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Review Paper

DNA topoisomerase-targeting antitumor agents and drug resistance

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A review of the chemotherapeutic agents which have been developed by targeting DNA topoisomerase I and II is presented. Camptothecins as topoisomerase I-targeting agents and newly developed topoisomerase II-targeting agents with unique properties are expected to be promising anticancer agents in the near future. An important issue is how cellular sensitivity to these agents is controlled. One approach is to establish and characterize drug-resistant human cancer cell lines, which would provide powerful tools to understand their intracellular target sites and also the mechanisms for acquirement of drug resistance to topoisomerase inhibitors. Drug resistance to topoisomerase-targeting agents appears to be closely correlated with two events, namely decreased expression and point mutation of topoisomerase genes.

Key words: Camptothecins, chemotherapy, DNA topoisomerases, epipodophyllotoxins.

Introduction

DNA topoisomerase catalyze conformational changes of DNA such as winding/unwinding, catenation/decatenation and condensation/decondensation. There are two major types of the enzymes in eukaryotes. Type I DNA topoisomerase (Topo I) catalyzes DNA by nicking/rejoining a single strand of DNA, and is closely coupled with replication, transcription and other DNA processes. Type II topoisomerase (Topo II) breaks both DNA strands, passes through a second double-strand DNA prior to resealing, and is coupled with DNA processes such as replication, transcription, chromosome condensation and decondensation.[1,2]

Topoisomerase-targeting drugs apparently interfere with the breakage–rejoining reaction of DNA topoisomerase. In the presence of these drugs, intermediate forms of drug–enzyme–DNA, 'cleavable complexes', accumulate (Figure 1). The cellular accumulation of these drug-induced cleavable complexes may induce cytotoxicity. However, although some topoisomerase-targeting drugs can induce cytotoxicity, they do not stimulate the formation of cleavable complexes.[3–6] In this review, the properties of camptothecins as Topo I-targeting agents and several Topo II-targeting agents of intercalating and non-intercalating types are presented. Analyzing mammalian cell lines resistant to these agents is also helpful in understanding how cellular sensitivity is controlled. We thus present some unique characteristics of human cancer cell lines resistant to camptothecin, etoposide, teniposide and other drugs.

Topo I-targeting camptothecins

Human Topo I, a 100 kDa monomeric protein, is encoded by a single copy gene located on human chromosome 20 and catalyzes the complete relaxation of both positively and negatively supercoiled DNA without energy dependence.[1] Topo I is covalently linked to the 3′-phosphoryl end of the broken DNA strand via a tyrosyl phosphate bond. This intermediate of the DNA–enzyme complex causes single-strand DNA breaks.

Camptothecin (Table 1), a well-characterized Topo I-targeting drug, is a plant alkaloid isolated from *Camptotheca acuminata* which induces DNA breaks by Topo I.

Camptothecin is cytotoxic against a number of experimental tumor cells, but severe side effects have been observed in preclinical and clinical studies. Kunimoto *et al.*[7] have further developed a

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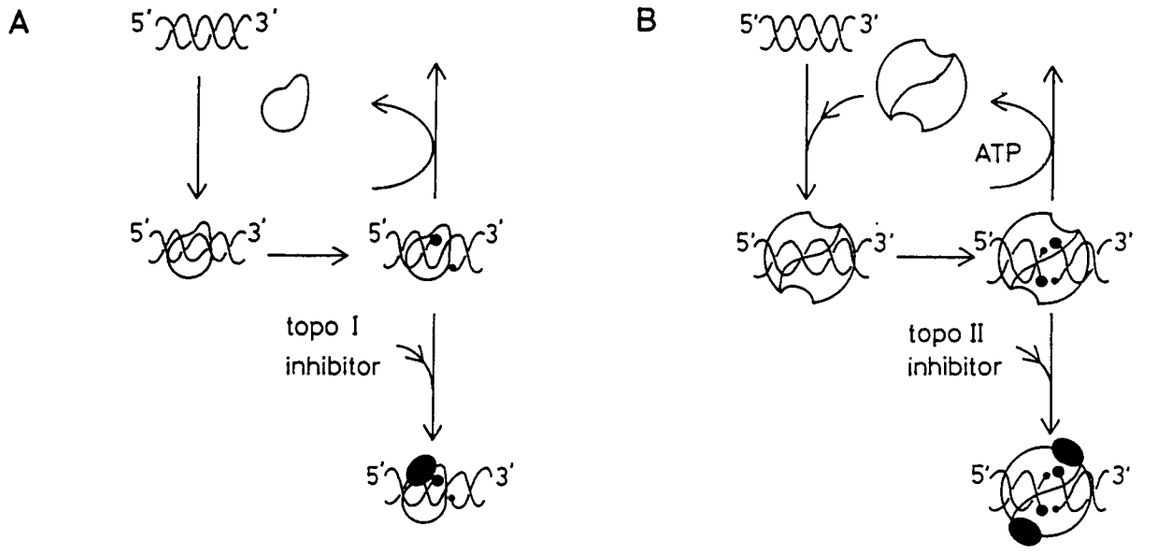

