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Targeting DNA topoisomerase II in cancer chemotherapy

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Summary

Recent molecular studies have greatly expanded the biological contexts where Top2 plays critical roles, including DNA replication, transcription and chromosome segregation. Although the biological functions of Top2 are important for insuring genomic integrity, the ability to interfere with Top2 and generate enzyme mediated DNA damage is an effective strategy for cancer chemotherapy. The molecular tools that have allowed understanding the biological functions of Top2 are also being applied to understanding the details of drug action. These studies promise a more refined ability to target Top2 as an effective anti-cancer strategy.

Introduction

An important reason why Top2 has held the interest of researchers studying cancer was the discovery that active anti-cancer drugs, notably etoposide and doxorubicin target Top2¹. These studies showed that most clinically active drugs that target Top2 generate enzyme mediated DNA damage²⁻⁴. Since etoposide and doxorubicin are highly active anti-cancer agents in many different settings, an identification of a critical target of these drugs was a major landmark in the pharmacology of anti-cancer drugs.

Recent work has shown that there may be contexts where the level of Top2 protein predicts clinical activity (as well as many contexts where it does not). With the understanding of mechanisms of drug action and improved patient survival rates has come the appreciation that clinical treatment with drugs targeting Top2 can lead to the dire consequence of secondary malignancies. An important goal of present and future work is to maximize therapeutic efficacy of therapy using Top2 targeting agents while minimizing the risks of secondary malignancy and other toxicities. This review highlights recent work that is relevant to maximizing the potential of Top2 as an anti-cancer drug target.

Inhibition of Top2 activity by anti-cancer agents

Drugs targeting Top2 are divided into two broad classes. The first class, which includes most of the clinically active agents including etoposide, doxorubicin, and mitoxantrone, lead to increases in the levels of Top2:DNA covalent complexes. Because these agents generate a “lesion” that includes DNA strand breaks and protein covalently bound to DNA, these agents have been termed Top2 poisons. A second class of compounds inhibits Top2 catalytic activity, but do not generate increases in the levels of Top2 covalent complexes. This second class of agents is thought to kill cells through elimination of the essential enzymatic activity of Top2 and is therefore termed catalytic inhibitors (Fig. 1).

There are several lines of evidence indicating the importance of the distinction between Top2 poisons and Top2 catalytic inhibitors. Studies in yeast and mammalian cells demonstrated that resistance to Top2 poisons is recessive, i.e., presence of a drug resistant Top2 in the presence of a drug sensitive allele results in cells that are drug sensitive (reviewed in^{5,6}). The importance of enzyme mediated DNA damage is also demonstrated by observations that Top2 poisons rapidly elicit DNA damage responses such as ATM phosphorylation and activation of downstream damage responses⁷⁻⁹. Resistance to Top2 targeting drugs in mammalian cells is frequently associated with reduced expression of Top2 isoforms⁶, suggesting that resistance is mediated through a reduction in enzyme mediated DNA damage, rather than through enhancing available enzyme activity (where resistance would arise from increased expression of Top2 isoforms).

The generation of high levels of Top2 DNA covalent complexes has profound effects on cell physiology. Top2 poisons effectively block transcription and replication. DNA strand breaks are rapidly detected following treatment with Top2 poisons, and most of the strand breaks are protein linked, as expected^{10,11}. Cells subsequently commit to apoptosis, in fact etoposide is a very commonly used agent to study apoptotic processes¹².

The pattern of responses observed with catalytic inhibitors of Top2 differ from that observed with Top2 poisons, albeit with several important complications. Most catalytic inhibitors of Top2 are not specific for Top2 inhibition (see Box 1) with the exception of bisdioxopiperazines. While bisdioxopiperazines generate DNA damage responses following long exposure¹³, they do not produce a DNA damage response following short term exposure¹⁴⁻¹⁷. Importantly, in cell culture experiments, catalytic inhibitors of Top2 antagonize the toxicity of Top2 poisons¹⁸, indicating that the agents act by separable mechanisms. An important and still unanswered question is whether Top2 inhibitors that are not poisons might be active anti-cancer agents. This issue is addressed in the concluding sections of this review.

Top2 poisons

As shown in Box 1, a diverse range of compounds leads to increased levels of DNA cleavage. The precise mechanism of action of Top2 poisons remains a critical unsolved question. It is instructive to consider the mechanism of camptothecins against topoisomerase I, since several drug:protein:DNA ternary structures have been solved^{19,20}. Camptothecins intercalate between the -1 and +1 bases of DNA in the ternary complex, where the -1 base is the nucleotide that is covalently bound to Top1. The intercalated drug makes several contacts with the protein by hydrogen bonding. The overall ternary complex is stabilized both by stacking interactions between the drug and the bases it intercalates between, as well as the drug:protein interactions. Gratifyingly, the amino acids that interact with camptothecins are sites that can lead to camptothecin resistance when the amino acids are mutated²¹. The net result of the presence of the drug is that (for Top1) the 5'OH is displaced relative to the 3'phosphotyrosyl, thereby preventing religation. This type of inhibition has been referred to as interfacial inhibition, i.e., the drug interacts at the interface between protein and DNA²². A similar picture can be applied for intercalating Top2 poisons. The intercalator is positioned between the -1 and +1 bases, and disrupts the geometry required for religation after strand passage. This localization of Top2 poisons was first suggested by an analysis of Top2 cleavage sequence preferences obtained by treating purified enzyme with defined DNA fragments in the presence of Top2 poisons^{23,24}. A direct demonstration of this type of positioning has been obtained using a photoactivatable analog of mAMSA and phage T4 topoisomerase II²⁵ (The phage topoisomerase is more similar in its reactions and spectrum of inhibitors to eukaryotic Top2 than to prokaryotic type II enzymes²⁶). The mAMSA derivative is found linked to the +1 base relative to DNA cleavage, suggesting intercalation at the site of cleavage. Since the photoactivatable analog of mAMSA does not react significantly with DNA in the absence of Top2, the enzyme plays some role in

stabilizing the position of small molecule so that it can react with DNA. Although direct information has not been obtained with other intercalating agents, it has been found that there is a clear base preference at cleavage sites, with the strongest preferences at either -1 or $+1$ ²⁷. These results are consistent with the presence of the intercalator at a protein:DNA interface, with a major effect of the inhibitor being a change in geometry of residues required for religating the DNA strand break. It is likely that this type of model also extends to non-intercalating Top2 poisons²⁴. For non-intercalating poisons an initial drug protein binary complex may be important for delivering drug to a site where a stable ternary complex can be formed²⁸⁻³⁰.

There are several important questions that remain in applying the interfacial inhibition model to Top2 poisoning. First, does the model imply that trapping double strand cleavage requires the action of two drug molecules at the site of cleavage? Osheroff and colleagues have argued that two etoposide molecules are required for enhanced double strand cleavage³¹. It is plausible that similar effects occur with intercalators, although this has not been examined carefully. It should be noted that double strand cleavage is not absolutely required for effective Top2 poisons; single strand cleavage by Top2 can also result in cytotoxicity³². Second, it has been suggested that many Top2 poisons do not block religation but instead generate high levels of Top2:DNA covalent complexes by stimulating cleavage³³. This mode of action is not easily explained by interfacial inhibition, and suggests that some agents can cause DNA (or protein) perturbations that lead to continuous cycles of cleavage.

