

Identification of a small topoisomerase I-binding peptide that has synergistic antitumor activity with 9-aminocamptothecin

Christopher D. Pond, Kathryn M. Marshall, and Louis R. Barrows

Department of Pharmacology and Toxicology, University of Utah College of Pharmacy, Salt Lake City, Utah

Abstract

The topoisomerase I (top1)-targeted camptothecin class of anticancer drugs is important in the treatment of several types of cancers. This class of drug inhibits the top1 enzyme during its catalytic DNA relaxation cycle, stabilizing the transient covalent top1-DNA complex by simultaneous noncovalent interactions with DNA and top1. We examined top1 using phage display because of the significance of this known top1-directed drug action. Several peptides that bind top1 were discovered and these were examined for top1 affinity, top1 catalytic and cleavage complex effects, and cytotoxic effects in cultured cell lines and in an *in vivo* tumor model. Although several peptides exhibited nanomolar and low-micromolar affinity for top1, none had cytotoxic effects when administered alone. However, in combination with 9-aminocamptothecin, one 15-mer peptide (SAYAATVRGPLSSAS) had synergistic cytotoxic effects with 9-aminocamptothecin both in the cytotoxicity assay and in nude mouse xenograft human tumor models. This report details the investigation of this peptide. [Mol Cancer Ther 2006;5(3):739–45]

Introduction

The camptothecin class of anticancer drugs has recently become important in the treatment of several types of cancers. Topotecan and irinotecan, two analogues of camptothecin, are in clinical use. In recurrent ovarian cancer, topotecan possesses antitumor activity similar to paclitaxel, with nonoverlapping side effects, and is an established treatment in second-line or salvage settings and is being investigated as a primary therapeutic alternative

(1). For metastatic colorectal cancer, irinotecan was first used in salvage and evaluated in polytherapy settings (2) and is now used in first-line therapy with fluorouracil, leucovorin, and oxaliplatin (3).

The exact mechanisms of toxicity are not yet resolved, but this class of drug specifically targets topoisomerase I (top1), a DNA unwinding enzyme responsible for relaxation of DNA during replication and transcription as well as other functions, including DNA repair and recombination. Top1 is also a kinase that participates in RNA processing.

The varied activities of top1 situate it in several protein complexes and in physical association with many proteins, including TATA-binding protein, p14ARF, PSF/p54, and p53 (4–7). Top1 has been detected in RNA polymerases I, II, and III transcription complexes (8–11) and in replication complexes (12, 13). Upon DNA damage, nucleolar top1 relocates (14) and associates with p53 where it may function in DNA repair processes (6). Ubiquitination and proteasome degradation play important roles in the removal of top1-DNA complexes and may play a role in cellular resistance to camptothecin (15). Poly(ADP) ribosylation inhibits top1 activity (16, 17), whereas phosphorylation by at least two kinases has been shown to increase top1 activity (18, 19). Top1 is also known to bind Werner syndrome helicase, a protein involved in recombinational repair and replication (12). Additionally, top1 controls transcription of some genes via interaction with *cis*-acting regulatory gene elements and regulates transcript processing via its phosphorylation of, and association with, serine/arginine-rich RNA splicing factors (20, 21).

The major toxicity of the camptothecin drugs is thought to arise during S phase by stabilization of the covalent top1-DNA nucleoprotein complex whereas a single-stranded scission is present in the DNA (22). This is termed the cleavable complex (22). Double-stranded DNA breaks result from replication runoff when this complex is encountered on the leading strand of DNA synthesis by the cell replication machinery (23), and these are thought to account for the major cytotoxic effects of the drug. Another cause of cytotoxicity may be that camptothecin poisoning induces apoptosis by targeting telomeric repeats that have multiple copies of the top1 cleavage sequence 5'-TT↓AGGG-3' (downward arrow denotes site of scission; ref. 24).

We examined top1 using phage display to identify top1-binding peptide ligands capable of binding top1 with high affinity. Next, we explored the possibility that these novel top1-binding peptides have pharmacologic effects because of the significance of the camptothecin class of drugs and the myriad of top1-protein interactions known. We hypothesized that top1 interacts with proteins via epitopes

Received 9/20/05; revised 12/2/05; accepted 1/11/06.

Grant support: University of Utah Technology Innovation grant.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Louis R. Barrows, Department of Pharmacology and Toxicology, University of Utah, 30 South 2000 East Room 201, Salt Lake City, UT 84112. Phone: 801-581-4547; Fax: 801-585-5111. E-mail: lbarrows@pharm.utah.edu

Copyright © 2006 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-05-0377

formed by topographically clustered amino acid sequences displayed on its surfaces, as it does with DNA (25). Short peptides that bind such epitopes may mimic a subset of the total protein-protein interactions and act as agonists or antagonists of select top1 activities. We hypothesized that the ability to selectively interfere with, or mimic, specific sites of protein-top1 interaction may yield new top1-directed therapeutics. Several peptides with high affinity for top1 were discovered and these were examined for top1 affinity, top1 catalytic and cleavage complex effects, and for cytotoxic effects in cultured cell lines. Although several peptides exhibited nanomolar and low-micromolar affinity for top1, none had cytotoxic effects when administered alone. However, in combination with 9-aminocamptothecin (9AC), one TAT-labeled 15-mer peptide had synergistic cytotoxic effects with 9AC both in the cytotoxicity assay and in a nude mouse xenograft human tumor model.