Figure 1. Topoisomerase-mediated cleavable complex formation with topoisomerase inhibitors. (A) Single-strand DNA breaks by topoisomerase inhibitor(s). DNA Topo I [◯] is covalently linked to the 3'-ends of the broken DNA strands in the presence of Topo I inhibitor(s) [●]. (B) Double-strand DNA breaks by Topo II inhibitor(s). DNA Topo II [◯] is covalently linked to the 5'-ends of the broken DNA strands in the presence of Topo II inhibitor(s) [●].

new camptothecin derivative, CPT-11, with higher antitumor activity, less toxicity and high aqueous solubility. Clinical trials with CPT-11 have recently demonstrated chemotherapeutic effectiveness against lung, ovary, uterus and colon cancers as well as leukemia.[8,9] Two other camptothecin derivatives, 9-amino-camptothecin (9-AC) and 10,11-methylenedioxy-camptothecin (Topotecan) (Table 1), produce long-term remission in nude mice carrying human colon cancer xenografts.[10] Camptothecin interferes with the breakage–rejoining reaction of Topo I by trapping the cleavable complex, especially by slowing the reclosure step of the breakage–rejoining reaction[11] (see Figure 1). The cleavable complex can be converted to Topo I-linked single-strand DNA breaks in the presence of sodium dodecyl sulfate or alkali. Dilution, addition of excess DNA or salt and exposure to high temperatures rapidly reverses the cleavable complexes,[12] suggesting that camptothecin-stabilized cleavable

Table 1. DNA topoisomerases-targeting antitumor agents

Targets	Drugs	
Topo I	Camptothecins	7-ethyl-10-[4-(1-piperidino)-1-piperidino] Carbonyloxy camptothecin (CPT-11) 9-amino-camptothecin (9-AC) 10,-11-methylenedioxy-camptothecin (Topotecan)
Topo II	Intercalators	
	acridines	amsacrine (*m*-AMSA)
	anthracyclines	adriamycin (doxorubicin) daunomycin (daunorubicin)
	anthracenediones	mitoxanthrone
	actinomycins	actinomycin D
	ellipticines	2-methyl-9-OH-ellipticiriuss acetate
	Non-intercalators	
	epipodophyllotoxins	etoposide (VP-16) teniposide (VM-26)
	isoflavonoids	genistein
	dioxapiperadines	ICRF-193
	thiobarbituricacid	merbarone

complexes are in dynamic equilibrium with non-cleavable complexes. Since camptothecin is a dramatic S-phase specific agent,[13] there should be a higher level of cleavable complexes in proliferating tumor cells than in quiescent cells.

Topo I is a phosphoprotein and one of its serine residues becomes phosphorylated *in vivo*, which results in increased enzymatic activity.[14] However, polyADP-ribosylation of the protein inactivates the enzyme and treatment with 3-aminobenzamide, an inhibitor of polyADP-ribose synthetase, results in enhancement of the cytotoxic activity of camptothecin.[15] Further study is required to determine how cellular levels of Topo I are regulated, and also how CPT-11 and other derivatives induce antitumor activity.