What do we know about the protein determinants of Top2 poisons? A standard approach to this question has been the isolation of drug resistant forms of the target enzyme. While numerous drug resistant alleles of Top2 have been identified, no clear pattern that might lead to the identification of a drug binding site has emerged. Many mutations that reduce Top2 catalytic activity can also lead to drug resistance, complicating the interpretation of possible drug binding sites. A large scale screen of drug resistant mutants of yeast Top2 failed to identify any mutants with separable effects on etoposide versus mAMSA sensitivity³⁴. However, many of the mutants in Top1 that led to camptothecin resistance were not understood until a three dimensional structure of a drug:DNA:enzyme ternary complex was determined³⁵. The newly described structure of Top2 bound to DNA may be one step forward in helping to rationalize why specific mutants confer drug resistance³⁶. For example, in previous structures, the TOPRIM domain was quite far from the active site tyrosine. During cleavage and religation, these two elements must interact, and therefore must be close to each other. Since Top2 poisons act at the point of cleavage and religation, the relevant drug binding pocket may be formed by residues at that use both the TOPRIM and winged helix domains (Fig. 2). The hypothesis that the winged helix and the TOPRIM domains come together to form a drug binding pocket is supported by studies of fluoroquinolone action against prokaryotic Top2 enzymes. Fluoroquinolone resistant mutants occur in both the TOPRIM and wing helix domains, and rarely occur in other parts of the protein (reviewed in³⁷). While this localization of mutants is consistent with a drug binding pocket consisting of these two domains of the protein, direct localization of fluoroquinolones by structural approaches has not yet been accomplished. It is likely that structural studies with the bacterial type II topoisomerases will be informative for understanding eukaryotic Top2 poisons as well³⁸.

A complementary approach to isolating drug resistant mutants of Top2 that has recently generated some success has been to identify alleles of Top2 that confer hypersensitivity to Top2 targeting drugs. This approach has led to the identification of mutations that are specific for classes of Top2 poisons (e.g., hypersensitivity to etoposide and no change in sensitivity to intercalating agents), and may generate mutant proteins that are suitable for structural studies of drug binding^{39,40}.

Generation of DNA damage by Top2: a requirement for processing Top2 DNA covalent complexes

Top1 trapped by camptothecin generates a reversible single strand break in DNA. When a DNA polymerase collides with a trapped Top1:DNA covalent complex, a (non-protein linked) double strand break can be generated^{41,42}. Although Top2p generates double strand breaks during its reaction cycle, the two subunits associate very strongly (at least for the eukaryotic enzyme⁴³). It is likely that processing reactions, either proteolytic or nucleolytic are required to generate a double strand break. Because the trapped enzyme:DNA covalent complex is processed into a double strand break, any collision that provokes processing has the potential to generate double strand breaks. Therefore, ongoing DNA replication is not required to generate double strand breaks in cells treated with Top2 poisons.

Repair of Top2 mediated damage must accomplish several tasks. Successful repair must effectively recognize a Top2 complex as DNA damage rather than an active enzyme that can dissociate from DNA. Repair then requires removal of the protein that is covalently bound to DNA, and repair of the DNA strand breaks. The recognition of a trapped covalent complex as damage probably occurs because the Top2 covalent complex is a roadblock to replication and transcription. One unusual aspect of Top2 covalent complexes as DNA damage is that drug:protein:DNA complexes may remain reversible until some processing intervenes. Therefore, if recognition and repair is not initiated, the Top2 covalent complex can reverse without deleterious effect. However, once repair is initiated, the Top2 covalent complex is probably irreversible, and cells are committed to either repair the DNA damage, or suffer cell death.

Nucleolytic excision of protein adducts

One way is to remove protein DNA adducts is to excise the lesion by a nucleolytic digestion of DNA that is covalently bound to the protein⁴⁴ (Fig. 3). This type of repair has been observed for Top1 covalent complexes by the protein tyrosyl DNA phosphodiesterase I (Tdp1)^{45,46}. This enzyme processes 3'phosphotyrosyl peptides linked to DNA, and leaves a 3' phosphate product. Other nucleolytic enzymes have also been proposed that could remove 3' phosphotyrosyl peptides from DNA⁴⁷⁻⁴⁹, although direct biochemical evidence has not yet been reported. Tdp1 is highly conserved among eukaryotes, but has not been found in prokaryotes. Although the original characterization of yeast Tdp1 indicated a specificity for removal of 3'phosphotyrosyl peptides from DNA⁵⁰, yeast *tdp1* mutants were found to be hypersensitive to etoposide, and the purified yeast protein was also able to remove 5' phosphotyrosyl peptides from DNA⁵¹. Interestingly mammalian cells lacking Tdp1 activity are not hypersensitive to etoposide, and purified human Tdp1 has minimal activity against Top2:DNA covalent complexes (JLN unpublished data). These results suggest that there are clear differences between the yeast and mammalian Tdp1 proteins. Both the yeast and human proteins have been crystallized^{52,53}, and the active sites show few differences. The biochemical basis of the difference between the two proteins remains an unanswered question.

Until recently, there has been little direct information about nucleolytic removal of Top2 covalently bound to DNA. However, processing of 5' phosphotyrosyl linked proteins (the same type of trapped complex formed by Top2) is critical for normal meiotic recombination. The processing of early intermediates of meiotic recombination may model pathways that occur in repairing drug induced Top2 damage in somatic cells. Spo11 is a type IIB topoisomerase homolog that initiates meiotic recombination. Although the details of the Spo11 reaction are poorly understood, the Spo11 protein becomes covalently bound to DNA by a 5' phosphotyrosyl linkage^{54,55}. Spo11 protein is removed from the DNA leading to a double strand break that is required for recombination. Since Spo11 linked to DNA closely resembles DNA damage arising from trapping Top2, it is plausible that the pathways that remove Spo11

may also function in repairing Top2 mediated damage. In yeast, nuclease deficient alleles of the MRN complex genes *MRE11* and *RAD50* are unable to remove Spo11 from DNA⁵⁶. In addition, yeast *SAE2* encodes an endonuclease that is required for Spo11 removal⁵⁷⁻⁵⁹. Mammalian homologs of Sae2 (termed CtIP) have recently been identified, and they also play key roles in processing recombination intermediates^{60,61}. Thus CtIP and the Mre11 complex are good candidates for endonucleolytic processing of both Top2 and Spo11 DNA covalent complexes. Keeney and colleagues applied a novel assay in yeast using tagged versions of Spo11 to identify potential nucleolytic processing pathways⁶². Having immunoprecipitated the tagged Spo11 and characterized the DNA covalently bound to the protein, they found a specific small DNA associated with Spo11. They suggested that this was the product of endonucleolytic removal of Spo11. The nucleolytic product required both wild type Mre11 and Sae2 suggesting that it was a bona fide processing intermediate. Although a processing intermediate was also detected in yeast cells treated with etoposide (examining labeled Top2), the processing was not dependent on either Mre11 or Sae2. An important qualification in the experiments with etoposide is the lack of clear evidence that the detected product was a processing intermediate. Nonetheless, the results strongly indicate that an endonucleolytic pathway represents one significant pathway available for repairing Top2 mediated DNA damage. These results are also supported by studies in a bacterial model system using SbcCD, a bacterial nuclease that is related to the Mre11 complex proteins, which has been shown to remove protein covalently bound to DNA^{44,63}.

Recent experiments using fission yeast have provided direct support for the hypothesis that Mre11 and CtIP play direct roles in removing Top2 covalently bound to DNA. Hartsuiker and colleagues used physical assays similar to those described above with Spo11 removal. They showed that treating *Mre11* and *CtIP* (*Ctp1* in *S. pombe*) mutants of *S. pombe* with the epipodophyllotoxin derivative TOP-53 resulted in elevated levels of Top2 covalent complexes compared to isogenic wild type strains⁶⁴. This work is of particular significance since it represents the first direct evidence for nucleolytic processing of a topoisomerase covalent complex in eukaryotic cells.

While the Mre11/CtIP pathway is clearly involved in nucleolytic processing of Top2 covalent complexes, it is likely to be one of several different nucleolytic pathways. For example, genetic studies in *S. cerevisiae* and *S. pombe* suggest that the XPG nuclease homologs Rad2 (Rad13 in *S. pombe*) also play a role in repairing Top2 covalent complexes. Repair of Top1 covalent complexes involves a multiplicity of repair pathways⁶⁵, a similar complexity of removing Top2 covalent complexes is also likely.

