and DNA, might provide a useful framework for new structure–activity studies of topoisomerase II-trapping drugs. Interesting results have been recently obtained in our laboratory studying the sequence-selective action of mitoxantrone, an intercalating topoisomerase II-trapping drug. Strikingly, mitoxantrone and VM-26 showed

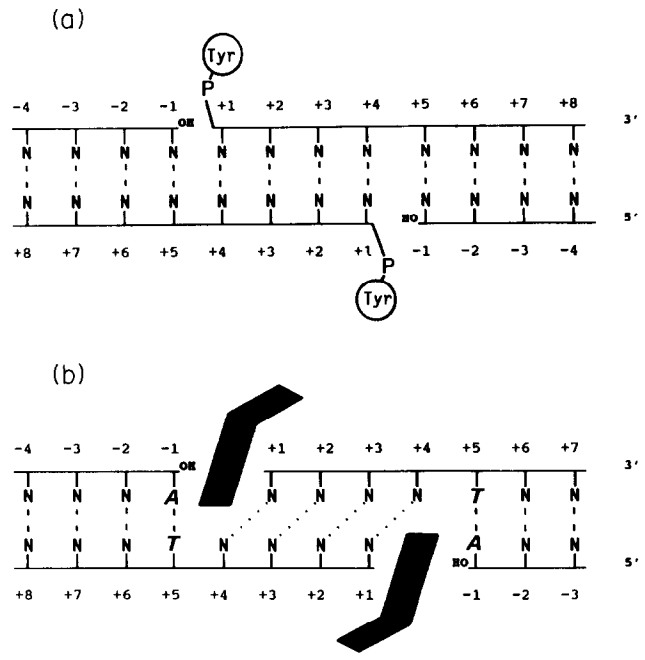

Fig. 1. Schematic representation of a topoisomerase II DNA cleavage site (a) and the proposed model for the ternary complex (b). Bases (N) are numbered from the cleaved bond on each strands. (a) Tyr indicates the tyrosine residue covalently linked to the 5'-DNA terminus of the strand break. (b) Doxorubicin molecules (black elongated symbols) are shown placed between the bases flanking the DNA cleavage, inducing a strand misalignment. A, Adenine; T, thymine.

very similar patterns of DNA cleavage sites in sequencing gels and the same local base requirements; moreover, in spite of structural resemblance and similar DNA binding affinity, sequence specificities of mitoxantrone and anthracyclines were completely different (unpublished data). These results further support the idea that intercalating and non-intercalating agents bind to the same site, and may suggest that mitoxantrone shares with VM-26 some specific interactions with the DNA and the enzyme in the ternary complex. When more information will be available on the local base selectivity of other topoisomerase II-trapping drugs, it might become clear that different chemical structures can share, at least in part, local base requirements suggesting similar interactions in the ternary complex. This information may provide a new way to classify topoisomerase II-trapping antitumour drugs. Moreover, investigations on local base sequence specificity of drug action can allow novel structure–activity relationship studies, which may provide insight into drug structural aspects important for trapping of the enzyme at specific DNA sites. This may ultimately lead to the design of new compounds that can direct topoisomerase II to cleave DNA at specific sites on the human genome.

RELATIONSHIPS BETWEEN DRUG CYTOTOXIC POTENCY AND DNA SITE SELECTIVITY OF TOPOISOMERASE II DNA CLEAVAGE IN CHROMATIN

Among close analogues, the cytotoxic potency of topoisomerase II-trapping drugs correlates with the extent and persistence of drug-stimulated cellular DNA breaks [7, 8, 10]. The same correlation was not found when drugs from different classes were compared [8, 27, 28]. Although drug classes usually differ for their DNA sequence selectivity, the role of this selectivity in drug cytotoxicity needs further investigations to be fully established.

The selective cytotoxicity of the antitumour drugs must reflect characteristics of topoisomerase action in living cells. In the case of camptothecin, it has been proposed that single-strand breaks in DNA regions which are actively being synthetised represent the most lethal lesions as a collision may occur between topoisomerases trapped on the DNA and moving replication forks [7]. The block of DNA polymerases would then result in irreversible double-strand cleavages, which may trigger cellular responses ultimately leading to cell death [7]. The functional state of the DNA may then play an important role in determing the lethal consequence of the topoisomerase-trapping by drugs.

Structural features influencing the DNA site of topoisomerase activities

Many factors probably modulate the DNA site of topoisomerases I and II activities in nuclear chromatin, including DNA sequence,chromatin structure and DNA binding proteins. They might also influence the DNA sequence-dependent action of drugs and, indeed, topoisomerase DNA cleavage patterns *in vivo* were somewhat different from those observed *in vitro* [29–31].

