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Mutagenic Activity of Topoisomerase I Inhibitors

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Abstract

Topoisomerase I-directed agents are now in Phase I and II clinical trials and show great promise as potentially important agents for cancer chemotherapy. Because of their mechanism of action they may also be potential mutagens; however, their mutagenicity and oncogenicity still remain to be elucidated. We have previously shown that VP-16, a topoisomerase II-directed agent, induces sister chromatid exchanges and gene deletions and/or rearrangements in vitro. These observations may account for both the cytotoxic effects of topoisomerase II-directed agents as well as their recently reported leukemogenic potential. To evaluate the potential mutagenicity of topoisomerase I-directed drugs, we measured mutant frequencies at the hypoxanthine phosphoribosyl transferase locus of the V79 Chinese hamster fibroblast cell line treated with the topoisomerase I-directed drugs camptothecin and topotecan, and compared these results with mutant frequency obtained with the topoisomerase II-directed drug VP-16 and an alkylating agent, N-methyl-N’-nitro-N-nitrosoguanidine (MNNG). All of these drugs showed a dose-dependent increase in mutant frequency at the hypoxanthine phosphoribosyl transferase locus. At a dose producing approximately 30% survival, VP-16, camptothecin, and topotecan induced mutant frequencies of \(11.3 \times 10^{-6}\), \(4.9 \times 10^{-6}\), and \(2.7 \times 10^{-6}\), respectively, whereas the spontaneous mutant frequency at this locus was \(0.3 \times 10^{-6}\). In contrast, the alkylating agent MNNG produced a mutant frequency of \(562 \times 10^{-6}\) at 26% survival dose. The molar mutagenic potencies, expressed as mutant frequency/mol-h exposure, for VP-16, camptothecin, topotecan, and MNNG at approximately 30% survival dose were 0.9, 8.2, 2.3, and 56.8, respectively. On Southern blot analysis after EcoRI, PstI, or HindIII digestion, 6 of 12 independent thioguanine-resistant mutants induced by topotecan showed gene deletions or rearrangements. In contrast, five of five independent spontaneous mutants and six of six independent mutants induced by MNNG demonstrated the same restriction pattern as the parental V79 cells. These results indicate that the mutant frequency and the mutagenic potential of topoisomerase I and II active agents are quantitatively similar. The results further demonstrate that topoisomerase I and II active agents introduce mutations characterized by gene deletions and rearrangements, whereas spontaneous mutations and those induced by alkylating agents appeared to be more characteristically associated with point mutations. Thus, clinical use of the topoisomerase I and II active agents is expected to cause similar mutagenic effects that could potentially lead to secondary malignancies.

Introduction

The eukaryotic topoisomerases are nuclear enzymes that catalyze alterations in DNA tertiary structure required for replication, transcription, chromosome condensation, and chromosome separation (1–3). Their mechanism of action involves the transient formation of a DNA strand break in which the topoisomerase becomes covalently linked to the DNA, maintaining the separated ends of the strand break in approximation while another DNA strand is passed through the break. Following this process of DNA strand passage, the topoisomerase rejoins the separated ends of the protein cross-linked DNA strand break and dissociates from the DNA (1–3). This process can join or separate single- or double-stranded DNA circles, decatenate highly linked groups of closed circular DNA, and interchange DNA between supercoiled and relaxed circular forms. Topoisomerase I forms a break in a single strand of DNA and facilitates passage of another single strand through the break, whereas topoisomerase II forms a double-strand break and facilitates passage of a double strand of DNA.

In recent years, agents such as VP-16,1 VM26, Adriamycin, and 4’-(9-acridinylamino) methanesulfon-m-anisidine whose intracellular target is topoisomerase II have been shown to be highly useful in cancer chemotherapy (3–7). More recently, a group of agents has been identified whose intracellular target is topoisomerase I (8, 9). The latter includes camptothecin and its analogues, topotecan, CPT-11, and 9-aminocamptothecin. In preclinical studies and now in Phase I and II clinical trials, the topoisomerase I active agents show great clinical promise (10–12). The mechanism of action of both the topoisomerase I and II active agents involves formation of a ternary complex of drug, enzyme, and DNA that interferes with normal dissociation of the topoisomerase from the DNA so that the protein cross-linked DNA strand break is stabilized (13). The term “cleavable complex” has been applied to those protein cross-linked DNA strand breaks since upon treatment of cells with protein-denaturing agents, they are converted to a frank