Materials and Methods

Isolation of Human Top1

Human top1 was obtained both from baculovirus-expressing insect cells and human placenta. Sf9 insect cells were grown in 1 liter cultures, infected with baculovirus stock (a gift of Dr. J. Champoux, University of Washington, Seattle, WA) and isolated by centrifugation 50 hours later (26). Top1 from the sf9 cells or from placenta was isolated and purified using the method of Holden et al. (27). Successful isolation of top1 was confirmed by DNA relaxation activity assays and SDS-PAGE/Western analysis.

Phage Display

Phage display was done as described by Parmley and Smith (28) with the random 15-mer library (29) generously supplied by Dr. G.P. Smith (Biological Sciences, University of Missouri, Columbia, MO), accessed through his website.² This library was constructed using a random 45 bp insert in the *pIII* gene with the fUSE5 vector, which displays a foreign 15-mer peptide near the terminus of each of the five copies of the *pIII* gene product (29). Briefly, top1 was first biotinylated with a biotin derivative containing a spacer engineered to reduce steric interference (EZ-Link Biotin-PEO-Amine, Pierce, Rockford, IL). The reaction was done at a 5:1 molar ratio of biotin/top1 in 10 mmol/L HEPES (pH 8.2)-buffered 500 mmol/L NaCl. The unreacted biotin was removed by gel filtration in a Biospin 30 column (Bio-Rad, Hercules, CA) and biotinylated top1 was assayed for activity using a pull-down method with streptavidin-coated beads. The biotinylated top1 maintained catalytic activity (data not shown). The phage display experiments were done in 30 mm polystyrene culture Petri plates prepared by adding 10 µg Neutravidin (Pierce) in 100 mmol/L sodium bicarbonate (pH 8.2), to the plate, on a rocker, at 4°C. After 1 hour, the plate was rinsed in TBS-0.5% Tween and then nonspecific binding was blocked for 1 hour by adding 1.0 mL of 3.0% bovine serum albumin in TBS. For phage display, 20 µg biotinylated top1 was reacted with a

mixture of 100 µg pBR322 and 10 µmol/L 9AC in 100 µL top1 reaction buffer [150 mmol/L KCl, 12 mmol/L MgCl₂, 2 mmol/L EDTA, and 50 mmol/L Tris-HCl (pH 7.5)] was applied to the above Neutravidin-coated (10 µg) plastic Petri dish. The dish was then blocked with 3.0% bovine serum albumin and 10 µL of a 10⁹/µL titer phage library in 100 µL TBS-0.05% Tween was added. Phage were allowed to associate with gentle rocking for 2 hours at 4°C, and then washed repeatedly over 2 hours at 4°C with TBS 0.1% Tween. The bound phage were then eluted in 0.1 N HCl-glycine and used to infect competent K91 Kan *Escherichia coli* and amplified. Phage were harvested, concentrated by polyethylene glycol precipitation, titered, amplified, and the phage display process was repeated. After two to three rounds of screening, phage clones were picked and their DNA was sequenced to determine the displayed peptides.

Analytic Titering

Ten microliters of amplified phage clones (~10¹² cfu) were incubated with 50 nmol/L biotinylated top1 in 100 µL of top1 reaction buffer for 1 hour at 4°C on a rocker. These reactions were then applied to bovine serum albumin-blocked, streptavidin-coated plates (above method). The plates were rocked while incubated at 4°C for another hour and then washed five times with 1 mL TBS-0.1% Tween. Competent *E. coli* were added directly to the washed plates and the output assessed by counting a 10⁻⁶ dilution of the culture on tetracycline plates (Fig. 1). Figure 1 (column 11) presents the control experiment titering of unselected phage library.

Peptide Synthesis

The peptides were synthesized at the University of Utah Core Oligonucleotide and Peptide Synthesis Facility. For relaxation assays and peptide affinity studies, the peptides were synthesized as sequenced from the phage. For the cell and mouse studies, a domain that enhances cellular uptake (called a TAT domain and signified as -T) was added with short spacer (alanine-glycine) to the COOH terminus of the peptide (30). Peptides not containing the added TAT domain had no detectable effects on cells in culture.

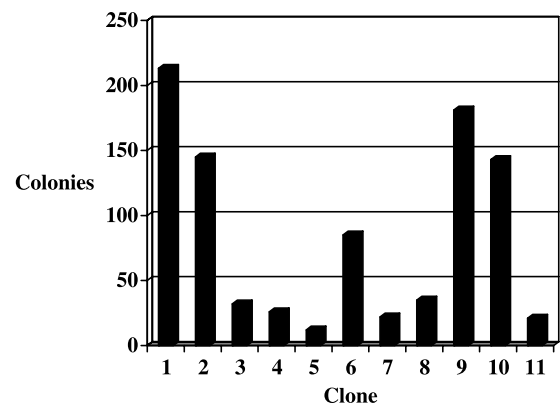


Figure 1. Analytic titering of 10-phage clones from top1-DNA-9AC biopanning screen and a similar titer of library (11) using only top1 as substrate. Clones that seemed to bind top1 (1, 2, 6, 9, and 10) above background (11) were sequenced, synthesized and designated T1BP3 (T1BP1, T1BP2, T1BP3, T1BP4, and T1BP5, respectively).

² www.biosci.missouri.edu:16080/smithgp/

Sequence Analysis

Similarity searches were done using Basic Local Alignment Search Tool at National Center for Biotechnology Information³ in the Swiss-Prot database of protein sequences (Swiss-Prot Release 44.5 of September 13, 2004).⁴ Only human sequences were used in the similarity analysis. With the exception of P54/nrb, all the top1-binding proteins referred to in this work are in the Swiss-Prot sequences. Sequence analysis (identification of top1-binding proteins with similar sequence) was done using the Basic Local Alignment Search Tool algorithm and the PAM 30 matrix, word size of two; expect value of 20,000 out of the entire Swiss-Prot database.