Consensus cleavage sequences have been determined by sequencing *in vitro* cleavage sites for topoisomerases I [32, 33] and II [16, 34, 35]. They are loose consensus, as they not always accurately predict the intensity of topoisomerase cleavage, and encompass at most 10–15 nucleotides. Consistently, DNase footprinting experiments showed that eukaryotic topoisomerases protect short DNA regions centered at the cleavage site [36, 37]. Moreover, topoisomerase II appeared to cleave preferentially purine/pyrimidine repeats [38]. It has to be pointed out that cleavage, binding and catalytic sites of topoisomerases are overlapping sets of sites and do not necessarily coincide. At one extreme, it has been reported for DNA gyrase, a bacterial topoisomerase II, that a strong binding site was not a cleavage site [39]. Nevertheless, strong binding often correlates with strong cleavage [2, 40], and DNA relaxation and binding activities of *Drosophila* topoisomerase II were dependent on the same DNA sequence [41]. Similar observations have been made for topoisomerase I by using a strong hexadecameric recognition sequence [42]. Therefore, localisation *in vivo* of a topoisomerase DNA cleavage site would likely indicate an important site for enzyme activity in cellular chromatin. The site of enzyme activity may be also modulated by several DNA structural features, such as topology [3, 43–45], bending [40, 46], DNA crossovers [47] and local twist angle variations [48]. Moreover, the accessibility of DNA sites to topoisomerase II in chromatin is likely restricted by the presence of histones [31, 49–53].

Therefore, although locally the site of DNA cleavage may be determined by the local base sequence, in the long range, DNA structural aspects and the chromatin structure may strongly modulate the site of topoisomerase DNA cleavage in the human genome.

Topoisomerase II DNA cleavage sites in the cellular genome

Sites of topoisomerase II DNA cleavage have usually been mapped in nuclear chromatin or in living cells by trapping the enzyme with VM-26. DNA cleavage has been localised in non-transcribed regions of cellular genes, at sites which often coincide with DNase I hypersensitivity regions of gene promoters [29, 31, 49, 54–56]. This may suggest a role for topoisomerase II in

the regulation of gene transcription and/or maintenance of an open chromatin structure.

A second type of *in vivo* DNA sites of topoisomerase II cleavage has been characterised. The eukaryotic topoisomerase II is a major component of chromosome scaffolds and interphase nuclear matrix [57–59] and DNA attachment to nuclear scaffolds probably occurs at specific sequences, termed SARs or MARs (scaffold- or matrix-attachment regions, respectively) [60, 61], which are enriched for sequences related to the *in vitro* topoisomerase II DNA cleavage consensus [59, 62–64]. Interestingly, AT-rich sequences related to MAR may trigger a cooperative binding of topoisomerase II to DNA which can influence the location of VM-26-stimulated DNA cleavage [65]. Recently, topoisomerase II DNA cleavage has been mapped in living cells within MARs of the chicken α-globin gene cluster [66, 67], and of the *Drosophila* histone gene repeat and in the centromeric satellite III repeat [31]. These results strongly indicate that topoisomerase II is intimately associated *in vivo* with DNA at MAR-related sequences. VM-26-trapping of topoisomerase II has been shown to excise from the genome a 20-kb DNA fragment representing an entire DNA loop or domain [67]. Interestingly, at the sequence level, *in vivo* DNA cleavage has been reported to occur specifically within a GC-rich core flanked by MAR-related AT-rich sequences and no obvious relation was found with *in vitro* cleavage consensus [31]. Moreover, strand breaks produced *in vivo* by topoisomerase II have been shown to be separated by six base pairs instead of four as seen in *in vitro* cleavage [31].

Therefore, two classes of topoisomerase II DNA cleavage sites have been identified in cellular genomes (sites within MARs and sites coinciding with DNase I-hypersensitive regions), which might correspond to separate pools of the enzyme in the nucleus (topoisomerase II proteins in the nuclear matrix and those extractable from nuclei at moderate ionic strength, respectively). Whether the sequence selectivities of drug actions on purified topoisomerase II is still the same in cellular chromatin remains to be determined.

NOVEL TOPOISOMERASE II-TRAPPING ANTITUMOUR DRUGS

Novel compounds have been recently shown to stimulate DNA cleavage by eukaryotic topoisomerase II (Table 1). Although new specific topoisomerase I-trapping drugs, besides camptothecins, have not been reported yet, some of these new agents seemed to stimulate topoisomerase I DNA cleavage as well (Table 1). Among the known classes of drugs, acridinecarboxamide and anthrapyrazole sub-families and new 4-substituted derivatives of etoposide have been shown to stimulate protein-linked DNA cleavage in cultured cells [68–70]; chrysophanol derivatives (of the anthracene class, Table 1) with alkylating capability have been shown to promote irreversible topoisomerase II DNA cleavage [71].