Degradation of Top2

A second distinct pathway for processing Top2 damage depends on proteolytic degradation of covalently bound Top2 as a first step in processing⁶⁶. Liu and colleagues found that treatment of HeLa cells with teniposide led to a rapid depletion of Top2 protein, especially Top2 β isozyme. Degradation of Top2 β depended on ubiquitination, and could be inhibited by proteasome inhibitors. Interestingly, transcription inhibitors could also prevent degradation. These results suggested that a response to transcriptional blocks by Top2 poisons is targeted degradation of Top2 β . However an alternate plausible explanation for the results is that trapping of Top2 covalent complexes leads to a degradation of Top2 β independent of whether it is trapped on DNA. To exclude this possibility, subsequent experiments connected degradation of Top2 β to the generation a DNA damage signal. Quiescent cells downregulate Top2 α and typically only express Top2 β . Therefore, postmitotic neurons were treated with etoposide in the presence of other inhibitors. In quiescent cells, Top2 β is degraded in response to etoposide. Concomitantly, there is an induction of both γ H2AX phosphorylation and autophosphorylation of ataxia telangiectasia mutated (ATM) at Ser1981⁶⁷, both proteins are involved in the response

to DNA damage. Treatment with either a proteasome or a transcription inhibitor prevented Top2 β depletion and also prevented both γ H2AX phosphorylation and autophosphorylation of ATM. These experiments demonstrate that the Top2 β degradation is needed for a DNA damage-inducing signal in non-dividing cells. A simple explanation is the DNA double strand breaks are not generated at a high level (in non-growing cells) in the absence of proteolysis.

Recent results have also suggested that the protease pathway acts against trapped Top2 α complexes⁶⁸. As was observed with Top2 β , Top2 poisons induce the degradation of Top2 α , although the overall level of degradation is substantially lower. The degradation could be blocked by either proteasome or transcription inhibitors, but not by the polymerase inhibitor aphidicolin. DNA damage signaling was attenuated by all of the inhibitors, but was not completely abolished. Finally, p53 induction was not substantially affected by any of the inhibitors. These results imply that there are (at least) two distinct pathways involved in processing Top2 damage: a transcription dependent pathway, where processing is initiated by proteolysis, and a replication dependent pathway, with processing mainly proteasome independent.

What is the effect of these pathways on cell killing? Treatment of cells with either a proteasome or transcription inhibitor plus a Top2 poison has little effect on cell survival⁶⁸. By contrast aphidicolin partly reduces cell killing⁶⁸. The effect of aphidicolin on etoposide cytotoxicity is not as pronounced as the complete blocking of the camptothecin cytotoxicity by aphidicolin^{42,69}. Nonetheless, the role of replication in enhancing the cytotoxicity of etoposide suggests that this agent may also exhibit some degree of schedule dependence⁶. Finally, proteolysis will not completely degrade all of the bound protein; the tyrosine (and perhaps a few additional amino acids) will remain covalently attached to DNA and require nucleolytic repair as described above.

The two pathways described above both are concerned with removal of the Top2 protein. For both pathways, the product of the reaction is frequently a double strand break. It is likely that the repair of double strand breaks follows the patterns of double strand breaks induced by other agents such as ionizing radiation or endonuclease cleavage. Cells lacking non-homologous end-joining are clearly hypersensitive to Top2 mediated damage (see Table 1). The importance of homologous recombination in repairing Top2 damage in mammalian cells is less clear, however some mutants that show defects in homologous recombination are hypersensitive to etoposide^{70,71}, and treatment with etoposide stimulates both NHEJ and homologous recombination⁷². Since NHEJ clearly repairs (some) Top2 damage, this pathway is likely to play some role in the oncogenic rearrangements induced by Top2 targeting agents⁷³.

Catalytic inhibitors of topoisomerase II

Several classes of compounds have been described that target topoisomerase II without stabilizing Top2 covalent complexes. The most important class of compounds, the bisdioxopiperazines include ICRF-159, ICRF-187, and MST-16. These drugs have two activities: they are potent chelating agents and they block Top2 in the catalytic cycle after strand passage but before the hydrolysis of the second ATP^{74,75}. At this point in the reaction cycle, Top2 encircles the strand that the enzyme had cleaved; this is the point in the reaction cycle where the enzyme is blocked by non-hydrolyzable ATP analogs⁷⁶. Bisdioxopiperazines have modest anti-tumor activity, and their clinical usage is primarily for reducing the cardiotoxicity of anthracyclines. However, they are important tools for studying the effects of Top2 inhibition because they are the most specific Top2 inhibitors that are not Top2 poisons.

The question of whether bisdioxopiperazines are pure catalytic inhibitors has been raised recently. First, bisdioxopiperazines trap Top2 on DNA, even though the trapping is as a non-covalent clamp around DNA. Results in yeast suggest that the trapped clamp can lead to

cytotoxicity independent of loss of catalytic activity⁷⁷. The clamp affects chromatin structure¹⁶, and alterations in chromatin structure may play some role in cell killing, again independently of loss of Top2 activity. In addition to the potential deleterious effects of Top2 clamps on DNA, it has also been suggested that bisdioxopiperazines can trap Top2 covalent complexes⁷⁸. This is a surprising result since bisdioxopiperazines do not provoke the DNA damage responses generated by drugs such as etoposide. Liu and colleagues have shown that bisdioxopiperazine “trapping” of Top2 as a closed clamp on DNA leads to degradation. The degradation is specific for Top2 β , and is blocked by transcription inhibitors⁷⁹. However, even if the closed clamp form of Top2 β can block transcription, treatment of cells with bisdioxopiperazine does not lead to degradation of the large subunit of RNA polymerase II, as occurs with other types of DNA damage. Since Snapka and colleagues found that denaturation conditions needed to trap Top2 covalent complexes differed from those used with conventional poisons, the observed damage may represent damage induced in part by the denaturation conditions.

It would be extraordinarily useful to have available other catalytic inhibitors of Top2 that act by different mechanisms. A particularly useful class of inhibitors would be potent specific ATP competitive inhibitors of Top2 (i.e., much improved versions of novobiocin). Inhibitors of this class would not interfere with DNA metabolism, except by causing a loss of Top2 activity. Very recently, a rationally designed ATP competitor inhibitor termed QAP1 was synthesized, and shown to inhibit both Top2 α and Top2 β {Chene, 2009 #598}. Although cellular activity of this compound has not yet been described, QAP1 can reduce DNA damage responses induced by doxorubicin, consistent with the agent acting as a Top2 catalytic inhibitor. Agents such as QAP1 are likely to be useful both for exploring cellular consequences of the loss of Top2 activity and answering whether catalytic inhibition of Top2 is an effective and safe anti-cancer strategy.

Targeting topoisomerase II in anti-cancer therapy

The ability of Top2 to generate DNA damage in the presence of Top2 targeting agents led to the hypothesis that an important determinant of drug sensitivity was the overall level of Top2. Early experiments with cell lines selected for drug resistance *in vitro* were consistent with this hypothesis, namely, cells overexpressing Top2 were drug hypersensitive, and cells with reduced levels of detectable Top2 were resistant to Top2 poisons^{5,80}. Similar results were obtained using expression of short sense or anti-sense RNAs derived from Top2 α , followed by selection for resistance to etoposide. Several anti-sense constructs led to reductions of Top2 α protein levels and resistance to multiple classes of Top2 poisons⁸¹. These experiments demonstrated that targeted changes in Top2 levels were sufficient to confer resistance to multiple classes of Top2 poisons. A more recent set of experiments using shRNA technology extended these observations to an *in vivo* setting⁸². In these experiments a library of shRNAs were screened for genes conferring resistance to doxorubicin. Three genes were identified that could lead to doxorubicin resistance: p53, Chk2, and Top2 α . In addition to demonstrating that a principal target of doxorubicin *in vivo* is Top2, a point of longstanding contention, the model system of using a library of shRNAs has the potential for identifying other genes conferring resistance to Top2 targeting drugs.

