1. INTRODUCTION

Camptothecins (CPTs) are a group of antineoplastic agents that specifically target DNA topoisomerase I (TOP-I) and are known as “TOP-I poisons.” The parent compound is a naturally occurring alkaloid found in the Chinese plant *Camptotheca accuminata*. This compound was first identified in the 1960s in a screen of plant extracts for antineoplastic drugs (1). Subsequently, many derivatives of the parent compound have been synthesized, including topotecan, irinotecan, 9-aminocamptothecin, 9-nitrocamptothecin, exatecan mesylate (DX-8951f), ST1481, and Karenitecin (BNP1350). Topotecan and irinotecan have been approved by the Food and Drug Administration for clinical use. Topotecan, a 10-hydroxyl modification of CPT, is approved for treatment of metastatic ovarian and small-cell...
lungs and for myeloid malignancies. Irinotecan (CPT-11) is a prodrug that is converted to the active compound SN-38 by plasma and cellular carboxylesterases, and is approved for use in the treatment of metastatic colorectal and rectal carcinomas. Food and Drug Administration approval for clinical use of both of these drugs was based on approximately 30% response rates with transient clinical responses (2,3). However, based on studies in mouse xenograft models, these response rates are disappointing (4–6). The mechanisms underlying de novo and acquired clinical resistance to CPTs are unclear. Similar to other drugs, clinical resistance to CPTs might be the result of inadequate metabolism and accumulation of drug in the tumor, alterations in the target (TOP-I), or alterations in the cellular response to the TOP-I–CPT interaction.

2. CELLULAR METABOLISM, ACCUMULATION, AND TRANSPORT OF CPTS

Cell culture data indicate that only brief exposures to submicromolar concentrations of CPT are required to target TOP-I and to kill proliferating cancer cells (7). Additionally, the lactone form of CPT is the active form of the drug (8). Achieving high enough intracellular concentrations of the active form of CPT is dependent on cellular uptake, metabolism, and efflux mechanisms (Table 1).

Few studies have addressed mechanisms of cellular CPT uptake. Both active and passive transport mechanisms are implicated in intestinal cell uptake of CPT (9). Furthermore, active efflux by P-glycoprotein (P-gp) and multidrug resistance–associated protein (MRP) in mammalian intestinal cells may also limit the oral absorption of CPT-11 (10). Another study found that ovarian cancer cells contain active transporters that are required for the influx of topotecan and SN-38 (11).

In addition to uptake, cellular metabolism may be particularly important for the prodrug CPT-11, which is converted to its active form, SN-38, by cellular carboxylesterases (12–15). Increased levels of cellular carboxylesterases correlate with increased cellular sensitivity to CPT-11 (12,16). However, a recent study found that carboxylesterase-mediated sensitization of human tumor cells to CPT-11 cannot override BCRP-mediated drug resistance (which is further discussed in the following sections) (17).

SN-38 is also conjugated and detoxified by UDP-glucuronosyltransferase (UGT) to yield an SN-38-glucuronide (18). SN-38 glucuronidation is specifically catalyzed by human liver UGT1A1, UGT1A3, UGT1A6, and UGT1A9 isoforms (19) and is associated with increased efflux of the drug from colon cancer cells (20). Furthermore, glucuronidation of CPTs has been associated with altered chemosensitivity of breast cancer cells and lung cancer cells (21,22).
Several ATP-binding cassette (ABC) proteins have been implicated in efflux and cellular resistance to CPTs in yeast and mammalian cells. A screen for mutations that suppress the cytotoxic effects of CPT in yeast cells resulted in the identification of an ABC protein, Snq2, which confers resistance to CPT (23). Furthermore, in mammalian cells, P-gp overexpression confers resistance to CPT derivatives, albeit to a lesser degree than to other substrates of P-gp, such as the anthracyclines (24). Also, antisense oligonucleotides directed against the MRP2 gene can increase cellular sensitivity to CPT-11 and SN-38 (25).