Binding Analysis

The binding analysis of the peptides was done on a BIAcore 2000 instrument at the University of Utah Core Protein Interaction Facility. Each channel of a four-channel CM5 sensor chip was derivatized with one of the T1BPs (T1BP1, T1BP2, and T1BP4) and a control surface left blank. Approximately 100 to 300 RU of each peptide were titrated onto the chip. Catalytically active top1 was used as the mobile or analyte phase and diluted in 150 mmol/L NaCl, 30 mmol/L HEPES, 0.05% P20, and injected at various concentrations (8.0, 4.0, 2.0, 1.0, and 0.4 nmol/L) through the microfluidics sample handling system of the instrument at a flow rate of 30 μ L/min. With each injection of a particular concentration of top1, association and dissociation data for three peptides were generated. Binding constants were determined using the BIAevaluation 2.1 software.

Top1 Relaxation/Cleavage Activity Assays

Peptides were assayed for their ability to interfere with or enhance relaxation of supercoiled DNA mediated by 100 kDa top1. Top1 (80–120 μ g) and pBR322 (500 ng) were combined in a top1 reaction buffer [150 mmol/L KCl, 12 mmol/L MgCl₂, 2 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 7.5)] at 37°C for 30 minutes with 1 μ mol/L peptide. The 20 μ L reactions were stopped with 1.0 μ L of 1% SDS. Digestion of protein was done by addition of 1.0 μ g proteinase K for 30 minutes at 37°C, loaded with 5 μ L of a standard 5 \times bromphenol blue/glycerol loading buffer and run in an 0.8% agarose gel in TAE (0.04 mol/L Tris-acetate and 1.0 mmol/L EDTA). DNA cleavage assays were done in 20 μ L volumes of reaction buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L KCl, 10 mmol/L MgCl₂, 0.5 mmol/L EDTA, and 0.2 mg/mL bovine serum albumin], 500 ng radiolabeled (³H thymidine) supercoiled replicative form M13 mp 19 DNA and 112 ng top1. The reactions were incubated for 30 minutes at 30°C and then treated with 1 μ L of 1 mg/mL proteinase K in 0.05% SDS for an additional 30 minutes at 37°C to transform the 9AC-top1-DNA complexes into nicked DNA. This was followed by electrophoresis in an 0.8% agarose-TAE gel containing ethidium bromide (0.5 μ g/mL). A control set of experi-

ments and log serial dilutions of peptide were examined for each peptide. Percentage cleavage was determined by liquid scintillation counting of excised bands using the method described by Marshall et al. (31).

Cell Localization Study

Part of each batch of peptide synthesized with the TAT domain was labeled with an immunofluorescent dye (FITC) and confocal microscopy experiments confirmed peptide uptake into live cells. HCT 116 (human colon tumor cells) were grown in McCoy's medium supplemented with 7.5% calf serum/2.5% FCS (Atlanta Biologicals, Lawrenceville, GA), 10.0 units/mL penicillin, and 100 μ g/L streptomycin. Cells grown to 30% confluence in 60 mm plates were washed in PBS and the labeled TAT linked peptide was diluted to 1 μ mol/L and added to the plate in unsupplemented McCoy's medium and allowed to incubate at 37°C for 1 hour. The plates were washed in PBS, supplemented medium reapplied and the cells visualized.

Cytotoxicity Assay

HCT 116 cells grown as monolayer cultures (as above) were harvested by trypsinization before confluence and seeded at 20,000 per well in 200 μ L Corning 96-well microtiter plates in the same medium. Cell viability was determined at 3 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described by Marshall and coworkers (31) adapted from Mosmann et al. (32). A2780 ovarian MDR+ and MDR– were obtained from Dr. J. Kopecek (University of Utah, Salt Lake City, UT) and grown as the HCT 116 cells except in supplemented α -MEM.

In vivo Mouse Studies

HCT 116 cells (4×10^6) were injected into the flanks of nude (BALB/c nu/nu) mice for both experiments. The animals were randomized when the tumors were staged at 50 mm³. In the first experiment, control animals were administered 0.1 mL of vehicle (α -MEM containing 0.05% methylcarboxycellulose and 2% DMSO) i.p., in the absence of peptide (PBS, control 1) or presence of T1BP2-T (control 2, 150 μ g in 0.1 mL PBS) or T1BP3-T (control 3, 150 μ g in 0.1 mL PBS) injected s.c. at site of tumor. No difference in tumor growth was observed for these control groups and the results were combined as control ($n = 15$). Animals treated with 9AC were divided into four groups, each containing five mice. T1BP-T and PBS s.c. injections were administered as described above at the site of the tumor, 30 minutes after the i.p. injection of 9AC in a 50 μ L volume. 9AC was dissolved in vehicle. Groups consisted of animals treated with a total of 3 mg/kg 9AC (low dose) in the absence or presence of T1BP2-T or a total of 8 mg/kg 9AC (high dose). Animals were treated with 1 or 2 mg/kg doses twice a week over 2 weeks.

In the second experiment, 18 mice were evaluated in three groups of six animals. Control animals were injected i.p. with 0.1 mL vehicle (α -MEM containing 0.05% methylcarboxycellulose and 2% DMSO). Treated mice received a total of 9 mg/kg 9AC, 3 mg/kg in 0.1 mL vehicle a week over 3 weeks, in the absence or presence of 150 μ g T1BP2-T in 0.1 mL PBS injected s.c. at the site of the

³ <http://www.ncbi.nlm.nih.gov/blast>

⁴ <http://www.expasy.org/sprot>

tumor 30 minutes after 9AC injection. Animals were sacrificed when their tumors exceeded 15% of their body weight (Institutional Animal Care and Use Committees approval UU 00-05004).

