Inhibitors of topoisomerase: Problems and prospects

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DNA topoisomerases, which solve topological problems associated with various DNA transactions, are the targets of many therapeutic agents. Various topoisomerase inhibitors especially, topo-poisons, camptothecin (topo-I) and etoposide (topo-II) are some of the drugs that are used in the current treatment protocols, particularly for the treatment of leukemia (AML, ALL etc). However, tumor resistance, normal and non-specific tissue cytotoxicity are the limitations for successful development of these drugs as one of the primary therapeutic agents for the treatment of tumors in vitro. This brief review presents the current understanding about cytotoxicity development and outlines various approaches to overcome the limitations for enhancing the efficacy of topo-poison based anticancer drugs.

Keywords: Camptothecin, Cleavable complex, 2-Deoxy-D-glucose, Etoposide, Topoisomerases

Topoisomerases are ubiquitous and evolutionarily conserved nuclear enzymes, which modulate the topological state of DNA by passing an intact DNA helix through a transient single (topo-I) or double (topo-II) strand break, facilitating vital nuclear transactions such as transcription, replication, chromosome segregation, mitotic division etc. Since the level of topoisomerases is generally elevated in malignant (cancer) and actively growing cells, these enzymes have been the specific targets of several anti-tumor drugs. Currently used topoisomerase inhibitors exert their cytotoxic effects either by stabilizing the covalent complexes between the enzyme and DNA (cleavable complex) or by inhibiting the catalytic activity (Fig. 1). Despite the development of specific and more effective inhibitors, with enhanced clinical activities, drug resistance by tumors as well as lack of specificity resulting in normal tissue toxicity has continued to limit the success of cancer therapy using topoisomerase poisons. Therefore, there is a great need to develop newer approaches, which selectively enhance the drug-induced toxicity in cancer cells.

One of the approaches to achieve this objective would be to synthesize novel, sequence specific DNA ligands, with associated topoisomerase inhibitory activity, that exhibit differential effects between tumor and normal cells. On the other hand, approaches that selectively modify the drug accumulation and consequences of drug-induced primary lesions in tumor cells, while sparing cells from the normal tissue, could be equally effective. Pre-target events including intracellular drug accumulation and distribution, drug-target interactions influenced by enzyme levels, sequence specificity and activity, status of nuclear organization etc., and post-target events like macromolecular synthesis, DNA repair, cell cycle perturbations and apoptosis, collectively contribute to the drug-induced cytotoxicity. Therefore, an understanding of the molecular mechanisms involved in the repair/reversal of cleavable complexes as well as the interactions of topoisomerases with DNA repair machinery, besides the induction of cell cycle perturbations and apoptosis will greatly facilitate the optimal use of topoisomerases as anticancer drugs.

Topoisomerases as molecular targets for anticancer therapy—The long stretch of DNA in eukaryotic cells is packed very efficiently to fit into nucleus as a result; the chromosomal DNA is twisted extensively. Therefore selected regions of DNA need to become sufficiently untangled and relaxed to allow vital nuclear transactions viz., transcription, replication, chromosome strand separation, mitotic division, recombination and chromatin remodeling. Topoi-
Topoisomerases maintain the topology and regulate the dynamics of DNA structure by not only relaxing, unknotting, interlinking of strands, decatenation of gapped or nicked DNA circles but also by performing catenation and knotting to facilitate the condensation and packing of DNA into higher order structure. In addition, these enzymes fine-tune the steady-state level of DNA supercoiling both to facilitate protein interactions with DNA and to prevent excessive supercoiling that is deleterious. Topoisomerases are known that specifically relax only negative supercoils, that relax supercoils of both signs or that introduce either negative (bacterial DNA gyrase) or positive supercoils into DNA (reverse gyrase). These enzymes accomplish this feat by either passing one strand (type-I subfamily) or by passing a region of duplex from the same or a different molecule through a double strand gap generated in DNA (type-II subfamily) followed by religation of the strand break. Several studies have demonstrated that topo I activity is sensitive to physiological, environmental and pharmacological DNA modifications and can act as a specific mismatch and abasic site nicking enzyme. Recently, increased in vitro cleavage by topo II has also been reported following the introduction of abasic sites into DNA substrates, analogous to PARP, which binds to single strands breaks and initiate DNA repair.