1 The abbreviations used are: VP-16, etoposide; VM26, teniposide; α-MEM, α-modified Eagle’s medium; AML, acute myelocytic leukemia; CPT, camptothecin; HAT, hypoxanthine-aminopterin-thymidine; HPRT, hypoxanthine phosphoribosyl transferase; MF, mutant frequency; MMP, molar mutagenic potency; MNNG, N-methyl-N’-nitro-N-nitrosoguanidine; SCE, sister chromatid exchange; β-TG, 6-thioguanine.
DNA strand break. Stabilization or "freezing" of these cleavable complexes not only produces a disruption in DNA integrity but also interferes with the orderly progress of replication, transcription, DNA repair, chromosome condensation, chromosome separation, and other processes that require DNA topological alterations for their normal progress (1). Recent studies have shown that drug-induced cleavable complex formation is necessary but not sufficient to cause cytotoxicity (14). Thus, both topoisomerase I and II active agents are essentially non-toxic to nonreplicating cells, and cells need to undergo DNA synthesis to manifest maximum toxicity (14–16).

We previously suggested that chromosomal aberrations created during replication in the presence of topoisomerase II inhibitors may be the more proximal cause of cytotoxicity (14, 17). We postulated that recombination processes must be initiated to bypass the replication blocks created by VP-16-stabilized complexes of topoisomerase II and that some of these events are expected to involve aberrant, illegitimate, or nonhomologous recombination, leading to cytotoxicity and/or mutation. Thus, nonhomologous recombination that produces deletions of part or all of an essential gene could result in the loss of gene products and cell death, whereas aberrant recombination or rearrangement that results in deletion of a suppressor gene or activation of a proto-oncogene could produce a cell with a growth advantage that would lead to development of cancer. This hypothesis is supported by the demonstration of high levels of SCE, mutation, and gene deletions and rearrangements caused by treating cells with agents such as VP-16 and VM26 (17–20). More important, this proposal predicts the development of secondary cancers in patients treated with the topoisomerase II active agents. This prediction has, in fact, been realized for topoisomerase II active agents such as VP-16 which has recently been associated with the development of a characteristic form of AML (21–23).

Topoisomerase I active agents can be predicted to produce similar molecular alterations to those caused by topoisomerase II active agents and may, therefore, be expected to cause similar clinical consequences. Thus, camptothecin analogues produce DNA strand breaks as a result of collisions between moving replication forks and topoisomerase I-stabilized cleavable complexes with resultant DNA fragmentation and cytotoxicity (2, 9). This process may also lead to an increase in recombinational events which is consistent with the demonstration that camptothecin analogues have been shown to produce marked increases in SCE (24, 25). The present experiments were carried out to determine whether CPT analogues behave in an analogous fashion to the epipodophyllotoxins in producing DNA deletions and/or rearrangements which could indicate their potential for development of drug-induced secondary malignancies, such as acute leukemia. To further evaluate their potential for causing secondary cancers, we determined the camptothecin- and topotecan-induced mutant frequency and compared them to those induced by the topoisomerase II inhibitor VP16 and the highly mutagenic alkylating agent, MNNG.

**Materials and Methods**

**Chemicals.** VP-16 was a gift from Bristol Laboratories (Syracuse, NY). Topotecan (SK&F 10486) was kindly provided by SmithKline Beecham (King of Prussia, PA). Camptothecin, 6-TG, and MNNG were purchased from Sigma (St. Louis, MO). Stock solutions of VP-16, CPT, topotecan, and MNNG were prepared in DMSO at a concentration of 1 mg/ml and were diluted in α-MEM just before use. 6-TG was dissolved at a concentration of 1 mg/ml in 10 mM NaOH, then diluted into α-MEM buffered to pH 7.2 with 25 mM HEPES immediately prior to use.

**Cell Culture.** Chinese hamster V79 cells were maintained in α-MEM supplemented with 7 mM glutamine, 10% heat-inactivated FCS, and penicillin-streptomycin buffered to pH 7.2 with 25 mM HEPES. Before mutant selection experiments, cells were maintained for 2 weeks in α-MEM containing 0.1 mM hypoxanthine, 0.4 mM aminopterin, and 16 mM thymidine (α-MEM-HAT) to eliminate spontaneous HPRT– cells.