Results

Phage Display

During each successive round of the biopanning process, the relative affinity of the phage for the top1 target complex were assessed by comparing the output of the top1-DNA-9AC plate or a control plate coated with Neutravidin and blocked with bovine serum albumin. Approximately 10-fold more phage were recovered from the top1-DNA-9AC-coated plate than from the control plate after the third round. One would expect that this output probably included phage that bound to DNA, top1, and DNA/top1 complexes. Because of difficulties in measuring interactions between three binding partners and our interest in phage that bound selectively to top1, a titrating experiment was done to identify selective binders. Several clones from the third round were subjected to analytic titrating against biotinylated top1 not in complex with 9AC or DNA. The results are presented in Fig. 1. The binding efficiencies of the clones to top1, as represented by the number of colonies recovered from the titrating experiment, were varied. Several of the phage that bound top1 above the control were sequenced, their displayed peptides were synthesized (denoted as T1BP1–T1BP5), and subjected to biophysical and biological assays to assess top1-binding affinity and *in vitro* and *in vivo* activity. These short peptide sequences were also queried for identities to known top1-binding proteins by Basic Local Alignment Search Tool searches (Table 1). This report focuses on the one peptide that had both *in vitro* and *in vivo* activity, SAYAATVRGPLSSAS (T1BP2).

T1BP2 Affinity for Top1

T1BP2 had remarkable affinity for top1 when assayed by surface plasmon resonance (Fig. 2A). When the kinetics of this interaction was modeled using the Langmuir model of association and dissociation, the K_D was determined to be 6.65×10^{-7} mol/L. Figure 2B illustrates that the model fits

Table 1. Examples of sequences of phage display–derived peptides that bind top1

Peptide name (clone from Fig. 1A)	Sequence	Number of times appeared/no. cloned sequences
T1BP1 (clone 1)	SSQVVGVPQLMQSSP	1/5
T1BP2 (clone 2)	SAYAATVRGPLSSAS	1/5
T1BP3 (clone 6)	DRVPLVHVIFNSFGY	1/5
T1BP4 (clone 9)	RNQGPKVMVFPIAPS	1/5
T1BP5 (clone 10)	EGQFTFPRGASE*	1/5

NOTE: T1BP1 to T1BP5 were isolated from the third round of biopanning against a top1-DNA-9AC complex.

*EGQFTFPRGASE was truncated due to stop codon.

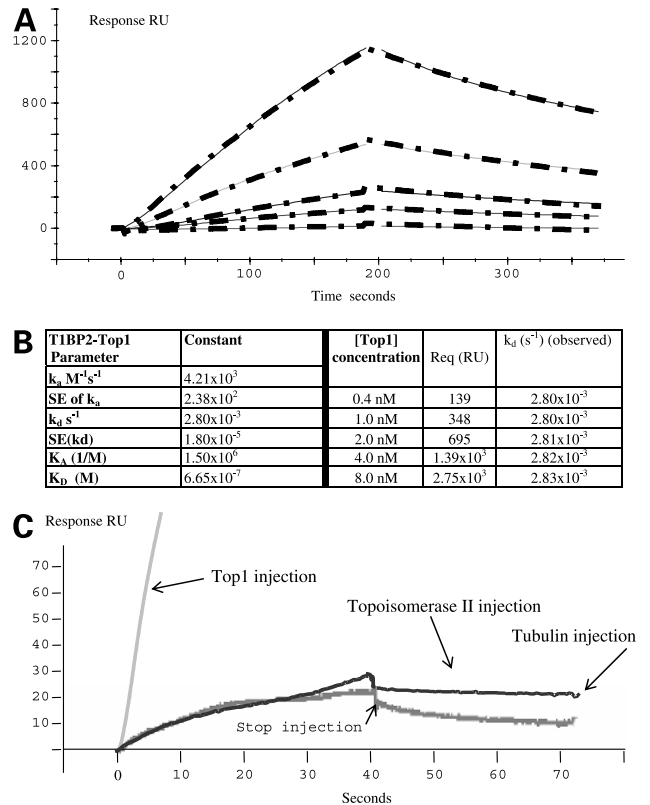


Figure 2. A, biosensor data generated from several concentrations of top1 (from top to bottom, 8.0, 4.0, 2.0, 1.0, and 0.4 nmol/L) injected over the T1BP2 surface. These top1 experiments were evaluated with BIAevaluation Software 3.1 using Langmuir kinetics to model the interaction. The accuracy of the fit to the model is shown by actual data (dashed lines) and fitted curves (narrow lines). B, the data indicate a low-micromolar affinity (K_D) of top1 for the novel peptide ligand. C, the biosensor response (summarized from three experiments) to the injection of 8.0 nmol/L top1, topoisomerase II, and tubulin over the channel derivatized with the T1BP2.

the data well; the association and dissociation curves that fit the peptide-top1 data were coincidental with curves generated by the model.

T1BP2-Binding Specificity for Top1

The biosensor surface, derivatized with T1BP2, was also used to quickly and efficiently evaluate the specificity of the peptide for top1 versus topoisomerase II (a functional analogue of top1) and tubulin (a protein unrelated to top1 but known to interact with many other proteins). The traces in Fig. 2C confirm the peptide specificity for top1 in the context of these other two proteins.

