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ORIGINAL ARTICLE

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Identification and proposed mechanism of action of thymidine kinase inhibition associated with cellular exposure to camptothecin analogs

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Abstract *Purpose*: To investigate the effects of several camptothecin analogs including 9-aminocamptothecin (9-AC), SN38, topotecan, and irinotecan (CPT-11) on the enzymes involved in the pyrimidine salvage pathway including thymidylate synthase (TS). A COMPARE analysis using the NCI 60 cell line drug-screening panel suggested that there were similarities in the mechanisms of action of camptothecin analogs and TS inhibitors. *Methods*: TS enzymatic activity was measured by both an in situ tritium release assay using both the H630 colon cancer cell line and the CEM human leukemia cell line, and by a radiolabelled in vitro assay using partially purified human TS as the enzyme source. Thymidine kinase (TK) activity was measure by a radiolabelled in vitro assay using H630 colon cancer cell lysates as the enzyme source. *Results*: In vitro studies indicated that none of the analogs directly inhibited TS enzymatic activity; however, utilization of a coupled TS/TK in situ assay with radiolabelled deoxyuridine as the precursor revealed marked inhibition by the camptothecin analogs. 9-AC, SN38, and topotecan yielded IC_{50} values of 1.3, 1.6, and 1.1 μ*M* respectively. In contrast, there was no inhibition detected when deoxycytidine was used as the radiolabelled nucleoside precursor, suggesting that the drug effect was through inhibition of TK, rather than inhibition of TS. In vitro studies using cell lysates from H630 human colon cancer cells to measure TK activity showed no decrease in TK activity after 9-AC treatment. In addition, no changes were detected in the dATP and dTTP nucleotide pools. Permeabilizing the cell membranes with saponin did not abolish the inhibitory effect of the camptothecins indicating that altered cell transport was not responsible for the decreased activity in the in situ assay in intact cells. *Conclusion*: These studies suggest that there is inhibition of TK in intact cells associated with topoisomerase I inhibition by camptothecin analogs, and the inhibition of TK is the result of an indirect effect not related to feedback inhibition by changes in dTTP pools.

Key words Thymidine kinase · Camptothecin analogs · Topoisomerase I · Cross-inhibition · Thymidylate synthase inhibitors

Abbreviations *9-AC* 9-aminocamptothecin · *5FU* 5-fluorouracil · *AZT* 3′-azido-3′deoxythymidine · *CPT-11* irinotecan · *dCyd* 2′-deoxycytidine · *dThd* thymidine · *dUrd* 2′-deoxyuridine · *FA* folinic acid · *PBS* phosphate-buffered saline · *TCA* trichloroacetic acid · *TK* thymidine kinase · *TS* thymidylate synthase · *top1* topoisomerase I

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Introduction

Thymidylate synthase (TS) (EC 2.1.1.45) has been shown to be essential for cellular growth and survival, and as such is a critically important target for treatment of various cancers. TS as well as thymidine kinase (TK) (EC2.7.1.21) are key enzymes in the biochemical pathway leading to the intracellular synthesis of thymidylate. TS is responsible for the de novo synthesis of dTMP, while TK is involved in the salvage of preformed nucleosides. Nucleoside salvage involves both transport and phosphorylation of the nucleoside by TK. While the

metabolic pathways of TS and TK are related, these two enzymes are thought to have different regulatory mechanisms [22, 26]. It has been reported that the regulation of TS is coupled with folate metabolism [8] while TK is regulated by the cellular levels of dTTP [2, 4].

A COMPARE analysis using the NCI 60 cell line drug-screening panel has suggested that camptothecin derivatives may share a similar mechanism of action with agents that inhibit TS [27]. Camptothecins are a group of compounds currently being used as therapeutic agents for the treatment of various cancers including those of the ovary and colorectum [26]. These compounds include 9-aminocamptothecin (9-AC), topotecan and SN38, the active anabolite of irinotecan (CPT-11). Camptothecins are the best-characterized inhibitors of topoisomerase I (top1) [5, 19, 20], an enzyme responsible for unwinding supercoiled double-stranded DNA [5, 20]. Camptothecins bind to the enzyme and stabilize the previously formed cleavable complex causing DNA strand breakage as the advancing DNA replication apparatus collides with the camptothecin-stabilized complex [5, 19].

In a study by Mullany et al. the growth inhibitory effect of adding the camptothecin analog SN38 with 5-fluorouracil (5FU) and folinic acid (FA) to adenocarcinoma cells was investigated [17]. When the cells were treated with the drugs simultaneously or with SN-38 followed by 5FU/FA, the cytotoxicity was much greater than when cells were treated with either agent alone or with 5FU/FA first. These results suggest that the scheduling of 5FU and SN38 may play an important role in determining an effective treatment regimen. These investigators also found elevated dTTP pools in cells treated with SN38 and suggested feedback as a possible mechanism of TK inhibition, although inhibition of this enzyme was not investigated.

