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Review

New Molecular Mechanisms of Action of Camptothecin-type Drugs

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Abstract. *Camptothecin (CPT) derivatives have emerged as a promising group of chemotherapeutic agents. The FDA has approved the CPT derivatives topotecan and irinotecan for second line treatment of ovarian cancer and metastatic colorectal cancer, respectively. These and other CPT derivatives have become part of the multi-million dollar industry that is dedicated to finding better chemotherapeutic agents with excellent tumor kill and less normal tissue toxicity. In order to reach this goal it is imperative to understand the details of the mechanisms of action and the targets of these drugs, as well as the cellular response to the drugs. Although investigations of CPT date back to the 1960's, most of the studies that have been added to our present knowledge were done in the last 10 years. The purpose of this paper is to review the latest insights into the CPT binding site, CPT-induced gene expression and CPT-induced pathways to apoptosis.*

The Camptothecin Molecule

Camptothecin (CPT) consists of five rings. The E ring, which is considered to be the most chemically reactive ring of the molecule, undergoes an equilibrium reaction between an active lactone ring and a less active carboxylate form. The original molecule was isolated from the *Camptheca acuminata* tree in China and was used in Chinese medicine. In 1958, Wall *et al.* were the first western scientists to isolate the compound (1). The original clinical trials proved camptothecin to have potent anti-cancer properties, but it was too toxic for practical use. Since then modifications have been made to the parent molecule in order to increase efficacy and decrease toxicity (2).

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The Binding Site of the Cleavable Complex

The anti-tumor activity of CPT and its derivatives is mainly due to its interaction with Topoisomerase 1 (TOP1), a ubiquitous enzyme involved in the regulation of DNA topology during replication, recombination and transcription. TOP1 forms a phosphotyrosine bond with DNA, catalyzing a forward reaction in which DNA is cleaved to allow unwinding, and a reverse reaction in which DNA is religated (3). CPT interferes with the religation step of this process by reversibly binding to and stabilizing the enzyme/DNA complex (4).

Scientists combined crystallographic observations with chemical and biochemical information to design a model for the binding of CPT within the three-dimensional TOP1-DNA complexes. X-ray crystallography revealed that the TOP1 enzyme resembles a clamp with two arms connected by a hinge. In this study it was discovered that the core of the enzyme contained three subdomains, with subdomains I and II on one side and subdomain III on the other. The DNA fitted in the grooves in the core of the enzyme, while subdomains I and III formed a salt bridge with DNA to completely envelop it. The spatial organization of the enzyme around DNA allowed the active site Tyr^{123} to form a covalent phosphodiester with the 3' end of the scissile DNA strand. It was hypothesized that CPT binding took place by displacement of the nucleotide that was at the 1+ base pair downstream from the cleavage site, allowing CPT to occupy the space (5, 6).

Further investigations have added information to the binding mechanism of CPT. A study performed with Topotecan revealed that the CPT analog, acting as a uncompetitive inhibitor, intercalated between the upstream (–1) and downstream (+1) base pairs at the DNA cleavage site. The intercalation resulted in a shift of base pairs and displacement of the 5'-OH strand away from the phosphotyrosine bond thus blocking religation. This binding

occurred whether E ring of Topotecan was in the closed lactone form or the open carboxylate form; however, a higher occupancy rate (63%) was seen with the lactone form. Further understanding of the interaction between the moieties on the CPT molecule and specific amino acids in the core of TOP1 may lead to appropriate modifications of the CPT rings to improve binding and cell kill (7).

Support for this configuration comes from studies looking at differences in cell sensitivity to CPT based on modifications made to the DNA molecule at the cleavage site. One study demonstrated that the presence of the N^2-ethyl-2'-deoxyquanosine (N^2-ethyl-dG) adduct (a biproduct of alcohol metabolism) enhances the CPT interaction with the TOP-DNA complex, especially when the adduct was located at the 1+ position. The O^6-methylguanine (O^6MG) molecule also increases cellular sensitivity to CPT. It is thought that N^2-ethyl-dG and O^6MG inhibit the religation reaction by sterically blocking the dissociation of CPT from the cleavable complex (8, 9).