Topo II α level changes during the cell cycle whereas, topo II β and topo I levels remain essentially similar throughout the cell cycle and distributed homogenously throughout the nucleus. More recent studies have mapped the enzyme linked cleavable complexes on the DNA loops bound to the nuclear matrix at matrix attachment regions (MARs). Topo II β and topo I remained completely extractable throughout the cell cycle, while scaffold associated topo II α does not appear to be involved in catalytic DNA turnover, although it may play a role in replication of centrioles, where it accumulates during M phase.

Levels of topoisomerases are generally elevated in malignantly transformed (cancer) and actively growing cells as compared to the non-malignant tissue, which is partly attributed to the higher rate of DNA replication, transcription and other cellular processes in transformed cells for a higher rate of growth and proliferation. Increased demand for a rate of cellular proliferation increases the topo levels manifolds (up to 200 folds), thereby allowing a differential sensitivity of tumors to drugs targeting topoisomerases as compared to normal cells. Higher levels of topo pool sizes has also been attributed to the over-expression of topoisomerase gene as a result of mutation during the process of transformation, although in most tumor types it appears to be as a result of higher requirement in proliferating tumor cells. However, topo pool sizes vary among different types of tumors, albeit to a lesser extent and this marked heterogeneity of enzyme levels has been correlated with drug sensitivity.

Fig. 1—Action of topoisomerase I and II and their inhibition
Higher levels of topoisomerase being a selective and differential marker of transformed cells make these enzymes an attractive molecular target for the development of antineoplastic chemotherapy. Currently several anti-tumor drugs like etoposide, doxorubicin, teniposide and camptothecin specifically target topoisomerase enzyme.

Types of topoisomerase inhibitors and development of cytotoxicity—Topoisomerase inhibitors include catalytic inhibitors of the enzyme and topoisomerase poisons. Topoisomerase poisons exert their cytotoxic effects by stabilizing the covalent complexes between enzyme and DNA (cleavable complex). These compounds interfere in the religation step of the enzyme catalysis, thereby, leaving the DNA strand breaks unligated. The protein-DNA strand breaks thus created are not efficiently repaired and induce apoptosis. On the other hand, the catalytic inhibitors inhibit the enzyme catalysis activity by not allowing the enzyme to function itself and therefore do not allow topoisomerases to create strand break. Several compounds, membrane, suramin, bisdioxopiperazines (ICRF), are known catalytic inhibitors of topoisomerase, while epipodophyllotoxins like etoposide (topo-II inhibitor) and camptothecin (topo-I inhibitor) are well known topoisomerase poisons. However, these compounds differ in the mechanism of their action as well as the cytotoxicity, for example, ICRF-193 does not have a significant cytotoxicity, while topoisomerase poisons like etoposide and camptothecin (CPT) have considerable amount of cytotoxicity. Not only the concentrations of DNA-protein cross links, but their life-time and stability also contribute to the cytotoxicity. Hypersensitive response to CPT induced by Cockayne’s syndrome has been correlated with the increased life-time of double strand breaks (DSBs) and cleavable complexes, predominantly at the site of replication in nascent DNA. Although, cleavable complexes are formed in all phases of cell cycle, S-phase cells are found to be more sensitive particularly to topo poisons. There is enough evidence that nascent DNA binds to the nuclear matrix is an important site of cleavable complex formation, and these complexes inhibit DNA synthesis by blocking the movement of nascent DNA away from replication sites on the nuclear matrix. It is well established that topo poisons mediated stabilized cleavable complexes collide with replication fork leading to the formation of lethal double strand breaks and hence causes cytotoxicity. On the other hand, collision of topo poisons mediated stabilized cleavable complexes with transcription bubble leads to the generation of gapped DNA fragments and hence the formation of single strand breaks.

Earlier studies have suggested the stabilization of cleavable complexes as a prerequisite for cytotoxicity of topoisomerase poisons with a direct correlation between cleavable complexes and cytotoxicity, as well as indirect evidences relating the rate of repair and the formation of cleavable complexes in the damage response. Many studies have revealed that the damage by topo poisons is processed similar to frank double strand breaks; but there are also evidences to believe that DNA-protein cross-links are probably handled differently. An unequivocal damage response pathway to topo poison induced cleavable complexes has not emerged so far.

