Cancer Chemother Pharmacol (2004) 53: 107–115
DOI 10.1007/s00280-003-0717-6

ORIGINAL ARTICLE

**Michael C. Nivens · Takita Felder
Amanda H. Galloway · Maria Marjorette O. Pena
Jeffery J. Pouliot · H. Trent Spencer**

# Engineered resistance to camptothecin and antifolates by retroviral coexpression of tyrosyl DNA phosphodiesterase-I and thymidylate synthase

Received: 10 April 2003 / Accepted: 29 August 2003 / Published online: 7 November 2003


**Abstract** *Purpose*: Gene transfer of cDNA sequences that confer drug resistance can be used (1) to protect hematopoietic cells against the toxic effects of chemotherapy, (2) for in vivo enrichment of genetically engineered cells and (3) to protect cytotoxic T lymphocytes in drug-resistant immunotherapy approaches for the treatment of cancer. We have previously developed strategies to confer resistance to agents targeting thymidylate synthase (TS) and have now expanded our drug resistance strategies to include retroviral expression of tyrosyl-DNA phosphodiesterase (TDP-I), an enzyme recently implicated in the repair of topoisomerase-I (Top-I)/DNA lesions induced by camptothecin (CPT). The combination of TS and Top-I inhibition has been shown to be an effective treatment for several types of cancer. *Materials and methods*: Retroviral vectors were generated that individually encoded TS and TDP-I or that coexpressed both enzymes. Murine fibroblast and Chinese hamster lung transfectants were generated with the vectors and resistance to TS- and Top-I-directed inhibitors was tested. Murine bone marrow progenitor cells were also transduced using recombinant retroviruses encoding TS and TDP-I and the degree of drug resistance conferred to gene-modified cells was tested. *Results*: Enforced expression of TDP-I increased TDP-I activity in gene-modified cells and conferred up to threefold resistance to CPT. The degree of resistance was dependent on the duration of drug treatment. Simultaneous expression of the TS gene encoding *E. coli* TS optimized for expression in mammalian cells (optecTS) and TDP-I conferred extremely high-level resistance to concurrent treatment with the TS-inhibitor BW1843U89 and CPT. Furthermore, by direct analysis of DNA fragmentation using the comet assay, substantial protection was conferred (fourfold) against DNA fragmentation associated with combination drug treatments by dual enzyme expression compared to non-modified cells. Hematopoietic progenitor assays of murine bone marrow cells transduced with retroviral vectors encoding TS and TDP-I showed that bone marrow cells could be protected from the cytotoxic effects of TS and Top-I inhibition. *Conclusions*: Enforced expression of optecTS and TDP-I conferred antifolate and CPT resistance to genetically modified cells. Additionally, this work further illustrated a role for TDP-I in the repair of dead-end Top-I complexes and implied that TDP-I expression analysis may aid in predicting the therapeutic effectiveness of the CPT class of compounds.

**Keywords** Camptothecin · Antifolates · Drug resistance · Thymidylate synthase · Tyrosyl DNA phosphodiesterase

T. Felder · A. H. Galloway · M. M. O. Pena
Department of Chemistry and Biochemistry,
University of South Carolina,
Columbia, SC 29208, USA

J. J. Pouliot
Laboratory of Molecular Biology,
National Institute of Mental Health,
Building 36, Room 1B08, Bethesda,
MD 20892-4034, USA

M. C. Nivens · H. T. Spencer (✉)
Department of Pediatrics, Division of Hematology/Oncology
and Bone Marrow Transplantation,
Emory University School of Medicine, 2040 Ridgewood
Dr. N.E., Atlanta, GA 30322, USA
E-mail: hspence@emory.edu
Tel.: +1-404-7128554
Fax: +1-404-7274455

## Introduction

Retroviral transfer of genes that confer drug resistance to hematopoietic cells that can protect an individual from the dose-limiting myelosuppressive side effects of specific chemotherapy regimens is currently being evaluated in clinical trials, and this strategy may also allow more aggressive chemotherapy dosing schedules (Sorrentino 2002; Koc et al. 1996). In addition, drug resistance can be used for in vivo enrichment of genetically modified cells and provide a mechanism for overcoming

low transduction efficiencies, which is a limitation in many gene transfer strategies. Enrichment of gene-modified cells has been accomplished using cDNAs encoding dihydrofolate reductase (DHFR), p-glycoprotein, alkylguanine-DNA alkyltransferase, and thymidylate synthase (TS) (Licht et al. 2000; Spencer et al. 1996; Allay et al. 1998; Davis et al. 2000; Shaw et al. 2001). Although many of these gene products are well characterized and are currently being used in clinical trials, an ideal drug-resistant marker has yet to be identified. Therefore, it is important to optimize drug resistance by evaluating novel genes that may allow for the attenuation of toxicity of additional neoplastic agents. The purpose of the current study was to characterize the use of tyrosyl-DNA-phosphodiesterase-I (TDP-I) as a potential drug-resistant gene and to illustrate the advantage of simultaneously expressing multiple genes to confer resistance to combinational chemotherapy regimens.

