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Topoisomerase II as a target for anticancer chemotherapy*

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Type II DNA topoisomerases are required for the segregation of genomic DNA at cell division in prokaryotic and eukaryotic cells, and inhibitors of these enzymes are potential cytotoxic agents in both prokaryotes and eukaryotes. The bacterial member of the topoisomerase II family, DNA gyrase, and the chemotherapeutic agents which target it are the subject of a recent review (Maxwell, A. *et al.*, 1993, in *Molecular Biology of DNA Topoisomerases*, Andoh, T. *et al.*, eds., pp. 21–30, CRC Press, Boca Raton). Here we present an overview of current knowledge of eukaryotic topoisomerase II and the anticancer agents which target this enzyme, focussing predominantly on new observations and recent reports and reviews.

1. STRUCTURE AND REACTION OF EUKARYOTIC TOPOISOMERASE II

Topoisomerase II enzymes pass one duplex DNA through a second duplex DNA. To accomplish this reaction, the enzyme forms a reversible covalent phosphotyrosine bond to a 5'-phosphate of each DNA strand thereby creating a transient break in each phosphodiester backbone. These breaks, which are formed with a 4 base-pair stagger, create a transient protein-linked gate through which a second duplex DNA can be transferred [2, 3].

The primary structure of the eukaryotic enzyme shows extensive homology in different species and has considerable structural and functional similarities with the homologous but tetrameric prokaryotic enzyme, DNA gyrase. This homology leads to a canonical model for enzymes of this family which is useful for understanding their structure, reaction, and interaction with drugs (Fig. 1).

Mammalian cells contain two isoforms of topoisomerase II, topoisomerase IIα and topoisomerase IIβ, which exhibit different sensitivity to inhibiting drugs [4] and distinct tissue distribution [5, 6]. The specific functions of these two isoforms have not been elucidated. Both isoforms undergo post-transcriptional modifications *in vivo* by phosphorylation of serine and threonine in the C-terminal domain [7–9], a region not required for enzymatic activity but which contains signals for nuclear targeting and possibly other essential functions *in vivo* [2, 10]. The localization of some functional regions in the polypeptide subunit is shown in Fig. 1.

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Abbreviations: ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; GM-CSF, granulocyte-macrophage colony stimulating factor; MRP, multidrug resistance-associated protein.

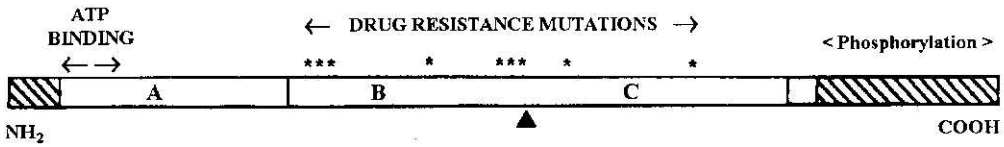

Fig. 1. A schematic representation of the polypeptide subunit of topoisomerase II.
Four domains can be defined in the native enzyme (A, B, C, and D) by sites of cleavage by protease V8 [2]. The N- and C-terminal regions (shaded) are not required for enzymatic activity *in vitro*; the C-terminal region contains signals for nuclear localisation and possibly for interaction with other proteins. The position of the active-site tyrosine to which the 5'-extremity of the interrupted DNA strand is covalently bound (▲) and of amino acid replacements which cause drug resistance in the human and *S. cerevisiae* enzymes (aligned according to the sequence comparisons in [2]) are indicated. The ATP binding region is positioned approximately based on studies of the homologous prokaryotic enzyme DNA gyrase and on the site of a mutation in the enzyme of *S. cerevisiae* [1, 2].