Previous studies have demonstrated that the chemical nature of the CPT molecule leads to binding at specific DNA sequence sites. Jaxel *et al.* found that CPT intercalated with the cleavable complex preferentially when guanine was located immediately 3' to the cleavage site. In the absence of CPT, TOP1 did not show the same sequence specificity for cleavage sites (10). A study investigating the presence of CPT-TOP1-DNA complexes in the telomeric regions of DNA found that TOP1 cleaved a specific G-rich nucleotide sequence, 5'GGGTT ↓ AGGGTT3', in the presence of CPT. The C-rich telomeres did not have these same sequences, nor did they undergo DNA cleavage by CPT. It was observed that binding of CPT on the G-rich telomeres led to apoptosis (11). Investigations into differences in telomere length in different cell types may help scientists to understand differences in sensitivity to CPT analogs.

Camptothecin, Topoisomerase I and S-phase Dependent Cell Death

Once the CPT molecule has intercalated into the TOP1-DNA complex (cleavable complex), the collision between the complex and the replication fork during S-phase is thought to result in DNA double strand breaks (DSBs) that eventually lead to cell death (Hsiang *et al.*, 1989). In fact it has been demonstrated that CPT-induced cell death does not occur in the presence of aphidicolin, a DNA polymerase inhibitor. Furthermore, studies have shown that the cleavable complex must be bound to the complementary strand of DNA synthesis in order for the potentially lethal collision to occur (12, 13).

Goldwasser *et al.* demonstrated that the factor that best correlates with the variability in growth inhibition between CPT-exposed cell lines is the formation of cleavable complexes; however, some cell lines displaying marked differences in growth inhibition had minimal differences in cleavable complexes. This study suggested that CPT cell kill was dependent on both the formation of the cleavable complex and a downstream mechanism (14). A study performed with SN-38, the active metabolite of irinotecan, suggested an immediate and a lagging phase of DNA DSBs of which only the former was inhibited by aphidicolin (15). The observed lagging phase supports observations that other processes besides collision with the replication fork lead to DNA DSBs and eventually apoptosis.

Alternative Pathways Leading to Cell Death

In addition to the S-phase dependent pathways, S-phase independent pathways have also been elucidated. One pathway is related to interference with transcription. Upon exposure to CPT one of the primary responses noted is rapid cessation of RNA synthesis (16). The blocking of RNA synthesis is thought to occur downstream from the promotor region, only when the cleavable complex forms on the template strand (17, 18). Additionally, Masesso *et al.* demonstrated induction of chromosomal aberrations in the G2-phase related to RNA polymerase. Cell lines, in which RNA synthesis was inhibited, showed a marked decrease in the chromosomal aberrations (19).

Other S-phase independent pathways unrelated to RNA synthesis have also been observed. In one study, neuronal cells were exposed to CPT with the hypothesis that CPT would not be toxic to differentiated, non-mitotic cell lines. Contrary to the hypothesis, neuronal cell lines underwent apoptosis when exposed to CPT. Apoptosis also occurred in the neuronal cells, when exposed separately to agents blocking DNA polymerase, RNA transcription, and DNA synthesis, suggesting a mechanism of cell death independent of S-phase. Further studies need to be done in order to gain full understanding of this mitosis-independent pathway (20).

Another possible mechanism of cell death by CPT is by blocking angiogenesis. One study showed that low doses of Topotecan delivered to a xenograft model of human hepatoblastoma resulted in increase of the endothelial cell apoptosis. This finding may be promising in developing an effective chemotherapeutic agent for advanced pediatric hepatomblastoma (21). Another study demonstrated inhibition of endothelial cell proliferation when exposed to SN38. Additionally, SN38 caused decreased expression of HIF-1 and VEGF, potent mediators of angiogenesis, in a time and dose-dependent manner (22).