Chemotherapy using 5-fluorouracil (5-FU, a precursor of FdUrd) and camptothecin (CPT, e.g. CPT-11), which are inhibitors of TS and topoisomerase-I (Top-I) respectively, is an effective treatment for late-stage colorectal cancer. Both TS and Top-I have been extensively studied and are well characterized, making them reasonable targets for drug-resistant gene therapy studies (Carreras and Santi 1995; Spencer et al. 1997; Champoux 2001). TS is an attractive target for chemotherapy because it is the rate-limiting step for the de novo synthesis of thymidylate, a required precursor for DNA synthesis. Because of the intrinsically high turnover rates of the hematopoietic and gastrointestinal compartments, mucositis and myelosuppression are dose-limiting for inhibitors of TS. The toxic effects of TS inhibition can be overcome by enforced expression of TS, effectively reversing the conditions of thymidine starvation. Recombinant *E. coli* TS (ecTS) has a lower affinity for antifolate-based TS inhibitors compared to the highly conserved mammalian enzymes (Fantz et al. 1998), and we have shown that when ecTS is optimized for mammalian expression (optecTS) high-level resistance can be conferred to murine fibroblast and hematopoietic cells against TS-directed inhibitors (Shaw et al. 2001).

Recently, novel mechanisms have been proposed describing the modulation of Top-I inhibition. These mechanisms present an opportunity to possibly modulate CPT toxicity using drug-resistant gene therapy strategies. The cellular toxicity resulting from Top-I inhibition develops from a series of increasingly harmful forms of DNA damage. In the absence of inhibition, Top-I cleaves one strand of a DNA duplex, forming a transient covalent intermediate between the enzyme's active site tyrosine and the 3′ end of the DNA (Nitiss and Wang 1996). After reducing the level of supercoiling, the enzyme safely reseals the DNA and dissociates (Champoux 2001). CPT and related compounds stabilize the Top-I/DNA complex, preventing the resealing step (Hsiang et al. 1989a; 1989b). In this way, chemical inhibition of Top-I acts in a similar manner to naturally occurring DNA lesions, such as abasic sites and oxidative products, that physically block the religation step of Top-I (Roffler et al. 1994; Lanza et al. 1996; Pourquier and Pommier 2001). If religation is inhibited, cellular toxicity occurs when a replication fork encounters a trapped Top-I complex (Knab et al. 1993), leading to DNA double-strand breaks (DSBs) that cannot be easily resealed because of the covalently bound protein on the 3′ end (Chen and Liu 1994; Froelich-Ammon and Osheroff 1995).

Recently, a mechanism for repair of this damage has been suggested by the discovery of an enzyme that can cleave the bond between Top-I and the 3′ end of DNA. (Yang et al. 1996). Several lines of evidence indicate that yeast tyrosyl-DNA-phosphodiesterase (TDP-I) functions in the removal of Top-I-linked DNA breaks and that such removal is part of a recombinational repair pathway (Pouliot et al. 2001) (Fig. 1A). TDP-I has been cloned from yeast (Pouliot et al. 1999) and humans (Interthal et al. 2001) and has obvious homologs in the genomes of several other species. The expression of TDP-I in mammalian cell lines, however, has not been extensively investigated.

CPTs are unique in that direct inhibition of Top-I activity accounts for only a small portion of the overall toxicity, with the majority of toxicity arising from DSBs as a result of interaction of the replication fork with the inhibited enzyme. The mechanism of CPT toxicity presents a unique opportunity to exploit a naturally occurring enzyme activity (TDP-I) using gene transfer techniques. In this study we showed that enforced expression of yeast TDP-I conferred resistance against CPT. The reduction of DNA damage induced by CPT in TDP-I-overexpressing transfectants demonstrates that the decreased toxicity was likely a result of a reduced incidence of DNA DSBs. Furthermore, because of the current clinical applications of Top-I inhibition combined with inhibitors of TS, we chose to overexpress TDP-I in combination with an optimized cDNA encoding ecTS. The results showed that extremely high-level resistance can be conferred on cells genetically engineered to express both enzymes.

## Materials and methods

### Generation of retroviral constructs

Retroviral constructs encoding human TS (hTS) and ecTS (Fig. 1B) have been previously described (Shaw et al. 2001). The retrovirus encoding optecTS was obtained by cloning the Sac II/Xho I optecTS cDNA fragment into the Sac II/Xho I MCS sites of pLXSN (Clontech, Palo Alto, Calif.), generating LOTS (Fig. 1B). Constructs encoding both optecTS and GFP were constructed by PCR cloning of the cDNA for eGFP into LOTS, generating LTG (Fig. 1B). The cDNA encoding the yeast TDP-I gene was propagated in the Novagen Pet 3B plasmid. The TDP-I cDNA was subcloned into the BamH I site of Qiagen PQE31, generating PYI. The TDP-I cDNA portion was then cloned into the BamH I site of pLXSN and screened for proper orientation,

