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Review

Sequence-specific interactions of drugs interfering with the topoisomerase–DNA cleavage complex

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Abstract

DNA-processing enzymes, such as the topoisomerases (tops), represent major targets for potent anticancer (and antibacterial) agents. The drugs kill cells by poisoning the enzymes' catalytic cycle. Understanding the molecular details of top poisoning is a fundamental requisite for the rational development of novel, more effective antineoplastic drugs. In this connection, sequence-specific recognition of the top–DNA complex is a key step to preferentially direct the action of the drugs onto selected genomic sequences. In fact, the (reversible) interference of drugs with the top–DNA complex exhibits well-defined preferences for DNA bases in the proximity of the cleavage site, each drug showing peculiarities connected to its structural features. A second level of selectivity can be observed when chemically reactive groups are present in the structure of the top-directed drug. In this case, the enzyme recognizes or generates a unique site for covalent drug–DNA binding. This will further subtly modulate the drug's efficiency in stimulating DNA damage at selected sites. Finally, drugs can discriminate not only among different types of tops, but also among different isoenzymes, providing an additional level of specific selection. Once the molecular basis for DNA sequence-dependent recognition has been established, the above-mentioned modes to generate selectivity in drug poisoning can be rationally exploited, alone or in combination, to develop tailor-made drugs targeted at defined loci in cancer cells.

Keywords: Topoisomerase; Antitumor drug; Poisoning; Sequence specificity; Isozyme selectivity

1. Introduction

DNA topoisomerases (tops) are essential enzymes present in all organisms [1,2]. They modify DNA topology in connection with a number of nuclear processes, such as replication, transcription, chromatin remodeling, chromatin condensation/decondensation, recombination and repair [3]. All tops can introduce breaks into DNA segments: type II tops transport a double helical portion of DNA through a cut involving both strands of another double-helical region; type I enzymes produce single-stranded breaks, and then either transport a DNA segment through the break (type I-A) or allow rotation of the broken strand around the intact strand (type I-B) [4,5]. In the human genome, five DNA tops are presently known: two type II tops (top2α and top2β), a type I-B enzyme (top1) and two type I-A enzymes (top3α and top3β) [1].

Top1 and top2 have been shown to represent the principal targets of effective antitumor drugs and, hence, have deserved investigation to understand the biochemical and pharmacological basis of drug action [6]. Indeed, topoisomerase poisons, such as antitumor drugs, transform these essential enzymes into lethal DNA-damaging agents. Circumstantial evidence indicates that top2 (and top1) can initiate cellular processes that eventually lead to chromosomal translocations and cell transformation [7,8]. Thus, a full understanding of molecular mechanisms of DNA topoisomerase functions leading to cell death and/or genome alterations may reveal unique opportunities to successfully treat human cancers.

2. The catalytic process and its poisoning

The enzymatic cycle of top1 [2], largely shared by top2 [9], can be divided into four steps: (1) binding of the enzyme to DNA, (2) DNA cleavage, (3) strand passage and (4) DNA

Abbreviations: top, topoisomerase; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; CPT, camptothecin; AmC, cytosine arabinoside; *m*-AMSA, amsacrine; ET, ecteinascidin 743; CL, clerocidin; XK469, 2-{4-[(7-chloro-2-quinoxalinyl)oxy]phenoxy}propionic acid; VM-26, teniposide; dh-EPI, 4-demethoxy-3′-deamino-3′-hydroxy-4′-epi-doxorubicin; da-IDA, 4-demethoxy-3′-deamino-4-deoxy-4′-epiaminodaunorubicin

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religation. Once the protein and the nucleic acid are bound to each other, the phenolic OH of a tyrosine residue in the catalytic pocket reacts with a DNA phosphodiester bond by a transesterification process, producing a covalent enzyme–DNA intermediate linked through a phosphotyrosine bond (cleavage complex), while the DNA strand is cleaved. Cleavage may occur simultaneously on both strands in the presence of top2 enzymes, whereas it involves only one strand in the presence of top1 enzymes. In type I-A and type II topoisomerases, the enzyme becomes linked to the 5′-end of the cleaved phosphodiester bond, whereas in type I-B enzymes, it is bound to the 3′-end. The third step consists in the strand passing process, which involves a conformational rearrangement of the enzyme to allow one or both strands of DNA to pass through the gap generated by the cleavage step. The final religation process corresponds to the attack of the free 5′- or 3′-hydroxyl terminus of the cleaved bond onto the phosphotyrosine linkage to reseal the original phosphodiester bond. Then, enzyme release occurs (Fig. 1). Given the energy requirements of the enzyme, ATP binding and hydrolysis has to be included in the catalytic cycle of top2.