Recently, the BCRP gene (also known as MTX or ABCP), an ABC half-transporter (26,27), was found to be overexpressed in cells selected for resistance to doxorubicin, mitoxantrone, or topotecan. Interestingly, these cells are cross-resistant to SN-38 and 9-aminocamptothecin, but not to CPT (21,28–30). Additionally, in some human cells treated with anthracyclines a mutant form of BCRP (R482G/T) was preferentially selected, which exhibits greater resistance to anthracyclines and relative lower resistance to topotecan compared to the wild-type protein (31,32). Furthermore, cells that overexpress both the native and mutant R482T form of BCRP are more resistant to and accumulate less 9-aminocamptothecin compared with a close analog, 9-nitrocamptothecin (33). Similarly, another study showed that a lipophilic 7–modified CPT analogue (ST1481) is not a substrate for BCRP (34). These results suggest that certain CPT analogs may be less susceptible to BCRP-mediated efflux. Furthermore, recent studies found that estrogen antagonists and agonists and novobiocin can enhance accumulation of CPT analogs in cells overexpressing BCRP, thereby overcoming BCRP-mediated drug resistance (35,36).

To date, there are relatively few published studies of BCRP gene expression in clinical samples. BCRP seems to be expressed at low levels in breast cancer and leukemic cells (37,38). Furthermore, the expression of BCRP in breast carcinoma cells does not seem to correlate with response to doxorubicin-based chemotherapy (37), nor is it upregulated in patients with breast

<table>
<thead>
<tr>
<th>Process</th>
<th>Protein</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Cellular uptake</td>
<td></td>
<td>9,11</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Carboxylesterase</td>
<td>12–15</td>
</tr>
<tr>
<td></td>
<td>UDP-glucuronyltransferase</td>
<td>18–20</td>
</tr>
<tr>
<td>Cellular efflux</td>
<td>P-glycoprotein</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>MRP2</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>BCRP</td>
<td>26–30</td>
</tr>
</tbody>
</table>
cancer that were previously treated with anthracyclines versus those patients that were not treated with these drugs (39). Another recent study found that BCRP protein expression was increased in leukemia cells from three of four patients after an infusion of topotecan and arabinoside-C (40). However, more clinical studies are needed to determine the role of BCRP overexpression and mutations in resistance to CPTs.

3. ALTERATIONS IN TOP-I THAT CONFER RESISTANCE TO CPTS

CPT causes DNA damage by stabilizing a normally transient covalent complex between TOP-I and DNA (41). Furthermore, genetic studies in yeast identified TOP-I as a unique cellular target for the CPTs (42–44). Therefore, it is not surprising that TOP-I mutations conferring resistance to CPT have been identified in various mammalian and yeast cells, and, more recently, in tumor tissue from a patient treated with irinotecan (45). Most of the point mutations can be found clustered in three regions of the protein, including one near the catalytic tyrosine at position 723 (46–55) (Fig. 1). Furthermore, crystal structures of the TOP-I–DNA and TOP-I-DNA-topotecan covalent complexes and models of the TOP-I-DNA-drug ternary complex have enabled structural mapping of these mutations (56–60). Structural models of the ternary complex implicate CPT in an intercalated position at the −1 and +1 base pairs of the cleavage site, with hydrogen bonding between the drug and both TOP-I and DNA, stabilizing the ternary complex. Mutations in the regions between amino acids 361–364, 533, and 722 explain resistance to CPT, because these regions of TOP-I are associated with the DNA and are in close proximity to the intercalated drug (57).

Some TOP-I point mutations, including Y723F, Y727F, R364H, G503S, N722S, and F361S, which confer CPT resistance, are also implicated in resistance to the indolocarbazoles, a group of TOP-I–targeting compounds structurally unrelated to CPT (61,62). These studies indicate that CPT and the indolocarbazoles may share binding sites in the TOP-I–DNA complex. In contrast, some TOP-I mutants, including N726S/A, that confer resistance to CPT retain sensitivity to the indolocarbazole rebeccamycin (61). In addition to point mutations, a mutant TOP-I containing an internal duplication of residues 20–609 has been described in a murine cell line that is enzymatically resistant to the indolocarbazole NB-506, and CPT (63).