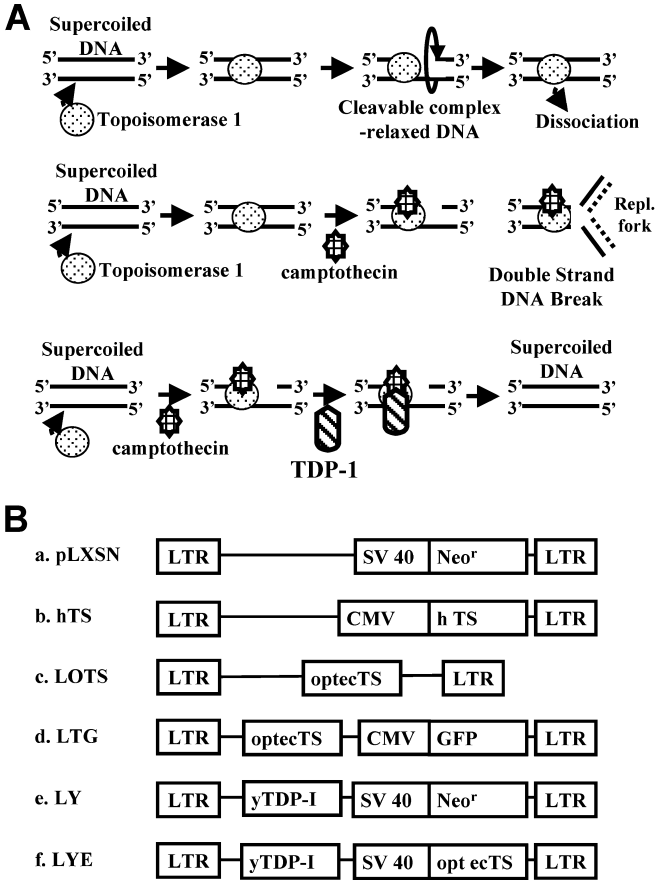


**Fig. 1A, B** Proposed schematic of TDP-I activity and constructs generated for drug-resistant gene therapy strategies. **A** Top-I catalyzes the transient covalent break in one strand of the DNA duplex to reduce the level of DNA supercoiling. Camptothecin induces stabilization of the covalent DNA/Top-I intermediate dead-end complex. TDP-I catalyzes the naturally occurring enzymatic activity that repairs this complex, and prevents subsequent damage by specifically cleaving the 3′ tyrosyl DNA-phosphodiester bond between Top-I and DNA. This activity prevents DNA fragmentation thought to occur when the dead end complex interacts with ongoing replication forks. Subsequent repair of nicked DNA occurs by existing enzymatic activities. **B** Retroviral vectors generated in the pLXSN backbone. Human TS (hTS) and LOTS were generally used as control vectors. LTG expresses both optecTS and eGFP from a single construct. LY expresses yTDP-I from the retroviral LTR and the neo-resistance gene from the internal SV40 promoter. LYE expresses TDP-I from the retroviral LTR, and optecTS from an internal SV40 promoter

generating LY (Fig. 1B). For coexpression of TDP-I and optecTS, LYE (Fig. 1B) was constructed by cloning an SV40-driven optecTS cDNA, which has previously been shown to confer resistance to BW1843U89 (Shaw et al. 2001) into LY, generating LYE (Fig. 1B).

## Cell lines and survival curves

All cells were grown in DMEM (Cellgro, Mediatech) containing L-glutamine (4 m*M*), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, Ga.). The LY plasmid was transfected into both NIH 3T3 and the TS-deficient Chinese hamster lung [CHL($TS^-$)] cell line RJK88.13 by calcium phosphate precipitation (Stratagene, La Jolla, Calif.). Stable transfectants were selected in G418 (800 μg/ml) (Life Technologies, Rockville, Md.). Both hTS and LYE were transfected into CHL($TS^-$) cells by calcium phosphate precipitation, and stable transfectants were selected for TS expression by blocking thymidine uptake using the nucleoside transport inhibitor dipyridamole at a concentration of 5 μ*M*. Survival curves were obtained using polyclonal populations of dipyridamole-selected cells seeded on six-well plates at a concentration of $1.5\times10^4$ cells per well. To generate survival curves using CPT, CPT was added at various concentrations up to 500 n*M* for either 24 h or continuously for 6 days. Surviving cells were counted using a hemocytometer 6 days after seeding or when the untreated cells reached confluency. The results are reported as the average of three separate experiments. For combinational chemotherapy treatments, survival curves were obtained using CHL($TS^-$) cells transfected with LYE, hTS and LTG. In combinational chemotherapy regimens, CPT was applied concurrently with the antifolate BW1843U89 at the concentrations indicated in the figures.

## TDP-I activity assay

The TDP-I activity assay was carried out essentially as described by Pouliot et al. (1999). The oHN279y (TCCGTTGAAGCCTGCTT-Ty) substrate was kindly provided by Dr. Howard Nash (National Institutes of Health), and contained a 3′-tyrosine covalently bound to the DNA sequence. Reactions were carried out in buffer containing 50 m*M* Tris, pH 8.0, 5 m*M* dithiothreitol (DTT), 100 m*M* NaCl, 5 m*M* EDTA, 10% glycerol, and 500 μg/ml bovine serum albumin. Briefly, 40 pmol of substrate was labeled with 40 pmol of 3000 Ci/mmol gamma [$^{32}$P]ATP using T4 polynucleotide kinase, and was purified on a G-10 spin column. Cell lysates were generated from CHL($TS^-$) cells by lysing a confluent 100-mm plate in cell disruption buffer (10 m*M* Tris, pH 7.9, 10 m*M* $MgCl_2$, 1 m*M* DTT, 1% Triton X-100, 5% glycerol, 1 m*M* EDTA, 1× protease inhibitor cocktail, and 0.1% Ipegal CA 630). The assay was carried out in 12 μl with serial dilutions from 0.15 to 0.006 μg/ml of total protein. Each reaction used 0.04 pmol of the purified substrate per reaction, and was allowed to proceed for 20 min at 30°C. Reactions were stopped by the addition of 5 μl sequencing dye, transfer to ice, and loading onto a 12% polyacrylamide gel. The resulting autoradiograms were analyzed by density scanning.