DNA damage by UV irradiation and photoproducts formation has shown an increase in the levels of topo I-DNA complexes and p53 simultaneously. Although, the role of p53 in response to DNA damage has been established, its interaction with endogenous topo I has also been implicated in repair of damaged genome. Recent studies have shown that although MCF-7 cells with wild type p53 showed an elevation in topo I-DNA complexes, p53 mutant SK-BR-3 cells and null HL60 cells do not show such induction. A physical interaction between p53 and topo I has also been investigated and recruitment of topo I by p53 has been proposed to cause an additional damage to genome, thereby committing cells to apoptosis.

Cellular responses to topo poisons—There are enough evidences to believe that, while the stabilized cleavable complexes can be repaired to a certain extent, moderate levels of the complex can mediate other cellular responses like cell cycle arrest, induction of apoptosis, mitotic cell death and senescence. However, necrosis and non-specific cytotoxicity are observed at higher doses of topo poisons. Indirect evidences have shown that cleavable complexes can be repaired by non-homologous end joining (NHEJ) repair pathway, while the role of homologous recombination is equivocal. Cells deficient in NHEJ repair pathway (Ku deficient) are more sensitive to topo poisons, which is correlated with higher amount of cleavable complexes. Cells deficient in double strand break (DSB) repair (ATM deficient) also sensitize them to topo poisons. Inhibition of patch strand repair also increases the cytotoxicity of...
topo poisons. The induced expression of DNA damage responsive genes DIN3 and RNR3 in TOP 1+ camptothecin treated yeast cells and deletion of RAD52 gene, which is required for recombinational repair of DSB, enhances cell sensitivity to camptothecin. The protein linked DSB doubled when incubated with 3-aminobenzamide (inhibitor of poly ADP ribose polymerase) in addition to camptothecin, shows that protein concealed strand breaks can be lethal lesions and intracellular activity of topoisomerase I and II may be regulated coordinately through poly ADP ribosylation.

Ubiquitin (E1 enzyme)/26S proteasome-dependent degradation of cleavable complexes has been suggested to be a unique repair process for the repair of topo I mediated DNA damage leading to its down regulation. Yong Mao et al. have shown that treatment of mammalian or yeast cells expressing human DNA topo I with camptothecin induces covalent modification of topo I by SUMO-1/Smt3p, a ubiquitin like protein. Since, Ubc9 mutant yeast cells expressing human DNA topo I are hypersensitive to CPT, it is suggested that UBC9/SUMO1 may be involved in the repair of topo I mediated DNA damage. The trimeric topo I-CPT-DNA cleavable complex seems to be the signal for SUMO1 conjugation with topo I, which is supported by the observation that majority of SUMO-1 conjugated topo I is covalently linked to DNA and mutant cell lines (CPT-K5 and U-937/CR) are defective in CPT induced SUMO-1 conjugation to topo I. Further, a ubiquitin mutant with substitution at Lys-63 appears to be deficient in DNA repair RAD6 pathway and also defective in its polymerization.

Brief exposure to topo poisons at moderate concentrations mediate a higher mitotic cell death in synchronized M phase cultures, leading to the formation of micronuclei, which correlate well with the amount of cleavable complexes. In asynchronous cell culture most of the topoisomerase inhibitors arrest the cell at G2/M cell cycle checkpoint, possibly facilitating the repair of cleavable complexes. G2/M cell cycle arrest primarily arises because of alteration in tyrosine dephosphorylation of p34cdc2 and cyclin B, leading to inactivation of cyclin B/p34cdc2 complex. Short exposure to topo poisons initiate ATM responsive p53 dependent, p21 and GAAD-45 mediated cell cycle arrest. However, DNA synthesis is required at the time of topo inhibitors treatment, since these cell cycle dependent changes are not observed when incubated simultaneously with aphidicolin (DNA synthesis inhibitor). Cleavable complex has also been found to induce cellular proliferation through induction of excision repair enzymes or otherwise p53/p21 mediated Bax synthesis and its translocation to the mitochondria resulting in membrane permeability transition leading to cytochrome c release culminating in apoptosis.