Recent studies also indicate that interactions between TOP-I and other proteins may affect cellular sensitivity to CPTs. The TOP-I–binding protein, nucleolin, may recruit TOP-I to the nucleolus as a result of the high rate of transcription in this region (64). Studies of a nucleolin orthologue in yeast, Nsr1p, indicate that the absence of this protein is associated with relocalization of TOP-I from a predominantly nucleolar localization to a
diffuse nuclear localization in resistance to CPT (65). Recently, nucleolin was found to redistribute from a predominantly nucleolar pattern to a speckled nuclear and perinuclear pattern in U937 cells exposed to CPT (66). TOP-I is also known to move rapidly from the nucleolus to the nucleus after cellular exposure to CPT (67,68). This relocalization has been associated with SUMO (small ubiquitin-like modifier) modification of TOP-I (69). This event may decrease TOP-I–DNA interactions and thus minimize TOP-I–mediated DNA damage induced by CPT (see further discussion in the following sections). Notably, altered localization of topoisomerase IIα (top2α) (as a result of loss of nuclear localization sequences) was identified in mammalian cell lines resistant to the top2-targeting drugs etoposide and mitoxantrone, which presumably results in resistance by decreasing interactions between the enzyme and DNA (70–73).

4. ALTERATIONS IN THE CELLULAR RESPONSE TO TERNARY COMPLEX FORMATION

CPT specifically targets TOP-I and induces the formation of CPT–TOP-I–DNA ternary complexes (74). CPT cytotoxicity is S-phase selective and can be ameliorated in cell culture by treatment with the DNA polymerase inhibitor, aphidicolin (75). Furthermore, collision of replication and transcriptional machinery with the ternary complex leads to double-stranded DNA breaks and is necessary for induction of cell death (74,76). However, relatively little is known about the pathways downstream to CPT-TOP-I-DNA ternary complex formation that ultimately lead to repair of DNA damage or cell death.

Studies in yeast and cell culture models have implicated several DNA replication, DNA damage checkpoint, and DNA repair proteins in the response to cleavable complex formation (Table 2). Cellular exposure to CPT results in the activation of S-phase checkpoint proteins, such as Chk1

Fig. 1. Schematic of resistance-conferring mutations of TOP-I. The catalytic Y723 is indicated, as are the three main clusters of mutations.
(77), ATR (78), ATM (79), and the DNA-dependent protein kinase (DNA-PK) multimer (79,80). Furthermore, loss of function of Chk1 or ATR is associated with increased cellular sensitivity to CPT (77,78). Several studies showed that loss of RAD9, a checkpoint protein that is activated by DNA damage and induces G2 arrest, enhances TOP-I-induced cell death (81,82).

In addition to checkpoint proteins, proteins involved in DNA replication are also involved in CPT cytotoxicity. A yeast screen for conditional mutants with enhanced sensitivity to TOP-I-mediated DNA damage led to the identification of the yeast replication proteins, Dpb11p and Cdc45p, as important determinants of CPT sensitivity (83). Dpb11p and Cdc45p are implicated in DNA polymerase switching from priming to processive replication (83). Also, studies in murine cells implicate the loss of the Werner syndrome protein in CPT hypersensitivity (84). The Werner protein is a helicase that interacts with TOP-I, and CO purifies with the DNA replication complex (85,86).

With regard to repair of CPT-induced DNA damage, both mismatch repair and base excision repair systems are implicated (Table 2). Cells lacking the mismatch repair protein, MSH2, are hypersensitive to CPT (87,88). Recently, Meijer et al. showed that a eukaryotic polynucleotide kinase, Pnk1, also plays a role in CPT-induced DNA damage repair, and cells