## Detection of DNA fragmentation by the comet assay

To assess DNA damage in individual cells the comet assay was performed essentially as described by Fairbairn et al. (1995). DNA fragmentation as a result of either CPT alone or in combination with BW1843U89 was assessed after plating $1\times10^6$ cells in six-well plates. Cells were treated 4 h after plating with no drug, with 300 n*M* CPT, or with 300 n*M* CPT plus 150 n*M* BW1843U89 for 14 h. After treatment cells were trypsinized and washed once with DMEM then with phosphate-buffered saline, then resuspended to a density of $2.5\times10^5$ cells/ml. A 500-μl aliquot of this solution was added to 1 ml 1% low-melting agarose type VII (Sigma, St. Louis, Mo.), and plated on a glass slide. The cells were lysed using a buffer comprising 100 m*M* EDTA, 2.5 *M* NaCl, 10 m*M* Tris-HCl (pH 10.5) and1% Triton X-100, followed by a wash with water, and treated with an alkali buffer containing 50 m*M* NaOH and 1 m*M* EDTA (pH 12.5). The cells were electrophoresed for 50 min at 9 V in the dark, neutralized in 0.5 *M* Tris (pH 7.5), washed in distilled water, stained with 2.5 μg/ml propidium iodide (Sigma), and allowed to dry overnight. Slides were analyzed using a fluorescence microscope (Olympus BX51) and Image-Pro plus software (Media Cybernetics, Silver Spring, Md.). The percentage of cells forming a visible comet was calculated based on the total number of cells, and the percentage of comet formation per cell type was determined from the average of a triplicate set of experiments using duplicate slides in each experiment.

Murine bone marrow transduction and clonogenic assays

To transduce bone marrow cells isolated from C57Bl/6 J mice, retrovirus was transiently produced by transfecting 6 μg each of Gag/Pol plasmid (Stratagene), Ecotropic envelope plasmid (Stratagene), and either LYE or LTG plasmids into 293T cells. Murine bone marrow was isolated as previously described (Spencer et al. 1996), and allowed to expand for 1 day after stimulation with rmIL-3 (20 ng/ml), rmIL-6 (10 ng/ml), and rmSCF (50 ng/ml). Viral supernatant (5 ml) was collected at 40 and 60 h after transfection and applied to $6.0\times10^4$ bone marrow cells in two viral applications (20 h apart), supplemented with polybrene at a concentration of 6 μg/ml. LYE-transduced cells were then challenged with 100 n*M* CPT for 24 h then plated in methylcellulose (M3435, Stem Cell Technologies, Vancouver) containing 100 n*M* BW1843U89.

## Results

### Resistance conferred by enforced expression of TDP-I

A putative function of TDP-I is the cleavage of the tyrosyl-phosphodiester bond joining Top-I to the 3′ end of DNA that results from natural DNA lesions or from Top-I inhibition (Fig. 1A). This model of the release of Top-I from the cleavable complex may circumvent the toxicity induced by CPT or natural DNA lesions that cause dead-end complexes to persist. To determine if enforced expression of TDP-I confers resistance against CPT, we constructed retroviral vectors encoding TDP-I only, and vectors that coexpressed TDP-I and optecTS (Fig. 1B).

Survival curves were generated using pLXSN or LY stable transfectants of NIH 3T3 or CHL($TS^-$) cells. Polyclonal NIH 3T3 populations were continuously challenged with increasing CPT concentrations for 6 days. Under these continuous treatment conditions, expression of TDP-I did not confer a survival advantage over mock transfectants (Fig. 2A). However, if challenged for only 24 h then allowed to grow in drug-free medium for 5 days, a moderate survival advantage was observed for LY transfectants compared to pLXSN mock transfectants (Fig. 2A). Under the same conditions, similar results were obtained for stable CHL($TS^-$) LY transfectants compared to pLXSN control cells, but a more dramatic protection was observed with a threefold increase in the CPT $IC_{50}$ value (Fig. 2B). The doubling times for all transfectanted cell lines were similar, approximately 15 h in log phase. Analysis of TDP-I activity of a polyclonal population of stable CHL($TS^-$) LY transfectants and two monoclonal stable transfectants showed that the increase in the $IC_{50}$ value was accompanied by an average threefold increase in TDP-I activity (Fig. 3). TDP-I activity of LY transfectants increased proportionately to the amount of protein used in the assay. In contrast, the activity of cell-free extracts from mock transfectants was at or below the sensitivity of the assay. These results show that resistance to CPT can be achieved by enforced retroviral expression of TDP-I under specific CPT treatment.

