Annu. Rev. Pharmacol. Toxicol. 2001. 41:53–77

TUMOR CELL DEATH INDUCED BY TOPOISOMERASE-TARGETING DRUGS

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Key Words topoisomerase, camptothecin, apoptosis, ubiquitin, SUMO

■ **Abstract** DNA topoisomerases are double-edged swords. They are essential for many vital functions of DNA during normal cell growth. However, they are also highly vulnerable under various physiological and nonphysiological stresses because of their delicate act on breaking and rejoining DNA. These stresses (e.g. exposure to topoisomerase poisons, acidic pH, and oxidative stresses) can convert DNA topoisomerases into DNA-breaking nucleases, resulting in cell death and/or genomic instability. The importance of topoisomerase-mediated DNA cleavage in tumor cell death and carcinogenesis has been recognized. This review focuses on recent findings concerning the molecular mechanisms of the stress responses to topoisomerase-mediated DNA damage. The involvement of ubiquitin/26S proteasome and SUMO/UBC9 in these processes, as well as the role of topoisomerase cleavable complexes in apoptotic cell death are discussed.

INTRODUCTION

The two major DNA topoisomerases in human cells, topoisomerase I and topoisomerase II, have been firmly established to be effective molecular targets for many antitumor drugs. The molecular mechanisms for topoisomerase inhibition by these antitumor drugs have been the subject of many reviews (1–5). This particular review discusses recent advances in our understanding of the cellular events that respond to topoisomerase poisoning. In particular, the downstream events leading to tumor cell death following topoisomerase inhibition are discussed.

HUMAN DNA TOPOISOMERASES

Currently, three type I DNA topoisomerases, topoisomerase I (hTOP1), topoisomerase IIIa (hTOP3α), and topoisomerase IIIβ (hTOP3β), have been identified in human cells. hTOP1, a type IB topoisomerase, functions as a swivel in DNA

replication, RNA transcription, and chromosome condensation and segregation (2, 5–7). It is a monomeric 100-kDa protein encoded by the gene located on human chromosome 20q12-13.2 (8, 9). The active site tyrosine (a.a #723) is covalently linked to the 3′-phosphoryl end of the transiently broken DNA strand during enzyme catalysis (2, 8, 9). The enzyme can be divided into four domains: (*a*) an NH2-terminal domain (24 kDa), which is known to be dispensable for activity; (*b*) the core domain (54 kDa); (*c*) a linker region (3 kDa); and (*d*) the COOH-terminal domain (10 kDa), which contains the active site tyrosine (10). X-ray crystallographic studies of the hTOP1-DNA complex have confirmed a long-held notion that the major protein-DNA contact is upstream of the site of cleavage (11, 12). TOP1 is essential, as is demonstrated in studies of *Drosophila* and knock-out mice (13, 14). TOP1 has been shown to interact with a number of nuclear proteins such as T antigen, TOPOR, nucleolin, p53, HMG1 and 2, TBP, and UBC9 (15–23). More recently, it has been shown to co-immunoprecipitate with the Werner syndrome gene product, WRN (24).

hTOP3α is a type IA topoisomerase and is essential for early embryogenesis from mouse knock-out studies (25). hTOP3α is encoded by a single-copy gene located on human chromosome 17p11.2-12 (26). It has been shown to interact with the Bloom's syndrome gene product (BLM), a RecQ-family helicase (27). A TOP3 isozyme, TOP3β, has been isolated from *Drosophila*, mouse, and human (28–30). Both TOP3α and TOP3β have been shown to interact with human RecQ5beta, a large isomer of RecQ5 DNA helicase (31). The function of TOP3 is still unclear. Their interaction with RecQ-family helicases suggests potential roles in decatenation, repair/recombination, and aging (31–33).

Two topoisomerase II isozymes, topoisomerase IIα (hTOP2α) and topoisomerase IIβ (hTOP2β), have been identified in human cells (5, 34). hTOP2α is a homodimer with two identical 170-kDa polypeptides encoded by a single-copy gene located on human chromosome 17q21-22 (35). hTOP2α catalyzes ATP-dependent strand-passing reactions and functions in DNA replication and chromosome condensation and segregation (2, 5). The active-site tyrosine of each subunit is covalently linked to one of the 5′-phosphoryl ends of the transient DNA double-strand break during enzyme catalysis (5, 35). The enzyme contains two major domains, the ATPase (N-terminal) and breakage/reunion (C-terminal) domains (5, 35). hTOP2α is essential for cell growth and is a cell proliferation and tumor marker (36). Its level reaches the highest in the late S/G2 phase of the cell cycle, consistent with its function in chromosome condensation and segregation (36). hTOP2α is also the major component of the nuclear protein scaffold (37). hTOP2β, which shares 72% amino acid identity with hTOP2α (5, 34), is also a homodimer with two identical 180-kDa polypeptides (34, 38). Despite exhibiting similar enzymatic activities in vitro, hTOP2β is regulated quite differently from TOP2α; hTOP2β is expressed at a constant level throughout the cell cycle (38). The function of hTOP2β remains unclear. TOP2β knock-out mice are defective in development of neuromuscular junctions, and the pups die shortly after birth (39). However, in human tissue culture systems, hTOP2β is apparently nonessential

for growth because hTOP2β is not detectable in a number of drug-resistant cell lines selected for resistance to hTOP2 poisons (40, 41). In addition, cell lines from TOP2β knock-out mice can be established in vitro (42).