**Drug Treatment and Selection of HPRT– Mutants.** At least 1 × 10⁷ logarithmic phase V79 cells maintained in HAT medium were treated with various concentration of MNNG for 1 h or with VP-16, camptothecin, or topotecan for 24 h. Following drug treatment, cells were washed twice with medium, and then allowed an expression time of 10 days during which they were subcultured in complete medium every 2 days. Finally, 1 × 10⁷ cells were replated at the density of 1 × 10⁵/ml in fifty 75-cm² flasks (2 × 10⁶ cells/flask) and exposed to 2 µg/ml 6-TG for 14 days to select mutant cell lines deficient in HPRT. After 14-day exposure to 6-TG, the number of TG-resistant colonies containing 50 cells or more were counted microscopically without staining. For Southern blot analysis, we picked 12 TG-resistant colonies induced by 0.05 µM topotecan from 12 different flasks, and 5 spontaneous TG-resistant colonies from 5 independent experiments and 6 MNNG (1.5 or 1.0 µg/ml)-induced TG-resistant colonies from 6 independent experiments, then expanded them in α-MEM containing 2 µg/ml 6-TG. At the end of 10-day expression time, 100 cells from each drug-treated condition or nontreated cells were replated in 25-cm² flasks in medium without 6-TG. Cells were grown for 7 days and the number of colonies were counted after fixation and staining with a solution containing 3 g methylene blue and 1 g NaOH in 750 ml 0.9% NaCl and 250 ml 37% (w/w) formaldehyde.

As shown by O’Neill et al. (26), cloning efficiency of HPRT– mutant cells are reduced by coincubation of HPRT+ cells because active metabolites of 6-TG produced by HPRT+ cells are transferred into HPRT– cells and kill them. We therefore examined the effect of HPRT+ cells on the recovery of HPRT– cells during simultaneous treatment with 6-TG. We checked cloning efficiency of HPRT– cells seeded with various numbers of HPRT+ cells in 2 µg/ml 6-TG-containing medium. One hundred HPRT– V79 cells were plated with different numbers of HPRT+ cells (V79 cells maintained with HAT medium for 14 days). After a subsequent 14-day incubation in 6-TG-containing medium, we enumerated the number of HPRT– colonies as shown in Fig. 1 and used this graph to correct cloning efficiency for effect of HPRT+ cell density (26). Mf was calculated as follows. Mf = cloning efficiency in the presence of 6-TG (corrected for the effect of HPRT+ cell density)/cloning efficiency in the absence of 6-TG. Molar mutagenic potency (Mf at particular survival/[drug dose at same survival × h of drug exposure]) was calculated according to...
Clive et al. (27). Mf value at the HPRT locus was log transformed for statistical analysis. Mf values among spontaneous and drug-induced mutation were compared by Student’s t test.

**DNA Isolation and Southern Blot Analysis.** High molecular weight DNA was extracted from parental V79 cells and HPRT mutant cell lines arising spontaneously or drug-induced according to methods described previously (28). The DNA obtained was quantitated by absorption spectroscopy and 20 μg DNA was completely digested with the restriction enzyme, PstI, HindIII, or EcoRI ( Gibco-BRL, Gaithersburg, MD). The full length hamster HPRT cDNA was isolated from the plasmid PHT20, obtained from American Type Culture Collection ( Rockville, MD), after PstI digestion and labeled with 32P-dCTP (specific activity, 3000 Ci/mmol) (Amersham Corp., Arlington Heights, IL) by using the random primer DNA labeling kit (Pharmacia, Piscataway, NJ) to a specific activity of 1 × 109 dpm/μg. Southern blotting was performed as described previously (28). Briefly, after restriction endonuclease digestion, DNA was electrophoresed in a 0.9% agarose gel for 16 h at 25 V. The DNA in the gel was depurinated by 250 mM HCl, then denatured with 0.5 N NaOH/1.5 N NaCl and neutralized with 0.5 M Tris-HCl/1.5 N NaCl at room temperature. DNA was transferred to a nylon membrane Hybond-N (Amersham) by capillary action in 20X SSC (1X SSC = 0.15 M NaCl and 0.015 M sodium citrate). The membrane was baked at 80°C for 2 h and prehybridized for 1 h in Quick hybridization Solution (Stratagene, La Jolla, CA) at 68°C. Hybridization to the 750-base pair HPRT cDNA was done at 68°C in the same solution containing denatured labeled DNA and salmon testis DNA. The membrane was washed three times in 0.1% SDS/2X SSC at room temperature for 15 min and twice in 0.1% SDS/0.2X SSC for 15 min at 60°C. Subsequently the membrane was exposed to XAR diagnostic film (Kodak, Rochester, NY) and developed with an M35A X-OMAT processor (Kodak).