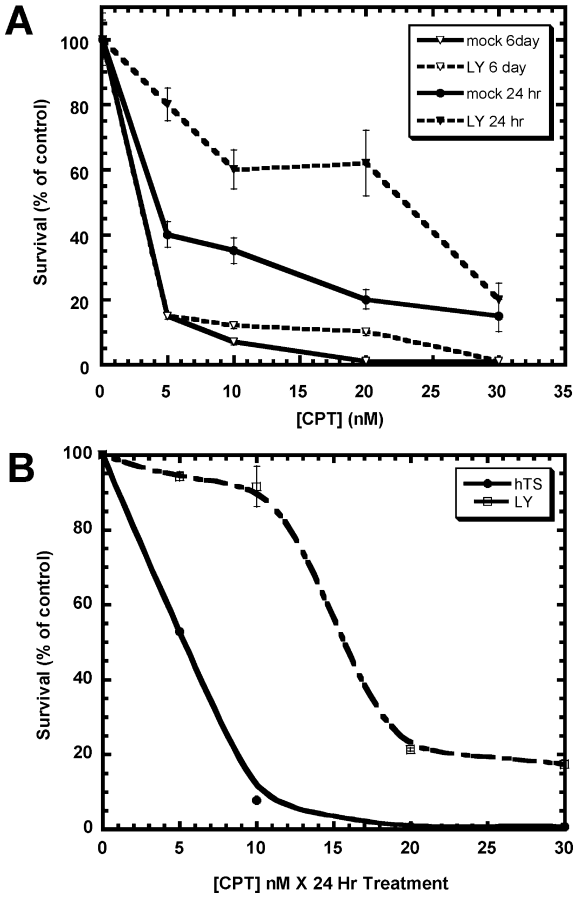


**Fig. 2A, B** Drug-resistant studies in NIH 3T3 and CHL($TS^-$) cells. **A** NIH 3T3 cells were transfected with either pLXSN or LY and selected in G418. Open symbols represent continuous drug treatment and closed symbols represent 24 hour treatments. Dashed lines represent TDP-I transfectants and solid lines represent mock controls. Under continuous CPT treatment no survival advantage was observed for LY transfectants, but survival differences were observed if transfectants were treated for only 24 h. **B** Resistance was observed for CHL($TS^-$) LY stable transfectants (*open squares*) treated with CPT for 24 h, as compared to hTS transfectants (*closed circles*)

### Resistance conferred against CPT and antifolates by coexpression of TDP-I and TS

We have previously shown that an ecTS construct (optecTS) optimized for mammalian expression confers significant resistance against TS-directed antifolates (Shaw et al. 2001). The antifolate BW1843U89 is among the most potent inhibitors of TS, and when used in conjunction with optecTS allows the greatest fold resistance to gene-modified cells compared to the use of other antifolate TS inhibitors. To determine if coexpression of optecTS and TDP-I conferred dual resistance against TS and Top-I inhibitors, we generated CHL($TS^-$) transfectants that express hTS, optecTS, or optecTS and TDP-I. Compared to stable transfectants expressing hTS, CHL($TS^-$) cells expressing optecTS alone or in combination with TDP-I were significantly more resistant to the TS-specific inhibitor BW1843U89

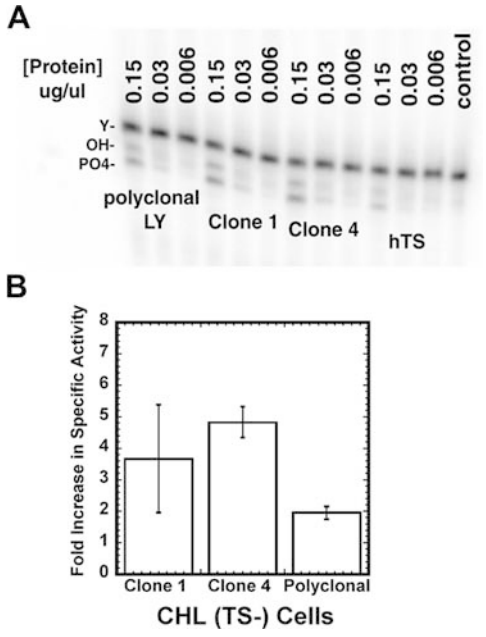


**Fig. 3A, B** TDP-I activity in CHL(TS$^-$) cells. **A** Total protein from stable transfectants was analyzed for TDP-I activity. The letter *Y* denotes the tyrosyl terminated TDP-I oligonucleotide substrate. The bands represented by *PO4* (phosphate-terminated) indicates the release of the tyrosyl moiety by TDP-I, and the bands denoted by *OH* indicate the hydroxyl terminated oligonucleotide. The phosphate-terminated product (*PO4*) is converted to OH by unknown enzyme activities after incubation in whole cell lysates. After three separate experiments, an average threefold induction of TDP-I activity was observed in a polyclonal population of stably transfected cells and two blindly picked clones (clones 1 and 4) compared to mock-transfected controls (**B**)

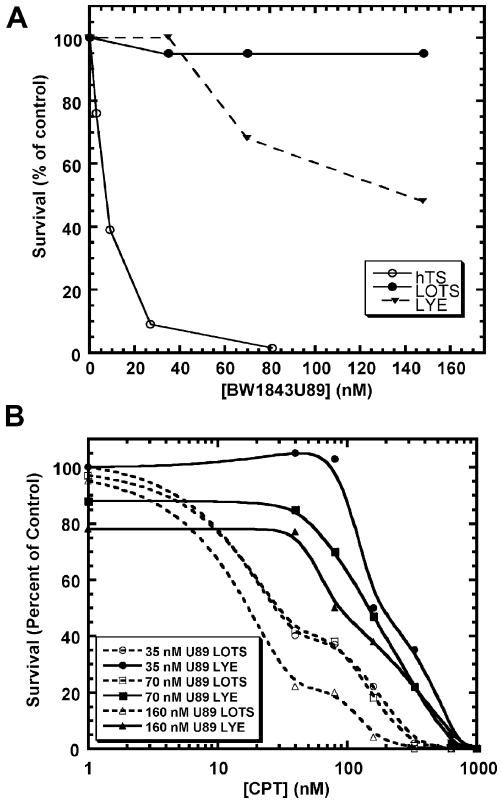


**Fig. 4A, B** Survival curves for hTS, LOTS and LYE stable CHL(TS$^-$) transfectants. Stable transfectants were generated using hTS, LOTS or LYE then grown in increasing concentrations of BW1843U89. **A** Cells expressing optecTS are >20-fold more resistant to BW1834U89 than cells overexpressing hTS. Expression of TDP-I in the bicistronic construct lowers ecTS enzyme levels which results in lower BW1843U89 resistance compared to cells overexpressing optecTS alone. **B** Stable transfectants expressing only ecTS (*dashed lines*) or both ecTS and TDP-I (*solid lines*) were challenged with increasing concentrations of CPT at various concentrations of BW1843U89 (*U89*). Increased survival was observed for cells expressing both enzymes compared to cells expressing only ecTS. Concentrations of drugs are shown in the figure

(Fig. 4A). As expected, stable transfectants expressing both optecTS and TDP-I were less resistant to BW1843U89 (Fig. 4A) due to a decrease in ecTS levels encoded by the dual expression vector, which were approximately twofold lower than constructs encoding only optecTS.