By combining recent structural and biochemical studies, an interesting picture of the topoisomerase II reaction mechanism is emerging. The molecule of human topoisomerase IIα is shown in Fig. 2 (upper), as seen by electron microscopy. Biochemical studies suggest that a complex sequence of intramolecular signalling and conformational changes occurs during the course of topoisomerase II reaction; after the covalent binding to DNA an allosteric change causes the N-terminal domains of the subunits to form a molecular clamp that captures the DNA segment to be transferred [11]. The enzyme can be visualized in this clamped conformation when the ATP binding site is occupied by a nonhydrolysable analogue, and the change of conformation which has occurred is shown in Fig. 2 (lower). The two forms which are observed both contain a tunnel, which may represent the passage through which DNA is transferred during the catalytic cycle.

2. ANTICANCER AGENTS TARGETING TOPOISOMEREASE II

Several agents employed in cancer chemotherapy target topoisomerase II. These agents

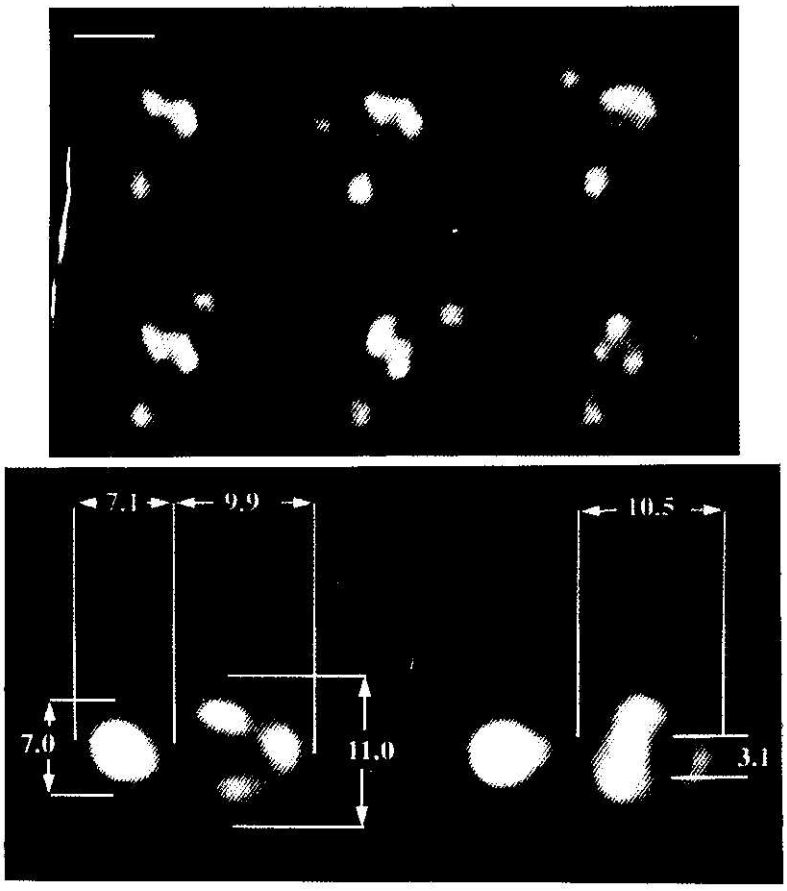

Fig. 2. Human topoisomerase IIα observed by scanning transmission electron microscopy.
(Upper) The molecule is composed of a major globular domain approx. 9 nm in diameter to which two smaller domains are connected by flexible arms. The small domains contain the N-terminal region of the 174 kDa polypeptide subunits. The bar represents 10 nm. (Lower) The enzyme after incubation with the nonhydrolysable ATP analogue ADPNP, which has been shown by biochemical studies to arrest the reaction after an allosteric transformation which results in the formation of a molecular clamp from the N-terminal regions, in which the DNA to be transferred is captured [11]. Two forms are observed which contain an approx. 3 nm-diameter tunnel in different positions, and may represent successive conformations during the transfer of the captured DNA between the enzyme's subunits. Dimensions are in nm. (Schultz, P., Oudet, P. & Hancock, R., in preparation).