In the presence of a drug which is able to affect the DNA cleavage-religation process by increasing the DNA cleavage rate or by reducing the DNA religation rate, the cleavage complex has a longer life and DNA breaks persist [10,11]. This action converts top2 into an endogenous toxin that stabilizes damage in the genome and triggers a series of apoptotic events that finally lead to cell death. There are several modes for a drug to affect the cleavage complex, but it is generally believed that a ternary complex is formed, in which the drug is bound to the DNA and the enzyme simultaneously [2,9]. Hence, there will be normally two pharmacophoric regions in a poison molecule, one facing (and interacting with) the enzyme and the other the nucleic acid.

3. Structures of topoisomerase poisons

A large number of compounds have been shown to interfere with top (principally top2) activity, and they exhibit a variety of structural features [2,9,12,13]. The structures of selected compounds are shown in Fig. 2.

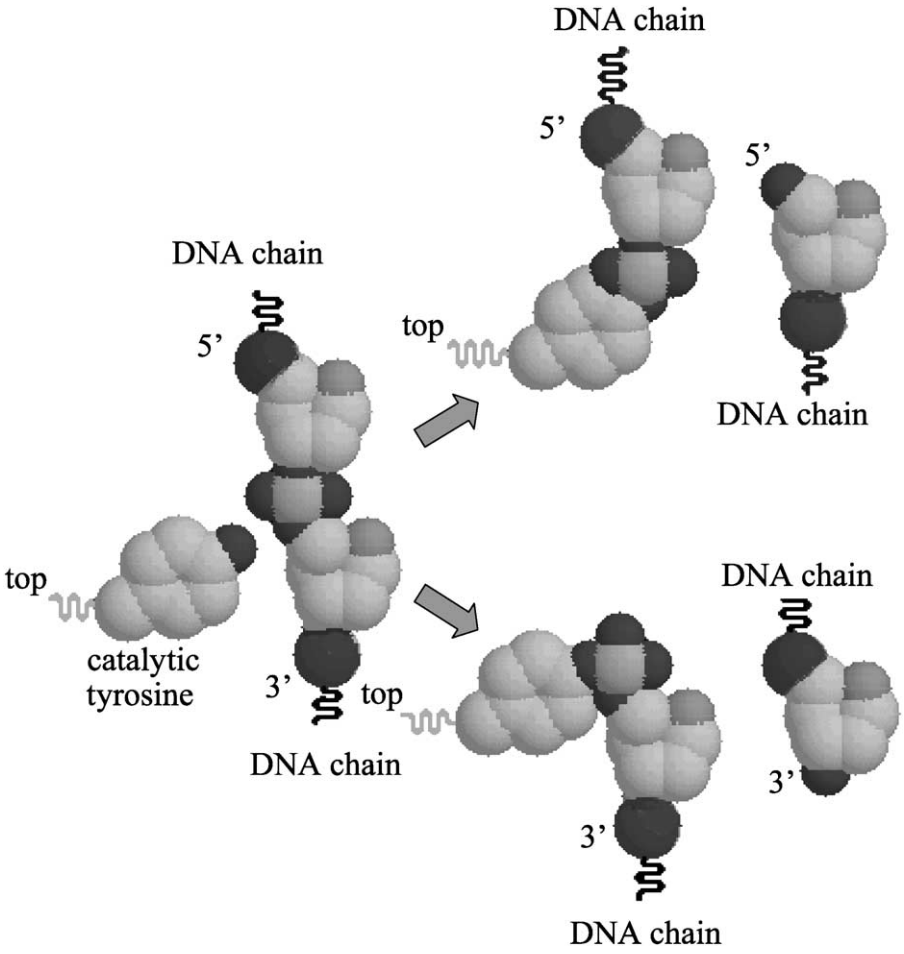

Fig. 1. Schematic representation of the chemical processes occurring in the topoisomerase catalytic cycle. The tyrosine residue at the active site (left) can attack a phosphodiester bond of DNA, giving a phosphotyrosine adduct linked either at the 3′ (upper reaction scheme) or at the 5′ (lower reaction scheme) position. This will produce a cut in the DNA chain leaving a free 5′ or 3′ hydroxyl group.