To determine if enforced expression of TS and TDP-I conferred dual resistance against BW1843U89 and CPT, CHL(TS$^-$) transfectants were treated with various concentrations of CPT (0–1 μ*M*) for 24 h, then exposed continuously to increasing concentrations of BW1843U89 (35, 70 and 160 n*M*). After 6 days, surviving cells were counted and the number compared to the number of control cells expressing only ecTS. As shown in Fig. 4B, at each concentration of BW1843U89, transfectants expressing both enzymes were resistant to CPT compared to transfectants expressing only optecTS.

Also, as expected, transfectants expressing hTS were progressively sensitive to BW1843U89 at increasing concentrations of CPT (Fig. 5A). Similarly, transfectants expressing optecTS only were progressively sensitive to BW1843U89 at increasing concentrations of CPT (Fig. 5B). However, transfectants expressing both optecTS and TDP-I were insensitive to increasing concentrations of CPT up to 80 n*M* (Fig. 5C). From these data, the fold difference in the BW1843U89 $IC_{50}$ value could be calculated for LYE compared to hTS at the various concentrations of CPT. The LYE construct conferred approximately 20-fold resistance to BW1843U89 over hTS in the absence of CPT, 110-fold at 40 n*M* CPT, and greater than 150-fold at 80 n*M* CPT. This resistance rapidly diminished at higher concentrations of drug.

## Comet analysis of CPT- and BW1843U89-induced DNA damage

Treatment of cells with CPT leads to the accumulation of double-stranded DNA fragmentation and initiates cell death (Wu et al. 2002; Lansiaux et al. 2001). BW1843U89 treatment has also been shown to cause double-stranded DNA fragmentation through the inhibition of TS (Backus et al. 2000). To determine if TDP-I

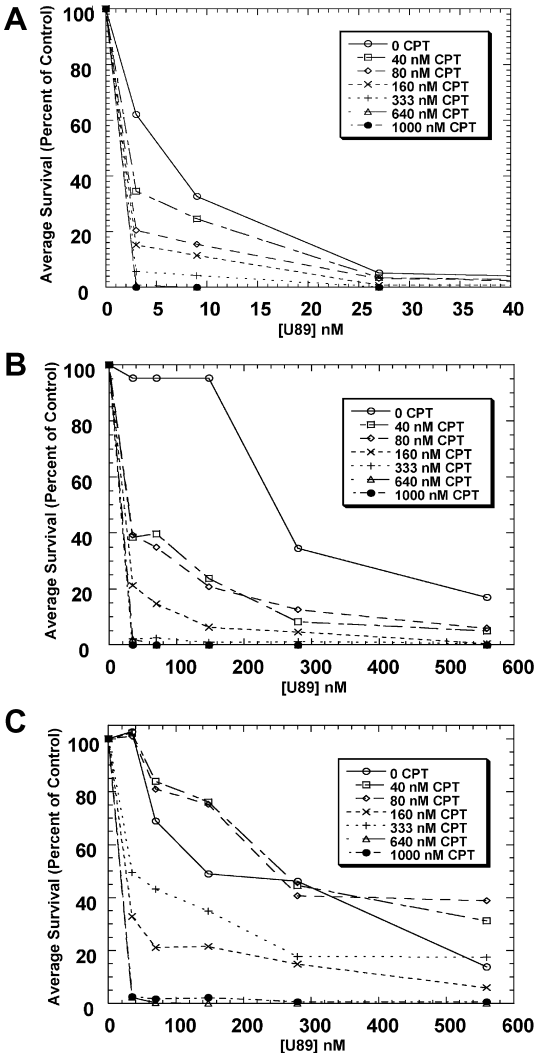


**Fig. 5A–C** Coexpression of yTDP-I and optecTS confers high level resistance to combined CPT and BW1843U89. Stable CHL(TS$^-$) hTS (**A**), LOTS (**B**), or LYE (**C**) transfectants were treated for approximately 20 h with various CPT concentrations then continuously with various concentrations of BW1843U89. **A** Cells expressing only hTS were increasingly sensitive to higher concentrations of CPT and BW1843U89. **B** Cells expressing optecTS were resistant to BW1843U89 alone but were sensitive to increasing concentrations of CPT. **C** Cells expressing both optecTS and TDP-I were insensitive to BW1843U89 at doses as high as 80 n*M* CPT