TOPOISOMERASE I–TARGETING ANTICANCER DRUGS

Camptothecin was originally identified as the antitumor component in the extract of the plant *Camptotheca acuminata* (43). In animal studies, camptothecin exhibited potent antitumor activity against a broad spectrum of tumors (44, 45). However, brief phase I/II trials in the early 1970s that used the sodium salt of camptothecin failed owing to excessive toxicity (44, 45). This failure is now attributed to the use of the inactive form of camptothecin (hydrolyzed camptothecin with the critical lactone ring open). In 1985 hTOP1 was identified as a molecular target for camptothecin, and the critical cellular lesion induced by camptothecin was shown to be a covalent reaction intermediate of the TOP1 reaction, the reversible cleavable complex (46). Subsequent studies have firmly established that human TOP1 is the sole antitumor target of camptothecin (47–49). The fact that TOP1 is a new molecular target has stimulated further interest in camptothecins (50, 51). Figure 1 lists some of the camptothecins in clinical use or development. Additional camptothecin derivatives such as homocamptothecin and silatecans are in various stages of preclinical development (52–54). Although the molecular target for camptothecin has been firmly established, the molecular basis for its potent antitumor activity against a broad spectrum of solid tumors remains to be determined. In addition to camptothecin, a myriad of TOP1-poisoning compounds have been identified (1, 50, 51). This review emphasizes our current understanding of the cell-killing mechanism(s) of TOP1 targeting drugs using camptothecin as a model.

Mechanism of Topoisomerase I Inhibition by Camptothecin

Camptothecin was initially shown to inhibit TOP1 by stabilizing a covalent reaction intermediate, the cleavable complex (46, 47). Exposure of the complex to strong protein denaturants such as SDS and alkali reveals the strand break, with TOP1 covalently linked to the 3′-phosphoryl end of the broken DNA strand (46, 47). Camptothecin appears to be an uncompetitive type of enzyme inhibitor, as it binds neither TOP1 nor DNA, but interacts with the TOP1-DNA complex in a reversible manner (55, 56) The reversible TOP1-camptothecin-DNA ternary complex is apparently nonproductive in catalysis (55, 56). Further studies have suggested that camptothecin specifically inhibits the religation step in TOP1 catalysis (57).

The molecular mechanism of TOP1 inhibition by camptothecin has also been probed with alkylating camptothecin derivatives (56, 58). In one case, 10-bromoacetamidomethyl-camptothecin (BrCPT) was shown to be covalently linked to TOP1 in the ternary complex (56). In another, 7-chloromethyl-10,11-methylenedioxy-camptothecin was shown to be covalenly linked to DNA (N3

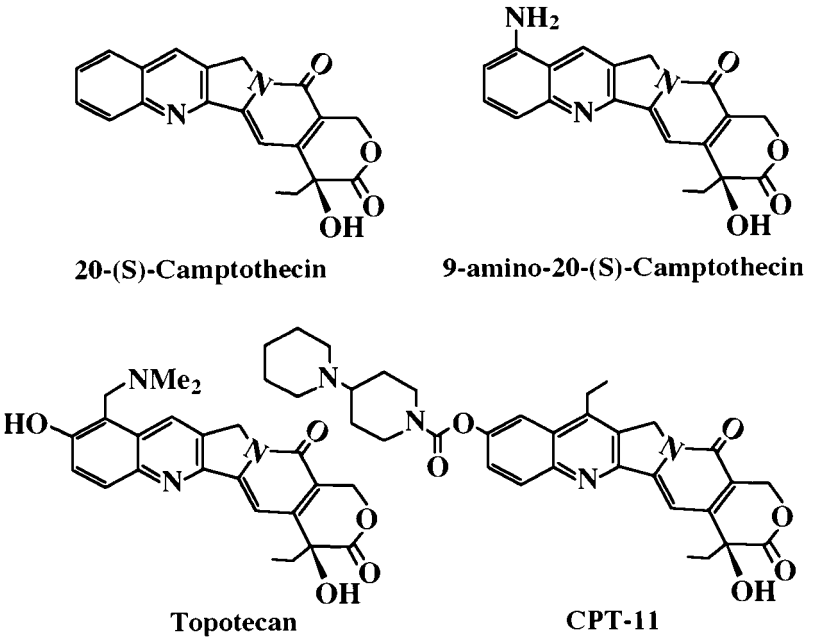

Figure 1 Chemical structures of camptothecins.

purine alkylation) (58). Consequently, it has been suggested that camptothecin binds to both enzyme and DNA at the enzyme-DNA interface (56, 58). These results, together with the results from X-ray crystallographic studies of the TOP1-DNA complex, have led to the proposal of a base-flipping model for the TOP1-camptothecin-DNA ternary complex (10, 11). In this model camptothecin intercalates at the +1 position, causing the +1 purine base to flip out of its helical stack (11). The flipped base not only accommodates camptothecin intercalation but also forms additional interaction with TOP1 in the ternary complex (11). More recent DNA binding studies of a camptothecin derivative, topotecan, have indicated that topotecan can indeed intercalate DNA (DS Pilch, personal communication). Molecular modeling studies of the topotecan-DNA complex, however, have suggested that the 7 position of camptothecin is in close proximity to the N3 position of the +1 purine without the +1 purine having to be flipped (DS Pilch, personal communication). These studies support the intercalation model but challenge the concept of base-flipping in the ternary complex.

Other Mechanisms of Topoisomerase I Inhibition

The intercalation model for camptothecin is attractive because it incorporates both camptothecin-DNA and camptothecin-topoisomerase interactions at the enzyme-DNA interface. It seems plausible that this model could be applicable to

intercalative TOP1 poisons such as actinomycin D (52), protoberberines, and nitidines (59–60). However, recent studies of a TOP1-poisoning DNA intercalator, nogalamycin, have demonstrated that nogalamycin binds at a distal upstream site (from -3 to -6) (61). In addition, a DNA bending sequence was able to mimic nogalamycin in inducing TOP1 cleavable complex at the same site (61). These studies suggest that DNA bending may underlie the mechanism of TOP1 poisoning induced by nogalamycin (61). Previous studies of a TOP1 binding/cleavage hotspot from *Tetrahymena* rDNA have also demonstrated the importance of DNA bending in TOP1 poisoning (62). Thus, it appears that multiple mechanisms of TOP1 poisoning may exist.