Quinolones have long been known to trap DNA gyrase, the bacterial topoisomerase II, and are currently used as bactericidal agents [2]. Some derivatives have been shown to specifically trap the mammalian topoisomerase II [72], which has led to the development of new quinolones as antitumour drugs. These results are instructive since they suggest that the bacterial and mammalian enzymes are closely related and that aspects of their actions have probably been conserved through evolution. Two new quinolones have been studied for the stimulation of topoisomerase II cleavage and shown to act in a different way from VM-26 and *m*-AMSA [72]. The quinolones were unable to inhibit the DNA religation step or the relaxation rate of *Drosophila* topoisomerase II. It has been proposed that they could stimulate DNA cleavage by enhancing the forward reaction [72].

Flavonoids are weak cytotoxic drugs, and those that stimulate topoisomerase II DNA cleavage may be isoflavonoids (genistein) [73–75] or flavones (quercetin) [74, 75]. Genistein did not show good antitumour activity in the NCI screening program [76]. Interestingly, the planar aromatic moiety of the drug appeared to be essential for stimulation of topoisomerase II DNA cleavage [74]. Recently, the sequence specificity of DNA cleavage of these compounds appeared to be somewhat similar to that of *m*-AMSA as seen by agarose gels [75]. However, an analysis by sequencing gels may provide useful information to definitively establish this point. As these agents are also tyrosine kinase inhibitors, it would be interesting to identify possible correlations among their abilities in inhibiting different enzymes.

The terpenoides, terpentecin and clerocidin, have been shown to have potent cytotoxic and antitumour activities [77]. These biological effects correlated with their ability, comparable to that of VP-16, to stimulate topoisomerase II DNA cleavage. The pattern of cleavage sites was distinct from those of VP-16 and *m*-AMSA [77]. This new class of topoisomerase II-trapping drugs is of particular interest, since the terpenoid molecule is characterised by the lack of an aromatic quasi-planar moiety (in this regard, terpenoids are the only exception among all topoisomerase-trapping drugs listed in Table 1). The authors suggested that the epoxide moiety of terpenoids could covalently react in the ternary complex, resulting in an irreversible DNA cleavage [77].

The 2-nitroimidazole derivative, RO-15-0216, and amonafide are probably the most selective topoisomerase II-trapping drugs known, since they appeared to stimulate DNA cleavage at only one site in the whole pBR322 DNA [24, 78]. An analog of amonafide has been reported to stimulate protein-linked DNA breaks in tumour cells [79].

Saintopin, a new antitumour anthraquinone compound, has been shown to trap both topoisomerases I and II [80], sharing this property with actinomycin D and indoloquinolinediones [81, 82]. Although saintopin stimulative effects were comparable to those of camptothecin, etoposide and *m*-AMSA, its DNA cleavage patterns were different from them [80].

Streptonigrin, a non-intercalative agent, has long been known for its antitumour activity and ability to induce cellular DNA breaks [83]. At least part of these DNA breaks may be due to stimulation of topoisomerase II DNA cleavage [84]. The DNA cleavage stimulated by streptonigrin was equally as reversible as that stimulated by etoposide, however, at high drug concentrations streptonigrin was less potent in DNA cleavage stimulation than etoposide [84].

Indoloquinolinediones have been reported to stimulate both topoisomerase I and II DNA cleavage [82]. The active compounds did not shown DNA unwinding activity, and the drug sequence specificity of cleavage stimulation remains to be definitively established [82].

CONCLUSION

New molecular aspects of drug–topoisomerase interactions have been elucidated [16, 19] and genomic sites of topoisomerase II action have been started to be identified in cellular chromatin [31, 67]. This information and the available molecular tools provide new opportunities for drug screening and for understanding the specific action of topisomerase-trapping antitumour drugs in living tumour cells. Several issues remain to be resolved

in the attempt to develop new therapeutic strategies and improve the efficacy of a therapy based on topoisomerase-trapping drugs. One of the most important is the definition of the molecular mechanisms by which the primary DNA lesion leads to cell death. Moreover, new lines of investigation will be prompted by the discovery of novel topoisomerases in living cells [85]. Some of them may have highly specialised functions, and the role of these novel topoisomerases in the antitumour activity of topoisomerase-trapping drugs remains to be clarified. The new eukaryotic enzymes might be candidates for the design of more selective antitumour drugs.

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