**Results**

Fig. 1 shows cloning efficiency of HPRT mutant cells incubated with various numbers of HPRT cells in 6-TG-containing medium. Because recovery of HPRT mutant cells incubated with 2 × 10^5 HPRT cells was 70%, we corrected the Mf value according to this cloning efficiency as previously indicated (26). Table 1 compares the Mf as a function of cytotoxicity induced by CPT, topotecan, VP-16, and MNNG at the hemizygous HPRT locus detected by development of TG resistance. Before treating cells with the potential mutagens, they were incubated for 14 days in HAT medium to remove all preexisting TG-resistant mutations. The success of this treatment was confirmed by demonstrating that the HAT-treated cells contained no TG-resistant mutants. Cells that were subsequently treated with DMSO and allowed an expression time of 10 days to serve as controls for the mutagen treatment yielded a background Mf of 0.3 × 10^-6 at the HPRT locus. As shown in Fig. 2, each of the agents tested (VP-16, camptothecin, topotecan, and MNNG) demonstrated significantly increased mutagenesis compared with controls (P < 0.05). As shown in Table 1 and Fig. 2, at 30% survival, VP-16, camptothecin, and topotecan produced Mfs of 11.3 × 10^-6, 4.9 × 10^-6, and 2.7 × 10^-6, respectively. In contrast, MNNG produced a Mf frequency of 562 × 10^-6 at 26% survival. Although the induced Mf was higher for VP-16 than for topotecan or camptothecin, both of the topoisoenzyme I and II active agents produced much greater Mfs at the HPRT locus than the background Mf (P < 0.05), yet one or two orders of magnitude less than the MNNG-induced Mf. We also compared these agents according to their MMP, which provides an assessment of their mutagenic potential on a molar exposure × time basis. The MMP at approximately 30% survival for VP-16, camptothecin, topotecan, and MNNG were 0.9, 8.2, 2.3, and 56.8 mutations/mol-h, respectively. From this viewpoint, MNNG still shows the highest mutagenicity; however, topoisoenzyme I active agents are more mutagenic on a molar basis than the topoisoenzyme II active agent VP-16.

Interestingly, both types of analysis indicate that topotecan is slightly less mutagenic than the parent compound camptothecin. However, the importance of the MMP comparison in cancer chemotherapy remains to be determined based on molar concentration and exposure times required for successful chemotherapy.

To evaluate the mechanism causing the mutation in these TG-resistant cells, we cloned TG-resistant colonies from control untreated cells representing background mutations and from each treatment representing drug-induced mutations and then compared the DNA restriction patterns of their HPRT genes to those in the parental V79. The parental cells exhibited characteristic DNA fragments of 8.8, 7.8, 6.0, 5.0, 3.2, and 2.6 kilobase pairs in PstI digests, of 13.0, 9.7, and 5.7 kilobase pairs in HindIII digests, and of 17.5, 11.3, and 1.2 kilobase pairs in EcoRI digests. The 5.0- and 2.6-kilobase pair fragments in the PstI digest, the 5.7-kilobase pair fragment in the HindIII digest, and the 1.2-kilobase pair fragment in the EcoRI digest are consistent with a previously identified pseudogene (29). Fig. 3 illustrates that all of the spontaneous mutants obtained from independent experiments showed the same HPRT restriction.
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<table>
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<tr>
<th>Drug</th>
<th>Drug concentration (µM)</th>
<th>Survival (%)</th>
<th>CL Ef (%)</th>
<th>Mf (X 10^-7)</th>
<th>MMP (Mf/mol-h)</th>
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<tr>
<td>CPT</td>
<td>0.0125</td>
<td>64.3 ± 6.7</td>
<td>74.3 ± 6.7</td>
<td>1.7 ± 0.3</td>
<td>5.7</td>
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<td>0.025</td>
<td>29.7 ± 1.5</td>
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<td>4.9 ± 0.9</td>
<td>8.2</td>
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<td>0.05</td>
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<td>79.8 ± 14.9</td>
<td>5.7 ± 1.5</td>
<td>4.8</td>
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<tr>
<td>Topotecan</td>
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<td>0.05</td>
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<td>0.08</td>
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<td>VP-16</td>
<td>0.3</td>
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<td>89.0 ± 5.1</td>
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<td></td>
<td>0.5</td>
<td>30.8 ± 0.9</td>
<td>95.1 ± 4.9</td>
<td>11.3 ± 3.7</td>
<td>0.9</td>
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<tr>
<td></td>
<td>0.8</td>
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<td>77.5 ± 3.5</td>
<td>27.2 ± 4.4</td>
<td>1.4</td>
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<td>MNNG</td>
<td>6.8</td>
<td>69.0 ± 5.6</td>
<td>88.5 ± 6.5</td>
<td>193 ± 23.3</td>
<td>29.2</td>
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<td></td>
<td>10.2</td>
<td>26.1 ± 2.0</td>
<td>64.0 ± 16.0</td>
<td>562 ± 87.5</td>
<td>56.8</td>
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As described in 'Materials and Methods,' cells were treated for 24 h with VP-16, CPT, and topotecan, or for 1 h with MNNG.