It has been demonstrated that DNA minor-groove binding bis- and terbenzimidazoles can poison TOP1 (63–67). The mechanism of poisoning has been correlated with their binding strength to the DNA minor groove (65–67). Studies of TOP1 cleavage hotspots induced by the minor-groove binding Hoechst 33342 have identified a cleavage consensus sequence of $T^{*}CATTTTT$ from -1 to $+7$ (1, 63). Mutational analysis has revealed that binding of Hoechst 33342 to the T stretch is responsible for stabilization of a TOP1 cleavable complex (T-K Li, DS Pilch LF Liu, unpublished results). The molecular basis for enhanced topoisomerase I cleavage induced by DNA minor-grove binders has been suggested to be related to ligand-induced DNA bending (64).

DNA adducts and DNA structural perturbations (pyrimidine dimers, 8-dihydro-8-oxoguanine, 5-hydroxycytosine adducts, AraC-substituted sites, benzo[a]pyrene carcinogenic adducts, abasic sites, base mismatches, and uracil substitution) have been shown to poison DNA TOP1 (68–73). It appears that the cleavage/religation reaction of TOP1 is highly sensitive to the local DNA structure. However, the precise molecular mechanism(s) of TOP1 poisoning by these various DNA adducts and DNA structural perturbations is still unclear.

Collisions Between Topoisomerase I Cleavable Complexes and Replication Forks

Camptothecin is known to kill S-phase cells selectively (74, 75). It is now well established that camptothecin kills S-phase cells by a mechanism involving collisions between advancing replication forks and TOP1 cleavable complexes (Figure 2). This collision triggers both S-phase-specific cell death and cell cycle arrest at the G2 phase of the cell cycle (74–76). Analysis of the aberrant replication intermediates in an SV40 cell-free replication system has suggested that the collision between the replication fork and TOP1 cleavable complex leads to three biochemically identifiable events: the formation of a double-strand break at the fork, irreversible arrest of fork movement, and formation of an irreversible TOP1-DNA covalent adduct (77). It has been deduced from the structure of the aberrant replication intermediates that the collision is dependent on the relative orientation of the cleavable complex and the advancing replication fork (77). Effective collision leading to these three biochemical events requires that the cleavable complex

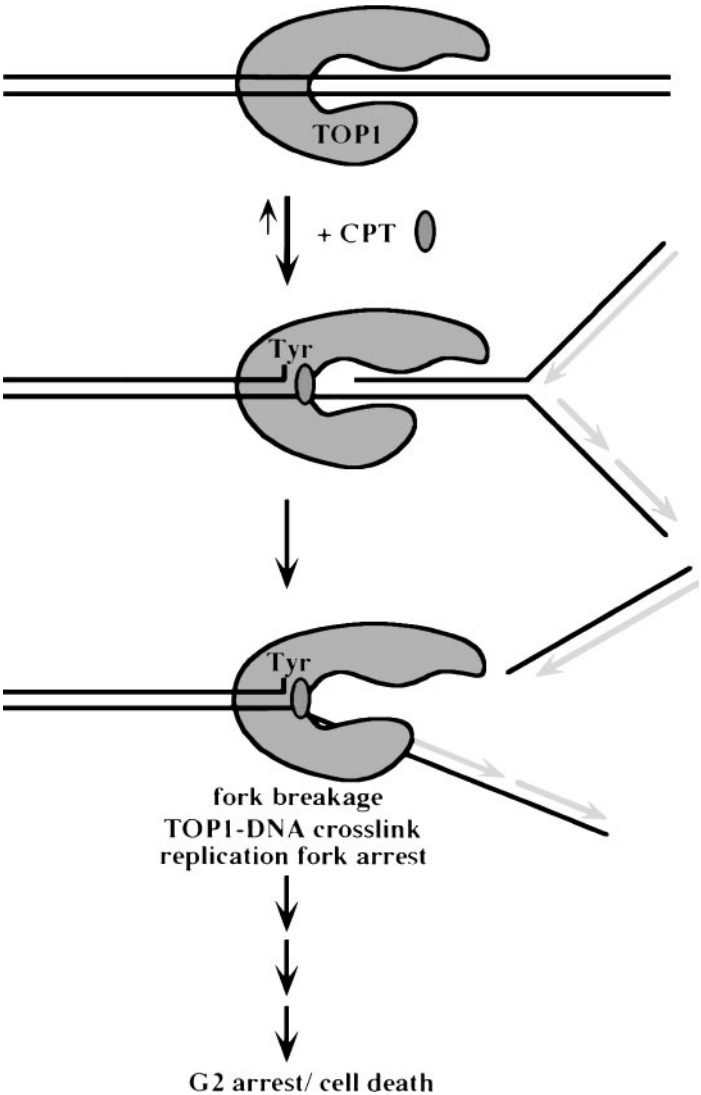

Figure 2 A replication collision model for camptothecin cytotoxicity.

form on the strand that is complementary to the leading strand of DNA synthesis (77). Indeed, more recent studies from analysis of the replication intermediates in tissue culture cells treated with camptothecin have also supported this notion (78). However, the relative importance of these three events in cell death and G2 cell cycle arrest has not been established. Studies of yeast have demonstrated that at least the double-strand break is important for camptothecin cytotoxicity (48).

In addition to cell death and cell cycle arrest, collisions between replication forks and TOP1 cleavable complexes have also been suggested to be responsible for elevation of p53, phosphorylation of Chk1 and RPA, and activation of NFκB (79–83). The precise roles of these molecules in camptothecin sensitivity/resistance remain undetermined.

A number of studies have demonstrated that camptothecin induces apoptotic cell death (80–82, 84–86). However, it is unclear whether this is the main mode of cell killing by camptothecin. It was demonstrated that apoptotic cell death induced by camptothecin only occurs at high concentrations of camptothecin (84–86). At lower concentrations, cell death occurs primarily in S-phase cells (see discussion above) and does not share all characteristics of apoptotic cell death (e.g. cell shrinkage, nucleosomal DNA laddering) (84–86). The relevance of apoptotic cell death at the clinical doses of camptothecin is questionable. Nevertheless, in vitro RNA transcription studies have demonstrated that high concentrations of camptothecin can induce double-strand breaks, possibly owing to two closely spaced TOP1 cleavable complexes located on separate strands (87). These TOP1-mediated double-strand breaks could be responsible for apoptotic cell death induced by high concentrations of camptothecin.