T1BP2 Effects on Top1-Mediated DNA Relaxation and Cleavage Complex Formation

Figure 3A is an image of a DNA relaxation assay run in the presence of T1BP2. Although other peptides discovered in our biopanning process had the ability to moderately interfere with or enhance the activity of top1, T1BP2 had only a slight ability to inhibit top1-mediated relaxation. In the cleavage complex assay (Fig. 3B), T1BP2 enhanced the formation of cleavage complexes at intermediate

concentrations of 9AC (data from 0.45 and 0.9 $\mu\text{mol/L}$; 0.45 $\mu\text{mol/L}$ data shown). At these concentrations of 9AC, through the range of peptide concentrations tested, T1BP2 increased cleavage complex formation by $\sim 50\%$.

Cellular Toxicity Studies

All five peptides were also assayed for cytotoxicity in tumor cell lines, but only T1BP2 had activity in cultured cells. The cellular toxicity data presented in Fig. 4 reveal that T1BP2-T had no toxicity when used alone, but acted to increase the cytotoxicity of 9AC when the cells were treated with the two together. Confocal fluorescence microscopy studies showed that FITC-labeled T1BP2-T localized to the cell nucleus (data not shown). The effect of T1BP2-T seems to be specific to top1 poisons because it did not significantly enhance the cytotoxicity of doxorubicin (Fig. 4C and D), etoposide, or UV light in HCT 116 cells (data not shown). The selective synergism with 9AC suggested that T1BP2-T is a top1 drug sensitizer with the potential for improving human cancer chemotherapy. Experiments with multidrug-resistant and normal ovarian cancer cells showed that drug sensitization is not likely due to MDR-mediated effects.

In vivo Activity of T1BP2 in Nude Mice

Nude mice bearing tumors derived from cultured HCT 116 cells and receiving 9AC chemotherapy were administered T1BP2-T. In two experiments, graphed in Fig. 5, the *in vivo* activity of T1BP2-T corresponded with the *in vitro* effects in the cleavage assay, (i.e., it increased the activity of 9AC). In the first experiment, T1BP2-T had no demonstrable effect in animals treated with 1 mg/kg 9AC on days 1,

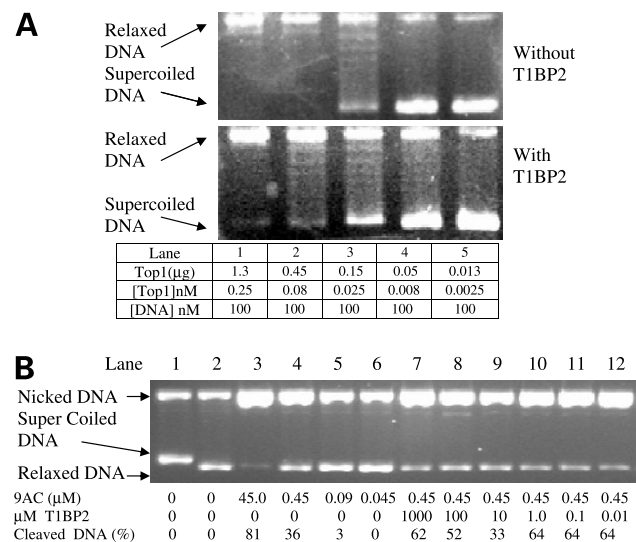


Figure 3. **A**, 100 kDa top1-DNA relaxation gels with (bottom) and without (top) 1 $\mu\text{mol/L}$ T1BP2. Inhibition of top1 relaxation (slight increase in supercoiled pBR322 DNA remaining) by T1BP2 activity was observed (lanes 1 and 2), which had relatively high concentrations of top1. **B**, cleavage complex assays containing T1BP2: lane 1, the ^3H -DNA substrate only (note that there is some endogenous nicked DNA). Lanes 2 to 12, 20 ng top1 and the indicated amounts of peptide and 9AC were added to each reaction. At 0.45 $\mu\text{mol/L}$ 9AC, the addition of peptide caused an increase in cleavage at all but one concentration in this experiment.

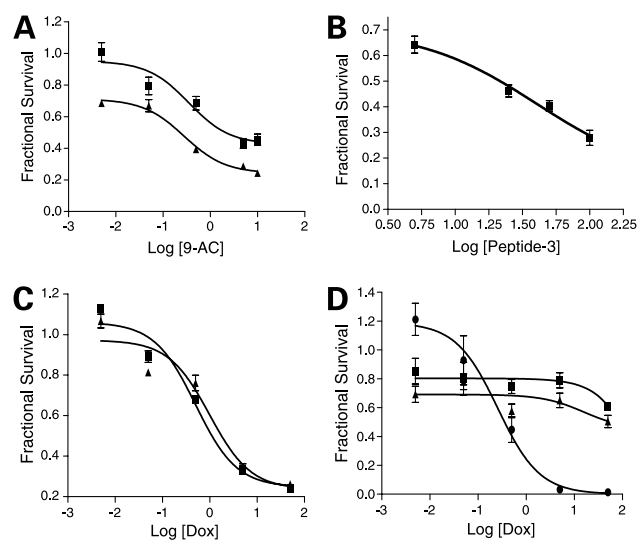


Figure 4. Cytotoxicity of T1BP2-T on HCT 116 colon cancer and MDR cells. **A**, 0.005 to 50 $\mu\text{g/mL}$ 9AC (\blacksquare), + 50 $\mu\text{g/mL}$ (16 $\mu\text{mol/L}$) T1BP2-T (\blacktriangle) in HCT 116. **B**, 0.5 $\mu\text{g/mL}$ 9AC + 0.01 to 100 $\mu\text{g/mL}$ (0.003–32 $\mu\text{mol/L}$) T1BP2-T in HCT 116. **C**, 0.005 to 50 $\mu\text{g/mL}$ doxorubicin (Dox; \blacksquare), + 50 $\mu\text{g/mL}$ (16 $\mu\text{mol/L}$) T1BP2-T (\blacktriangle) in HCT 116. **D**, 0.05 to 50 $\mu\text{g/mL}$ doxorubicin (\blacksquare), + 50 $\mu\text{g/mL}$ (16 $\mu\text{mol/L}$) T1BP2-T (\blacktriangle) in A2780 ovarian cells MDR+; 0.05 to 50 $\mu\text{g/mL}$ doxorubicin (\bullet) in A2780 ovarian cells MDR–.