If enhanced levels of Top2 targeting drugs lead to hypersensitivity to Top2 targeting drugs, then tumors expressing higher levels of Top2 might be expected to be particularly good candidates for therapy using Top2 poisons. Most disease classes have given equivocal results when clinical outcome is compared to Top2 levels⁶. However, it had been observed that patients with amplified ERBB2 (Her2/neu) often showed very good responses to drugs targeting Top2, especially anthracyclines. Since Top2 is located close to *ERBB2* on chromosome 17, the levels of Top2 α gene copy number were assessed in samples with

ERBB2 amplification. In several cases, co-amplification of Top2 α was observed, with a good correlation of enhanced sensitivity to various Top2 poisons⁸³⁻⁸⁵.

Amplification of Top2 α has several interesting properties. In general, amplification is specific for tumors that have amplified *ERBB2*, but also occurs in some breast tumors lacking this amplification⁸⁶. Top2 α and *ERBB2* are independent amplicons in the sense that the copy number of Top2 α and *ERBB2* frequently differ⁸⁷. One way this may occur is if *ERBB2* is amplified first with a secondary round of amplification that includes Top2 α . Interestingly, there are also deletions of Top2 α as well as amplification. While one might predict that the deletions of Top2 might reduce anthracycline sensitivity, in some cases the deletion is also associated with positive responses⁸⁶. Whether Top2 α amplification leads to higher Top2 protein levels is controversial. An important caveat of studies examining Top2 levels is that most of the studies used immunohistochemistry (IHC), the sensitivity and quantification of which is certainly less than the FISH used to study gene amplification.

Although the role of Top2 α amplification in conferring sensitivity to anthracyclines continues to be examined vigorously, it has been suggested that anthracyclines fail to provide a long term benefit in tumors where Top2 α is amplified⁸⁸. That chemosensitivity can be conferred by an amplified gene is unusual. Typically, one thinks about amplification conferring resistance, and providing a selective advantage to treated cells. In this instance, amplification confers sensitivity, and it is likely that treatment with anthracyclines selects for cells that have lost amplification. Since anthracyclines induce DNA damage, it seems possible that replication blocks may lead to alterations in Top2 α copy number. Given the importance of Top2 α for cells to proceed through mitosis, it would be interesting to determine whether there is any relationship between Top2 α levels and sensitivity to anti-mitotic agents such as taxanes.

Obstacles to the increased use of Top2 therapeutics

While Top2 targeting drugs are active in many contexts, there are important negative consequences of using these agents that are especially critical for this class of agents. Most important are the observations that treatment with Top2 targeting drugs can result in a wide spectrum of secondary malignancies. The first recognition that Top2 poisons could lead to secondary malignancies came from acute myeloid leukemia (AML) as a complication of chemotherapy regimens that included etoposide and teniposide⁸⁹⁻⁹². A large body of evidence supports the hypothesis that Top2 poisons have a high potential for generating translocations that can lead to secondary malignancies (reviewed in^{93,94}; for a different view see⁹⁵).

A novel insight into secondary malignancies induced by Top2 targeting drugs has come from studies using a transgenic mouse model that carried skin specific ablation of Top2 β . Use of organ specific ablation was needed because Top2 β null mice are inviable. Etoposide applied to skin can generate malignancies, mainly melanomas. In skin lacking Top2 β , etoposide induced melanomas are reduced in frequency⁹⁶. Loss of Top2 β also led to reduced NHEJ induced by etoposide. Taken together, these results suggest that Top2 β is responsible for an important fraction of malignancies induced by etoposide. A plausible model is that Top2 β is trapped on DNA perhaps in non-replicating cells. Proteasomal degradation uncovers a double strand break, which will be repaired by NHEJ, in some cases leading to oncogenic translocations.

Whether bisdioxopiperazines influence the induction of secondary leukemias has been the subject of intense debate. In a trial for pediatric Hodgkin's disease, it was reported that adding bisdioxopiperazines to a regimen that included etoposide and doxorubicin led to a slight increase in secondary malignancies⁹⁷, although most of the reported effects were below statistical significance⁹⁷⁻⁹⁹. A more recent pediatric trial in ALL found no enhanced risk of

secondary malignancies when bisdioxopiperazines were added to regimens containing Top2 poisons¹⁰⁰.

Top2 β may also be the villain in anthracycline-induced cardiomyopathy. Bisdioxopiperazines (here ICF-187) have been widely used as a cardioprotectant against anthracycline-induced cardiomyopathy. The mechanism of cardioprotection had been widely thought to be due to anthracycline-induced generation of reactive oxygen species¹⁰¹. In cardiac myocyte culture, anthracyclines induce a DNA damage signal that can be blocked by bisdioxopiperazines. The DNA damage signal can be blocked by the proteasome inhibitors bortezomib and MG132. Importantly, the DNA damage signal induced by anthracyclines can also be attenuated in MEFs derived from Top2 β -null mice compared with MEFs that express Top2 β ¹⁰². As described above, bisdioxopiperazines lead to degradation of Top2 β . The effect seen in Top2 β -null MEFs may arise from specific degradation of the enzyme, preventing toxic damage.

Can the sensitivity to Top2 targeting drugs be exploited?

Top2 poisons generate DNA damage that interferes with critical cellular processes, and leads to types of damage that require the interplay of several repair pathways. It is likely that alterations in repair capabilities represent a major determinant of *in vivo* response to Top2 targeting drugs. An important goal is to be able to specifically disrupt pathways in cancer cells, leading to enhanced clinical response. This concept is central to designing new ways of using Top2 targeting agents and in rationally designing combinations with agents targeting other processes. Although there are experimental drugs targeting repair pathways (e.g., an inhibitor of DNA-PKcs, which has been shown to confer hypersensitivity to Top2 poisons¹⁰³), further exploitation of this concept will require new small molecules targeting repair pathways as well as an enhanced ability to assess repair capability (see Box 2). One pathway that may be closer to clinical exploitation is the proteasome dependent repair pathway. The proteasome inhibitor bortezomib has been tested in combination with anthracyclines with some potential^{104,105}.

The future of Top2 as a drug target

Is there a need for new and different Top2 drugs? The first answer to this question is a resounding yes, since Top2 targeting is clearly successful in a wide variety of contexts. It is clear from broad clinical experience that Top2 targeting drugs can be safely and effectively combined with many other agents. The Top2 targeting drugs in clinical use were identified not based on their activity against Top2, but mainly on empirical anti-tumor activity. Therefore, it would be expected that rational screening would lead to potent and specific Top2 poisons. It would be very desirable to know if greater potency and specificity would enhance clinical response.

At the time etoposide and doxorubicin were approved for use, we did not know of the existence of Top2 β . The results reviewed in this article suggest that the targeting of Top2 β leads to several undesirable consequences and little clear benefit. The negative effects of targeting Top2 β include the induction of cardiotoxicity, and potentially a major role in secondary malignancies. On the other hand, there are potential benefits of targeting Top2 β , especially the ability to kill non-proliferating cells. While targeting Top2 β may contribute to toxicity, it may also be important for eliminating cancer cells that function as cancer stem cells.

An important question is whether isotype specific Top2 poisons can be identified, since the two enzymes share catalytic mechanisms, and a great deal of amino acid homology in their catalytic domains. It has been previously suggested that the intercalators mAMSA and mitoxantrone confer cytotoxicity mainly due to targeting Top2 β ¹⁰⁶. More recently, a novel intercalator NK314 has been reported to be highly specific for Top2 α ^{107,108}. Toyoda and colleagues also suggested that etoposide and doxorubicin generate greater cytotoxicity by

targeting Top2 α . Taken together, these results suggest that agents specific for Top2 α may be possible, and may be useful for having both greater anti-tumor activity, and reduced toxicity.