In the study reported here we investigated the effect of several well-characterized camptothecin compounds on TS and TK. The objective of our study was to determine what effect, if any, the camptothecin analogs have on the biochemical pathways involved in pyrimidine synthesis and to determine the mechanism(s) accounting for the changes associated with camptothecin exposure.

Materials and methods

Materials

[5-^{3}H]dUrd (23.0 Ci/mmol), [6-^{3}H]dCyd (21.0 Ci/mmol), [6-^{3}H]dUrd (11.2 Ci/mmol), [5-^{3}H]dUMP (21.1 Ci/mmol), [methyl-^{3}H]dThd (20 Ci/mmol), [methyl-^{3}H]AZT (10.9 Ci/mmol) were obtained from Moravek Biochemicals (Brea, Calif.). Activated charcoal, bovine serum albumin, dextran, saponin, aphidicolin, doxorubicin, ATP, $MgCl_2$, and dThd were purchased from Sigma Chemicals (St. Louis, Mo.). Trichloroacetic acid (TCA) was purchased from Mallinkrodt (Paris, Ky.). Biosafe II scintillation cocktail was purchased from RPI (Mount Prospect, Ill.). VP16 was obtained from Bristol Myers (Syracuse, N.Y.). The camptothecin analogs 9-AC, topotecan, and 20S-CPT were provided by the Developmental Therapeutics Program, NCI. SN38 and CPT-11 were obtained from Pharmacia-Upjohn (Columbus, Ohio).

Cell culture

Human 630 colon cancer cells were maintained in RPMI-1640 medium (Life Technologies, Gaithersburg, Md.) and supplemented with 10% dialyzed fetal bovine serum (Life Technologies). Human leukemia cell lines, the CEM parent wildtype and the camptothecin-resistant CEM/C2 with a mutation in the top1 enzyme, have been previously described [9]. The cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. All cell lines were kept at 37 °C in an atmosphere containing 5% CO_2.

In situ tritium release assay

A modification of a previously published method was used to determine TS and TK activity in intact cells [29]. H630 colon cells (0.5×10^6) were plated in six-well Costar plates. After a 48-h incubation, 500 µl of fresh medium was added, and the cells were incubated in the presence or absence of camptothecin analogs for 45 min at 37 °C. The assay was initiated by the addition of 10^6 dpm [5-^{3}H]dUrd or [5-^{3}H]dCyd at a final concentration of 38 n*M*, and the incubation allowed to proceed for 15 min. As shown by the pathway in Fig. 1, when dUrd is used, the precursor must first be phosphorylated by TK before proceeding to TS for synthesis of thymidylate. However, when dCyd is used as the precursor the pathway proceeds to the final TS step without the need for TK. The use of both dUrd and dCyd allows a determination of whether TK or TS is the enzyme responsible for the inhibition of tritium release by drug treatment [28].

The reaction was terminated by the transfer of 250 µl to a microfuge tube containing 250 µl of a 15% activated charcoal suspension in 20% aqueous TCA. The tubes were vortexed and centrifuged in a Sorvall microfuge for 5 min. A 250-µl aliquot of the cleared supernatant was counted in a Beckman liquid scintillation counter to determine the amount of tritium released. The release of the tritium was expressed as a percentage of the total amount of radioactivity added. Samples containing only medium and labelled precursor were used for background counts which consistently represented approximately 1% of the total counts added.

IC_{50} values were determined from concentration-response curves and represent the concentration of camptothecin analog necessary to inhibit the release of tritium by 50%. The in situ tritium release assay was also performed using the human leukemia CEM parent and CEM/C2 cell lines, both of which grow as suspensions. Approximately 10^6 cells were removed from a T75 flask (Falcon/Becton Dickson, Franklin Lakes, N.J.) and centrifuged for 10 min at 200 *g* in a Sorvall low-speed centrifuge. The cell pellet was resuspended in 3 ml medium and distributed equally among the wells of a six-well plate. The cells were then treated with camptothecin analogs and the labelled precursor as described above. The reaction was terminated by removing 250 µl of the cell mix to the microfuge tube containing the charcoal suspension. The final determination of the tritium released was performed as described above for the adherent cells.

Time curve for drug exposure

H630 cells were plated as described above. The cells were exposed to 5 µ*M* 9-AC for various times (5, 10, 20, 30 and 45 min). For the control time-point, the labelled precursor was added to cells for 5 min with no drug pretreatment. For the 5-min time-point the label and the drug were added at the same time. For all other time-points the label was added 5 min prior to the end of the desired drug exposure time. The in situ assay was then performed as described above.