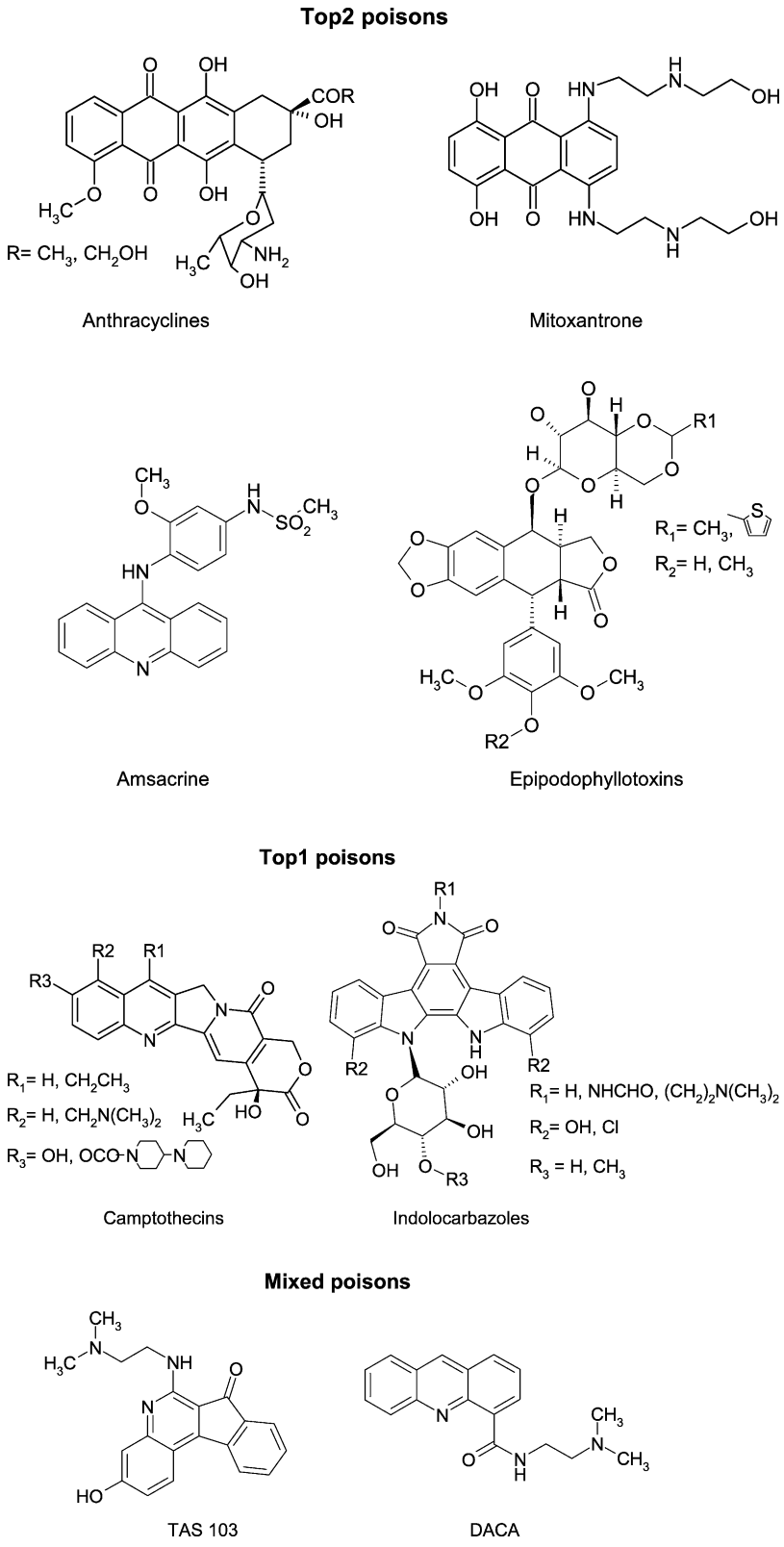

Fig. 2. Selected chemical structures of topoisomerases I and II poisons.

Clinically useful top1 poisons include the natural compound camptothecin (CPT) and its synthetic analogues topotecan and irinotecan. In addition, minor groove binders, such as the bisbenzimidazoles Hoechst 33258 and Hoechst 33342, and the indolocarbazoles derived from rebeccamycin, were found to be top1 inhibitors.

Top2 poisons include drugs from different families, such as epipodophyllotoxins, anthracyclines, acridines, anthraquinones, ellipticines, bisantrene, actinomycin D, terpenoids, quinolones and flavonoids. It is difficult to envisage common structural features among them. In fact, some of the compounds do not bind to DNA, while others exhibit a high affinity for the nucleic acid. In particular, very effective are the intercalators (anthracyclines, anthracenediones, acridines, bisantrene, actinomycin). They are characterized by a planar polycyclic DNA-intercalating region, to which polar side chain groups are eventually attached. The planar region is supposed to be able to slip between DNA bases and generate efficient stacking interactions with them. This is likely to represent the DNA-binding domain in the cleavage complex. In addition, to further stabilize contacts with the nucleic acid, the side-chains are likely to interact with the enzyme, thus, acting as enzyme-recognition elements. Indeed, in addition to their chemical structure, the relative position of the (planar ring/side chain) pharmacophores plays a major role in modulating nucleic acid binding and enzyme poisoning effects [14–16]. Non-DNA-binders, like epipodophillotoxins, can also stabilize the cleavage complex. In this case, perhaps, stronger contacts are formed with the enzyme to compensate for poor interaction with the nucleic acid. Indeed, recent studies show that both the recombinant N-terminal ATPase domain and the B′A′ core domain of human top2α bind etoposide specifically, even in the absence of DNA. [17].

Dual top1 and top2 inhibitors have finally been described. Among others, they include saintopin, intoplicine, aclarubicin [18], acridine-4-carboxamide derivatives [19], bis(phenazine-1-carboxamides) [20], indeno-quinolines, [21] acetyl-boswellic acids [22] and fluorinated lipophylic epipodophylloids [23]. As with top2 poisons, different structural types are found, including intercalating and nonintercalating agents.