4, and 8 (3 mg/kg 9AC). However, in animals treated with higher concentrations of 9AC, T1BP2-T had a statistically significant $P < 0.05$ augmentation of the antitumor effect of 9AC (day 15). The second experiment was similar except that mice received 3 mg/kg 9AC injections, with or without T1BP2-T, on days 1, 8, and 18. Significantly improved ($P < 0.05$) response was observed on days 12, 18, 21, and 26.

Discussion

Low-micromolar concentrations of T1BP2 had the ability to alter the *in vitro* catalytic rate of top1-mediated DNA relaxation. It also had the ability to increase the amount of cleaved DNA at certain 9AC-top1 concentrations. Most significantly, although nontoxic when administered alone, T1BP2 sensitized tumor cells to 9AC, both in cellular-based assays and in a mouse tumor model. T1BP2 increased top1-mediated DNA cleavage complex formation *in vitro* and the *in vivo* effect of T1BP2 significantly increased drug-specific killing of tumor cells.

Specific killing of tumor cells by 9AC is thought to be primarily due to processing of 9AC-top1-induced DNA single-strand breaks into DNA double-strand breaks during DNA replication in S phase. Therefore, the most direct explanation of the increased killing of the tumor cells with the combination of T1BP2 and 9AC is that T1BP2 stabilizes top1-DNA-9AC and this leads to more double-strand breaks. This hypothesis is consistent with the DNA cleavage assay results, but does not rule out other potential mechanisms of sensitization.

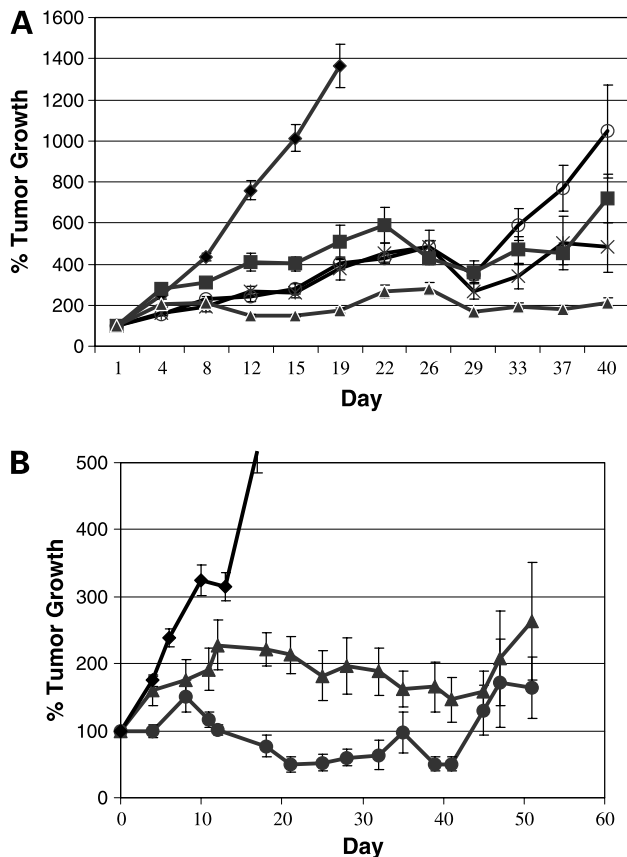


Figure 5. The results of *in vivo* experiments. **A**, control HCT 116 tumor growth (◆), 8 mg/kg 9AC (■), 8 mg/kg 9AC + T1BP2-T (▲), 3 mg/kg 9AC (○), and 3 mg/kg 9AC + 7.5 mg/kg (~2.5 μmol/L) T1BP2-T (×). At all doses tested, 9AC was found to have significant antitumor effects. Animals treated with 8 mg/kg 9AC and 7.5 mg/kg T1BP2-T had significantly lower ($P < 0.05$) tumor growth than animals treated with 8 mg/kg 9AC alone (day 15). **B**, control HCT 116 tumor growth (◆), 9 mg/kg 9AC (▲), and 9 mg/kg 9AC + 7.5 mg/kg T1BP2-T (●). Tumor growth in T1BP2-T-treated animals ($P < 0.05$) was observed at days 12, 18, 21, and 26, compared with mice receiving 9AC alone.

The sequence alignments between T1BP2 and known top1-binding proteins by analysis of the Swiss-Prot human sequence database may provide clues into such other potential mechanisms of sensitization that may act in tandem with cleavage complex modulation. When the Swiss-Prot database was queried with six residue fragments of the peptide sequence, several interesting and high-similarity matches were found. Figure 6 illustrates these identities. The NH₂ terminus of the peptide (YAATDR) was found to align with RNA polymerase II (largest subunit), transcription factor IID 55 (found in transcriptional complexes with top1), and Werner syndrome helicase, a top1 and p53-binding protein (12, 33). The COOH-terminal sequence of the peptide has similarity to p53, BRCA2, DNA polymerase II B, top2α, and DNA and RNA polymerases; all of which have been found in complexes with top1, shown to interact with top1 and/or be modulated by top1 (34, 35). The PLSS sequence,

although short and not unique, is found in only ~1 of 200 proteins in the Swiss-Prot human database. YAATDR was found less frequently.