The role of p53 and other apoptosis modulators on camptothecin sensitivity/resistance has been controversial (80, 85, 88–90). Use of apoptotic-specific assays showed that p53 plays a positive role in camptothecin-induced apoptosis (89, 91). However, use of clonogenic survival assay showed that p53 plays the opposite role in camptothecin sensitivity (85, 88, 91). This apparent paradox can be explained if apoptosis is only a minor mechanism of cell killing by camptothecin. Alternatively, p53 and other apoptotic modulators may only modulate the kinetics of apoptotic cell death without affecting the commitment step of apoptosis (91).

Collisions Between Transcription Elongation Complexes and Topoisomerase I Cleavable Complexes

Earlier studies have demonstrated that camptothecin inhibits both DNA and RNA synthesis (92–95). Inhibition of DNA synthesis is mostly irreversible, whereas inhibition of RNA synthesis appears to be reversible (92–95). Studies of HeLa cells have demonstrated that camptothecin treatment results in rapid transcription arrest and redistribution of the RNA polymerase elongation complexes (96). The RNA polymerase elongation complexes accumulate to a higher level at the 5′ end of the gene and gradually decrease in density toward the 3′ end. This result suggests that camptothecin specifically inhibits transcription elongation (96–99). The molecular mechanism of transcription-elongation inhibition by camptothecin has been revealed by in vitro studies. Use of a purified T7 transcription system has shown the simultaneous presence of camptothecin and TOP1 to cause transcription arrest and premature termination (99). This result is consistent with the notion that the TOP1-camptothecin-DNA ternary complex forms a road block on the transcription

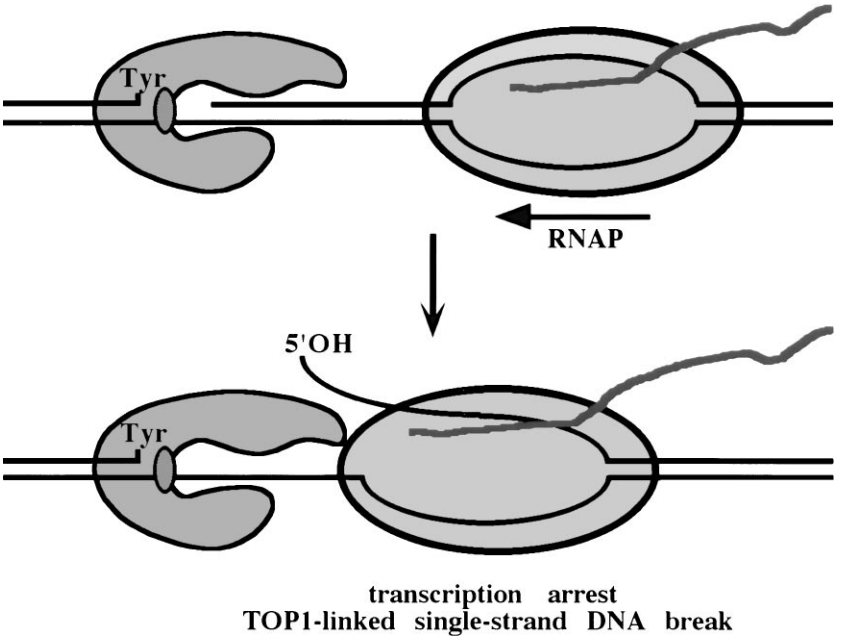

Figure 3 A transcription collision model: conversion of topoisomerase I cleavable complexes into topoisomerase I–linked single-strand breaks.

template. Further analysis has suggested that this road block is orientation-specific relative to the transcribing RNA polymerase, a situation analogous to the collision event between the replication forks and TOP1 cleavable complexes described above. In this case, the road block is effective only if the TOP1 cleavable complex is formed on the template strand (99). More recent studies using the same T7 transcription system have also demonstrated that transcription elongation can process the reversible TOP1 cleavable complexes into "irreversible" TOP1-DNA covalent complexes (87). The role of these long-lived TOP1-DNA covalent complexes in camptothecin cytotoxicity has only begun to emerge. Figure 3 schematically shows a transcription collision model for camptothecin.

Repair of Topoisomerase I–Mediated DNA Damage

Because of its rapid reversibility, the TOP1 cleavable complex by itself should not be viewed as a form of DNA damage. As discussed above, cellular processes (e.g. replication forks, transcription elongation complexes, and DNA helicases) can convert the reversible TOP1 cleavable complexes into some forms of DNA damage (e.g. double-strand breaks at the replication forks and TOP1-linked DNA single-strand breaks within the transcribed regions). Consequently, the TOP1 cleavable complex can be viewed as a unique form of cellular stress that can be converted into

some forms of DNA damage, collectively called TOP1-mediated DNA damage, by certain cellular processes.

As discussed above, the double-strand break repair pathway is involved in the repair of TOP1-mediated DNA damage (48). Presumably, the double-strand breaks are primarily generated by the collision between the reversible TOP1 cleavable complex and the replication fork (74–77). Transcription-coupled repair has also been shown to be involved in the repair of TOP1 cleavable complexes (100). In this case the TOP1-mediated DNA damage is presumably generated by collisions between transcription elongation complexes and reversible TOP1 cleavable complexes.