The search for improved Top2 targeting drugs will require further advances in both the biochemistry and structural biology of drug action. While the structures that have already been determined have provided important insights into the biochemistry of Top2, the only structure of Top2 bound to a drug that has been determined is the ATPase domain of Top2 bound to ICRF-187¹⁰⁹. The goal for understanding the biochemistry of a drug like etoposide is the determination of a ternary complex between drug, protein, and DNA. Hopefully, the structures of the breakage/reunion domains of Top2 α and Top2 β , especially their DNA bound forms, will be solved soon.

An interesting question related to drug development is whether catalytic inhibitors of Top2 might be active anti-cancer agents. Much of the literature on the action of Top2 poisons implicitly assumes that they inhibit Top2 activity. Compared to many other enzyme inhibitors, any of the currently described Top2 targeting agents has relatively poor potency (for example, the K_i of etoposide for Top2 is in the 5-20 μM range, the K_i for ICRF-193 is in the 1-2 μM range). The availability of crystal structures provides the tools for addressing whether Top2 inhibition will be a valuable strategy (and will provide tools needed to answer many important biological questions).

The recent biological insights in transcription, replication and checkpoint control also offer ways to better understand drug action and resistance. Since cancer cells can clearly present with altered topoisomerase levels, whether by amplification or changes in gene regulation, these alterations provide an opportunity for enhanced therapeutic index. Finally, active anti-cancer therapy requires an understanding of how cancer cells 'make a living', and topoisomerases clearly are central to many of these core biological functions.

At a glance

- Top2 is the target of several important classes of anti-cancer drugs, including the epipodophyllotoxin etoposide, and the anthracycline doxorubicin.
- Most clinically active drugs that target Top2 kill cells by trapping an enzyme intermediate termed the covalent complex. Therefore, the principal action of Top2 targeting drugs currently used are to generate enzyme mediated DNA damage.
- A recent structure of the breakage reunion domain of Top2 bound to DNA has been determined. This structure is likely to be of great use in understanding the protein determinants of the action of drugs targeting Top2. A drug:protein:DNA ternary complex would be extremely valuable, but has not yet been determined.
- Top2 mediated DNA damage is repaired by multiple pathways. The DNA damage includes DNA strand breaks and proteins covalently bound to DNA. Repair of Top2 damage requires double strand break repair pathways, and other pathways specific for the removal of protein:DNA adducts.
- Sensitivity to Top2 targeting drugs depends in part on levels of Top2 protein. Cells overexpressing Top2 are hypersensitive to Top2 poisons while cells expressing low levels of Top2 are relatively drug resistant. Top2 α is frequently co-amplified with *ERBB2*. This can lead to some tumors with elevated levels of Top2 α .
- An important side effect of targeting Top2 with Top2 poisons are secondary malignancies arising from drug induced translocations. Top2 β may be the Top2 isoform that is most responsible for secondary malignancies caused by Top2 targeting drugs.

- Anthracycline use is limited by cardiotoxicity. Although the mechanism of the cardiotoxicity is poorly understood, recent results suggest that anthracyclines acting against Top2 β may contribute significantly to cardiotoxicity. There may be considerable benefit to developing Top2 targeting drugs specific for the Top2 α isoform.
- Catalytic inhibition of Top2 may also be a useful anti-cancer strategy. New compounds are being developed to test this possibility.

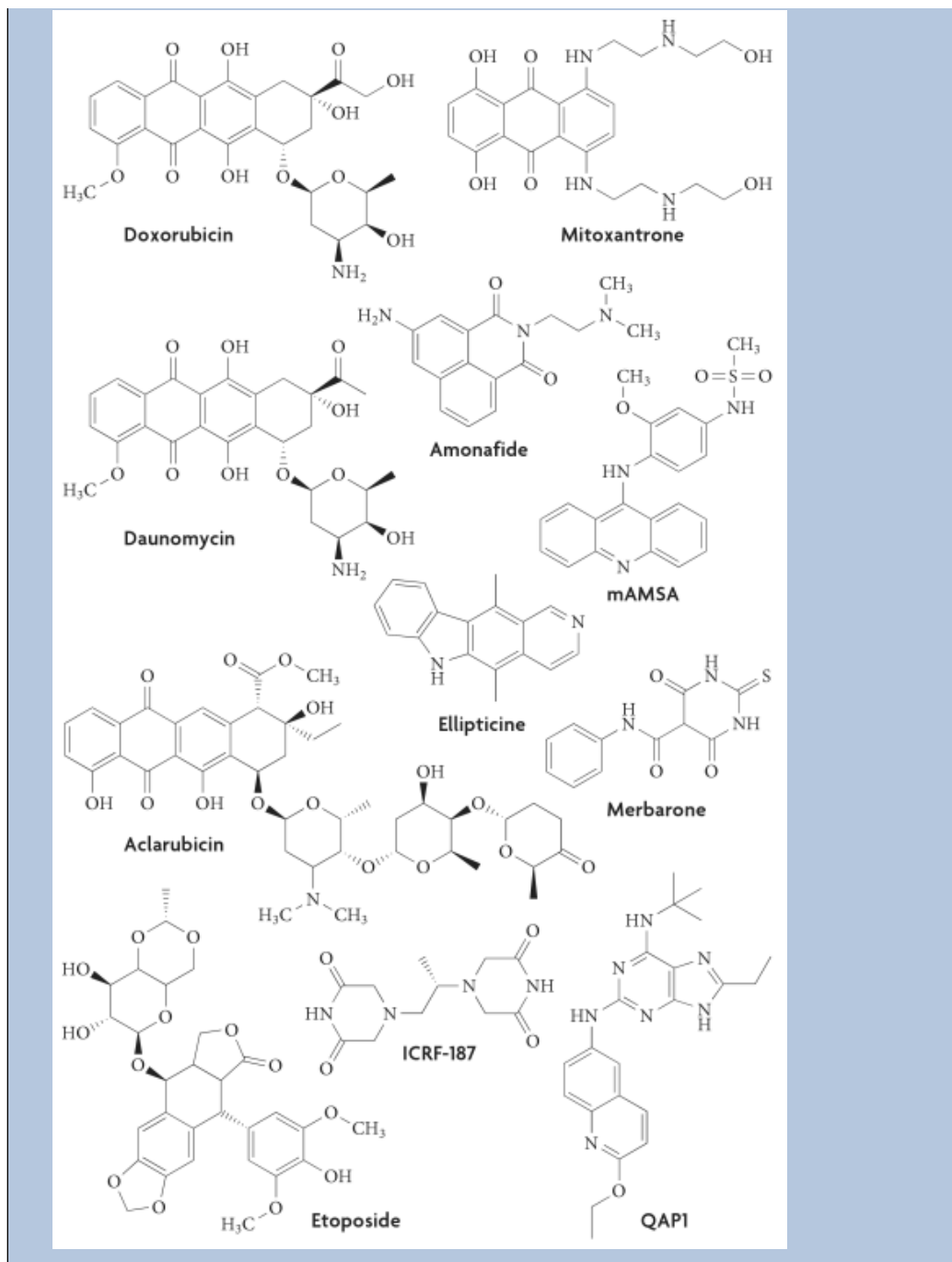
Box 1. Many different classes of compounds target Topoisomerase II

Drugs targeting topoisomerase II fall into two categories, Top2 poisons and Top2 catalytic inhibitors. Many Top2 poisons have demonstrated anti-cancer activity. Top2 poisons can be further sub-divided into intercalating and non-intercalating poisons. The intercalators are chemically diverse, and include doxorubicin and other anthracyclines, mitoxantrone, mAMSA, and a variety of other compounds that are not currently in clinical use such as aonafide and ellipticine⁵. Other than their ability to intercalate in DNA, there is no obvious chemical similarity that could explain the ability of these compounds to trap Top2.

Importantly, some compounds, such as oAMSA and ethidium bromide have little ability to poison Top2, suggesting that intercalation of a small molecule is insufficient to trap Top2 as a covalent complex on DNA^{1,110}. Some of the intercalating Top2 targeting drugs, notably the anthracyclines, produce a variety of effects on cells, including many effects that are independent of their action against Top2. For example, doxorubicin is known to produce free radicals, to cause membrane damage, and to induce protein:DNA crosslinks. Whether Top2 is the most important target of anthracyclines remains a controversial issue, (reviewed in¹¹¹), although some of the results presented in the text support the hypothesis that Top2 is the most relevant target for both clinical response and cardiotoxicity. For alternate hypotheses, see¹¹²⁻¹¹⁴.