fall into two classes, the demethylepipodophyllotoxins VP-16 (etopside) and VM-26 (teniposide), which target topoisomerase II specifically [3], and the intercalating agents doxorubicin (adriamycin), daunomycin, mitoxa- trone, and amsacrine, which also inhibit other functions of DNA. All of these drugs appear to slow the DNA religation reaction of the enzyme, thereby arresting the reaction at a step in which the enzyme is covalently bound to DNA. Details of the interaction between these drugs and topoisomerase II-DNA reaction intermediates are not understood. Amino acid replacements which confer resistance to single or multiple topoisomerase II-targeting drugs are located in the two central domains B and C of the polypeptide subunit [2, 12, 13], the region which contains the active-site tyrosine residue to which DNA is covalently bound during the catalytic cycle (Fig. 1) and which is believed to form a pocket in the native enzyme. The location of these mutations is consistent with the view that drugs interact with this region of the enzyme in its DNA-bound conformation. Interestingly, the vast majority of resistance-associated mutations involve topoisomerase IIα, suggesting that this isoform may play a predominant role in the action of these agents in mammalian cells.

Several new classes of inhibitors have been described over the past few years (Table 1). Some of these agents target different steps of the topoisomerase II reaction. Particularly interesting in this regard are the bisdioxopiperazines, which trap an intermediate enzyme conformation but do not cause accumulation of the covalent DNA-enzyme complex [14], and the fluoroquinolones, which appear to cause accumulation of topoisomerase II-DNA adducts by enhancing the rate of the initial transesterification reaction [15]. Whether these agents will have unique activity when they

Table 1
New inhibitors of eukaryotic topoisomerase II

Class and agent	Step inhibited	Reference
Demethylpodophyllotoxins:		
1- and 4-β-alkyl derivatives	After cleavage,	[85]
Aza-analogue (azatoxin)	before religation	[86]
Intercalating agents:		
9-Anilinoacridines	After cleavage, before religation (?)	[87]
N-methylpyrrolecarboxamideanilinoacridine	After cleavage, before religation (?)	[81]
Quinobenzoxazines	Before cleavage	[88]
Naphtalimide:	After cleavage,	
Amonafide*	before religation	[89]
Bis-polysulfonated naphthylurea		
Suramin	Before cleavage	[51]
Anthrapyrazoles:		
DUP941*	Before cleavage	[90]
Bisdioxopiperazines:	Trap an intermediate enzyme conformation;	
ICRF-193		[19]
MST-16*	no cleavage	
2-nitroimidazole:	After cleavage,	
Ro 15-0216	before religation	[82]
Fluoroquinolone:		
CP-115,953	Enhances cleavage	[15]
Pyrimido[1,6-α]benzimidazole:	After cleavage,	
RO 47-3359	before religation (?)	[91]

* In clinical trials

enter the clinic is unknown. Clinical trials of some of these new agents are described in Section 5.

3. MECHANISMS OF CYTOTOXICITY OF TOPOISOMERASE II-TARGETING AGENTS

Because a better understanding of events leading from inhibition of topoisomerase II to the cytotoxic outcome might suggest ways to improve the therapeutic usefulness of topoisomerase II-targeted agents, considerable attention has been focused on the mechanism by which these agents kill cells. Two major models of cytotoxicity have been put forward, an enzyme inhibition model and an enzyme poisoning model.

The enzyme inhibition model suggests that topoisomerase II-directed agents kill cells by abolishing the enzyme's activity. Studies of temperature-sensitive topoisomerase II mutants in yeast and of the effects of topoisomerase II inhibitors on mammalian cells establish that topoisomerase II activity is required for the formation and segregation of mitotic chromosomes during the late G2 period of the cell cycle [16–20], a period when a replicated chromosomal DNA molecules undergo topological transformations that allow the formation and separation of the two daughter chromatids. If these topoisomerase II-mediated processes are arrested while other events involved in cell division continue, chromosome nondisjunction results followed by formation of micronucleated, nonviable daughter cells [18–20], an effect closely correlated with cell death (R. Hancock, unpublished). This mechanism almost certainly accounts for the cytotoxicity of inhibitors which do not stabilize topoisomerase II-DNA adducts [19] and has also been proposed to explain the cytotoxicity of agents which do stabilize these adducts.