This sequence similarity information suggests several signaling pathway-dependent mechanisms that could cause sensitization of cancer cells to killing by 9AC. Upon induction of ionizing radiation-induced double-strand break lesions in cells, top1 is very quickly delocalized from the nucleolus, ribosylated, complexed with p53, and relocalized at discrete foci (14, 17). Although PLSS is not in the reported binding domain of p53 for top1 (reported to be p53 residues 302–321; ref. 36), the PLSS region has been implicated in SH3-mediated interactions functioning in base excision repair of DNA damage and p53-mediated apoptosis in response to genotoxic stress (37). It is also a site of mutational activation of p53 (38). The similarity of T1BP2 to p53 suggests other possible mechanisms of cell sensitization. Along with other proteins, p53 helps to activate the two major DNA damage-dependent cellular checkpoints, the G₁-S and the G₂-M, as well as direct apoptosis. These mechanisms, respectively, allow DNA repair or commit cells to death, depending on the severity, type, and cell cycle timing of DNA damage. Tumor cell killing could be attributed to increased sensitivity to apoptotic stimuli or attempted replication of a severely damaged genome in cells exposed to a p53 mimic peptide. Additionally, given the potential lethality of top1 lesions on DNA and the economy of top1 demanded by its various cellular roles, top1 access to DNA at potential cleavage complex sites may be an important factor. If p53 simply sequesters top1 to reduce potential replication fork-induced double-strand breaks;

	T1BP2	SAYAATVRGPLSSAS
		YAAT
WSH:		2 YAAT 215
RNA polymerase II (largest subunit)	871	YDATVR
		YA+TVR
TFIID 55 kDa subunit:	24	YASTVR 29
		V+G--PLSS+
TopIIα	1385	VKGSVPLSSS
		PLSS+
P53:	92	PLSSS 96
		PLSS+
PCNA:	220	PLSST 225
		PLSS
BRCA2	2800	PLSS 2805
		+ PLSS
DNA polymerase II Subunit B	54	KQPLSS 59
		SA + PLSS
RNA polymerase III	371	SA---PFTQPLSS

Figure 6. T1BP2 similarities to top1-interacting proteins (proteins that have been found in complexes with top1, immunoprecipitate with top1, found to bind top1, or affect top1 function *in vitro*, as described in the text).

interruption of the p53-top1 interaction could cause the cell killing effects observed.

BRCA2, a scaffolding protein also involved in DNA repair, has not been shown to directly interact with top1 protein but a recent study found BRCA2 to increase top1 activity in cell extracts and modulate top1-mediated sensitivity to camptothecin (39).

In summary, this work describes the discovery of a short peptide T1PB2, which binds top1 with low-micromolar affinity, inhibits top1 DNA relaxation, increases top1-mediated cleavage complex formation, and increases tumor sensitivity to 9AC. The peptide also has interesting sequence similarity to several critical top1-binding, DNA-metabolizing enzymes, which may help explain the observed sensitization to 9AC.

Acknowledgments

We thank Dr. Joseph A. Holden.