Non-intercalating Top2 poisons include the epipodophyllotoxins etoposide and teniposide, and fluoroquinolones, which are mainly active against prokaryotic type II topoisomerases. Since non-intercalating Top2 poisons do not interact strongly with DNA, it has been suggested that protein drug interactions play key roles in their ability to trap Top2 covalent complexes^{115,116}.

Several classes of compounds have been described that inhibit Top2 activity but do not increase DNA cleavage. Most prominent are the bisdioxopiperazines, which inhibit the enzyme ATPase activity non-competitively and trap Top2 as a closed clamp^{74,117,118}. ICRF-187, a bisdioxopiperazine, is used as a cardioprotectant in some patients treated with anthracyclines. Other Top2 catalytic inhibitors include novobiocin¹¹⁹⁻¹²¹, merbarone¹²², and the anthracycline aclarubicin¹²³. All three compounds have significant targets besides Top2^{121,124,125}; therefore these compounds have not been useful in assessing the feasibility of using catalytic inhibitors of Top2 as an anti-cancer therapy. Merbarone has attracted interest because it is the only agent that has been found to inhibit Top2 cleavage of DNA but not affect protein:DNA binding¹²⁶. QAP1 is a newly described purine analog that was rationally designed to target the Top2 ATPase activity¹²⁷. This compound may be particularly useful in assessing the effects of catalytic inhibition of Top2. Several other catalytic inhibitors have been described, however, their detailed mechanism of action has not been explored.



Box 2. Combining Top2 targeting drugs with other agents

Most drugs targeting Top2 have been in clinical use for many years. Etoposide, doxorubicin, and mitoxantrone have been used with many different agents including DNA damaging agents (including platinum compounds), anti-mitotics (including Vinca alkaloids and taxanes), and more recently developed targeted therapies (such as monoclonal antibodies targeting EGFR and Her2/neu). In most cases, combinations have been derived based on the clinical activity of single agents, the desire to reduce the toxicity of active combinations, or other primarily clinical considerations. Therefore, much of the vast literature on

combinations of Top2 targeting drugs with other classes of chemotherapeutic agents is usually divided by tumor type (for a small sampling of current examples, see ¹²⁸⁻¹³⁰).

An approach that has been frequently used in clinical trials is to combine several different Top2 targeting drugs, especially etoposide and an anthracycline. As noted in Box 1, there has been controversy whether anthracyclines mainly act against Top2 or some other targets. The action of anthracyclines against other targets may provide an important rationale for combining these compounds with other Top2 inhibitors. However, the different toxicity profiles (e.g., the relative absence of cardiotoxicity with epipodophyllotoxins) provide an additional important justification for combining different Top2 targeting agents.

There are cases where combinations may be well justified on mechanistic grounds. One combination of particular interest has been the combination of a Top1 and a Top2 inhibitor. The rationale for this combination was originally based on the hypothesis that these drugs (in part) inhibit the catalytic activity of topoisomerases, and that since Top1 and Top2 have overlapping functions in DNA metabolism, targeting both enzymes might increase anti-tumor activity. It was also felt that the combination of a Top1 and a Top2 poison would help to prevent mechanism based drug resistance, since resistance to camptothecins can be due to down-regulation of Top1 thereby leading to hypersensitivity to etoposide (due to a possible increased expression of Top2 to compensate for Top1 down-regulation). Early trials of combinations of etoposide and topotecan or irinotecan generated concerns with toxicity and complicated schedule dependence ¹³¹⁻¹³⁴ More recent results have been more promising ¹³⁵⁻¹³⁷.

Agents that interfere with DNA repair pathways are likely to be candidates for combination therapy with Top2 targeting drugs. Bortezomib is a small molecule that inhibits proteasome degradation, and would be expected to block the proteasome dependent pathway of repairing Top2 covalent complexes. Bortezomib has been tested in combination with etoposide and doxorubicin^{104,138-140}. Inhibitors of DNA dependent protein kinase catalytic subunit have also been described^{141,142}, and shown to sensitize cells to etoposide¹⁰³.

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Glossary

MRN complex

A protein complex consisting of Mre11, Rad50, and Nbs1. This complex is required for checkpoint signaling and for double strand break repair. In yeast, the

Nbs1 component is replaced by a protein termed Xrs2, and the yeast complex is termed the MRX complex. The yeast complex is required for removing Spo11 from DNA during meiotic recombination.

TOPRIM domain

A conserved domain found in topoisomerases, primases, and other DNA metabolic enzymes. The Toprim domain adopts a Rossman fold, and is involved in divalent cation binding.

Top2 α and Top2 β isozymes

In lower eukaryotes, such as yeast, insects, vertebrates such as *Xenopus*, there is a single Top2 isoform. Mammals have two Top2 isoforms termed " α " and " β ". The α isoform is preferentially expressed in proliferating cells, and is essential for all growing cells. The β isoform is expressed in quiescent cells, and is not essential for all cells, although it is required for viability in mice.

Top2 catalytic inhibitor

A Top2 targeting agent that does not generate elevated levels of covalent complexes, and presumably is cytotoxic by depriving cells of an essential enzyme activity, rather than by generating enzyme mediated DNA damage (see Top2 poison).

Top2 poison

Drugs targeting Top2 that lead to elevated levels of Top2:DNA covalent complexes.