Table 2

<table>
<thead>
<tr>
<th>Process mutated gene</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Checkpoint ATM, MEC1, MEC2</td>
<td>79,81,115</td>
</tr>
<tr>
<td>ATM</td>
<td></td>
</tr>
<tr>
<td>ATR</td>
<td>78</td>
</tr>
<tr>
<td>CHK1</td>
<td>77</td>
</tr>
<tr>
<td>RAD9, RAD17</td>
<td>81,82</td>
</tr>
<tr>
<td>DNA repair CAS, CSB</td>
<td>116</td>
</tr>
<tr>
<td>RAD6</td>
<td>81</td>
</tr>
<tr>
<td>TDP</td>
<td>90</td>
</tr>
<tr>
<td>TRF4</td>
<td>117</td>
</tr>
<tr>
<td>MSM2, MSM3, MSH2</td>
<td>87,88,118</td>
</tr>
<tr>
<td>XRCC</td>
<td>91</td>
</tr>
<tr>
<td>PNK1</td>
<td>89</td>
</tr>
<tr>
<td>DNA replication CDC45, DPB11</td>
<td>83</td>
</tr>
<tr>
<td>WRN</td>
<td>84</td>
</tr>
<tr>
<td>Ubiquitylation/sumoylation UBP1</td>
<td>102</td>
</tr>
<tr>
<td>DOA4</td>
<td>103</td>
</tr>
<tr>
<td>26S proteasome, ubiquitin</td>
<td>101</td>
</tr>
<tr>
<td>C9, SUMO</td>
<td>104,119</td>
</tr>
</tbody>
</table>
lacking this gene are hypersensitive to CPT (89). Additionally, Nash and colleagues identified a tyrosine-DNA phosphodiesterase that specifically cleaves TOP-I that is covalently linked to DNA (90). Studies in yeast indicate that loss of tyrosine-DNA phosphodiesterase in the presence of mutant RAD9 confers hypersensitivity to CPT (90). Importantly, although most of these studies indicate that loss of function of a DNA repair protein enhances cellular sensitivity, to date only a single study has demonstrated that overexpression of a DNA repair protein confers CPT hypersensitivity. Park et al. have shown that overexpression of a protein involved in base excision repair, X-ray repair cross-complementing gene I protein (XRCC), leads to CPT resistance in cells (91).

Cellular processes, such as apoptotic pathways, downstream from DNA damage may also be important in the resistance to CPT (92). Studies have shown that proapoptotic proteins, such as p53 and Bax, are upregulated after CPT treatment, whereas bcl-2 expression is decreased (93). Additionally, CPT resistance has been associated with downregulation of apoptotic pathways involving Bcl-2, caspases, Akt, necrosis factor-κB (NF-κB), and transforming growth factor (TGF)-β (94). Overexpression of bcl-2 and p21Waf1/Cip1 have been associated with relative resistance to CPT (92,95). Furthermore, CPT treatment of cells leads to the activation of caspases and cleavage of TOP-I, a substrate for caspase-3 (93,96). Suppression of NF-κB leads to reduced CPT cytotoxicity by a p21-dependent mechanism (97) and proteasome inhibition-mediated stabilization of NF-κB is associated with enhanced CPT cytotoxicity (98). Finally, a recent study found that HER2 and HER3 cause a phosphoinositide-3 kinase–dependent activation of AKT that leads to CPT and multidrug resistance (99). Taken together, these data indicate that proapoptotic and antiapoptotic proteins may regulate the cellular response to CPT.

Recently, posttranslational modifications of TOP-I were reported after CPT treatment of cells and may be involved in resistance. TOP-I is ubiquitylated and degraded after cells are treated with CPT, which appears to occur in the context of the ternary complex rather than free TOP-I (100). Recently, tumor cells deficient in CPT-induced TOP-I downregulation were found to be more sensitive to CPT, implicating ubiquitylation of TOP-I as an important determinant of cellular sensitivity (101). Additionally, CPT-induced TOP-I–DNA covalent complex formation results in transcriptional arrest and 26S proteasome-mediated degradation of TOP-I and the large subunit of RNA polymerase II. Degradation of the transcriptional machinery then initiates transcription-coupled repair. Furthermore, recovery from transcriptional arrest depends on degradation of TOP-I and functional transcription-coupled repair, affecting cellular sensitivity to CPT (76). In yeast, two proteins related to the ubiquitylation pathway were discovered using genetic screens for mutants that alter CPT sensitivity. Overexpression
of a ubiquitin specific protease, Ubp11, confers resistance to TOP-I–mediated DNA damage (102) and loss of DOA4, a 26S proteasome-associated C-terminal ubiquitin hydrolase, sensitizes cells to TOP-I–mediated DNA damage (103).