Camptothecin treatment has been shown to reduce the cellular content of TOP1 in peripheral-blood mononuclear cells (101). In tissue culture cells camptothecin was also shown to reduce the cellular content of TOP1 (102). The reduction of TOP1 cellular content is dependent on the presence of active E1 (102). In addition, inhibition of 26S proteasome by proteasome inhibitors abolishes the reduction of TOP1 content in camptothecin-treated cells. More recently, the presence of ubiquitin-TOP1 conjugates has been detected in cells treated with camptothecin (SD Desai, M Yong, LF Liu, unpublished results). Together, these results suggest that camptothecin induces ubiquitin/26S-proteasome-dependent degradation of TOP1, a phenomenon referred to as TOP1 downregulation (102).

TOP1 downregulation may serve two roles. First, reduction of TOP1 cellular content is an effective way to confer cellular tolerance to further camptothecin treatment. Second, TOP1 downregulation may be part of a repair pathway for repair of TOP1-mediated DNA damage. Recent studies have demonstrated that TOP1 downregulation is a transcription-dependent event (SD Desai, D Rodriguez, LF Liu, unpublished results). Figure 4 schematically shows a model for TOP1 downregulation in the repair of TOP1-mediated DNA damage. In this model transcription elongation complexes process the reversible TOP1 cleavable complexes into long-lived TOP1-mediated DNA strand breaks. The covalently bound TOP1 is then multi-ubiquitinated and degraded by 26S proteasome. The tyrosine DNA phosophodiesterase may be involved in this pathway by removing the covalently bound multi-ubiquitinated TOP1 from DNA either prior or subsequent to 26S proteasome–mediated degradation. Repair of the single-strand breaks is presumably carried out by either transcription-coupled repair or other repair pathways.

Tumor cells exhibit great variability in their camptothecin sensitivity (85, 89, 103). This large variability is not due to the amount of cellular TOP1 cleavable complexes and is not shared by VP-16, a TOP2-specific drug (85, 103). For example, studies using a panel of breast cancer cell lines have demonstrated over 500-fold variation in their camptothecin sensitivity (85). No single drug-resistance parameter can explain this variability (85). However, TOP1 downregulation shows a significant correlation with camptothecin resistance in these breast cancer cell lines (SD Desai, LF Liu, unpublished results). These results suggest that the ubiquitin/26S proteasome pathway could be a significant determinant for camptothecin sensitivity/resistance.

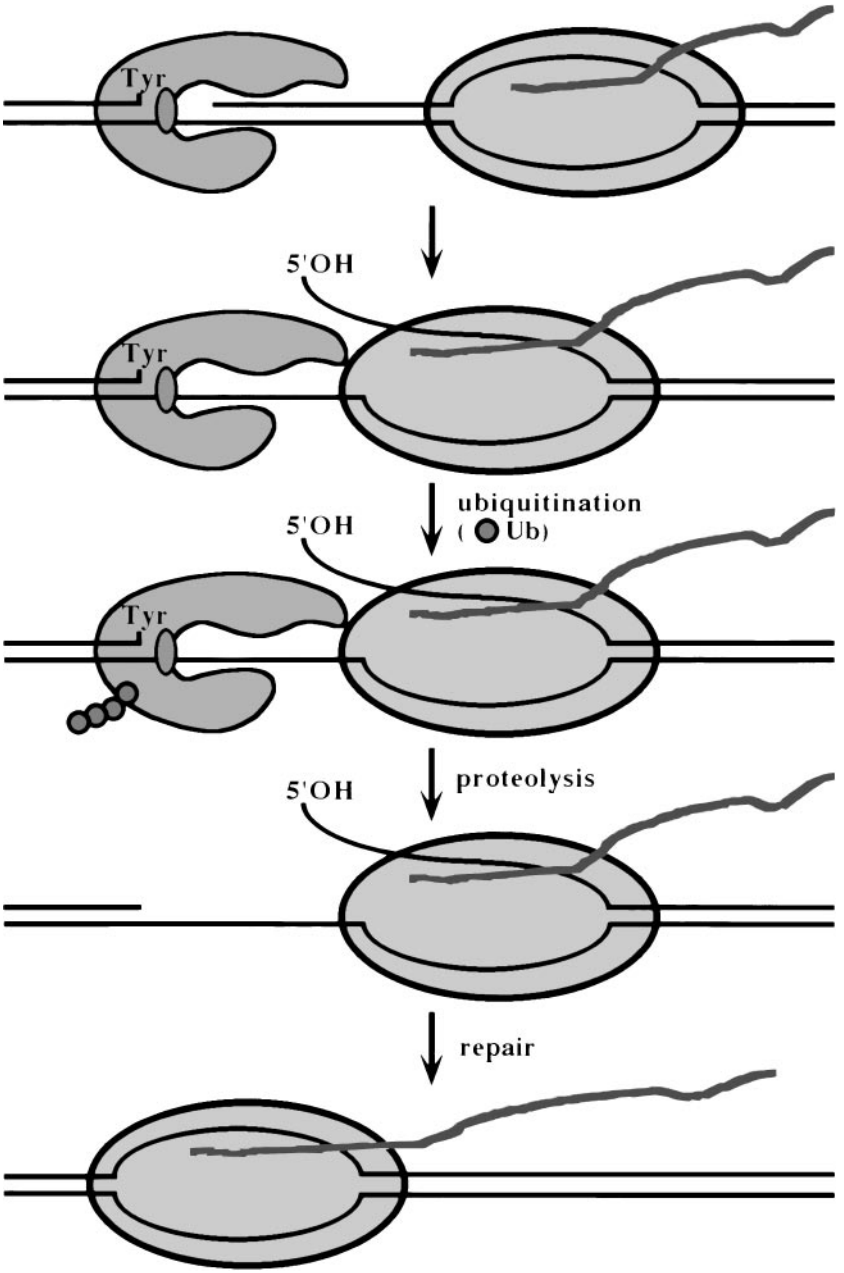

Figure 4 Topoisomerase I downregulation: roles of transcription and ubiquitin/26S proteasome.