4. DNA sequence specificity of topoisomerase poisoning

4.1. Classical drugs

It has become evident that poisoning of the top–DNA cleavage complex by anticancer drugs does not occur at random along the DNA chain, but follows well defined rules, as demonstrated by the sequence analysis of the DNA regions where cutting had been stimulated by the top poison [24–26]. Top activity on the genome exhibits per se a certain degree of specificity. Indeed, significant nucleotide preferences were found in the regions flanking the cleavage site, often corresponding to alternating purine–pyrimidine tracts [27,28]. However, site selectivity exhibited by the poisons is more efficient and dependent on the poison's nature. Compounds of different chemical classes stimulate specific cleavage patterns in DNA fragments, which do not comprise all of the sites recognized by the enzyme. Moreover, the effects of specificity are principally due to the base immediately preceding (-1) or following ($+1$) the cleavage site [29]. These findings confirm that the drugs are localized at (or very close to) the enzyme active site, and possibly contact both components of the cleavage complex. Localization of amsacrine at the active site has been demonstrated, using a covalently binding azido derivative, which was found to localize the drug molecule at the -1 and $+1$ positions in the presence of top2 [30]. Another similar example is a reactive CPT derivative bound to top1 [31].

A peculiar case seems to be that of nogalomycin [32], which has been proposed to bind to an upstream site (from position -6 to -3) to provide a DNA structural bend that stimulates highly specific top1-mediated DNA cleavage. In this case, the drug would act at a distance from the catalytic site, like an allosteric effector.

An interesting mechanism is exhibited by the antimetabolite cytosine arabinoside (AraC) [33]. Lesions generated when the drug is incorporated into chromosomal DNA behave as position-specific top2 poisons and stimulate DNA cleavage mediated by the human type II enzymes. Moreover, additive or synergistic increases in DNA cleavage were observed in the presence of AraC lesions and etoposide, pointing to the possibility that the sequence-specific lesions might result from a combination of processes.

A question which arises when considering drug sequence specificity in producing protein-mediated DNA damage is related to possible species-related effects. This issue was recently addressed by Strumberg et al. [34], who performed a molecular analysis of drug interactions in yeast and human type II tops. Similarities and differences in DNA cleavage patterns and nucleic acid sequence preferences were observed between the human, yeast and *E. coli* top2 enzymes in the presence of the nonintercalators fluoroquinolone (CP-115,953), etoposide and azatoxin and the intercalators amsacrine and mitoxantrone. Additional base preferences were generally observed for the yeast, when compared with the human top2α, enzyme. Preferences in the immediate flanks of the top2-mediated DNA cleavage sites were, however, consistent with the drug-stacking model for both enzymes. Homologous mutations in yeast and human top2 decreased the reversibility of the drug-stabilized cleavage sites and produced consistent base sequence preference changes. These data indicate that the structure of the enzyme/DNA interface plays a key role in determining the specificity of top2 poisons and cleavage sites for both the intercalating and nonintercalating drugs. These effects must be borne in mind when comparing results obtained with topoisomerase enzymes of different origin. The principal base preferences exhibited by the drugs, thus, far investigated are summarized in Table 1.

Confirming the above-mentioned location of the drug at the enzyme active site, the principal preferences for DNA cutting correspond to the bases located at positions $-1/+1$.

Table 1
Base preferences of selected topoisomerase poisons

−1[a]		+1[a]	
Poison	Base preference	Poison	Base preference
Doxorubicin[b]	A	Amsacrine[b]	A
Etoposide[b]	C(T)	Bisantrene[b]	A
Mitoxantrone[b]	C/T	Camptothecin[c]	G
Ellipticine[b]	T	Indolocarbazole[c]	G
Amonafide[b]	C	Saintopin[d]	G
Genistein[b]	T		

[a] Position from the cleavage site.
[b] top2 poisons.
[c] top1 poisons.
[d] Dual poison.

Generally, drugs having a strong −1 preference do not exhibit a strong +1 preference. This represents an indication that effective recognition of DNA by a drug is carried out either on one side or on the other of the cleavage complex, the drug molecule being principally located either upstream or downstream the cleaved phosphodiester bond.

A closer examination of individual specificities shows that a purine residue is invariantly found to be preferred at +1, both by top1 and top2 poisons. Considering the large planar portions of these drugs, this could indicate that DNA–drug stacking effects are operating in the ternary complex with the enzyme. Instead, the majority of −1 specific agents accept both pyrimidines and purines in the cut strand. Although the list of −1 specific agents includes nonintercalating agents, the majority of the compounds in this list are also intercalators, which prefer stacking interactions, so that the presence of pyrimidine preferences are not easily explainable at first glance. However, if we consider that all −1 specific agents are top2 poisons, and that the −1 base remains essentially paired to the complementary base located in the intact strand, the poison at −1 would always have a complete base pair to make contacts with, irrespective of the presence of a purine or a pyrimidine at the scissile strand.