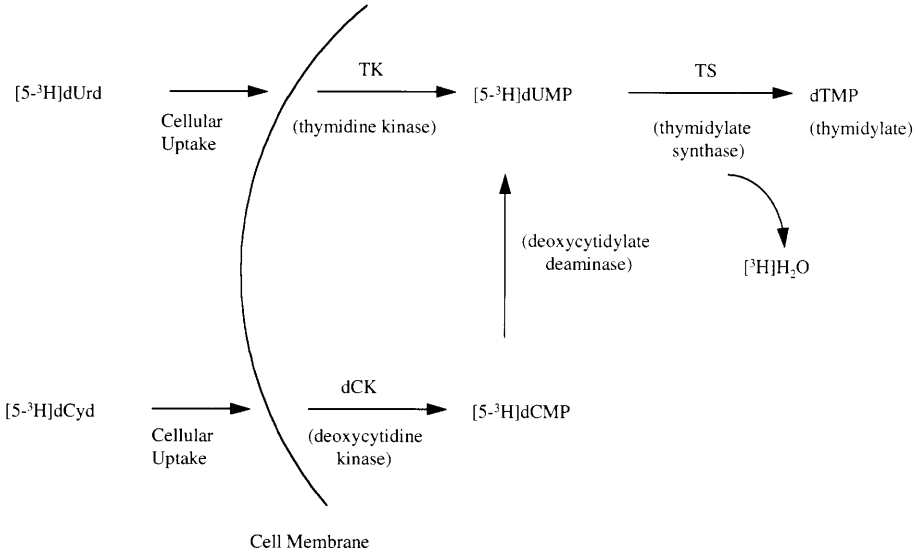

Fig. 1 Biochemical pathway of events represented in the in situ tritium release assay. The radiolabelled nucleotide precursors are taken up by the cell and phosphorylated by the corresponding enzyme. For [5-^{3}H]dCyd uptake, [5-^{3}H]dCMP must be deaminated to [5-^{3}H]dUMP by deoxycytidylate deaminase. The [5-^{3}H]H_2O is displaced from the [5-^{3}H]dUMP during the reaction catalyzed by TS. The tritiated water is released into the collected aqueous mixture and counted

TS catalytic assay

The previously described catalytic assay [1] was performed in a final volume of 200 µl containing, 10^{-5} *M* [5-^{3}H]dUMP, 2000 pmol dUMP, 200 pmol methylene tetrahydrofolate, 20 µ*M* *β*-mercaptoethanol and 3 µl of partially purified human TS [6]. Samples were incubated at 37 °C for 30 min. The reaction was terminated by the addition of 100 µl ice-cold 20% TCA. Unmetabolized [5-^{3}H]dUMP was removed by the addition of 200 µl of an albumin-coated activated charcoal solution. The samples were vortexed and allowed to stand at room temperature for 10 min. The charcoal was then settled by centrifugation at 13,000 *g* for 30 min. A 250-µl aliquot of the supernatant was counted in a Beckman liquid scintillation counter to determine the amount of radioactivity representing the [^{3}H]H_2O.

TK assay

TK activity was measured in lysates from H630 cells as previously described [15]. H630 colon cells were plated in T75 flasks at a density of 1–2 × 10^6 cells and allowed to grow for 48 h (70% confluency). The cells were washed with PBS (Biofluids, Gaithersburg, Md.) three times and incubated with 20 m*M* EDTA for 10 s. After removal of the EDTA, 10 ml PBS was added and the cells were incubated at 37 °C until the cells dislodged. The cells were spun at 200 *g* for 10 min. The cell pellet was resuspended in 350 µl buffer (50 m*M* Tris, 1 m*M* EDTA, 10% glycerol and 5 m*M* *β*-mercaptoethanol) and subjected to three 5-s sonication bursts and centrifuged at 12,000 *g* for 30 min. The supernatant was removed and used for assay measurement. The assay was performed in a total reaction volume of 200 µl containing 50 m*M* Tris, 10 m*M* ATP, 5 m*M* $MgCl_2$, 0.1 m*M* unlabelled dThd, 10 m*M* sodium fluoride, 500,000 dpm [^{3}H]dThd, and cell cytosol representing 150 µg of protein. Drug-treated samples also contained various concentrations of camptothecin analogs. Background and total available substrate controls did not include cytosol while assay-positive controls included cytosol but not camptothecins. The samples were incubated for 30 min at 37 °C. The reaction was quenched by placing the samples in boiling water for 1 min. DE81 filters were spotted with a 50-µl aliquot of each sample and air-dried for 15 min. The filter discs (excluding total available substrate controls) were washed three times in distilled water by gentle agitation on an orbital shaker (30 ml/filter for 10 min). The discs were placed in a scintillation vial with 1 ml 0.2 *N* HCl/0.4 *M* KCl solution and agitated gently on an orbital shaker for 15 min. Scintillation cocktail was added and the samples were allowed to equilibrate overnight before counting in a liquid scintillation counter. The TK activity was also measured using cell lysate treated with various concentrations of camptothecin analogs for 45 min prior to harvesting for assay use. H630 colon cells were plated as described above. After treatment with 10 µ*M* 9-AC for 45 min the cells were washed three times with PBS and the lysates were harvested and the assay was performed as described above.