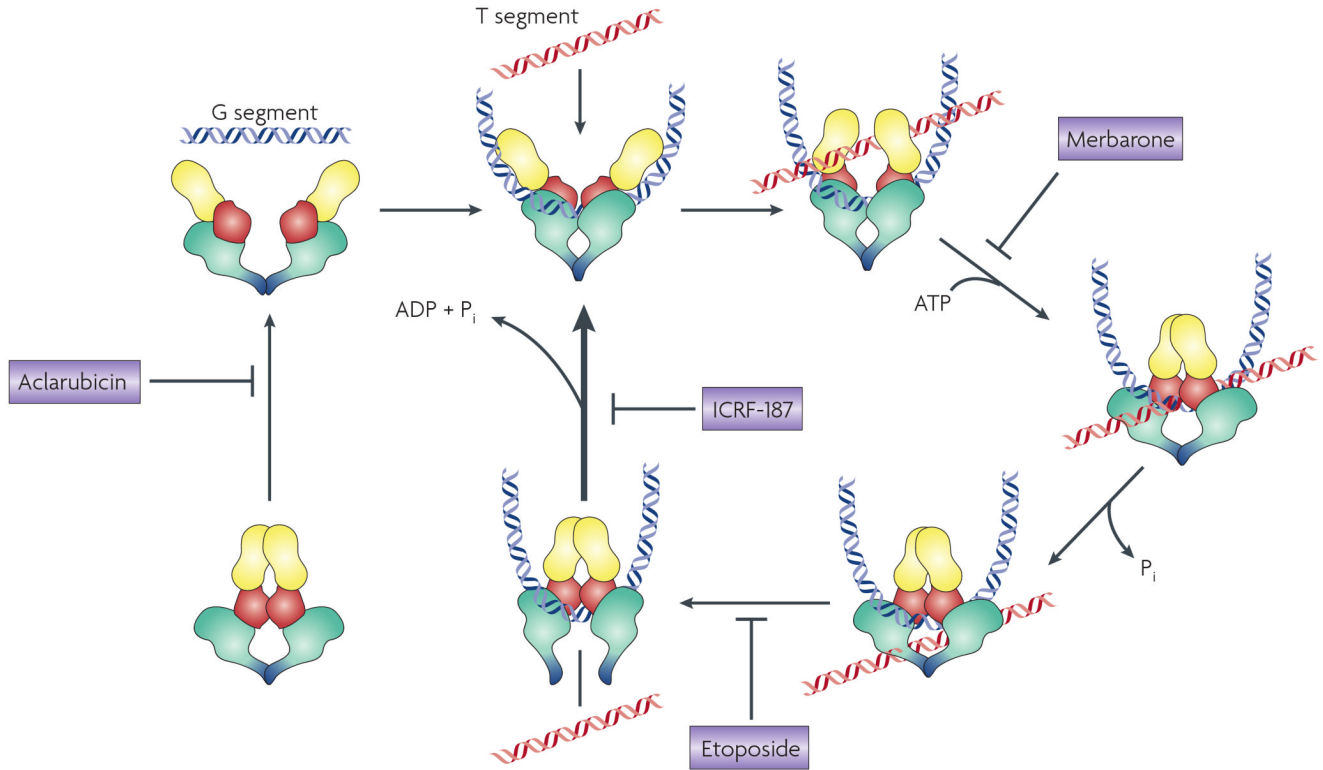


Figure 1. Mechanisms of inhibiting of Top2

Top2 can be inhibited at several different points in the enzyme reaction cycle, which can generate different biochemical and cellular consequences. One simple mode of inhibition is to inhibit a step early in the enzyme reaction cycle. For example, competitive inhibitors of ATP binding prevent strand passage, and do not generate enzyme mediated DNA damage. While agents such as novobiocin and coumermycin (not shown on the figure) inhibit both prokaryotic and eukaryotic Top2s, they are either less potent as well as relatively nonspecific (e.g., novobiocin) or are poorly taken up by mammalian cells (e.g., coumermycin). Similar effects would occur with inhibitors that prevent the binding of Top2 to DNA such as aclarubicin. Agents that prevent DNA cleavage by Top2, such as merbarone would also be expected to act as simple catalytic inhibitors. While merbarone clearly prevents DNA cleavage by Top2¹²⁶, merbarone clearly affects other targets besides Top2. A second mode of inhibition is blocking the catalytic cycle after DNA is cleaved but prior to DNA religation. This mode of inhibition occurs for most currently used Top2 targeting agents including anthracyclines and epipodophyllotoxins, as well as for agents that target prokaryotic type II topoisomerases. These agents prevent enzyme turnover, and therefore greatly inhibit the enzyme catalytic activity, however, the clearest effect is the generation of high levels of Top2:DNA covalent complexes. Therefore, these inhibitors generate DNA damage, and interfere with many DNA metabolic events such as transcription and replication. Since agents of this class convert Top2 into an agent that induces cellular damage, they have been termed topoisomerase poisons. Top2 can be inhibited after strand passage is completed, but prior to ATP hydrolysis and dissociation of N-terminal dimerization. Bisdioxopiperazines such as dexrazoxane (ICRF-187) inhibit both ATP hydrolysis and maintain Top2 as a closed clamp⁷⁴. As is the case with Top2 poisons, bisdioxopiperazines inhibit Top2 catalytic activity mainly by blocking enzyme turnover. Although these agents are frequently termed catalytic inhibitors, they leave Top2 trapped on DNA, and may interfere with DNA metabolism in ways distinct from the inhibitors described

in pathway (A). Nonetheless, since bisdioxopiperazines are relatively specific for Top2, they are the most commonly used catalytic inhibitors of Top2 in mammalian cells ¹⁴³.

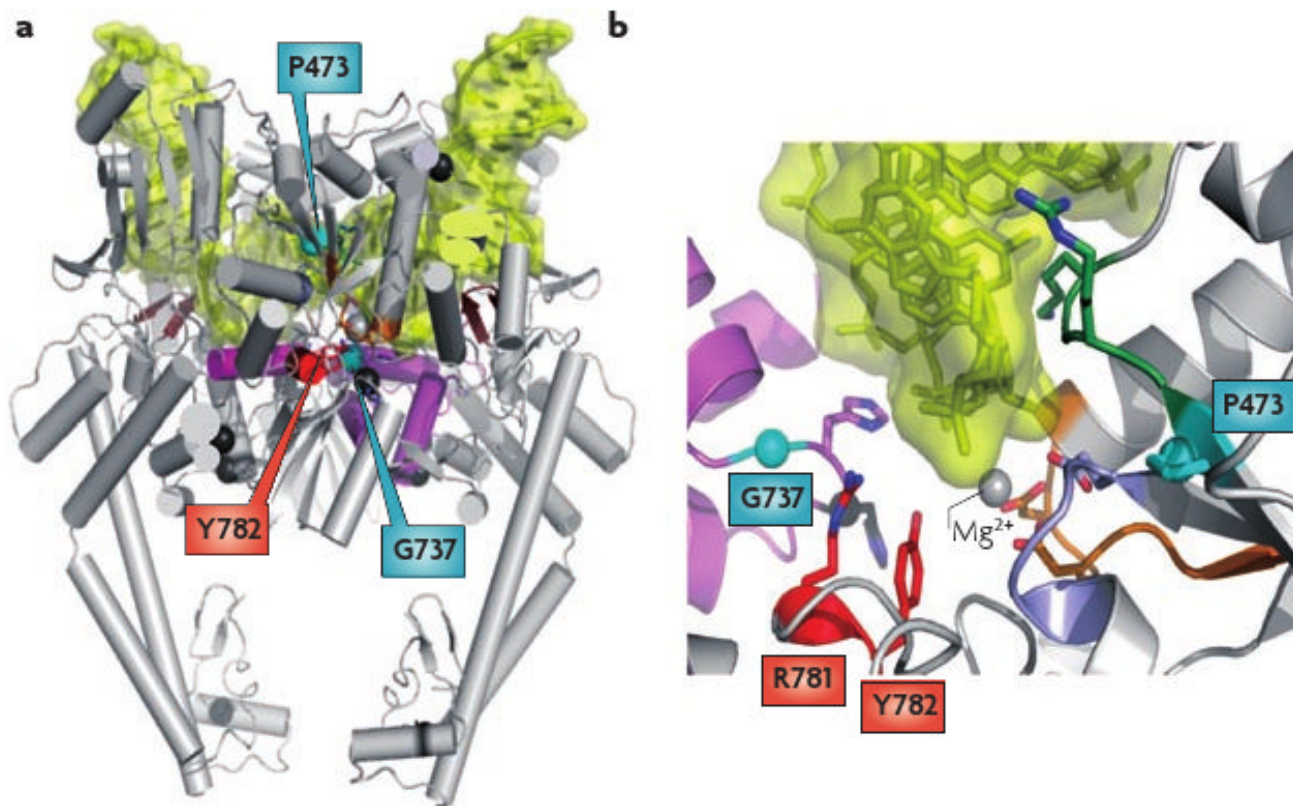


Figure 2. Structure of Top2 bound to DNA

Dong and Berger have described a structure of the breakage reunion domain of yeast Top2 bound to DNA³⁶. As described in the text, a key feature of the structure is the large bend induced in the DNA. Another key feature is the proximity of the TOPRIM domain and the active site tyrosine. Panel A shows the overall structure of yeast Top2 bound to DNA; the DNA is shown in yellow, while the winged helix domain helices are shown in purple. Previous studies have shown that drug resistant mutants occur near both the TOPRIM domain and the active site tyrosine. Residues labeled in blue are amino acids that are altered in drug hypersensitive top2 mutants. Pro473, is distant from the tyrosine in the primary sequence, but is close to Tyr782 in this structure. Pro473Leu is hypersensitive to the intercalator mAMSA. Gly737 and Ser740 are both in the winged helix domain; Ser740Trp is hypersensitive to etoposide, while G737 is hypersensitive to mAMSA. **Panel B** shows just the region around the active site Tyr, note the presence of a Mg ion complexed within the TOPRIM domain. The figure is adapted from Rogojina and Nitiss³².