Other Stress Responses to Topoisomerase I Cleavable Complexes

As discussed above, the TOP1 cleavable complex represents a unique form of cellular stress. It can induce DNA damage responses upon its processing by cellular machineries such as DNA replication and RNA transcription complexes. In addition to the DNA damage response, the TOP1 cleavable complex may also induce other stress responses unrelated to DNA damage but related to the covalently bound TOP1. Recent studies have pointed to such a possibility. Camptothecin was shown to induce rapid SUMO-1 (also SUMO-2/3) conjugation to TOP1 (104). SUMO-1 conjugation is carried out by an E2 enzyme, UBC9 (104). SUMO-1 conjugation to TOP1 occurs on TOP1 that is covalently linked to DNA (104). Although both ubiquitin and SUMO-1 conjugation to TOP1 are triggered by TOP1 cleavable complexes, they appear to represent two distinct cellular responses to the TOP1 cleavable complex. First, ubiquitin/proteasome-dependent degradation of TOP1 is dependent on active transcription, whereas SUMO-1 conjugation to TOP1 is not (104). Second, ubiquitin/proteasome-dependent degradation of TOP1 appears to be defective in many tumor cells, whereas SUMO-1 conjugation to TOP1 appears normal in all tumor cells examined (98; Y Mao, LF Liu, unpublished results).

Recent studies of SUMO-1 conjugation to TOP2 have suggested that the signal for SUMO-1 conjugation to TOP1 may arise from TOP1 rather than the disjoined DNA ends (105). These studies have demonstrated that both VP-16, a TOP2 poison, and ICRF-193, a TOP2 activity inhibitor, induces SUMO-1 conjugation to TOP2 (105). In the case of VP-16, SUMO-1-conjugated TOP2 is covalently linked to DNA, whereas in the case of ICRF-193, SUMO-1-conjugated TOP2 is not (105). In addition, stresses that lead to protein conformational changes (e.g. heat shock and oxidative stresses) have also been shown to induce rapid SUMO conjugation to nuclear proteins (105, 106). Consequently, it has been suggested that SUMO-conjugation to topoisomerases may be signaled by topoisomerase conformational changes rather than the disjoined DNA ends (105).

The importance of SUMO conjugation to topoisomerases in camptothecin cytotoxicity is still unclear. However, yeast cells defective in Ubc9 have been shown to be hypersensitive to camptothecin (104). The possible role of SUMO conjugation to topoisomerases could be to inactivate or redistribute topoisomerases so that SUMO-conjugated topoisomerases cannot participate in the formation of the potentially lethal topoisomerase cleavable complexes. The possibility that SUMO-conjugated topoisomerases may be targeted to PODs (nuclear bodies) has been suggested (104). At present, very little is known about the role(s) of SUMO conjugation to topoisomerases. However, the ease of manipulating the SUMO conjugation reaction using topoisomerase inhibitors could suggest the use of topoisomerase inhibitors to study the role of SUMO in nuclear protein processing.

Recently, another interesting cellular response to TOP1 cleavable complexes has been demonstrated (107). In the presence of camptothecin, the linking numbers of episomal plasmids in both mammalian and yeast cells have been shown to increase.

Within minutes, a large increase in plasmid linking number (over 10 linking numbers in a 5–10-kb plasmid) is completed (107). This large change in plasmid linking number is indicative of major chromatin structural alteration (107). As in the case of SUMO-1 conjugation to TOP1, linking-number change of episomal DNA is not affected by aphidicolin, a replication inhibitor, DRB, a transcription inhibitor, or 3-AB, a poly (ADP-ribose) polymerase inhibitor (107). The chromatin structural alteration could represent a novel stress response to TOP1 cleavable complexes. Its role in camptothecin sensitivity/resistance has yet to be determined.

TOPOISOMERASE II–TARGETING ANTICANCER DRUGS

TOP2 has been identified as the molecular target for many clinically useful anticancer drugs such as doxorubicin, daunorubicin, mitoxantrone, m-AMSA, and etoposide (1–3). These TOP2 poisons stabilize a covalent intermediate of the TOP2 reaction, the cleavable complex (1–3). Exposure of the cleavable complex to strong protein denaturants such as SDS and alkali reveals the double-strand break and the covalent linking of each TOP2 subunit to the 5′-phosphoryl ends of the broken DNA strand (1–3). The significance of TOP2 cleavable complexes probably extends beyond their role in mediating tumor cell killing by anticancer drugs. A number of physiological stresses such as acidic pH, oxidative stress, elevated calcium concentrations, and thiol stress have also been shown to increase TOP2 cleavable complexes (108–112). The possibility that TOP2 may be important for carcinogenesis and apoptotic cell death has also been raised (108–111).

Multiple Mechanisms of Topoisomerase II Inhibition

Like TOP1, TOP2 can probably be poisoned by a number of mechanisms (see Figure 5). Earlier studies of DNA intercalators have clearly demonstrated the importance of the intercalative mode of DNA binding (113–115). A rotational misalignment model has been proposed to explain the mechanism of action of these intercalative TOP2 poisons (2, 114). In this model binding of homodimeric TOP2 to DNA creates an isolated topological domain. Drug intercalation within this isolated topological domain misaligns the two ends of the transiently broken DNA strand (2, 114). It has also been suggested that drug-enzyme interaction is not necessary but that the drug has to be sterically tolerated in the active site (2, 114). Steric tolerance has been used to explain the difference between o-AMSA and m-AMSA (115).