Nucleotide pool analysis

Approximately 10^6 H630 colon cells were plated in T75 flasks. After a 48-h incubation the cells (with a cell count in the 3–5 × 10^6 range) were incubated in the presence or absence of 1 µ*M* 9-AC for 45 min at 37 °C in 10 ml fresh medium. Nucleotide extraction was performed as previously described [12]. Briefly, the cells were gently rinsed twice with ice-cold PBS/10 µ*M* dipyridamole solution. The cells were extracted with 3 ml ice-cold 0.5 *N* perchloric acid. The soluble fraction was neutralized, lyophilized and resuspended in deionized H_2O. The ribonucleotide triphosphate pools were quantitated by an anion-exchange HPLC method [11]. Samples used to measure the dTTP and dATP pool levels were treated as described for the ribonucleotide pools. The dTTP and dATP pools were measured by a DNA polymerase assay [14, 24]. For the experiments looking at AZT nucleotide analysis approximately 10^6 H630 colon cells were plated in T75 flasks. After a 48-h incubation the cells (with a cell count in the 3–5 × 10^6 range) were incubated with 5 µ*M* 9-AC, CPT-11, and 20S-CPT for 30 min at 37 °C in 10 ml fresh medium. Control flasks were not treated with any drug. All samples were then exposed to 1 µCi of [^{3}H]AZT for an additional 15 min. The cells were then washed and nucleotide extraction was performed as described above. The AZT nucleotides were separated and analyzed by HPLC [11].

Permeabilization of cellular membrane

H630 cells were plated in six-well Costar flasks as described above. The cell walls were permeabilized by treatment with saponin at a final concentration of 0.01% for 5 min [16]. The cells were washed twice with PBS. Following the addition of 500 µl fresh medium the cells were treated with 5 µ*M* 9-AC followed by [5-^{3}H]dUrd. Tritium release levels were measured using the in situ assay as described above.

Results

In situ tritium release assay in the H630 cell line

The effect of camptothecin analogs on intact H630 colon cells was measured using the in situ tritium release assay

(Fig. 1). The observed inhibitory activities of camptothecin analogs on the release of tritium are presented in Table 1. When [5-^{3}H]dUrd was used as the precursor, tritium release was inhibited by 9-AC, SN38, and topotecan with IC_{50} values of 1.3, 1.6 and 1.1 μM, respectively. The top1-inactive derivative CPT-11 did not inhibit tritium release activity at concentrations up to 20 μM. Two other drugs, the DNA synthesis inhibitors doxorubicin and VP16, also did not exhibit inhibitory activity in this assay when tested at concentrations up to 5 μM. Concentration-response curves for the effects of the drugs with inhibitory activity as well as for CPT-11 are illustrated in Fig. 2. The three camptothecin analogs 9-AC, SN38, and topotecan had comparable inhibitory effects on the release of tritium, while CPT-11 showed no inhibitory activity. The use of [5-^{3}H]dCyd as the nucleoside precursor produced very different results. As shown in Table 1, none of the drugs tested in the in situ assay showed any inhibitory effects on the release of tritium at concentrations up to 10 μM.

To rule out selective alterations in membrane transport of the various nucleosides, cells were permeabilized with saponin prior to drug treatment. Exposure to this compound did not alter the inhibitory effect of 9-AC on tritium release (data not shown).