Drug resistance to camptothecin

Many camptothecin-resistant cell lines have been isolated from human cancer cells and characterized. Two types of mechanisms (altered expression of Topo I and mutation of the Topo I gene) appear to be involved in the acquirement of drug-resistance to camptothecin (Table 2). A CPT-11 resistant cell line, CPT-K5, from a human T cell derived acute lymphoblastic leukemia cell possesses an altered form of Topo I.[16] The mutant enzyme has equivalent specific activity in relaxing plasmid DNA and in decreased cleavable complex formation. A point mutation, $A_{1598} \rightarrow G$, causes Asp_{533} to change into Gly at a conserved sequence in CPT-K5 cells, resulting in an altered sensitivity to the cytotoxic agent. The mutation site is supposed to be critical for the catalytic activity of Topo I. However, other human cancer cell lines resistant to CPT-11, such as PC-7/CPT, HT-29/CPT and St-4/CPT, have decreased levels of Topo I (Table 2). PC-7/CPT, a CPT-11 resistant human lung cancer cell line, shows pleiotropic changes: decreased levels of Topo I, low affinity of the drug to Topo I and low activity of carboxylesterase which catalyzes CPT-11 to active metabolite, SN-38.[18-19] A549/CPT shows about 3-fold resistance to CPT-11 derived from human lung cancer A549 cells.[20,21] A549/CPT cells have similar levels of Topo I to A549 cells, but the content of Topo II and its mRNA are elevated in A549/CPT over those in the parental cells. It remains unknown whether the elevated level of Topo II is correlated with the acquirement of CPT-11 resistance in A549/CPT cells. In a CPT-resistant cell line (HT-29/CPT) from human colon cancer HT-29, lower and higher levels of Topo I and II, respectively, have been observed.[20,21] The decreased expression of Topo I appears to be compensated for by elevated expression of Topo II, suggesting a functional complementation of these two enzymes. Further analysis of the regulatory mechanism of Topo I gene expression are required to understand how expression of the Topo I gene is reduced in CPT-resistant cell lines.

Topo II-targeting agents

Human Topo II, a homodimeric protein, consists of 170 (Topo IIα) and 180 kDa (Topo IIβ) forms.[22] Topo IIα and Topo IIβ share considerable sequence homology, but they are the products of different transcripts: Topo IIα is encoded on human chromosome 17 and Topo IIβ is encoded on chromosome 3.[22,23] Both Topo IIα and IIβ form stable covalent complexes with DNA and their responses to teniposide are identical. Topo IIα is highly enriched in mitotic cells and is located in condensed chromosomes. In contrast, Topo IIβ is much more uniformly distributed between mitotic and non-mitotic cells and is neither localized nor enriched in condensed chromosomes.[24] These two forms appear to be involved in different functions. Mammalian Topo II is thought to be involved in diverse biological processes, but the biological functions are not completely understood. The mitotic *Xenopus* egg extract system demonstrates evidence for the

Table 2. Biochemical changes in human cancer cell lines resistant to CPT-11

Class	Cell lines	Topo I		Topo II	Derivations
		activity	content	content	
Expression type	PC-7/CPT	↓	↓		18
	A549/CPT		→	↑	20,21
	HT-29/CPT	→	↓	↑	20,21
	St-4/CPT		↓		20,21
Mutation type	CPT-K5	→ $A_{1598} \rightarrow G$ Asp → Gly			16,17

involvement of Topo II in chromosome condensation.[25] In the SV40 cell-free replication system, Topo II is essential for segregation of completely replicated daughter molecules.[1] Strand-passing activity of Topo II is also important for DNA replication fork movement. Either Topo I or II can remove the topological interaction of the two parental DNA strands during the elongation step of SV40 DNA replication *in vitro*. Transcription of closed circular DNA is stimulated when negative supercoils are introduced by the addition of Topo II and supercoiling factor.[26] Topo II mediates some forms of illegitimate recombination in the cell.[27-29]

Topo II initiates a catalytic cycle by binding to DNA. The nucleotide sequence of DNA dictates the sites at which Topo II binds the double helix,[30] and also defines the sites of enzyme-mediated DNA cleavage and catalytic activity.[31,32] Following recognition of its nucleic acid substrate, Topo II rapidly establishes a double-strand DNA cleavage/religation equilibrium,[33] which highly favors religation. DNA cleavage and religation require the presence of a divalent cation such as magnesium.[34] Topo II makes a staggered break in DNA which results in four-base 5′-overhangs and several Topo II recognition/cleavable sites have been identified; however, no consensus sequence fits all of the known sequence.[35] Once Topo II creates a double-strand break in the nucleic acid backbone, it passes a separate double-strand segment of DNA through the break. Strand passage is completely dependent on the binding of magnesium and ATP,[34,35] and the ATPase activity is greatly stimulated by the presence of DNA.[33]