The topoisomerase II poisoning model emphasizes a different set of observations. First, cytotoxicity of agents which stabilize the topoisomerase II-DNA covalent intermediate is observed in after exposure of cells to drugs at low concentrations which do not stabilize many topoisomerase II-DNA adducts in intact cells (e.g. [21]) and do not inhibit topoisomerase II catalytic activity in cell-free systems [22]. Second, enzyme molecules covalently integrated into DNA are released when the inhibiting drug is removed [23], but cells nevertheless proceed to die. Third, examination of cells that survive treatment with these agents reveals the presence of sister chromatid exchanges and chromosome rearrangements (e.g. [18]) whereas chromosome breakage might be expected from inhibition of topoisomerase II catalytic activity during mitosis. Finally, topoisomerase II is not required for DNA replication or transcription during the G1 and S phases of the cell cycle [16–19], a conclusion supported by the observation that replication of the circular DNA genome of SV40 virus [24, 25] and transcription by RNA polymerase I and II of circular DNAs microinjected into *Xenopus* oocytes [26] are not affected by VM-26, yet the demethylepipodophyllotoxin and other topoisomerase II-targeting agents are particularly toxic during the S phase [27–29]. All of these observations are difficult to explain if cytotoxicity results solely from the inhibition of topoisomerase II activity. Instead, it has been suggested that the drugs in question transiently stabilize covalent adducts between small numbers of topoisomerase II molecules, which appear to be continuously probing genomic DNA *in vivo*, and DNA and that subsequent collisions of components of the transcription and replication machinery with these adducts result in the formation of DNA breaks at these sites, an effect termed "poisoning" [30]. This model appears to explain all of the observations presented above. Recent results of Howard *et al.* [31] using purified helicase and purified topoisomerase II provide direct evidence for this model *in vitro*. What is lacking at present, however, is direct evidence that these events occur in intact cells treated with drugs at doses which produce only a modest (one- to two-log) reduction in viability.

At later stages after exposure to topoisomerase II-targeting agents some cell types show characteristics of apoptosis, including the appearance in extracted DNA of fragments approx. 50 kb in length and of mono- and oligonucleosomal fragments [32, 33]. Recent work suggests that the approx. 50 kb fragment result from DNA cleavage in regions where chromatin loop domains are attached to the nuclear matrix or scaffold, cleavage mediated by topoisomerase II preferentially located in these regions and trapped by the drug while covalently bound to DNA [34, 35]. The gener-

ation of oligonucleosomal fragments, on the other hand, is not directly mediated by topoisomerase II and is seen in cells treated with a variety of unrelated cytotoxic agents [32]. Events linking the stabilization of topoisomerase II-DNA complexes and subsequent apoptosis are a subject of current investigations.

4. GENOME REARRANGEMENTS CAUSED BY TOPOISOMERASE II-TARGETING AGENTS

Growth of cultured cells in the presence of demethylepipodophyllotoxins or intercalating agents can lead to mutations, especially gene deletion and amplification (e.g. [36, 37]). Several recent studies suggest ways in which topoisomerase II might be involved in the generation of these mutations. Studies in cell-free systems indicate that helicases can convert reversible topoisomerase II-DNA adducts into complexes containing single-stranded DNA covalently bound to one active site of the topoisomerase II dimer [31]. Independent experiments indicate that this type of intermediate can transfer the bound DNA to any double-stranded DNA which contains a free 3′-hydroxyl group, albeit with varying efficiency depending upon other structural features at this 3′ end [38]. If the acceptor 3′-hydroxyl group comes from the DNA which was originally bound to the enzyme, elongation or nucleolytic shortening of this extremity prior to religation might explain the formation of small deletions and insertions like those observed in the *aprt* gene of teniposide-treated CHO cells [39]. If the acceptor group comes from a foreign DNA, nonhomologous recombination of the type described by Bae and coworkers [40] will result.