Topo poisons can also mediate senescence, which correlates to a cytostatic but not quiescent cell population resting in cell cycle blocks. Induction of p53 dependent expression of p21, which appears to be necessary for cell cycle arrest, can also induce senescence. These studies further substantiated that the loss of clonogenicity of cells does not necessarily result from loss of cell viability.

DNA degradation during etoposide induced apoptosis results in formation of high molecular weight DNA fragments, but not oligonucleosomal DNA fragments, which is accompanied by down-regulation of caspase-activated DNase (CAD). Further it is not affected by z-VAD-fmk (caspase inhibitor), suggesting that caspase is not involved in the excision of DNA loop domains. It appears that topo II is involved in caspase-independent excision of DNA loop domains during apoptosis, and represents an alternative pathway of apoptotic DNA disintegration different from CAD driven caspase–dependent oligonucleosomal DNA cleavage. Furthermore, there is evidence that several oncogenes including ras, myb and p53 interact with sequences within the top II α promoter.
Pre-clinical studies—Pre-clinical studies in different solid tumors have established etoposide as a primary treatment modality in lung tumors and leukemia especially in Hodgkin’s and non-Hodgkin’s Lymphoma. Complete response in 12% of small cell lung tumors, significant response in acute amyloid leukemia and Hodgkin’s lymphoma has also been observed. A overall response rate (14%) has been observed in combination with cisplatinum. Studies with human xenografts have shown an unprecedented efficacy for 9-aminocamptothecin with complete remissions in colon, breast and non small cell lung carcinoma as well as in melanoma cell lines.

Clinical studies—A clear association between topo II α and tumor cell proliferation has been established in many of the human tumors. Higher levels of expression of topo I has been observed in colon, ovary, prostate, lung and colorectal tumors as compared to the levels in the corresponding normal tissues. Clinical studies with topo II inhibitor, etoposide have reported good responses primarily in patients with Ewing’s sarcoma, Hodgkin’s disease, neuroblastoma, rhabdomyosarcoma, small cell lung cancer, testicular cancer, gestational choriocarcinoma, acute myelogenous leukemia and Kaposi’s sarcoma, besides a spectrum of recurrent malignant solid tumors. A composite single agent response rate of greater than 20% has been observed in some of these tumors. Topo inhibitors have also been used as one of the primary drugs among multi-drug therapy in lung, testicular cancer and leukemias. Combination therapy with cyclophosphamide and vincristine in recurrent and refractory pediatric tumors have shown 95% responses with 35% complete responses, 40% very good partial responses and 20% partial responses. Similarly, 86-95% responses have been observed in lung cancer in combination with cisplatin.

Approaches for overcoming limitations—Therapeutic efficacy of topoisomerase inhibitors is limited primarily by the resistance of tumors at lower doses and toxicity to the normal tissues at moderate to higher doses. Among several factors that contribute to the resistance of human tumors is decrease in intracellular drug availability due to multiple drug resistance (MDR) gene product, P-glycoprotein (Pgp) mediated drug efflux process appears to be common to many drugs, although camptothecin, a topo I inhibitor seems to be an exception. Besides drug efflux, alterations or mutations in drug’s cellular target or binding sites, distribution of cleavage sites, increase in efficient repair of drug-induced damage (cleavable complexes) and cross resistance to other structurally and mechanistically related drugs also contribute to the drug resistance. Lower levels of topoisomerase in slow growing tumors and reduction of its expression due to hypermethylation have been reported.

Ubiquitin mediated repair and mutations in critical proapoptotic and antiapoptotic regulatory proteins like p53 and Bcl2 family or alterations in cell cycle machinery, which activate checkpoints and prevent initiation of apoptosis can also be the cause of resistance. Coordinately regulated detoxifying systems, such as DNA repair, glutathione S-transferase and cytochrome P450 mixed-function oxidases can also confer resistance to drugs. Resistance can also result from defective apoptotic pathways. A brief summary of the factors that limit topo inhibitors as effective anti-cancer drugs and suggested approaches to overcome them is presented in Table 1.