Gene Expression and Apoptosis

Several studies have added insight into how CPT leads to apoptosis. Hayward *et al.* showed that SN38 produces an

increase in production of pro-apoptotic factors such as p53, Bax, Bcl-xl, and p-21/WAF-1 in colon cancer cells (23). Up-regulation of the pro-apoptotic proteins bax, bf11, bak, pRb2, c-jun and jun-b, and down-regulation of the cell cycle proteins cdk4, cyclinB1, wee1, CRAF-1 and DP1 has been observed in human myeloid leukemia cell lines exposed to CPT (24). In accordance with these observations, resistant cell lines demonstrate up-regulation of antiapoptotic factors. One study showed that CPT-induced DNA-damage resulted in activation of NF-κB, an anti-apoptotic factor, in resistant cell lines. The expression of NF-κB was dependent on DNA DSBs created during S-phase. It was demonstrated that CPT-induced apoptosis was enhanced when NF-κB was inhibited (25). Another study examining gene expression showed that genes involved in both NF-κB transactivation and epidermal growth factor receptor (EGFR) signaling pathways were important for cell survival after CPT exposure (26).

Several studies have implicated the cell cycle checkpoint pathways ATR/Chk1 and ATM/Chk2 in the cellular response to CPT (27, 28). A study by Flatten *et al.* suggested that differential expression of genes associated with the ATR/Chk1 pathway may play a more important role. Cell hypersensitivity to SN-38 was related to down-regulation of Chk1, but not Chk2 (29). Wang *et al.* also demonstrated the importance of ATR/Chk1 in the S-phase checkpoint response observed in cells exposed to CPT and suggested that the collision with the replication fork rather than the DSBs activated ATR kinase. Ku80 appeared to be an important regulator of ATR binding to chromatin for the S checkpoint, because cells deficient in Ku80 showed higher activity of ATR/Chk1 (30).

Differences in the cellular response to CPT may depend on the cell cycle position. In one study accumulation of aphidicolin-dependent expression of p53 in the nucleus and induction of Bax was seen in S-phase cells, while aphidicolin independent p53 expression and induction of p21WAF were observed in G1-phase cells. This information may lead to further understanding of why some cell lines demonstrate sensitivity to CPT and undergo apoptosis, while other cell lines are more resistant and repair the DNA damage (31).

In an attempt to better understand the differences in gene expression, Mariadason *et al.* used gene array analysis to identify 130 genes that correlated positively and 178 that correlated negatively with CPT-sensitivity in colon carcinoma cell lines. In comparing the response of cell lines to CPT and 5-FU and the gene profiles of each agent, there was correlation of 32% and 24%, respectively. This study highlights the complexity of the interaction between cell processes and chemotherapeutic agents. Not only is the inherent mechanism of action of the chemotherapeutic agent important, but equally as important is the universal response of the cell to these agents. Gene profiling may help physicians to decide which chemotherapeutic agents work best with specific cell types (32).

Mechanism of Repair Response

Research indicates the cells respond to CPT-induced damage by destroying the cleavable complex. It is thought that the TOP1 in the cleavable complex undergoes multi-ubiquination, which in turn stimulates destruction *via* a 26S proteasome-dependent pathway. Interestingly, one study showed that although 90% of the cellular TOP1 was trapped in the cleavable complex, only 10% was conjugated to ubiquitin (33).

Results of another study by Desai *et al.* revealed that collision of the cleavable complex with RNA polymerase II is an important component in the initiation of topo1 degradation, and that the collision is linked to the ubiquination and subsequent 26S proteasome-dependent degradation of both TOP1-DNA complexes and RNA polymerase. The degradation of the cleavable complex and RNA polymerase is thought to be an important step preceding transcription coupled repair of the DNA single strand breaks (34).