CL Ef, cloning efficiency of drug-treated cells with normal medium which does not contain 6-TG.

These Mfs are already reduced by subtracting the spontaneous Mf of .

Mutant frequency/drug dose × h of exposure.

Discussion

VP-16 therapy has recently been associated with the development of a unique form of treatment-related AML which differs from the secondary AML arising after alkylating agent therapy (21-23, 30, 31). Thus, the AML that sometimes follows VP-16 therapy is usually of the M4 or M5 monocytic or myelomonocytic types and preceded by a latency of 4-5 years, during which time the patient frequently shows characteristics of a myelodysplastic syndrome. In contrast, the AML that follows VP-16 therapy is often of the M4 or M5 monocytic or myelomonocytic types, usually occurs early, within approximately 2 years of therapy, and is not preceded by a myelodysplastic syndrome. Chromosomal abnormalities in the leukemias that follow alkylating agent therapy may involve unbalanced abnormalities of chromosomes 5 and 7.
Fig. 3  Southern blot hybridization of a full-length hamster *HPRT* gene probe to genomic DNA from parental V79 cells and five separate spontaneous TG-resistant clones. *Upper panel*, treated with *PstI*; *middle panel*, treated with *HindIII*; and *lower panel*, treated with *EcoRI*. Kb, kilobase.

Fig. 4  Southern blot analysis as in Fig. 3. Genomic DNA from parental V79 cells and six separate MNNG-induced TG-resistant clones. *Upper panel*, treated with *PstI*; *middle panel*, treated with *HindIII*; and *lower panel*, treated with *EcoRI*. Kb, kilobase.
Induction of nonhomologous recombination and its consequences can be explained by the following scheme: a topoisomerase active agent stabilizes the drug-topoisomerase-DNA ternary complex. The ternary complex blocks replication fork progress. The cell attempts to bypass these obstructions by recombination processes which involve SCE and include homologous and, in some cases, nonhomologous recombination. The latter can result in gene deletions and/or rearrangements. If an essential gene is deleted then the cell will die once that essential gene product is exhausted. Other deletions or rearrangements can cause activation of a proto-oncogene or deletion of a tumor suppressor gene, resulting in development of a secondary malignancy.

The abnormal molecular consequences of treatment with topoisomerase I active agents are qualitatively and quantitatively similar to those induced by topoisomerase II active agents. This suggests that the topoisomerase I active agents have the potential to cause secondary, treatment-related malignancies in a fashion similar to the topoisomerase II active agents. Thus, camptothecin analogues stabilize a ternary complex including topoisomerase I, DNA, and drug. The stabilized complexes block replication fork progress, leading to DNA strand breaks (2, 9). Cells treated with camptothecin analogues undergo a high level of recombination as illustrated by a marked increase in SCE. Previous studies at the thymidine kinase locus suggest also that camptothecin induces a mutation process in a fashion similar to those caused by VP-16 (32). We have now shown that camptothecin, topotecan, and VP-16 have similar mutagenic activities and that these induced mutations at the HPRT locus frequently involve gene deletions and/or rearrangements.

Camptothecin analogues, including topotecan, CPT-11, and 9-aminocamptothecin, are highly promising chemotherapeutic agents with antitumor activity against a broad spectrum of cancers (33). Their usefulness is likely to be further enhanced by the fact that they have a unique target, topoisomerase I, and, therefore, tumors are not likely to show cross-resistance between the topoisomerase I active agents and other commonly used chemotherapeutic agents with different mechanisms of action. In addition, the camptothecins are minimally affected by the p-glycoprotein, suggesting that they will not be affected by the multidrug-resistant phenotype (34, 35). Camptothecin-resistant cell lines identified to this date have generally been due to a decreased amount of topoisomerase I (36, 37), chromosomal rearrangement (38), or point mutations in topoisomerase I (39–41). Thus, the topoisomerase I active agents promise to become important components of the chemotherapeutic armamentarium despite their potential for causing secondary, treatment-related malignancies. Although this complication has not yet been reported at the clinical level, it will be important to carefully follow patients receiving topoisomerase I-directed agents for the possible development of secondary malignancies, to define their clinical characteristics, and to report them early so that potential relations to dose-scheduling and/or other agents used in combination regimens can be defined and controlled in future trials.
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References


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