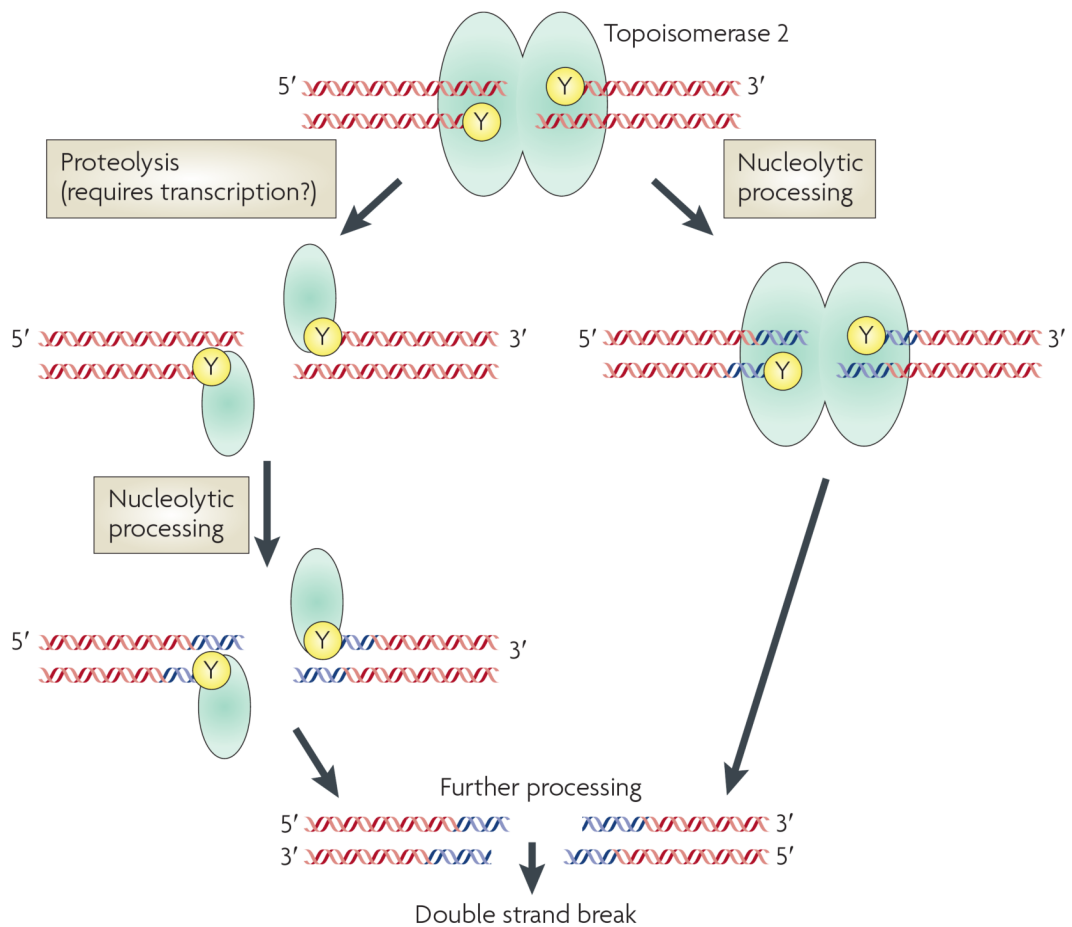


Figure 3. Pathways for the repair of Top2 mediated DNA damage

Following recognition of Top2 covalent complexes (perhaps by collision with replication forks), collision with other tracking proteins, such as RNA polymerase, or other undiscovered surveillance processes, repair can be initiated by proteolysis or by nucleolytic processing. Proteolysis will not completely remove the protein, since the phosphotyrosyl linkage to DNA cannot be removed by proteases. Therefore, after proteolysis, a nucleolytic processing step is still required. As illustrated, the product of nucleolytic processing is a DNA molecule containing a double-strand break. Note that Top2 can be trapped as a single strand break, since the two subunits break DNA in an independent, but coordinated process¹⁴⁴. For simplicity, the trapped structure that is illustrated shows a double strand break. Processing of a covalent complex with a single strand break might generate either a single strand break, or a double strand break. Recent experiments have demonstrated that a Top2 enzyme that can only generate single strand breaks can confer cytotoxicity in the presence of Top2 poisons³². In the case of a double strand break, repair is carried out mainly by homologous recombination or non-homologous end-joining. Repair can also take place by error-prone single strand annealing pathways. The error prone repair of Top2 generated DNA double strand breaks can generate translocations that lead to secondary malignancies. Repair of single strand breaks arising from Top2 covalent complexes have not been carefully explored.

Table 1
Genes participating in the repair of topoisomerase II mediated DNA damage^{a, b, c}

Gene	Function	Experimental system	Reference
Artemis	Nuclease participating in some NHEJ reactions	Mutant cell line	145-147
ATM	Checkpoint signaling (Protein kinase)	Numerous	9,125,148
ATR	Checkpoint signaling (Protein kinase)	Biochemical assay, chemical inhibition	125,149,150
BRCA1	Mutated in familial breast cancer, DNA damage sensing, transcription, possible modulator of Top2 activity	Mutant cell line	71
BRCA2	Mutated in familial breast cancer, homologous recombination, DNA repair DNA damage sensing	Mutant cell line	71
CHK1	Checkpoint signaling (Protein kinase)	Biochemical assay, siRNA	150,151
CHK2	Checkpoint signaling (Protein kinase)	Biochemical assay	151,152
CtIP	BRCA1 binding protein that functions in the MEN pathway, nuclease, mammalian homolog of Sae2	Biochemical assay	64,153
DNA-PKcs	DNA dependent protein kinase catalytic subunit, required for NHEJ	Mutant cell line, siRNA ^d , chemical inhibition	103,146,154,155
H2AX	Histone H2A isoform, phosphorylation marks double strand breaks	Mutant cell line	156
KU70	Subunit of a DNA binding complex (with DNA-PKcs required for NHEJ)	Numerous	154,157-159
KU80	Subunit of a DNA binding complex (with DNA-PKcs required for NHEJ)	Numerous	154
LIG4	DNA ligase IV, a ligase specific for NHEJ	siRNA	155
MRE11	Component of the MRN complex, required for DNA damage signaling and double strand break repair	Numerous	160-162
NBS1	Component of the MRN complex, required for DNA damage signaling and double strand break repair	Biochemical assay	163,164
PLK1	Polo-like kinase	Biochemical assay	165
RAD50	Component of the MRN complex, required for DNA damage signaling and double strand break repair	Biochemical assay	166
RAD52	Homologous recombination, repair of double strand breaks by homologous recombination	Yeast	160,167,168
Rb1	Retinoblastoma susceptibility protein, control of cell cycle progression	Mutant cell line	169
TDP1	Tyrosyl DNA phosphodiesterase I	Yeast ^e	51,170
XPC	Nucleotide excision repair	siRNA	155,171
XPG	Nucleotide excision repair	Yeast ^f	51

^aTopoisomerase II targeting drugs have been frequently used in studying apoptosis. Therefore genes that affect sensitivity to Top2 targeting drugs mainly by affecting apoptosis are not included in this table.

^bSeveral genomic screens have been carried out using yeast to identify genes that confer sensitivity to Top2 targeting drugs^{158,172,173}. These genes have not been discussed here unless similar genes or processes have been identified in mammalian cells.

^cThis table was assembled mainly using data using etoposide as a Top2 targeting drug. This was done to avoid the possible complication of effects of drugs (especially anthracyclines) on targets other than topoisomerase II.

^dDiscordant results have been reported for the sensitivity of DNA-PKcs deficient cells to etoposide. More recently, a DNA-Pk inhibitor has been reported to confer sensitivity to etoposide¹⁰³.

^eTdp1 mutants have been shown to confer sensitivity to etoposide in yeast cells, but not in mammalian cells.

^fSensitivity to etoposide has been demonstrated in yeast *rad2* mutants. *RAD2* is the yeast homolog of XPG. Sensitivity of XPG mutants in mammalian cells has not been reported.