References

- Coleman RL. Emerging role of topotecan in front-line treatment of carcinoma of the ovary. *Oncologist* 2002;7:46–55.
- Hobday TJ, Goldberg RM. Perspectives on the role of sequential or combination chemotherapy for first-line and salvage therapy in advanced colorectal cancer. *Clin Colorectal Cancer* 2002;2:161–9.
- Grothey A, Sargent D, Goldberg RM, Schmoll H-J. Survival of patients with advanced colorectal cancer improves with the availability of fluorouracil-leucovorin, irinotecan, and oxaliplatin in the course of treatment. *J Clin Oncol* 2004;22:1209–14.
- Karayan L, Riou JF, Seite P, et al. Human ARF protein interacts with topoisomerase I and stimulates its activity. *Oncogene* 2001;20:836–48.
- Straub T, Grue P, Uhse A, et al. The RNA-splicing factor PSF/p54 controls DNA-topoisomerase I activity by a direct interaction. *J Biol Chem* 1998;273:26261–4.
- Mao Y, Mehl IR, Muller MT. Subnuclear distribution of topoisomerase I is linked to ongoing transcription and p53 status. *Proc Natl Acad Sci U S A* 2002;99:1235–40.
- Gobert C, Bracco L, Rossi F, et al. Modulation of DNA topoisomerase I activity by p53. *Biochemistry* 1996;35:5778–86.
- Hannan RD, Cavanaugh A, Hempel WM, et al. Identification of a mammalian RNA polymerase I holoenzyme containing components of the DNA repair/replication system. *Nucleic Acids Res* 1999;27:3720–7.
- Desai SD, Zhang H, Rodriguez-Bauman A, et al. Transcription-dependent degradation of topoisomerase I-DNA covalent complexes. *Mol Cell Biol* 2003;23:2341–50.
- Rose KM, Szopa J, Han FS, et al. Association of DNA topoisomerase I and RNA polymerase I: a possible role for topoisomerase I in ribosomal gene transcription. *Chromosoma* 1988;96:411–6.
- Wang Z, Roeder RG. DNA topoisomerase I and PC4 can interact with human TFIIIC to promote both accurate termination and transcription reinitiating by RNA polymerase III. *Mol Cell* 1998;1:749–57.
- Lebel M, Spillare EA, Harris CC, Leder P. The Werner Syndrome gene product co-purifies with the DNA replication complex and interacts with PCNA and Topoisomerase I. *J Biol Chem* 1999;274:37795–9.
- Loor G, Zhang SJ, Zhang P, et al. Identification of DNA replication and cell cycle proteins that interact with PCNA. *Nucleic Acids Res* 1997;25:5041–6.
- Thielmann HW, Popanda O, Staab HJ. Subnuclear distribution of DNA topoisomerase I and Bax protein in normal and xeroderma pigmentosum fibroblasts after irradiation with UV light and γ rays or treatment with topotecan. *J Cancer Res Clin Oncol* 1999;125:193–208.
- Desai SD, Li T-K, Rodriguez-Bauman A, et al. Ubiquitin/26S proteasome-mediated degradation of topoisomerase I as a resistance mechanism to camptothecin in tumor cells. *Cancer Res* 2001;61:5926–32.
- Bauer PI, Buki KG, Comstock JA, Kun E. Activation of topoisomerase I by poly [ADP-ribose] polymerase. *Int J Mol Med* 2000;5:533–40.
- Smith HM, Groszovsky AJ. PolyADP-ribose-mediated regulation of p53 complexed with topoisomerase I following ionizing radiation. *Carcinogenesis* 1999;20:1439–44.
- Pommier Y, Kerrigan D, Hartman KD, Glazer RI. Phosphorylation of mammalian DNA topoisomerase I and activation by protein kinase C. *J Biol Chem* 1990;265:9418–22.
- Yu D, Khan E, Khaleque MA, et al. Phosphorylation of DNA topoisomerase I by the c-Abl tyrosine kinase confers camptothecin sensitivity. *J Biol Chem* 2004;279:51851–61.
- Rossi F, Labourier E, Forne T, et al. Specific phosphorylation of SR proteins by mammalian DNA topoisomerase I. *Nature* 1996;381:80–2.
- Merino A, Madden KR, Lane WS, et al. DNA topoisomerase I is involved in both repression and activation of transcription. *Nature* 1993;365:227–32.
- Hsiang YH, Lihou MG, Liu LF. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res* 1989;49:5077–82.
- Strumberg D, Pilon AA, Smith M, et al. Conversion of topoisomerase I cleavage complexes on the leading strand of ribosomal DNA into 5'-phosphorylated DNA double-strand breaks by replication runoff. *Mol Cell Biol* 2000;20:3977–87.
- Kang MR, Muller MT, Chung IK. Telomeric DNA damage by topoisomerase I: a possible mechanism of killing by camptothecin. *J Biol Chem* 2004;279:12535–41.
- Redinbo MR, Stewart L, Kuhn P, et al. Crystal structures of human topoisomerase I in covalent and noncovalent complexes with DNA. *Science* 1998;279:1504–13.
- Stewart L, Champoux JJ. Purification of baculovirus-expressed human DNA topoisomerase I. *Methods Mol Biol* 1999;94:223–34.
- Holden JA, Rolfson DH, Lowe RL. DNA topoisomerase I from human placenta. *Biochim Biophys Acta* 1990;1049:303–10.
- Parmley SF, Smith GP. Filamentous fusion phage cloning vectors for the study of epitopes and design of vaccines. *Adv Exp Med Biol* 1989;251:215–8.
- Nishi T, Tsurui H, Saya H. Construction and application of a phage random peptide library. *Exp Med* 1993;11:1759–64 (in Japanese).
- Vives E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* 1997;272:16010–7.
- Marshall KM, Matsumoto SS, Holden J, et al. The anti-neoplastic and novel topoisomerase II-mediated cytotoxicity of neomaphimedine, a marine pyridoacridine. *Biochem Pharmacol* 2003;66:447–58.
- Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63.
- Blander G, Kipnis J, Leal JFM, et al. Physical and functional interaction between p53 and the Werner's syndrome protein. *J Biol Chem* 1999;274:29463–9.
- Carty SM, Greenleaf AL. Hyperphosphorylated C-terminal repeat domain-associating proteins in the nuclear proteome link transcription to DNA/chromatin modification and RNA processing. *Mol Cell Proteomics* 2002;1:598–610.
- Czubaty A, Girstun A, Kowalska-Loth B, et al. Proteomic analysis of complexes formed by human topoisomerase I. *Biochim Biophys Acta* 2005;1749:133–41.
- Gobert C, Skladanowski A, Larsen AK. The interaction between p53 and DNA topoisomerase I is regulated differently in cells with wild-type and mutant p53. *Proc Natl Acad Sci U S A* 1999;96:10355–60.
- Jiang M, Axea T, Holgate R, et al. p53 binds the nuclear matrix in normal cells: binding involves the proline-rich domain of p53 and increases following genotoxic stress. *Oncogene* 2001;20:5449–58.
- De Vries EM, Ricke DO, De Vries TN, et al. Database of mutations in the p53 and APC tumor suppressor genes designed to facilitate molecular epidemiological analyses. *Hum Mutat* 1996;7:202–13.
- Rahden-Staron I, Szumilo M, Grosicka E, et al. Defective BRCA2 influences topoisomerase I activity in mammalian cells. *Acta Biochim Pol* 2003;50:139–44.

Molecular Cancer Therapeutics

Identification of a small topoisomerase I-binding peptide that has synergistic antitumor activity with 9-aminocamptothecin

Christopher D. Pond, Kathryn M. Marshall and Louis R. Barrows

Mol Cancer Ther 2006;5:739-745.

Updated version Access the most recent version of this article at:
<http://mct.aacrjournals.org/content/5/3/739>

Cited articles This article cites 38 articles, 17 of which you can access for free at:
<http://mct.aacrjournals.org/content/5/3/739.full#ref-list-1>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://mct.aacrjournals.org/content/5/3/739>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.