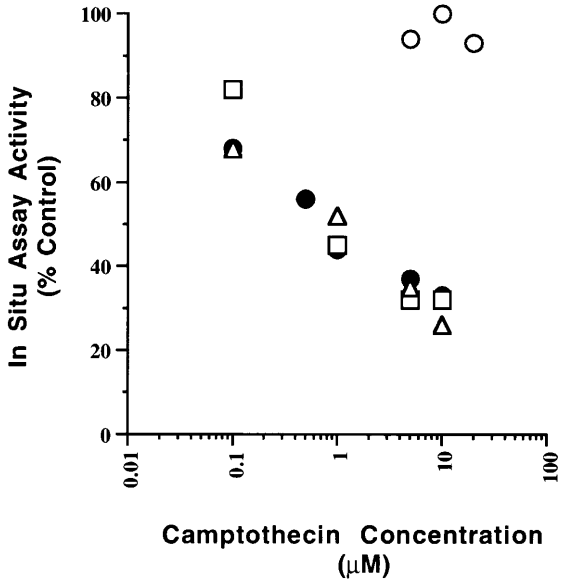

Fig. 2 Concentration-response curves for the inhibition of tritium release by camptothecin analogs. H630 cells were treated with various concentrations of 9-AC (●), SN38 (△), topotecan (□), and CPT-11 (○) for 45 min at 37 °C followed by the addition of [5-^{3}H]dUrd for 15 min. The amount of tritium released is expressed as percentage of control and determined as described in Materials and methods

Drug exposure time for camptothecin effect on tritium release

Figure 3 illustrates the effect of drug exposure time on the inhibition of tritium release by 9-AC. H630 cells were exposed to 5 μM 9-AC for 5, 10, 15, 30 or 45 min. The effect of the 9-AC was maximal within 15 min of drug exposure. There was no inhibition detected when drug and label were added simultaneously or when the drug was added for 5 min prior to the precursor. After 15 min of drug exposure with the final 5 min including the labelled precursor, only 50% of the tritium released in control cells was detected. Exposing the cells to drug for longer times did not further increase inhibition.

Table 1 Effect of camptothecin analogs in intact H630 colon cells using the in situ assay. H630 colon cells were preincubated with various concentrations of drugs at 37 °C for 45 min before the addition of either radiolabelled deoxyuridine or deoxycytidine for an additional 15 min. Tritium release was measured as described in Materials and methods, and the IC_{50} (μM) values (mean ± SD) shown were determined from dose response curves

Drug	Precursor	
	[5-^{3}H]-Deoxyuridine	[5-^{3}H]-Deoxycytidine
9-AC	1.3 ± 0.51	> 10
SN38	1.6 ± 0.32	> 10
Topotecan	1.1 ± 0.39	> 10
CPT-11	> 20	> 10
Doxorubicin	> 5	> 10
VP16	> 5	> 10

In situ tritium release assay in the CEM cell line

In order to test whether top1 cleavage complexes were required for camptothecin-induced TK inhibition, a camptothecin-resistant cell line (CEM/C2) was used. CEM/C2 cells are highly resistant to camptothecins because of a mutation [9] and silencing of the normal top1

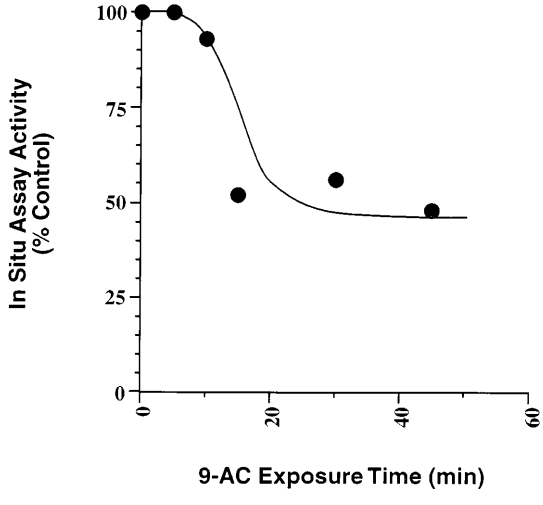

Fig. 3 Time dependence of the inhibition of tritium release by 9-AC. H630 cells were treated with 5 μM 9-AC for 5, 10, 20, 30 and 45 min. [5-^{3}H]dUrd was added for 5 min prior to the end of the desired drug exposure time. For the 5 min time-point, the labelled precursor and the 9-AC were added simultaneously. The amount of tritium release was expressed as percentage of control and was determined as described in Materials and methods

alleles [10]. The effect of the camptothecin analog 9-AC on the release of tritium in the in situ assay was examined in the both the CEM wildtype and CEM/C2 top1 mutant cell lines. As indicated in Table 2, in the wildtype cells, 5 μ*M* 9-AC had strong inhibitory activity (approximately 80%), as in the case of the H630 cells. In contrast, 9-AC had no inhibitory effect on tritium release in the CEM/C2 resistant cell line. As indicated, CPT-11 had little inhibitory effect in either the CEM or CEM/C2 cell lines. When [5-^{3}H]dCyd was used as the precursor, neither 9-AC nor CPT-11 had a significant inhibitory effect on tritium release in either the wildtype or the top1 mutated cell line. These results demonstrate that the presence of drug-induced top1 cleavage complexes is important for the effects of camptothecins on TK. To further test the necessity for top1-associated DNA fragmentation on TK inhibition, we exposed H630 cells to 10 μ*M* aphidicolin prior to camptothecin treatment. Treatment with this compound also did not alter the inhibitory effect of 9-AC on tritium release in the in situ assay (data not shown).