All clinically useful TOP2 posions (e.g. doxorubicin, daunorubicin, etoposide, mitoxantrone, m-AMSA) are highly sensitive to ATP and the ATP/ADP ratio for their poisoning activity (109, 116–118). For example, in the presence of ATP or AMPPNP, the poisoning activity of VP-16 or VM-26 is about 50- to 100-fold higher (109, 116–118). It has been shown that the AMPPNP-bound conformation of TOP2 is a circular protein clamp (119), which is capable of one-dimensional

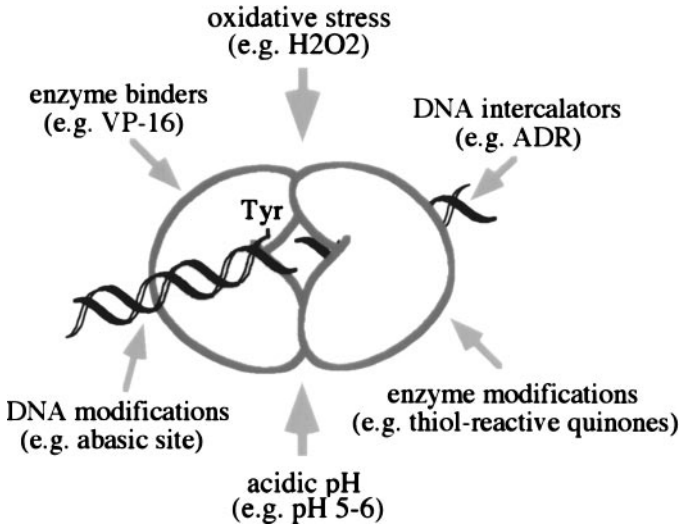

Figure 5 Multiple mechanisms for topoisomerase II poisonsing.

diffusion on DNA (118). It appears that the molecular target of these clinically useful drugs may be ATP-bound TOP2.

Selection of resistance to one TOP2 poison (e.g. VP-16, mitoxantrone, or m-AMSA) often leads to cross-resistance to all other TOP2 poisons (40, 120). This drug-resistance phenomenon has been termed atypical multidrug resistance to differentiate it from MDR1-mediated multidrug resistance (120). Recent studies have demonstrated that atypical multidrug resistance is due to mutation(s) on TOP2 (40, 117, 118, 120). Interestingly, these mutant TOP2 enzymes are cross-resistant to TOP2 poisons only in the presence of ATP. In the absence of ATP, they exhibit sensitivity to TOP2 poisons similar to the wild-type TOP2 (117, 118). These results suggest that the loss of ATP dependence in drug-stimulated cleavage is the underlying mechanism for atypical multidrug resistance (117, 118).

Alkylation of TOP2 in the TOP2-DNA complex has also been shown to trap TOP2 cleavable complexes (111, 118). For example, alkylation of thiol(s) on TOP2 by β-lapachone and naphthoquinones has been shown to be responsible for trapping TOP2 cleavable complexes (118). Thiol-specific alkylating agents such as N-ethylmaleimide (NEM), thiol binding metal ions such as Ni^{++}, and a number of organic disulfides, including glutathione disulfide, have been shown to poison TOP2 (118). The effect of ATP appears to be minimal for these TOP2 cleavage reactions (118). Hydrogen peroxide has also been shown to poison TOP2, possibly by a mechanism similar to that of thiol alkylating agents (108). Under certain conditions, hydrogen peroxide is highly effective in trapping TOP2 cleavable complexes, resulting in fragmentation of chromosomal DNA into 50–100-kb high-molecular-weight DNA fragments (108).

Acidic pH (e.g. pH 5–6) has been shown to be quite efficient in trapping TOP2 cleavable complexes (109, 110). TOP2 appears to be at least in part responsible for acidic pH–induced mutagenesis (H Xiao, T-K Li, LF Liu, unpublished results). It is interesting to point out that acid reflux (gastro-esophageal reflux) has been linked to Barrett's esophagus, a preneoplastic condition of the esophagus and the only known risk factor for the rising incidence of esophageal adenocarcinoma (121). These studies suggest that TOP2 poisoning by acidic pH could be important for Barrett's esophagus.

Certain DNA structural perturbations have been shown to trap TOP2 cleavable complexes (122–127). Abasic sites located near the site of cleavage have been shown to be effective site-specific TOP2 poisons (122–125). Alkylation of the base to perturb base pairing has also been shown to lead to TOP2 poisoning (126). In addition, incorporation of araC (cytosine arabinoside) into DNA also poisons TOP2 (127). It appears that both topoisomerases I and II are quite sensitive to DNA structural perturbations.

Bisdioxopiperazines (e.g. ICRF-193 and ICRF-187) represent a distinct class of TOP2 inhibitors. Unlike TOP2 poisons, they inhibit TOP2 catalytic activity without poisoning TOP2 (119, 128, 129). In the presence of ATP they appear to inhibit ATP hydrolysis and stabilize ATP-bound TOP2 in the circular clamp form (119).

Processing of Topoisomerase II Cleavable Complexes

Like TOP1 cleavable complexes, TOP2 cleavable complexes are reversible. It is reasonable to assume that TOP2 cleavable complexes have to be processed by cellular machineries to exert their lethality. Studies in cultured cells have demonstrated that both DNA replication and RNA transcription may be involved in processing TOP2 cleavable complexes (74). Studies in vitro have demonstrated that both DNA helicases and the replication forks can perturb the cleavable complexes (130). More recent studies have demonstrated that, like topoisomerase I cleavable complexes, TOP2β cleavable complexes are efficiently processed by the ubiqitin/26S proteasome pathway leading to downregulation of TOP2β (Y Mao, LF Liu, unpublished results). It is unclear what the role of TOP2β downregulation is in response to TOP2 poisons. However, it seems plausible that, like topoisomerase I downregulation, TOP2β downregulation represents a repair response to some irreversible forms of TOP2 cleavable complexes.

Studies in yeast have suggested that double-strand break repair pathways are involved in the repair of TOP2 cleavable complexes (3, 48). TOP2 downregulation by the ubiquitin/26S proteasome pathwa may represent one pathway that converts TOP2 cleavable complexes into DNA double-strand breaks. These double-strand breaks are then subject to double-strand break repair. On the other hand, studies of SPO11 (a TOP2-like enzyme that is known to produce protein-linked DNA breaks necessary for initiation of meiotic recombination) (131–134) have suggested that the Rad50/Mre11/Xrs2 complex may be involved in removing SPO11 from the

covalent SPO11-DNA complexes. It seems possible that multiple mechanisms may exist for the removal of TOP2 from the covalent TOP2-DNA complex.