Other proteins have been implicated in the TOP1 degradation process. It has been demonstrated that CPT stimulates conjugation of SUMO-1 (small ubiquitin-like modifier) to TOP1 when it is bound to DNA. SUMO-1 shares a sequence homology of 18% with ubiquitin and is associated with unique E1 and E2 enzymes, particularly the UBC9 E2 enzyme. Human DNA TOP1 expressing yeast cells with mutant UBC9 are hypersensitive to CPT. The details of the UBC9/SUMO-1 pathway are still unclear, but it is thought to be related to the repair of TOP1 mediated DNA damage (35). Cullin 3 (Cul3) is another component of the TOP1 degradation pathway. In one study, Cul3 overexpression was related to an increase in the degradation of covalent complexes and a reduction in cell death without identifiable change in basal TOP1 levels. Assessment of Cul3 expression in human tumors may help to determine response to CPT derivatives (36).

As the cell removes the TOP1 it also attempts to repair the damage. Collision of the replication fork with the cleavable complex and the subsequent formation of DSBs are thought to induce phosphorylation of H2A (γH2AX). A study by Furuta *et al.* demonstrated that the up-regulation of γH2AX in the presence CPT trapped cleavable complexes during S-phase resulted in the recruitment of repair genes, MRE11, Rad50 and Nbs1. Cells that were deficient in H2AX showed increased sensitivity to camptothecin (37).

The phosporylation of replication protein A2 (RPA2) may be another process that regulates cellular response to CPT-induced DNA damage. When human colon cancer cells were exposed to CPT, extensive (30-50% of total RPA)

and rapid phosporylation of RPA2 through the DNA-PK pathway was observed. Cells deficient in DNA-PK demonstrated less RPA2 phosphorylation, less DNA synthesis arrest and were more sensitive to CPT. It is thought that the phosphorylation of RPA2 is an important signal for the repair response (38).

The DNA DSBs can be repaired by the cell through homologous repair (HR) or nonhomologous end joining (NHEJ). The NHEJ pathway may play a role in CPT-induced cell death. Adachi *et al.* found that cells lacking the DNA ligase IV and KU70 (important proteins in NHEJ) were resistant to CPT (39). Alterations in mismatch repair (MMR) may also affect cellular response to CPT-induced DNA damage. Certain colorectal cancer cell lines have inactive MMR genes and microsatellite instability (MSI). These cell lines have also displayed increased sensitivity to CPT and significant decrease in ability to repair non-complementary DSBs through NHEJ. The researchers suggested that the NHEJ may cause unfavorable joining of DSBs that ultimately lead to cell death, but felt that this was not the sole explanation for the CPT hypersensitivity seen in MMR deficient cells. Although the exact mechanism of CPT sensitivity in these colorectal cell lines is unclear, screening for MSI may allow better selection of patients who could benefit most from treatment with a CPT analog (40, 41).

Conclusion

The last 10 years of research on the mechanisms of action of camptothecins have yielded an amazing amount of information. X-ray crystallography studies have revealed that the three-dimensional structure of topoisomerase I resembles a clamp with multiple domains that interact to encase DNA and allow for the formation of a phosphodiester bond between the enzyme and DNA. CPT intercalates into the TOP1-DNA complex by intercalating between the upstream and downstream base pairs of the DNA cleavage site. Scientists have been able to manipulate different aspects of the chemical interactions at the cleavage site to change the sensitivity of cells to CPT. Once the cleavable complex is stabilized by CPT, it interacts with various cell processes including DNA replication and RNA transcription. These interactions stimulate gene expression and a cascade of reactions leading to cell death. The presence of CPT stimulates the cell to respond to the damage by destroying TOP1 through a 26-S proteasome-pathway. Although these studies have added greatly to the general understanding of the mechanisms of action of CPT, they stimulate more questions as to the nature of the variability in the pathways between cells. Hopefully in the future, this knowledge will aid physicians in choosing the right chemotherapeutic agent based on cell characteristics and individual gene expression.

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