The mutagenic effects of demethylepipodophyllotoxins and mechanistically similar agents have immediate implications for the clinical use of these drugs. Treatment with these agents have been associated in a percentage of patients with the subsequent development of a distinctive form of acute myelogenous leukemia bearing a translocation in the MLL/ALL gene at chromosome 11q23 (e.g. [41–45]). Why this particular gene is uniquely susceptible to the mutagenic effects of these agents in bone marrow stem cells is currently unknown. It is likewise unknown whether the therapeutic and mutagenic effects of these agents can be separated.

5. TOPOISOMERASE II-TARGETING DRUGS IN CLINICAL CHEMOTHERAPY

Topoisomerase II-targeting drugs are among the more widely used antineoplastic agents. Although they are not curative as single agents, they contribute to the effective treatment of many cancers where long remission and cures are possible with combination chemotherapy. Etopside, for example, is used to treat testicular cancer, high-grade lymphoma, acute leukemia and lung cancer. Teniposide has contributed to dramatic improvement in cure rates of children with acute lymphocytic leukemia. The intercalating agent doxorubicin (adriamycin) is active against breast cancer, ovarian cancer, sarcoma, aggressive lymphoma, and acute leukemia.

Clinical development of new topoisomerase II-directed agents continues. Of the agents shown in Table 1, the naphthalimide amonafide has undergone the most extensive clinical testing to date. The results of these trials highlight some typical issues that are raised as a new topoisomerase II-directed agent enters the clinic. Phase I (dose-finding) trials indicated that the maximum tolerated dose was 300–400 mg/m^2 per day for 5 consecutive days [46] or 800–1100 mg/m^2 as a single infusion every 28 days [47], that suppression of the bone marrow limited further dose increases, and that the agent was cleared with a half-time of 3–5 h. Most of the subsequent phase II (efficacy) trials, of which 24 have been published to date, involved daily administration of amonafide for five consecutive days. These studies demonstrated that amonafide produces few regressions in patients with previously untreated melanoma, sarcoma, or carcinoma of the colon, stomach, esophagus, pancreas, lung (non-small cell), or endometrium. Likewise, amonafide was inactive against relapsed leukemia, hormone-refractory prostate cancer, and platinum-resistant ovarian cancer. Bone marrow suppression was observed in a significant percentage of patients in all of these negative studies, suggesting that the lack of antitumor activity was not due to inadequate dosing. In contrast, amonafide causes partial regression in patients

with previously untreated metastatic breast cancer when administered on either schedule [48, 49], and the response rate might be as high as 50% in patients who are exposed to enough drug to cause severe marrow toxicity. Why breast cancer is uniquely responsive to this agent is currently unknown.

Clinical studies of suramin raise a different set of questions. This bis-polysulfonated naphthylurea, which has a long history as a trypanocide, has shown activity against low-grade lymphomas as well as carcinomas of the prostate and adrenal cortex (reviewed in [50]). Studies by Bojanowski *et al.* [51] indicate that suramin can not only inhibit topoisomerase II at a step that precedes covalent complex formation, but also that this highly charged agent can penetrate cells. On the other hand, suramin also inhibits a number of other nuclear enzymes including DNA polymerase α, DNA primase, and RNA polymerase, abolishes the binding of the growth factors PDGF, NGF, IGF-1, EGF, interleukin-2, and transferrin to their receptors, inhibits enzymes involved in hydrolysis of extracellular matrix glycosaminoglycans (a step required for metastasis), and alters signal transduction (reviewed in [50, 52]). Demonstrating which of these many effects is responsible for the clinical antitumor activity of this agent is likely to prove difficult.