Topo II is a phosphoprotein.[36,37] Casein kinase II and protein kinase C can phosphorylate Topo II, resulting in enhancement of the enzymatic activity *in vitro*.[38,39] A serine/threonine kinase has been also identified as being a Topo II-associated kinase in mouse mammary cancer cells and this enhances Topo II activity about 10-fold over that of the unmodified enzyme *in vitro*.[37] Further characterization of this enzyme has not yet been reported. We have isolated etoposide-resistant epidermoid cancer cell lines (KB/VP-1 and KB/VP-2) from human cancer KB cells[40] (Table 3). Cellular levels of Topo II in KB/VP-1 and KB/VP-2 cells are 10% or less than that in KB cells, but Topo II in the resistant cell lines similarly decatenates kinetoplast DNA as that in KB cells. In contrast, etoposide–DNA–topo II complexes are formed in nuclear extracts of KB cells but not in those of KB/VP-2 cells. The relative specific phosphorylation of Topo II is about 15-fold higher in the resistant cell lines and serine is phosphorylated.[40] Serine and threonine kinase appears to be a requisite for the decatenation activity of Topo II, but not for Topo II-induced cleavable complex formation. This phosphorylation of Topo II in KB and KB/VP-2 cells is not inhibited by kinase inhibitors (H7 and H8) and the activity is not further enhanced by phorbol ester (unpublished data). Novel serine/threonine kinases might be involved in the activation of Topo II through phosphorylation.

A number of antitumor Topo II-targeting agents have been developed (Table 1). Most of them stabilize Topo II-induced DNA cleavable complexes[1,13] whereas others fail to stimulate the formation of Topo II-mediated DNA cleavage.[3-6] Mammalian Topo II inhibitors interfere with the cleavage–rejoining reaction by trapping the cleavable complexes.[1] Except for non-intercalators, such

Table 3. Biochemical changes in human cancer cell lines resistant to Topo II-targeting agents

Class	Cell lines	Selected agent	Topo II				Derivations
			activity	content	mRNA	mutation	
Expression type	KB/VP-2	etoposide	→	↓	↓		(40)
	KB/VM-4	teniposide	↓	↓	↓		(52)
	KB/40a	etoposide	↓	↓	↓		(61)
	SK3/VP	etoposide	↓	↓	↓		(62)
	Jurkat/ADR	adriamycin	↓	↓	↓		(63)
	Jurkat/AMSA	*m*-AMSA	↓	↓	↓		(63)
Mutation type	CEM/VM-1	teniposide	↓	→		$G_{1346} \rightarrow A$ Arg → Gin	(59,60)
	HL-60/AMSA	*m*-AMSA	→	→		$G_{1457} \rightarrow A$ Arg → Lys	(55)
	KBM-3/AMSA	*m*-AMSA				$G_{1457} \rightarrow A$ Arg → Lys	(56)

as epipodophyllotoxins and isoflavonoids, all known mammalian Topo II inhibitors are DNA intercalators that insert part of their plane structures between two adjacent base pairs in duplex DNA, causing a reduction in the rotation angle between adjacent base pairs by 10°–30° (see Table 1).[41] There appears to be an excellent correlation between the strength of intercalation by the intercalative inhibitors and the efficiency of cleavable complex formation.[42,43]

The modes of action of non-intercalative Topo II inhibitors such as epipodophyllotoxin and genistein remains unclear. However, the supercoiling reaction induced by both Topo II and supercoiling factor is inhibited by a relatively low concentration of etoposide, whereas no other reactions of Topo II, such as relaxation of supercoiled DNA, are inhibited.[44] Other classes of Topo II-targeting drugs such as ICRF-193, merbarone, aclarubicin and fostriecin have been recently developed and do not stimulate formation of DNA cleavable complexes.[3,6] In particular, dioxopiperazine derivatives such as ICRF-154 and ICRF-193 inhibit the activity of purified Topo II without formation of a cleavable DNA–protein complex.[45] These derivatives, however, block etoposide-induced DNA strand breaks in human T cell leukemic cells,[3] suggesting that they interact with the same site of Topo II as etoposide. These derivatives cause accumulation of G2 and early M phases of the cell cycle with fewer condensed and entangled chromosomes, and they have different inhibitory effects on cell growth than the conventional cleavable complex-forming Topo II-targeting agents.[3,45] A new antitumor compound, saintopin, isolated from *Paccilomyces* can induce both Topo I- and Topo II-mediated DNA cleavage and the DNA cleavage intensity pattern by saintopin with Topo II is different from that by camptothecin.[46] Saintopin represents a unique compound that acts upon both Topo I and Topo II, but further study is required to understand its molecular mechanism and antitumor activity.