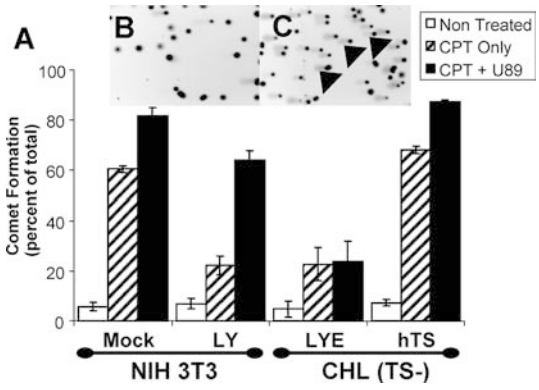


**Fig. 6A–C** Comet analysis as a measure of drug-induced DNA damage after 16 h drug treatment. Because CPT treatment causes DNA fragmentation, the comet assay was used as an indicator of CPT- and U89-induced toxicities. **A** Stable NIH 3T3 transfectants were generated with pLXSN as controls and LY, or CHL(TS$^-$) transfectants were generated with LYE, and hTS transfectants were used as controls. Comet-forming cells resulting from CPT treatment, CPT and BW1843U89 treatments or no treatment were blindly counted and recorded from three separate experiments. A positive comet was any cell having a visible comet (*arrows* in **C**). **B** Representative comet formation of CHL(TS$^-$) cells expressing both yTDP-I and optecTS after treatment with 300 n*M* CPT and 150 n*M* BW1843U89 treatment. **C** Control cells after an identical CPT/BW1843U89 treatment. *Arrows* indicate comet formation resulting from double-stranded DNA damage. Expression of TDP-I alone is sufficient to protect two cell lines from CPT-induced toxicity, but not against combined CPT/BW1843U89 treatment. Coexpression of TDP-I and optecTS protects NIH 3T3 cells and CHL(TS$^-$) cells from DNA fragmentation

and optecTS expression reduced DNA damage, we performed a comet assay to directly measure the extent of DNA fragmentation after drug treatment (McCarthy et al. 1997) and were able to quantify the damage induced by treatment with CPT alone and in combination with BW1843U89. As shown in Fig. 6A, transfectants expressing TDP-I were protected against CPT-induced DNA damage in both NIH 3T3 and CHL(TS$^-$) cells. As expected, TDP-I expression alone did not provide protection against the combination of CPT and BW1843U89, as shown by a threefold greater comet formation between mock-transfected cells expressing only hTS and cells expressing TDP-I (Fig. 6A). However, coexpression of TDP-I and optecTS conferred nearly complete protection from DNA damage compared to nontransfected cells (Fig. 6). These results provide direct molecular evidence of DNA protection against CPT/BW1843U89-induced DNA damage.

## Bone marrow transduction and protection of bone marrow progenitor cells

To determine if retroviral transduction of TS and TDP-I from the LYE construct confers resistance to clonogenic progenitor cells, recombinant retrovirus was generated with LYE and LTG. LTG transduction of 293T cells were analyzed by FACS to confirm production of viable virus using the method of Galipeau et al. (1999) and allowed a viral titer of $5\times10^5$ infectious particles/ml to be estimated. Consistent with our previous results (Shaw et al. 2001), expression of optecTS in hematopoietic cells protected transduced bone marrow progenitors against BW1843U89 (Fig. 7; LTG-transduced vs control). But transduction of murine bone marrow with LTG conferred little resistance to the combination of BW1843U89 and CPT. However, compared to mock-transduced controls, increased survival was observed in transduced progenitor cells coexpressing TS and TDP-I (Fig. 7; LYE-transduced vs control). Progenitor cells coexpressing both enzymes were more than fivefold more resistant to the BW1843U89/CPT combination. These results show that high-level resistance against TS

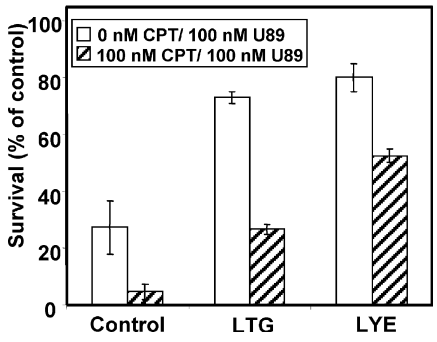


**Fig. 7** Progenitor survival assay. Murine bone marrow cells were isolated from C57Bl/6J mice and transduced with recombinant retrovirus encoding either LTG or LYE, or mock-transduced. In this assay, $6.0\times10^4$ cells were treated for 24 h with 100 n*M* CPT and 100 n*M* BW1843U89, and then plated in methylcellulose containing 100 n*M* BW1843U89. Progenitor colony counts were performed in triplicate 10 days after plating. The data show that transduction with a bicistronic retroviral vector encoding TDP-I and optecTS can protect hematopoietic progenitor cells from the toxicity of combined CPT/BW1843U89 treatment

and Top-I inhibitors can be conferred to hematopoietic cells by coexpressing TDP-I and optecTS.

## Discussion

Cellular resistance to single-dose chemotherapy schedules has prompted the use of combination regimens, where coadministration of multiple chemotherapy agents is now the standard treatment for most cancers. The coadministration of TS-directed inhibitors with inhibitors of Top-I has been shown to be an effective treatment for several cancers, most notably colon cancer. Recently discovered CPT homologs, such as irinotecan (CPT-11), have proven to be excellent inhibitors of Top-I, and are promising chemotherapeutic agents (Ychou et al. 2002). However, a major shortcoming is the extreme myelosuppressive side effects associated with coadministration regimens. It has been predicted that the transfer of genes that confer drug resistance into hematopoietic cells can decrease the myelosuppressive toxicity associated with chemotherapy treatment, and the use of MDR-1, DHFR and MGMT for this purpose has advanced to clinical trials.

In addition, the use of cDNA sequences that confer drug resistance can be used for in vivo selection of genetically engineered cells. However, with respect to in vivo selection, current selectable markers have specific limitations. For example, variants of alkylguanine-DNA alkyltransferase confer resistance to combinations of 6-benzylguanine and BCNU, which are extremely toxic to stem cells, and the use of the MGMT cDNA to encode these variants is arguably the most effective method for selecting genetically modified stem cells in vivo. But the effectiveness of this system is burdened by secondary cancers that can arise as a result of using alkylating agents such as BCNU as the selecting regimen. We therefore initiated studies to determine if hematopoietic cells could be genetically engineered to resist the toxic effects of TS and Top-I inhibition. Because these agents are also toxic to hematopoietic stem cells under defined conditions, it is predicted that engineered resistance to these agents could be used to select genetically modified stem cells in vivo.