It is very difficult to rationalize coupling of drug structures with corresponding specificities. The task could be made much easier, having tridimensional structures of the cleavage complex or, even better, of the ternary complex containing the drug.

Combining the biochemical and structural evidence, two models of the receptor site of CPT on the covalent complex of human top1 and DNA have been proposed [35,36]. Both attempt to explain the results of numerous CPT structure activity studies, and of investigations on drug-resistant top1 mutants. One model [35], schematically shown in Fig. 3A, uses the crystal structure of the human top1–DNA covalent complex and places the CPT molecule into the DNA duplex in a position that partially overlaps with the +1 guanine of the scissile strand; this placement requires the guanine base to flip out of the duplex and stack on the planar CPT. A second model [36] foresees intercalation of CPT into a B-form DNA duplex and models the positions of the phosphotyrosine723 and Asn722 residues as well (Fig. 3B). CPT occupies very different positions in the two models, neither of which can fully explain the experimental findings. In particular, no indication emerges for the requirement for a guanine at the +1 position of the scissile strand, as established by early investigations [25,26]. A theoretical structural model has been obtained very recently [37] by docking CPT into the top1–DNA cleavage complex obtained from X-ray data. The model of the lowest energy complex (Fig. 3C) is consistent with intercalation of the drug at the cleavage site, with the A-ring directed toward the major groove and the E-ring pointing into the minor groove. As shown in Fig. 3, the drug's orientation differs remarkably in the previous intercalation model [36], while it is similar in the guanine-flipping model [35]. Perhaps, publication of the X-ray structure of the ternary cleavage complex with topotecan, announced by Stewart et al. [38] at the 2001 AACR meeting will settle the question.

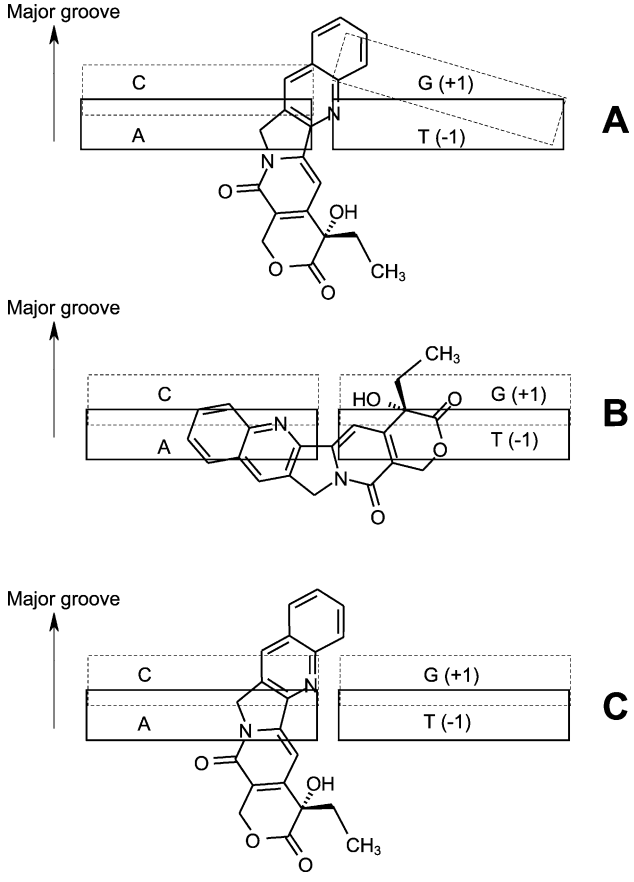

Fig. 3. Proposed orientations of CPT bound to DNA in the ternary CPT–DNA–top1 cleavage complex. DNA bases are viewed from the helix axis and schematically drawn as rectangles. Solid lines correspond to the pair at the −1 position (see text), while dashed lines correspond to the pair at +1. CPT is located between the −1 and +1 bases. (A) Flipping guanine model from Ref. [35]. (B) Intercalation model from Ref. [36]. (C) Intercalation model from Ref. [37].

An example of modulation of cleavage specificity is represented by homocamptothecin, containing a seven-membered β-hydroxylactone ring in place of the conventional six-membered α-hydroxylactone ring found in CPT [39]. Besides stimulating cleavage by top1 at preferred T.G sites, the homo derivative stabilizes cleavage at specific AAC.G sequences as well. Hence, notwithstanding the conserved primary G + 1 requirement, the ring homologation procedure generates a ternary complex structure which can discriminate up to three bases upstream the cleavage site. Clearly, further studies are required to fully elucidate the molecular interactions leading to specific DNA damage stimulation by the CPT family.