Some of the topo II poisons induce acute and delayed toxicities in normal tissue that include bone marrow suppression, mild nausea or vomiting and hair loss with myelo-suppression being the dose limiting toxicity. Mucosities, liver toxicity, fever and chills are also observed with high dose regimens. Although, most of the side effects occur within 1-10 days of drug administration, delayed induction of secondary tumors mainly in the form of leukemias have been observed. De novo translocations leading to fusion proteins involving topo I and MLL break point cluster regions and transcriptional activation of short interspersed elements (SINES, like Alu elements) by...
topo II poisons have recently been implicated for induction of secondary malignancies. There has been a constant effort for the development of newer class of drugs with higher sequence specificity. Many different analogues of camptothecin CPT-11 have been evaluated in pre-clinical studies and are under clinical trials. Strategies using MDR inhibitors to enhance intracellular drug retention and agents that leads to induction of topo II levels eg. Hoechst-33342 etc are under investigations. Combinations of topo inhibitors with NF-kB inhibitors or GM-CSF to reduce myelo-suppression and related toxicities are currently under investigations. Combinations of cyclophosphamide with etoposide and vincristine for pediatric solid tumors have been in phase I/II clinical trial. Use of topo poisons also potentiates the radioimmunotherapy of tumors. Combinations of proteasome inhibitors PS-341 with topo poisons has also enhanced the efficacy of these drugs with little or no normal tissue toxicity in vitro are currently under clinical trials. Etoposide, ifosamide and cisplatin combination therapy has been in clinical use for refractory childhood solid tumors.

Energy-linked modification of cellular responses to topo poisons using the glycolytic inhibitor 2-deoxy-D-glucose—The repair of cleavable complex, the primary lesions responsible for cytotoxicity as well as the drug retention linked to Pgp pump mediated efflux process are both known to be energy dependent processes. Since cancer cells derive a major portion of the energy (ATP) through glycolysis, it has been suggested that inhibitors of glycolysis, like 2-deoxy-D-glucose (2-DG) may selectively enhance the cytotoxicity of topo poison in cancer cells.

Studies carried out in human glioma (BMG-1 & U-87) and squamous carcinoma (4197 & 4451) cell lines have shown that the cytotoxicity of etoposide, a topo II inhibitor, camptothecin, a topo I inhibitor and bisbenzimidazole derivative, Hoechst-33342 (H-342), an AT specific minor groove binding DNA ligand that inhibits both topo I and topo II can be significantly enhanced by 2-DG under certain conditions.

Presence of 2-DG (5 mM, equimolar to glucose concentration in the media) for 2-4 hr following exposure to the topo inhibitors enhanced the cell death by a factor of nearly 2 with all the three drugs (Fig.3), implying that 2-DG (in fact the metabolic stress created by 2-DG) facilitates certain common processes that are responsible for cytotoxicity of topo inhibitors. These include enhanced drug retention, inhibition of repair of cleavable complexes, manifestation of cytogenetic damage and up-regulation of damage dependent apoptosis as well as alterations in damage related cell cycle delays.

Interestingly, when 2-DG has been added along with the topo inhibitors, a small but significant decrease in the cell death is noticed in case of etoposide, while no effect is observed with camptothecin (Fig.3). Since functioning of topo II is ATP dependent, these observations suggest that 2-DG, induced fall in energy status (ATP), may reduce the formation of cleavable complexes if the two are added simultaneously or 2-DG before etoposide. Reduced etoposide toxicity in glucose regulated protein (GRP) induced cells following long exposure to 2-DG (4-6 hr) has also been reported. Irrespective of the mechanisms involved in reduced toxicity of etoposide by 2-DG, our results as well as earlier findings suggest that the efficacy of topo II inhibitors can be enhanced by 2-DG, only when 2-DG is added (administered) after the cells are treated with the inhibitor, i.e. when significant amounts of cleavable complexes have been formed.

Analysis of the DNA damage using the nuclear halo assay revealed that under these conditions the presence of 2-DG significantly enhanced the halo diameters of BMG-1 cells implying a higher level of DNA strand breaks under these conditions (Fig.4). Although, the mechanisms involved in the repair of cleavable complex are not completely understood, cells defective in double strand break repair are found to be more sensitive to topo II inhibitors.