Cell-free TS and TK activity

To determine if the camptothecin analogs were affecting either TS or TK in a direct manner, we tested the camptothecins as inhibitors of both enzymes using in vitro assay systems. The camptothecin analogs 9-AC, SN38, and CPT-11 at concentrations of 10 and 50 μ*M* had no inhibitory effect on TS activity (data not shown), and 9-AC at concentrations of 1 and 5 μ*M* also had no inhibitory effect on TK activity (data not shown). This was also true when the cells were first pretreated for 45 min with 9-AC before harvesting the lysates for use in the TK assay.

Effect of 9-AC on AZT phosphorylation in H630 cells

To determine if the camptothecins had an effect on the phosphorylation of AZT, in which TK is utilized, H630 cells were treated with 5 μ*M* 9-AC for 15 min followed by a 30-min exposure to 1 μCi [^{3}H]AZT. As shown in Fig. 4, the levels of mono- and diphosphate AZT pools were 31% and 32% lower in the cells treated with 9-AC and 20S-CPT, respectively, compared with control cells. No AZT triphosphate pools were detected in either the control or the camptothecin-treated cells.

Table 2 Effect of 9-AC in wildtype CEM and camptothecin-resistant CEM/C2 human leukemia cells using the in situ assay. CEM wildtype (*WT*) and CEM/C2 cells were preincubated with 5 μM 9-AC at 37 °C for 45 min prior to the addition of either radiolabelled deoxyuridine or deoxycytidine for an additional 15 min. Tritium release was measured as described in Materials and methods, and the values shown are percentages of the control (mean ± SD, $n = 34$ experiments)

Drug	Precursor			
	[5-^{3}H]-Deoxyuridine		[5-^{3}H]-Deoxycytidine	
	CEM/WT cells	CEM/C2 cells	CEM/WT cells	CEM/C2 cells
9-AC	20 ± 5.7%	88 ± 13%	100%	100%
CPT-11	83 ± 5.3%	100 ± 0%	100%	100%

Analysis of nucleotide triphosphate pool distribution

H630 cells treated with 1 μ*M* 9-AC were analyzed for ribonucleotide triphosphate pool changes and for changes in pool size of dTTP and dATP. Table 3 shows the various ribonucleotide pool levels of cells pretreated with 9-AC for 45 min compared to those of untreated cells. A 1.12–1.40-fold increase was detected for each of the four ribonucleotide triphosphates investigated ($P < 0.02$ for GTP, CTP, and UTP pools). In separate experiments, the effect of 9-AC on the dTTP and dATP nucleotide pools was also studied. No differences in these triphosphate pool levels were detected following drug treatment, as shown in Table 3.

Discussion

These results suggest that camptothecin analogs do not have a direct effect on TS, but do inhibit TK through an indirect mechanism which is not associated with early changes in the dTTP nucleotide pool. To study the inhibitory effects of camptothecins on TS and TK we used the in situ tritium release assay. This assay which has been successfully adapted for the measurement of TS activity in intact mammalian cells measures the release of tritiated water when dUMP is converted to dTMP by TS. Although utilized mostly for the measurement of TS activity, the assay is a coupled assay and relies on TK to phosphorylate dUrd to dUMP, thus an effect on TK activity may also be detected by this assay. To separate inhibitory effects on TS from those on TK, we performed the assay with both dCyd and dUrd as radiolabelled precursors. Because these two precursors utilize different kinase enzymes, dCyd kinase (dCK) and TK, respectively, for phosphorylation, they may be used to distinguish the inhibitory effects on TK from those on TS [28].