The presence of common pharmacophores for similar specificities of cleavage stimulation, and the importance of the relative positions of the planar ring system and of the side-chain groups, has been recently addressed by preparing amsacrine (*m*-AMSA)/bisantrene hybrids and bisantrene isomers [14]. The new compounds were able to poison DNA top2 with an activity intermediate between those of bisantrene and *m*-AMSA. Moving the side-chain from the central to a lateral ring (from C-9 to C-1/C-4) only slightly modified the drug DNA affinity, whereas it dramatically affected local base preferences of poison-stimulated DNA cleavage. In contrast, switching the planar aromatic systems of bisantrene and *m*-AMSA did not substantially alter the sequence specificity of drug action. A computer-assisted steric and electrostatic alignment analysis of the test compounds suggested that bisantrene, *m*-AMSA and 9-substituted analogs share a common pharmacophore, whereas the 1-substituted isomer showed a radically changed pharmacophoric structure.

A further question arising from this investigation, approached using the same set of compounds, is a possible relationship between sequence specificity for DNA in the presence and in the absence of the enzyme. We should remember that most of the known top2 poisons are also good DNA binders. The bisantrene analogues exhibited different DNA sequence preferences, depending on the locations of the side-chain groups, which suggests a major role for the side-chain position in generating specific contacts with the nucleic acid, even in the absence of enzyme [40]. More interestingly, the observed preferences compare well with the alteration of base specificity found for the top2-mediated DNA cleavage stimulated by the isomeric drugs. This confirms that not only DNA affinity, but also DNA-binding specificity, represents per se an important determinant for the recognition of the topoisomerase–DNA cleavable complex by the drug, at least for poisons belonging to the amsacrine–bisantrene family.

Thus, it has been concluded that the relative space occupancy and electron distribution of putative DNA binding (aromatic rings) and enzyme binding (side-chains) moieties are fundamental in directing the specific action of top2 poisons and in determining the poison pharmacophore.

4.2. Drug conjugates containing DNA-recognition elements

A useful approach to confer a desired sequence specificity is to synthesize drug conjugates with structural elements known to recognize well-defined features of DNA. Since the target for topoisomerase is a double-stranded helix, the following targeting elements can be used in combination with normal drugs: groove-binding AT specific structures, such as the antibiotics netropsin and distamycin [41–43], hairpin pyrrole–imidazole polyamides [44] and triplex-forming oligonucleotides [45,46]. In the first two, the conjugates are tethered to the minor groove; in the third, major groove binding occurs. The success of the above approaches rests on the ability to target the nucleic acid without impairing binding of the topoisomerase. The AT-binder conjugates with CPT proved to be poorly effective, due to their limited sequence-recognition properties [43].

Other top1 poisons were covalently attached to triple helix-forming oligonucleotides. Besides increasing the drug affinity for DNA, the conjugates were shown to stimulate top1-mediated DNA cleavage in a triplex-directed sequence-specific manner [46]. In addition, the results obtained with drug–polyamide conjugates showed that they were able to recruit top1 to produce DNA cleavage in high yield [44]. Hence, the new compounds could be considered as new artificial nucleases. It will be now interesting to verify whether conjugates targeted to the coding regions of selected genes will be able to regulate transcription elongation through a top1-dependent mechanism.

5. Drugs producing covalent adducts in the cleavage complex

Poisoning of the cleavage complex by the presently used drugs is a reversible process, and normal processing of the DNA by the enzyme is restored once the drug is removed [47]. Since triggering of the cell death events is initiated by the presence of the cleavage complex, the extent of this process will depend upon the drug's in and off kinetics. The longer the drug is bound to the cleavage intermediate, the more effective will be induction of apoptosis. Hence, if the drug becomes permanently linked to the DNA–enzyme complex, apoptosis signaling should be most prominent.

Compounds bearing DNA-reactive groups, such as the alkylators [48], usually produce generalized damage to the nucleic acid and to other cellular structures. Although the toxicity generated by these compounds is being used for the treatment of cancer, nonetheless the related side effects are quite marked. In addition, sequence selectivity is in general poor, as a large number of reactive group(s) in the DNA chain are available for targeting at the same time. It would be much more specific if the drug were to react with the nucleic acid only when topoisomerase is present. An enhancement in top activity at specific DNA alkylation sites has been found using *N*-methyl-*N*′ -nitro-*N*-nitroso-

guanidine [49]. This suggested a role for top1 poisoning by alkylated bases in the antiproliferative activity of alkylating agents, as well as in the DNA lesions resulting from endogenous and carcinogenic DNA modifications.

Other interesting examples are represented by the drugs psorospermin, ecteinascidin and some analogues [50–53]. These are weak DNA alkylators in the absence of top2 in the first case and top1 in the second. Significantly, the alkylation reactivity of psorospermin at specific sites on DNA increased 25-fold in the presence of top2 [50]. In addition, psorospermin trapped the top2-cleaved complex at the same sites. These results imply that the efficacy of psorospermin is related to its interaction with the top2–DNA complex. The site of alkylation of psorospermin is within the top2 gate site, as for other derivatized intercalators used to pinpoint the site of drug action in the cleavage complex.