Topoisomerase II Cleavable Complexes and Carcinogenesis

Clinical studies have demonstrated that treatment with TOP2 poisons such as etoposide leads to secondary leukemia (135–138). These treatment-related acute myelogenous leukemias (t-AML) are associated with translocation of the human myeloid-lymphoid leukemia gene, MLL (also called ALL-1, Htrx1, or HRX) (135–138). Translocation occurs within the 8.3-kb breakpoint cluster region (BCR) (135–139). Recent studies have suggested that translocation within BCR may be mediated by TOP2 owing to poisoning of TOP2 by TOP2 poisons (139).

The strongest piece of evidence supporting the role of TOP2 in translocation of the MLL gene comes from a recent report that topoisomerase-targeting drugs can induce reversible TOP2 cleavable complexes within the BCR of the MLL gene in cells (139). However, studies from another group suggest that cleavage within BCR in cells treated with TOP2 poisons is not mediated by TOP2 but by a nuclease activated during apoptosis (140). It was shown that many apoptotic stimuli and chemotherapeutic agents that are not TOP2 poisons induced cleavage within the BCR of the MLL gene (140). It remains to be clarified whether TOP2 cleavable complexes within BCR are responsible for t-AML.

TOP2 cleavable complexes have also been suggested to be responsible for infantile acute leukemia because translocation of the MLL gene is also associated with this disease (141, 142). Maternal ingestion of TOP2 poisoning bioflavonoids in utero has been suggested to be responsible for infantile acute leukemia (141, 142).

The role of TOP2 in DNA sequence rearrangements has been demonstrated in a number of systems (143–145). The conversion of TOP2 cleavable complexes into double-strand breaks could be responsible for TOP2-mediated DNA sequence rearrangements. It should be noted, however, that a different mechanism has been proposed for T4 phage TOP2-mediated deletion formation (146, 147).

Topoisomerase II Cleavable Complexes and Apoptosis

It is well established that DNA double-strand breaks can trigger apoptotic cell death (148–150). In fact, TOP2 poisons such as etoposide have been used extensively as an apoptosis inducer (108,151–153). However, it is unclear whether TOP2 plays any role in mediating apoptosis induced by other apoptotic stimuli such as TRAIL (tumor necrosis factor–related apoptosis-inducing ligand)- and FAS ligand–induced apoptosis (153, 154). Recent studies have suggested that TOP2 can be poisoned under a variety of cellular stresses (e.g. oxidative stress, thiol stress, acidic pH stress, and intracellular calcium increase) (108–112). All these cellular stresses are also known to be elevated during apoptotic cell death (155). In addition, each of these cellular stresses is known to induce apoptotic cell death (155–159). Consequently, it has been suggested that TOP2 cleavable complexes may be involved in the commitment step of apoptotic cell death (108). Figure 6

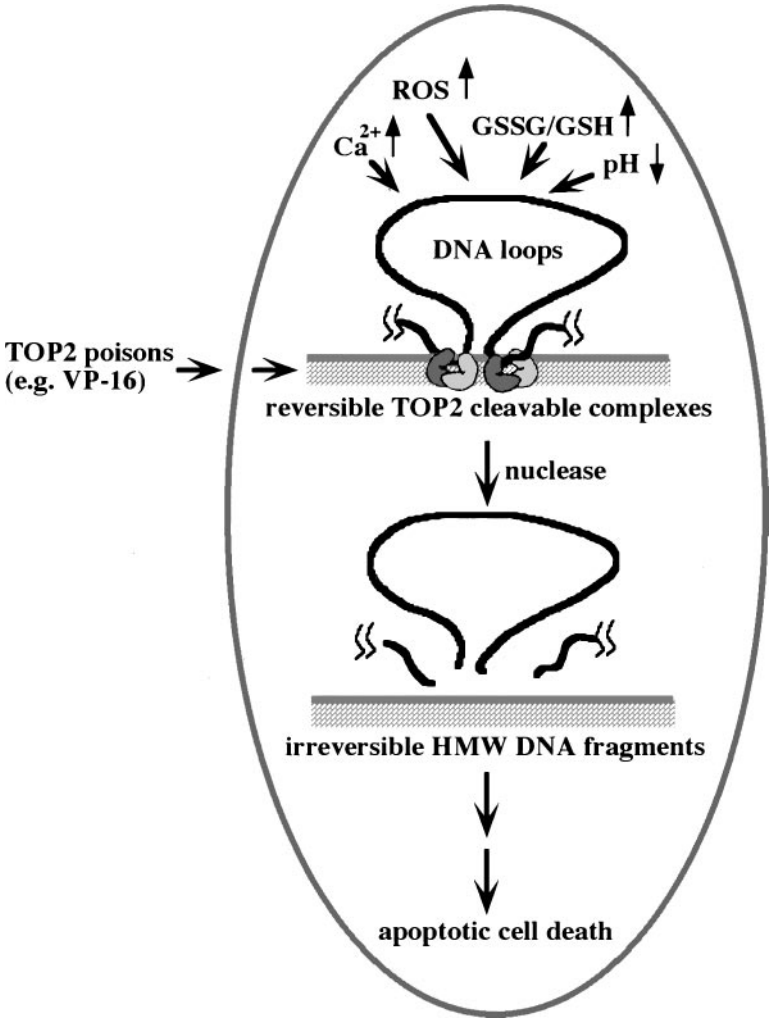

Figure 6 Topoisomerase II poisoning in cells under physiological stresses: role in apoptotic cell death.

shows a schematic diagram illustrating the potential role of TOP2 in apoptotic cell death.

ACKNOWLEDGMENTS

This work was supported by NIH grants GM27731, CA77433, and CA39662. We are grateful to Dr. Shyamal Desai, Dr. Yong Mao, and Ms. Sai-peng Sim for their helpful discussions.

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