Fig. 3—Effects of 2-DG (5mM) on the cytotoxicity of topoisomerase inhibitors etoposide, camptothecin and Hoechst-33342 in exponentially growing a human glioma cell line (BMG-1).
More recently, ubiquitination mediated protein (topoisomerases) degradation resulting in the formation of frank DNA strand breaks, facilitating the completion of repair by strand break repair pathways has been suggested^{35,43,45}. Therefore, it appears that 2-DG does not interfere in the conversion of cleavable complex into frank strand breaks, while it clearly inhibits the repair of strand breaks. Inhibition of DNA double strand break repair and excision repair following UV as well as ionizing radiation by 2-DG has been reported earlier^{37,92}.

Studies carried out to investigate the role of cytogenetic damage clearly show that, 2-DG enhances the topo inhibitor induced micronuclei formation by 40% and 60% respectively with etoposide and H-342, while a 20% increase has been observed with camptothecin (Fig.5 b,d). These observations are similar to the earlier results on the enhancement of radiation-induced micronuclei formation in tumor cell lines as well as short-term organ cultures of tumors^{88,89,92-95}. Modifications of the topo inhibitor induced cell death and micronuclei formation by 2-DG observed here (Fig.5d) imply that enhanced mitotic death is partly responsible for chemosensitization by 2-DG in these cells. Interestingly, it has also been found that 2-DG enhances the topo inhibitor induced delayed apoptosis, particularly in case of topo II inhibitor etoposide (Fig.5 c,e). However, no significant induction of early apoptosis can be observed under these conditions, implying thereby that the enhanced cytotoxicity is primarily due to an increase in the mitotic death.

Further investigations have also shown that 2-DG significantly enhanced the cell cycle delay induced by all the three topo inhibitors. Since the extent of cell cycle delay arising on account of the functioning check points^{47} is related to the level of DNA damage, the additional delay observed also indicates the presence of a higher level of DNA damage in cells treated with 2-DG following exposure to topo inhibitors.

Taken together, the available evidences so far indicate that the presence of 2-DG for a few hours following exposure to topoisomerase inhibitors can significantly enhance their cytotoxicity, by inhibiting the reversibility of cleavable complexes, the primary lesions responsible for the cell death. A higher level of DNA strand breaks accumulated under these condi-

![Image](https://via.placeholder.com/150)

Fig. 4—Effect of 2-DG (5mM, 2hr) on etoposide induced DNA damage studied by nuclear halo assay in a human glioma cell line (BMG-1). [a] Photo-micrographs of nuclear halo [b] Frequency distribution of halo diameters following various treatments.
Etoposide H-342 Camptothecin in
30:00
0:00
10
20
30
40
50
60

Fig 5—Photomicrographs of DAPI stained BMG-1 cells showing treatment-induced micronucleus (arrowhead) and apoptosis (arrow). [a] Control; [b] Etoposide (10 μM); [c] Etoposide (10 μM) - 2-DG (5 mM) in a human glioma cell line (BMG-1). Enhancement of etoposide induced micronuclei formation [d] and apoptosis [e] by 2-DG observed at 48hr after treatment are also shown.

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Future perspectives
Considerable progress has been made in the last decade towards the understanding of molecular and cellular mechanisms underlying the cytotoxicity of topoisomerase inhibitors, as well as natural and acquired tumor cell resistance to these drugs. These advancements should facilitate the design of new topo poisons as well as adjuvant biological response modifiers that selectively enhance the sensitivity of neoplastic cells. One of the challenges for the future is to develop predictive assays and to individualize therapy to benefit patients from the tumor specific therapeutic regimens. Further, development of appropriate in vitro and in vivo models to predict major toxicity is expected to facilitate the development of agents and strategies for ameliorating treatment induced toxicity with topo poisons.

In conclusion, topoisomerase inhibitors form an important class of anticancer drugs. They could be expected to play a greater role in the future, when limitations associated with their use are overcome by some of the approaches outlined here.

Acknowledgement
We are grateful to Gen T Ravindranath, Director INMAS for his keen interest and constant encouragement in this work. We thank Dr Seema Gupta and Dr Shailja Singh for helpful discussions during the preparation of this manuscript. These studies have been supported by DRDO project (INM-280), Ministry of Defence, Govt. of India. Mr. Rohit Mathur is thankful to CSIR, New Delhi for awarding JRF.
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INDIAN J EXP BIOL, JULY 2004


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