Ecteinascidin 743 (ET), a potent antitumor agent from the Caribbean tunicate *Ecteinascidia turbinata*, can alkylate selectively guanine N2 in the DNA minor groove, a process reversed by DNA denaturation. However, it differs remarkably from other DNA alkylating agents presently in the clinic by both its biochemical activities and its profile of antitumor activity in preclinical models [52]. A 100-kDa protein was found to bind to DNA alkylated by ET and was identified as top1 [52]. DNA alkylation was essential for the formation of top1-mediated cleavage complexes by ET, and the distribution of the drug-induced top1 sites was different for ET and camptothecin, although they still share a G+1 preference. These data indicate that DNA minor groove alkylation by ET is required to produce top1-mediated protein-linked DNA breaks. Other studies, however, question the relevance of top1 poisoning by ET (and its synthetic analogue phthalascidin) as they show that in vivo top1 cross-linking is poor and that ET can be cytotoxic independently of top1 expression [53,54].

A more striking example is represented by clerocidin (CL) [55–58], reported to produce top2-mediated irreversible cleavage compatible with G-1 preference [57], a process unusual for topoisomerase poisoning. Since CL contains chemically reactive epoxy and carbonyl groups, studies were made to detect possible reaction with DNA. Indeed, the drug was able to nick negative supercoiled plasmids per se [58]. Covalent adducts with guanines at N7 position were formed in topoisomerase-free media, which were able to trigger phosphodiester bond cleavage at the modified site. Only single-stranded or distorted double-helical regions of DNA underwent CL alkylation. The guanine-alkylating ability of CL suggests an unprecedented mechanism of top2 sequence-specific poisoning, according to which the enzyme renders the drug reactive toward DNA by changing the local structure of the nucleic acid and by bringing drug and DNA reactive moieties in close proximity.

In conclusion, the combination of reversible (the drug must recognize the cleavage complex) and irreversible (drug covalent binding occurs only when the drug is appropriately located at the cleavage site in close contact with the target reactive group in DNA) effects will contribute to subtly modulate the drug's efficiency in stimulating DNA damage at selected sites.

6. Topoisomerase II isoform specificity

It has recently become evident that top2 is not a single enzyme in eukaryotic cells, but is present as two isoforms: a 170-kDa one, referred to as α, and a 180-kDa one, referred to as β. They differ in subcellular localisation and biochemical properties [59], suggesting that each isozyme has a distinct cellular function. Hence, studies aimed at evaluating the differential susceptibility to inhibition by anticancer drugs and site selectivity are of great help to better understand the role and actions of the various drugs. In addition, the relative level of expression of the α and β isoforms may contribute to the degree of tumor responsiveness to different chemotherapeutic agents.

The relationship between expression of top2 isoforms and established prognostic factors and pathological variables was examined in over 50 primary breast tumor samples [60]. The expression of the two top2 genes was apparently not coordinately regulated in these tissue samples. Immunohistochemical analysis revealed that top2β was widely distributed (>90% positive tumor cells), but that top2α expression was less widely expressed. This suggested that top2α, but not tp2β, expression is dependent upon cellular proliferation status, but that the more widely expressed top2β protein may play a significant role as a target for antitumor therapy. An immunofluorescence technique was also devised to visualize and quantify isoform-specific cleavable complexes in situ [61].

A number of studies have been performed on the major classes of anticancer drugs, including acridines, anthracyclines, anthraquinones, epipodophyllotoxins, 9-OH-ellipticine [62–70]. The methodological approaches include the use of human cancer cell lines having defined profiles of expression of the two enzymes (breast cancer, acute lymphoblastic leukemias, sensitive and resistant leukemia HL-60), viz. noncycling cells, where the α isoform is down-regulated, transgenic cells lacking one isoform, or resistant cells transfected with a plasmid carrying one of the two isoforms. In addition, biochemical studies were carried out with the human recombinant enzymes overexpressed and purified from yeast. All drugs were able to affect both the α and the β isoform, although to different extents, indicating that both enzymes are good targets. Hence, the observed differences are quantitative, not qualitative, and the drugs presently in use exhibit a low level of discrimination. However, it appears that the anthracyclines prefer the α enzyme, and amsacrine and mitoxantrone the β enzyme.

While no very selective top2α poison has been found as yet, an almost pure top2β poison has been recently identified [71]. Indeed, the synthetic quinoxaline phenoxypropionic

acid derivative XK469 possesses unusual solid tumor selectivity and activity against multidrug-resistant cancer cells. In band depletion assays, XK469 caused little or no depletion of top2α and a significant dose-dependent reduction of top2β. In addition, exposure of subconfluent MCF-7 human breast cancer cells to the drug resulted in substantial cross-linking of top2β, but not top2α. Inhibition of superhelical DNA relaxation was also very effective in the presence of top2β. These findings indicate that the primary target of XK469 is top2β. Preferential targeting of this enzyme may explain the solid tumor selectivity of XK469 and its analogs because solid tumors, unlike leukemias, often have large populations of cells in the G(1)/G(0) phases of the cell cycle in which top2β is highly expressed whereas top2α is not.