When deoxyuridine was used, we detected high levels of inhibition of tritium release from the camptothecin analogs SN38, topotecan, and 9-AC, whereas CPT-11 was ineffective. The three active compounds had similar inhibitory effects with IC_{50} values ranging from 1.1 to 1.6 μ*M*. Data from the NCI Developmental Therapeutics Program has shown that 9-AC and topotecan have similar potency in producing 50% cytotoxicity in nine colon cell lines tested in the NCI 60 cell-line screen, while SN38 is approximately fourfold more potent. When deoxycytidine was used as the precursor, which is phosphorylated by dCK, no inhibition of tritium release was detected with any of the camptothecins at

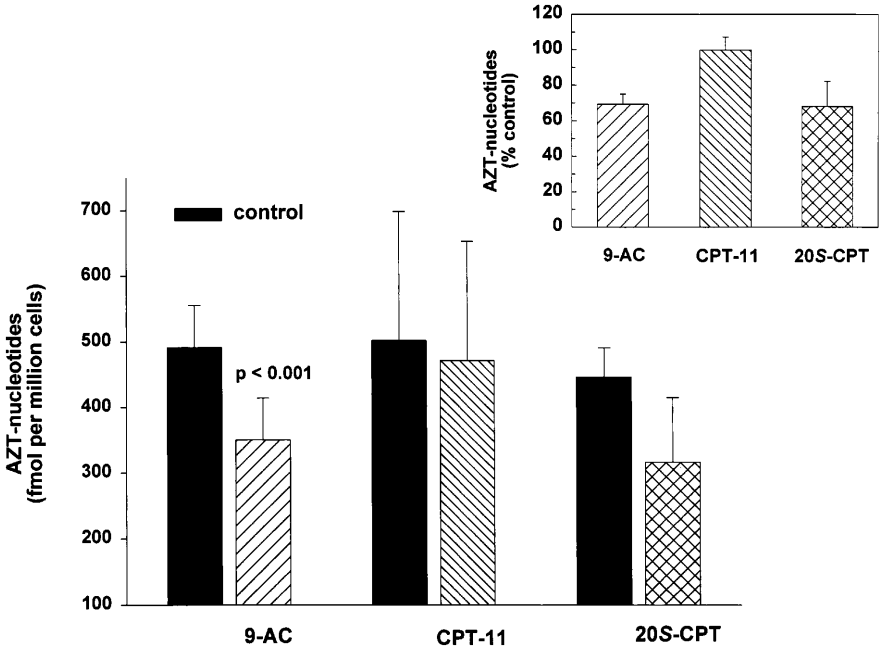

Fig. 4 Effects of camptothecins on the phosphorylation of AZT. H630 cells were treated with 5 μ*M* 9-AC, CPT-11, and 20S-CPT for 30 min followed by a 15-min exposure to 1 μCi [^{3}H]-AZT. Control flasks were not treated with drug prior to [^{3}H]AZT exposure. The cells were harvested and the nucleotides were extracted as described in Materials and methods. The AZT nucleotides were analyzed by HPLC and the amounts of nucleotides (in femtomoles per million cells) detected are shown. The *inset* represents the phosphorylation of AZT for the three drug-treated samples expressed as percentage of the control

Table 3 Effect of 9-AC on nucleotide triphosphate pool distribution in H630 human colon cancer cells. H630 colon cells were incubated with 1 μ*M* 9-AC at 37 °C for 45 min and harvested for nucleotide pool extraction as described in Materials and methods. The ribonucleotide pools were determined by an HPLC assay and the dTTP and dATP pools were measured by a DNA polymerase assay

	Nucleotide pool					
	Ribonucleotide (nmol/10^6 cells)[a]				dTTP (pmol/10^6 cells)[b]	dATP (pmol/10^6 cells)[b]
	UTP	CTP	ATP	GTP		
Control	2.60 ± 1.79	0.76 ± 0.54	6.64 ± 4.50	2.06 ± 1.27	153.3 ± 20.1	37.6 ± 4.1
9-AC	3.17 ± 2.10	1.06 ± 0.64	7.43 ± 5.25	2.56 ± 1.48	145.1 ± 14.4	37.9 ± 4.5
Fold increase	1.22	1.40	1.12	1.24	–	–

[a] Means ± SD (n = 10 experiments)
[b] Means ± SEM

concentrations up to 20 μ*M*. These results suggest that TK is the enzyme affected by camptothecin exposure. The cell-free TS catalytic assay also indicated that TS was not inhibited by the camptothecin analogs. Results of the TK cell-free catalytic assay likewise revealed that TK was not inhibited directly by the camptothecin analogs. In addition, we found no inhibition of TK when cells were pretreated before harvesting the lysate for use in the in vitro assay, suggesting that inhibition was not due to changes in protein levels induced by the drug exposures.

Since differential alterations in nucleoside transport could also explain the apparent inhibition patterns noted in this study, we addressed this possibility by investigating inhibition after permeabilization of the cell membrane by saponin. Since permeabilization had no effect on the pattern of inhibition associated with exposure to the camptothecin analogs, we conclude that the inhibition detected in the in situ assay was due to an indirect inhibitory effect on TK. Our experiments examining the impact of the camptothecin analogs on the phosphorylation of AZT by TK provide additional proof that inhibition of TK is associated with exposure to these analogs.