Drug resistance to Topo II-targeting agents

Many mammalian cell lines resistant to Topo II-targeting antitumor agents have been isolated and characterized. Cell lines carrying altered Topo II are often cross-resistant to a wide variety of Topo II-targeting agents. Drug-resistance to Topo II-targeting agents is associated with either normal or altered expression and catalytic activity of Topo II, suggesting that pleiotropic mechanisms underlie the acquirement of drug resistance. In Table 3, human cancer cell lines resistant to etoposide, teniposide, adriamycin and *m*-AMSA are presented. Two different mechanisms are mainly involved in acquiring drug resistance to Topo II-targeting agents, i.e. expression and mutation types. One class of resistant cell line with an altered expression type has decreased levels of Topo II mRNA. Decreased contents and activity of Topo II accompany decreased activity of DNA–protein cleavable complexes, resulting in the acquirement of drug resistance (Table 3). However, a mutant resistant to etoposide (KB/VP-2), which has decreased levels of both mRNA and Topo II, has similar decatenation activity to the parental cells.[40] The acquirement of drug resistance to etoposide/teniposide appears to be closely coupled with cellular levels of the cleavable complex, but not with decatenation activity of kinetoplast DNA. Many human cancer cell lines resistant to Topo II-targeting antitumor agents have decreased levels of Topo II mRNA (Table 3). Analysis of the regulatory mechanisms for expression of the Topo II gene is necessary to understand how drug resistance to Topo II-targeting agents is developed at the molecular level.

Expression of the Topo I gene is activated in response to phorbol ester in normal fibroblasts.[51] The expression of the Topo II gene is also activated in human cancer KB cells when exposed to high temperatures, suggesting that the Topo II gene is a SOS signal (unpublished data). However, exposure of KB cells to etoposide and teniposide also induces activation of the human multidrug resistance gene-1 promoter.[64–66] Topo II inhibitor-mediated stress appears to cause promoter activation of other drug resistance genes.

During the development of drug resistance to etoposide/teniposide, drug-resistant human cancer cell lines acquire various phenotypes that are closely correlated with etoposide/teniposide resistance. The teniposide-resistant cell lines, KB/VM-a, KB/VM-b, KB/VM-2, KB/VM-3 and KB/VM-4, show 3-, 6-, 12-, 16-, 74- and 95-fold higher resistance, respectively, than the parental KB cells after exposure to increasing doses of the drug.[52] KB/VM-a cells show temperature sensitive growth at 41.5°C, KB/VM-1 cells show decreased accumulation of etoposide and KB/VM-2 cells show reduced expression of the Topo II gene. The reduced expression of the Topo II gene combined with decreased permeability of the drugs and the temperature sensitive phenotype can account for the acquired teniposide resistance, but an altered site responsible for the temperature sensitive growth is not known.[52]

Cellular permeability and other changes should be also considered as possible mechanisms for drug resistance to Topo II-targeting agents besides Topo II itself. Combination with 1,4-dihydropyridine compounds potentiates the cytotoxicity of etoposide/teniposide against KB/VM-4 cells through enhanced permeability of the drugs.[53] These dihydropyridine compounds also potentiate the antitumor activity of etoposide against leukemia-bearing mice.[54] The permeability control of these podophyllotoxins can contribute to the further development of new chemotherapeutic methods with etoposide/teniposide.