Although most of the agents shown in Table 1 target the manipulation of DNA by topoisomerase II in one way or another, the enzyme's ATP--binding site also appears to be a potential therapeutic target. One major class of inhibitors, the coumarins, targets this region of DNA gyrase [1]. Novobiocin likewise appears to target the ATP-binding site of topoisomerase II in mammalian cells [53]. Preclinical studies indicate that novobiocin enhances the cytotoxicity of DNA cross-linking agents [54] and there is evidence that this is mediated through an effect on topoisomerase II [55], although effects mediated through other targets [56–58] also cannot be ruled out. Clinical studies utilizing novobiocin in combination with these DNA cross-linking agents are currently in progress [59], and whether this approach will improve the efficacy of DNA cross-linking agents will require large comparative (phase II) trials.

6. RESISTANCE TO TOPOISOMERASE II-TARGETING DRUGS

The realisation that existing topoisomerase II-directed agents are inactive in many human tumors has prompted research into mechanisms of resistance to these drugs. In cultured cells, resistance has been correlated with a number of different physiological changes [60–62] (Table 2). In yeast whose topoisomerase II level has been manipulated by expressing a transfected gene, diminished levels of topoi-

Table 2

Changes in topoisomerase II correlated with drug resistance in cultured cell lines

	Resistant to		Reference
	Epipodophyllotoxins	Intercalators	
Reduced topoisomerase II:			
α isoform	++	++	[63]
α isoform	0	++	[64]
both isoforms	++	++	[65, 92]
Mutation:			
α isoform	++	++	[2]
α isoform	++	0	[15]
α deletion Ala429	++	+	[93]
New variants (α isoform):			
C-truncated	++	++	[13, 94, 95]
truncated	++	++	[96]
Phosphorylation (α isoform):			
reduced	+	–	[97]
increased	+	+	[98]

somerase II are associated with drug resistance (e.g. [63]). Consistent with these studies, diminished levels of topoisomerase II are frequently observed in mammalian cells selected for resistance to drugs which stabilise topoisomerase II-DNA adducts. This change can affect the level of the α-isoform alone (e.g. [64]) or both isoforms [65]. The fact that mammalian cells can survive this decrease in topoisomerase II implies that the normal cellular level of this enzyme is in excess of that required for growth (Section 3).

In addition to these quantitative changes, point mutations in the gene for topoisomerase II have been observed in cultured cell lines selected for resistance to intercalating agents, to demethylepipodophyllotoxins, or to drugs of both classes [12, 13, 60] (Fig. 2). As noted above, these point mutations probably indicates the location of amino acids that are important in drug binding (Fig. 1).

In discussing resistance to topoisomerase II-directed drugs, it is important to realize that these agents must enter cells and accumulate within the nucleus in order to exert their effects. Cell lines selected for resistance often show decreased steady-state drug accumulation as a consequence of enhanced efflux mediated by the *mdr*1 multidrug transporter [66] or other systems (e.g. [67]). A wide variety of agents that can affect this type of resistance have been identified (reviewed in [68]).

In considering whether the changes described above contribute to resistance in the clinical setting, several concepts need to be kept in mind. First, it is likely that low levels of resistance (2- to 10-fold) might be enough to convert a responsive tumor to a non-responsive tumor *in vivo* (e.g. [69, 70]). Second, it is possible that the resistance encountered clinically might be multifactorial. Third, the growth kinetics of cells in clinical cancers differ substantially from those of cells in culture and of transplantable mouse tumors, which are selected for rapid proliferation [71]. This last point becomes important in light of the observation that topoisomerase II levels are low in G0 phase cells (e.g. [27, 72, 73] and references therein) and the observation that topoisomerase II-DNA adducts are most toxic during the S phase (Section 3).

The role of alteration in drug accumulation has received extensive attention (reviewed in [68]). It appears that expression at high levels of the *mdr*1 multidrug transporter occurs in cancer of the colon, liver, adrenals, and kidney, neoplasms which are typically resistant to topoisomerase II inhibitors. At the time of relapse, elevated levels of this transporter are also seen in some leukemias and lymphomas, breast cancers, and sarcomas. In view of the observation that drugs such as doxorubicin can also induce transient expression of this transporter [74], it is possible that examination of specimens obtained at relapse may underestimate the role of this transporter in resistance. The participation of multidrug resistance-associated protein (MRP) and other transporters in clinical drug resistance is currently being defined.