Several studies have shown that the catalytic activity of TK is feedback-inhibited by an increase in the dTTP nucleotide pool [3, 4, 15]. In a study by Xu and Plunkett the effect of dTTP pool elevation in CCRF-CEM cells associated with the treatment of CEM leukemia cells with DNA synthesis inhibitors was examined [28]. While drug treatment resulted in increases in dTTP pools, the degree of TK inhibition was much greater than could be accounted for by the change in dTTP levels. The authors concluded that the elevated dTTP pool levels and subsequent feedback inhibition were not the primary mechanism affecting TK, but that another undefined mechanism must be contributing to the noted effects. Our studies are consistent with this notion, since we saw no changes in the dTTP pool in cells after brief exposures to 9-AC. Although we did note elevations in the ribonucleotide triphosphate pools, none of these has been associated with feedback effects on TK enzymatic activity.

The enzymes involved in DNA synthesis have been extensively studied and the term "replitase complex" has been coined to describe a multienzyme complex which occurs primarily in S phase [21, 23]. The authors suggested that allosteric interactions occur between the enzymes because of their close proximity when contained within the replitase complex [18]. This interaction is postulated to be the cause of "cross-inhibition", the phenomenon in which the inhibition of one enzyme in a complex leads to the inhibition of a second unrelated enzyme through an allosteric interaction. Our observations showing inhibition of TK via an indirect mechanism and its association with an obligate inhibition of top1 are consistent with the phenomenon of cross-inhibition. It has been demonstrated that both the TK and top1 enzymes are associated with the replitase complex [23]. The requirement for top1 inhibition is illustrated in our experiments utilizing the top1-inactive camptothecin derivative CPT-11 as an inhibitor, and in the experiments utilizing the top1-mutated CEM/C2 cell line as an enzyme source. The CPT-11 analog resulted in no detectable inhibition. In the CEM/C2 cell line, which is resistant to camptothecins, we found that exposure to top1 inhibitors did not result in any significant inhibition. Consistent with our hypothesis concerning TK inhibition, previous studies have shown that in the top1-mutated CEM/C2 cell line, camptothecin-induced cleavable complex formation is dramatically reduced after exposure to camptothecin, as compared to the wildtype parent CEM cells. The CEM/C2 cell line has also been shown to be almost 1000-fold more resistant to camptothecin compared to wildtype cells [9].

Given that TK is inhibited via an indirect mechanism, we wished to determine whether this was a global cellular response to DNA fragmentation resulting from top1 inhibition as opposed to cross-inhibition. We found that pretreatment of cells with aphidicolin, which blocks cells in S phase and abrogates camptothecin-associated DNA damage [25], did not impact TK inhibition associated with exposure to the camptothecin analogs. Previous studies have shown that pretreatment of cells with aphidicolin before camptothecin exposure results in an abolition of camptothecin cytotoxicity, but does not change the level of DNA strand breaks or the formation of cleavable complexes [13]. This result suggests that top1 cleavable complexes in the absence of replication-induced DNA damage are sufficient to suppress TK. This finding supports the hypothesis that the noted TK inhibition is the result of allosteric interactions between enzymatic partners in the replitase complex, rather than the result of a global cellular response to camptothecin-mediated DNA damage. Adding further support to this hypothesis is the comparison of the time-course of inhibition of tritium release to that of top1 inhibition by the camptothecins. As illustrated in Fig. 3, the inhibition of tritium release was rapid with maximal levels reached within 15 min of drug exposure. In previous studies, treatment with 1 μM 9-AC has been shown to result in the formation of top1-associated single strand breaks within 10 min of drug addition [7]. The strong correlation between the time courses of tritium release and top1 inhibition further suggests that the mechanism of inhibition of TK and top1 are related.

The results of the COMPARE analysis suggest that the camptothecins may ultimately deplete thymidylate since the COMPARE program suggests relationships amongst compounds based on their mechanism of action. The most common compounds that cause depletion of thymidylate are inhibitors of TS. The association between TS inhibitors and camptothecin analogs identified by the COMPARE analysis suggests that thymidylate depletion may occur as a late event associated with camptothecins given the inhibition of TK associated with camptothecin exposure.

In conclusion, our studies demonstrate that cellular exposure to the camptothecin analogs results in inhibition of TK. This inhibition occurs via an indirect mechanism not associated with changes in dTTP pools. We propose that this inhibition may be due to the phenomenon of cross-inhibition between enzymatic partners in the replitase complex. Given the inhibition of TK associated with the camptothecin analogs, this study provides an additional rationale for combining agents that inhibit the de novo synthetic nucleotide pathways such as the fluoropyrimidines and certain antifolates with the camptothecin analogs.

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