Another mutation causing a change in drug resistance to Topo II-targeting agents is a point mutation of the Topo II gene (Table 3). An HL-60 cell line selected for resistance to *m*-AMSA (HL-60/AMSA) contains similar Topo II activity as the parental cell line, but *m*-AMSA-induced cleavable complex formation is dramatically reduced.[55] Etoposide-induced cleavage complex formation is similar to that of the parental HL-60 cells. There is a point mutation ($G_{1457} \rightarrow A$) in the Topo II gene of HL-60/AMSA which is responsible for a change of Arg_{486} to Lys (Figure 2). An identical change of amino acid at 486 is reported in another cell line resistant to *m*-AMSA (KBM-3/AMSA).[56] An Arg residue at 486 in the dinucleotide binding site is thus supposed to be important for *m*-AMSA-mediated trapping of the covalent Topo II–DNA complex.[55,56]

Another point mutation at a different site of the Topo II gene from the above mentioned cell lines has been also reported. Human leukemic cell line (CEM/VM-1) which is selected for resistance to teniposide displays cross-resistance to etoposide, doxorubicin, *m*-AMSA and actinomycinD.[49,57,58] Compared with the parent line, CEM/VM-1 cells exhibit decreased Topo II activity; a decrease in drug sensitivity and in the level of nuclear matrix Topo II, an increased ATP requirement by Topo II, a single mutation in Topo II resulting in a change of Arg_{449} to Gln at a highly conserved position in an ATP binding consensus site (see Figure 2), and decreased Topo II phosphorylation.[58–60] It remains unknown if the point mutation at the consensus B sequence of Topo II can account for all five altered phenotypes as described above. The analysis of these mutants resistant to *m*-AMSA and teniposide provide support for the notion that the polymerase chain reaction (PCR) with known sequences at the above hot spots for mutation will be useful in diagnosing the presence or absence of such mutations in clinical tumors.

Conclusion

Topo I and II are important cellular targets of a number of antitumor agents, which include camptothecin and its derivatives, epipodophyllotoxins, anthracyclines and ellipticines. A derivative of camptothecin, CPT-11, is now in clinical phase trials and has improved effects. A dioxopiperazine derivative, ICRF-193, is a Topo II inhibitory antitumor agent, but does not form Topo II-mediated DNA cleavable complexes. Saintopin is unique because it induces both Topo I- and Topo II-mediated cleavable complexes. The establishment of human cancer cell lines resistant to Topo I- and II-targeting

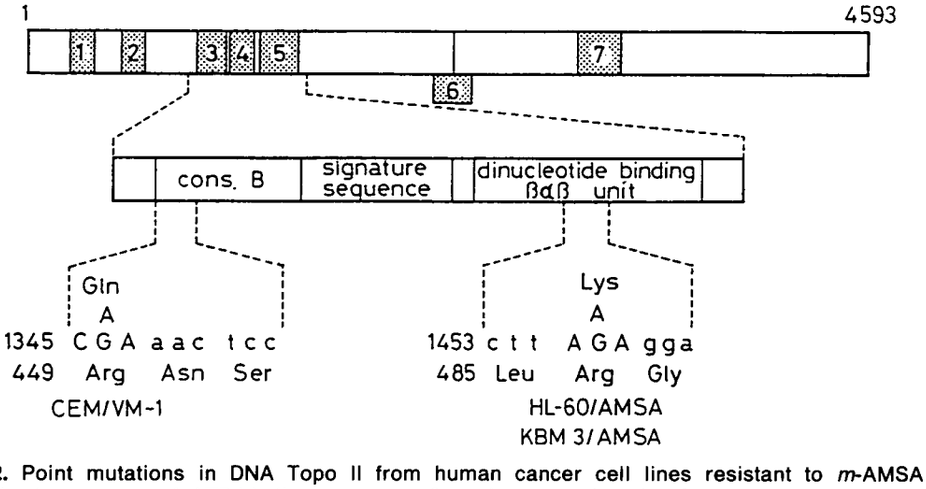

Figure 2. Point mutations in DNA Topo II from human cancer cell lines resistant to *m*-AMSA or teniposide: 1, consensus A ATP-binding sequence; 2, nuclear targeting site; 3, consensus B ATP-binding sequence; 4, Topo II signature sequence; 5, dinucleotide-binding $\beta\alpha\beta$ unit; 6, reactive tyrosine in a transient covalent bond to DNA; 7, leucine zipper.[56,59]

agents provides invaluable knowledge of how cells acquire resistance to them. It is also possible to develop further potent agents which can sensitize drug resistance to these Topo I- and II-targeting anticancer agents.

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