Topoisomerase II itself has been examined in a much more limited group of clinical samples. Using a highly sensitive DNA catenation assay or Western blotting, Holden and coworkers [75, 76] were able to demonstrate the expression of topoisomerase II in one or two specimens from a number of different tumor types. These studies demonstrate the widespread occurrence of this enzyme in human neoplasms but do not address the issue of why different patients with similar neoplasms respond differently to topoisomerase II-targeting drugs. To address this latter issue, it is necessary to compare samples from multiple patients with one or two neoplasms. In the first major study of this type, Potmesil and coworkers [77] compared topoisomerase II levels in samples of chronic lymphocytic leukemia (CLL) and acute lymphocytic leukemia (ALL) by Western blotting. Topoisomerase II levels were much lower in CLL samples, providing a partial explanation for resistance of this neoplasm to anthracyclines and demethylepipodophyllotoxins. Subsequent analysis has revealed that topoisomerase II mRNA levels also vary widely between different samples of ALL [78]. Interestingly, topoisomerase II mRNA was observed to closely parallel the level of mRNA for histone H3, an S phase restricted transcript, raising the possibility that topoisomerase II expression detected by methods such as Western and Northern blotting might simply provide a measure of the number of cells that are actively traversing the cell cycle.

Topoisomerase II has also been examined in marrow samples from patients with acute myelogenous leukemia [72]. Levels of topoi-

somerase IIα and β detected on Western blots varied over a 20-fold range. Interestingly, there was no evidence for reduced topoisomerase II levels or gene mutations at the time of relapse. Further examination on a cell-by-cell basis revealed marked heterogeneity; the percentage of leukemia cells that stained with affinity-purified anti-topoisomerase IIα antibody ranged from 1% to 40% in different specimens. Interestingly, treatment of patients with granulocyte-macrophage colony stimulating factor (GM--CSF) caused concomitant increases in the number of leukemia cells traversing the cell cycle and in the number staining positive for topoisomerase IIα.

Several conclusions can be drawn from the limited studies published to date. First, samples obtained at the time of relapse do not appear to contain topoisomerase II mutations of the type observed in drug-resistant cultured cell lines [72, 79]. Second, there is no evidence for diminished topoisomerase II levels at the time of relapse so long as samples containing a similar proportion of tumor cells at diagnosis and relapse are compared [72]. Third, there is marked cell-to-cell heterogeneity of topoisomerase IIα expression within each sample [72, 80] which appears to reflect the large number of G0 phase cells in clinical tumor specimens. This cell cycle-related heterogeneity is likely to frustrate attempts to correlate the bulk topoisomerase II content of tumor specimens with clinical sensitivity to chemotherapy. Accordingly, the hypothesis that alterations affecting topoisomerase II contribute to resistance in the clinical setting must be considered an attractive but currently unproven concept.

7. FUTURE PERSPECTIVES

The diverse chemical structure of agents that inhibit different steps of the topoisomerase II reaction (Table 1), and the number and complexity of these steps, suggest that the full potential of topoisomerase II-inhibiting drugs is yet to be explored. Several observations illustrate the subtlety of the interaction between these drugs and intermediates in the catalytic cycle. First, different drugs which cause accumulation of covalent topoisomerase II-DNA complexes have different effects on the selection of integration sites (e.g. [81–84]). Second, replacement of a single amino acid such as lysine for arginine can affect the sensitivity of topoisomerase II to different drug classes in a differential manner [2]. These observations raise the possibility that comparably subtle modifications of drug structure might increase efficiency or confer sensitivity on drug-resistant enzymes. These strategies will be facilitated by progress in modelling the structure of the enzyme, its complexes with DNA, and the binding of inhibitory drugs.

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