TOP-I is also modified by SUMO after CPT treatment (104). Sumoylation of TOP-I is associated with relocalization of the protein from the nucleolus to a more diffuse nuclear pattern after CPT treatment (69), whereas, TOP-I mutants that cannot be sumoylated remain more concentrated in nucleoli of cells even after CPT treatment (105). Together these studies strongly suggest that sumoylation regulates TOP-I localization in the nucleus and that sumoylation of TOP-I may function to decrease TOP-I–DNA interactions and thus minimize TOP-I–mediated DNA damage induced by CPT.

Although the mechanisms related to CPT-induced TOP-I ubiquitylation and sumoylation are unknown, recent studies of a TOP-I–binding protein, named topors, suggest that this protein may be involved (106). Topors is a RING protein that functions in vitro as an E3 ubiquitin ligase (107) and as an E3-type SUMO ligase (108). Moreover, topors sumoylates TOP-I in vitro (108). In cells, topors is associated with promyelocytic leukemia (PML) nuclear bodies and rapidly disperses to a diffuse nuclear pattern on cellular treatment with CPT, similar to TOP-I (109). It is possible that topors plays a role in the cellular response to CPT, and regulates the function of TOP-I after CPT-induced DNA damage (Fig. 2).

5. RESISTANCE TO CPT IN THE CLINICAL SETTING

More studies of clinical specimens are needed to determine whether the resistance mechanisms detected in yeast and cell culture models are clinically relevant. In addition, cellular metabolism, via carboxylesterases and UGTs, plays an important role in the cytotoxicity of CPT-11 in cell culture models. Little is known regarding the clinical relevance of this finding, although varied carboxylesterase activity has been reported in clinical specimens (13,14). BCRP seems to be expressed at low levels in breast cancer cells and leukemic cells (37,38). Recently, BCRP protein expression was found to increase in leukemia cells obtained from patients following infusion of topotecan and arabinoside-C in patients (40). To date, altered expression of BCRP in clinical samples has not been proven to correlate with altered CPT sensitivity.

There is a limited number of clinical studies that have analyzed clinical specimens (tumor tissue or surrogates) for mutations in TOP-I and most have yielded negative results (51,110). Recently, Tsurutani et al. reported a TOP-I mutation in a tumor specimen from a patient with large-cell carcinoma of the lung (45). The mutation results in two changes, a stop codon at position 736 and a glycine to serine missense mutation at codon 737. Inter-
estingly, the patient with this mutation did not respond to a chemotherapy regimen consisting of cisplatin and irinotecan (45). However, it remains to be determined if these mutations result in enzymatic resistance to CPT.

Other studies using clinical specimens found alterations in TOP-I and TOP-2α levels after treatment with CPT. Analyses in clinical study of 11 patients with nonhematological malignancies treated with oral CPT for 14 days showed decreases in TOP-I protein levels in nonmalignant peripheral blood mononuclear cells (PBMCs) that were not the result of cleavable complex formation, suggesting that TOP-I is degraded after CPT exposure in nonmalignant cells (111). Analyses in a similar clinical study of nonma-
lignant PBMCs in patients treated with a 72-hour infusion of 9-amino-
camptothecin (9-AC) also indicated decreases in TOP-I protein levels at 48
or 72 hours in two of three patients (112). In contrast, two of four patients
with leukemia who were treated with a 72-hour infusion of 9-AC showed no
change in TOP-I protein levels in their malignant blast cells at 48 or 72 hours
(113), suggesting that if TOP-I degradation occurs in these cells, the timing
is distinct from that of nonmalignant PBMCs. It is possible that the apparent
difference in 9-AC–induced TOP-I degradation may relate to alterations in
ubiquitin-proteasome pathways in malignant versus nonmalignant cells.
These findings are consistent with the observation that malignant and non-
malignant cultured cells differ in their capacity to degrade TOP-I (101).
Interestingly, topors protein and mRNA expression are decreased in
tumor tissues versus matched normal tissues, suggesting that topors may
be involved in the apparent differences in CPT-induced TOP-I degradation
in these tissues (114).

Other mechanisms of CPT resistance that have been identified in yeast
and cell culture models need to be evaluated clinically, including the role of
TOP-I localization and specific repair processes. Pharmacogenetic and bio-
chemical understanding of clinical CPT resistance will improve the use of
CPTs in the treatment of malignancy.

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