It will be very important to define the molecular determinants for preferential top2βtargeting by XK469, in order to define structure–activity relationships for quinoxaline phenoxypropionic acid derivatives, and to design new classes of anticancer agents, perhaps endowed with prominent solid tumor cell killing potency.

The sequence selectivity of isoenzyme-mediated DNA cleavage for amsacrine, teniposide (VM-26) and an anthracycline derivative were determined using the human recombinant enzymes [72,73]. Local base preferences for DNA cleavage exhibited by the above drugs were essentially the same for both isozymes and corresponded to those determined using the murine enzyme. The identical drug sequence specificities suggest that molecular interactions of the tested drugs in the ternary complex are likely similar in the two isozymes. Evidently, the contacts formed between the tested drugs and the enzyme concern residues conserved in both isoforms. The high degree of sequence homology found at the top2 active site [74] justifies the above results. Drugs which stimulate α and β cleavage at different sites could possibly be designed by exploiting differential interactions with the nonconserved residue(s) of the protein's active site. To do so, the structure of the cleavage complex must be appropriately modeled.

7. In vivo specificity of topoisomerase poisoning

A final issue that deserves to be addressed deals with in vivo sequence-specific stimulation of top-mediated DNA cleavage and its relationship to in vitro findings. In vitro experiments are performed with the purified enzyme and a double helical DNA fragment. The situation in vivo is much more complicated, as a crowding of different enzymes and proteins occurs in chromatin, so that topoisomerase has to find its way by competing with other biological macromolecules. In addition, the structure and topology of DNA in vivo is greatly affected by specific interactions with histones and DNA-processing enzymes, which could greatly affect cleavable complex formation and drug interference both quantitatively and qualitatively. Hence, studies aimed at defining the exact location of drug-stimulated damage in living cells are very useful for a proper assessment of the biological significance of cell-free topoisomerase poisoning experiments. In addition, specific (different) cleavage locations generated by each drug along the eukaryotic genome might substantially contribute to differentiate the anticancer potential of the drug.

Recent in vivo studies are described by Borgnetto et al. [75] who investigated DNA cleavage sites stimulated by three poisons with diverse sequence specificity, CL, VM-26 and the anthracycline dh-EPI in two genomic regions of *Drosophila melanogaster* Kc cells. CL-stimulated DNA cleavage sites were rarer than those of VM-26 at the satellite locus. In the histone H2A–H2B intergenic region, CL and dh-EPI stimulated cleavage, whereas VM-26 was only weakly effective. As observed in vitro, some CL cleavage sites did not undergo spontaneous reversion, indicating that this agent can stimulate irreversible cleavage in vivo. Direct genomic sequencing showed that many CL and dh-EPI sites, although distinct, mapped to the transcription start and to the proximal promoter of the H2A gene. Hence, the in vivo specificity of topoisomerase poisoning is likely to arise from a combination of chromatin accessibility and local drug-related preferences.

DNA cleavage sites stimulated by the two anthracyclines dh-EPI and da-IDA were investigated at the histone gene cluster of cultured Drosophila Kc cells [76]. The two agents produced closely related patterns of double-stranded DNA cleavage in Kc cell chromatin. Analyses of numerous base sequences of dh-EPI and (fewer) of da-IDA sites showed that both compounds largely followed the in vitro requirement of (5)′ TA at 3′ ends of cleaved strands. Nonetheless, a modulation in DNA cleavage was found when using human top2 isoforms in vitro. Human top2α promoted cleavage patterns that were much more similar to those of Drosophila top2 than human top2β. Moreover, da-IDA showed a marked site-dependent preference for human top2 β, suggesting that, notwithstanding the similarities found comparing the in vivo and in vitro data, differential effects on the two isozymes might influence drug activity in human cells.

8. Conclusions

Specificity is a fundamental feature of effective drugs, to prevent simultaneous recognition of multiple targets and, hence, the onset of undesired side effects. Although the treatment of cancer often requires the use of potent cytotoxic compounds, poorly selective between normal and cancer cells, important knowledge is being acquired on the mechanisms of recognition of biological targets by effective drugs. In this context, topoisomerases are useful targets to be exploited for specificity. Available results indicate that the biologically relevant specific DNA-binding properties of topoisomerase poisons occur through a protein-mediated process. Hence, the cleavage complex interface represents a unique structural feature, differing from DNA-sequence to

DNA-sequence. Small molecules are able to fit into this intermediate and generate site-selective contacts with the enzyme–DNA adduct. By properly exploiting the drug's ability to recognize more specifically its target, it should be possible to rationally design compounds able to transform the topoisomerase into an endonuclease that cleaves DNA at very few selected sites along the genome, thus, triggering the apoptotic process in malignant cells only. This will hopefully yield new powerful anticancer agents, able to distinguish between mutated and normal DNA, hence, to spare healthy cells from death. Although we are still far from being able to do so, the combined information on reversible, irreversible, type and isoform specificities exhibited by the topoisomerase poisons is rapidly yielding the seminal knowledge to successfully proceed in that direction.

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