We have recently shown that the transfer of an optimized ecTS cDNA confers extremely high-level resistance against TS-directed antifolates, and are now expanding the resistance to additional drugs by coexpression of multiple resistant genes. Previous studies have indicated that the enzyme TDP-I is involved in the repair of dead-end Top I complexes in yeast (Pouliot et al. 1999, 2001; Yang et al. 1996). We hypothesized that overexpression of TDP-I could possibly repair dead-end Top-I complexes generated by CPT treatment. It has been shown that TDP-I can indeed protect against CPT toxicity, but the degree of protection is dependent on the duration of drug challenge. Resistance conferred by TDP-I is ablated during long-term continuous drug exposure, suggesting that the accumulation of DNA lesions under continuous CPT treatment eventually overwhelms the DNA repair capacity of the cell regardless of the level of TDP-I. These findings suggest that the timing of CPT dosage will have to be carefully controlled during future in vivo gene therapy studies, which may limit the usefulness of strategies employing TDP-I. Although several mechanisms of CPT resistance are known, such as CPT transporter upregulation, downregulation of Top-I, or induction of TDP-I-independent pathways of DNA repair, our results suggest that resistance to short-term CPT treatment in TDP-I-overexpressing cells is not the result of such cellular changes.

Previous experiments in yeast have demonstrated the effect of TDP-I on the viability of cells challenged with Top-I damage (Pouliot et al. 1999, 2001). In yeast containing a disrupted *RAD9* gene, expression of TDP-I increases cell survival when challenged by CPT, but these studies did not directly measure the relative levels of DNA fragmentation resulting from CPT treatment in relation to TDP-I expression. In the present study, comet assays demonstrated direct evidence of the mechanism of protection conferred by TDP-I overexpression, showing that DSBs are reduced in CPT-treated cells overexpressing TDP-I. Recent biochemical and genetic studies have indicated that, in yeast, TDP-I repair occurs after DSBs (Pouliot et al. 2001). If also true in mouse cells, the decreased number of DSBs in the TDP-I transfectants suggests that TDP-I cleavage is an important step in the repair of Top-I-induced DNA damage.

It is proposed that DSB repair is rapidly facilitated by overexpression of TDP-I, which circumvents apoptotic events. Studies in yeast also have shown that strains with TDP-I deletions are not particularly sensitive to CPT and must be combined with mutations in the *RAD9* checkpoint gene before there is a significant difference in survival between wild-type cells and TDP-I mutants

(Pouliot et al. 1999, 2001). It has been suggested that there are multiple pathways of DSB repair and that *RAD9* is probably involved in a parallel as-yet-undefined pathway to TDP-I. Nevertheless, there is a clear correlation between *RAD9* function and the ability of TDP-I to modulate CPT toxicity (Pouliot et al. 1999, 2001). Interestingly, TS-directed chemotherapy agents induce cell cycle arrest by preventing progression past S-phase, in a similar manner to yeast *RAD9* mutations (Backus et al. 2000). The cell cycle delay of cells treated with TS-directed inhibitors may result in similar cellular effects to those produced by *RAD9* allowing dead-end Top-I lesion repair, and for this reason one would predict that combined TS/TDP-I gene therapy might be synergistic. The extremely high resistance observed when TS and TDP-I are overexpressed could result from the repair of such lesions.

In this study we showed that the cDNA encoding TDP-I confers substantial protection against the DNA damage and resulting cell death induced by CPT treatment, and that coexpression of TDP-I and optecTS results in a dramatic resistance to dual chemotherapy treatments. Together these results (1) extend the cellular importance of TDP-I to higher eukaryotes, (2) demonstrate that overexpression of TDP-I is not toxic in mammalian cell cultures, (3) indicate that overexpression of TDP-I protects cells against cell death induced by CPT, and (4) show that coexpression of TDP-I and ecTS protects genetically modified cells against inhibitors of Top-I and TS. In addition, these studies indicate that TDP-I is responsible for repairing at least one form of Top-I lesion in vivo and suggest that examination of differential expression of TDP-I might allow for the prediction of CPT effectiveness and toxicity in a clinical setting. This study, therefore, validates TDP-I as a useful marker for potential drug-resistant gene therapy strategies and suggests that the further use of nontraditional genes can extend the scope of such strategies by incorporating additional drugs that are already in clinical use.

## References


Allay JA, Galipeau J, Blakley RL, Sorrentino BP (1998) In vivo selection of retrovirally transduced hematopoietic cells. Nat Med 4:1136

Backus HH, Pinedo HM, Wouters D, Kuiper CM, Jansen G, van Groeningen CJ, Peters GJ (2000) Differences in the induction of DNA damage, cell cycle arrest, and cell death by 5-fluorouracil and antifolates. Oncol Res 12:231

Carreras CW, Santi DV (1995) The catalytic mechanism and structure of thymidylate synthase. Annu Rev Biochem 64:721

Champoux JJ (2001) DNA topoisomerases: structure, function, and mechanism. Annu Rev Biochem 70:369

Chen AY, Liu LF (1994) DNA topoisomerases: essential enzymes and lethal targets. Annu Rev Pharmacol Toxicol 34:191

Davis BM, Koc ON, Gerson SL (2000) Limiting numbers of G156A O(6)-methylguanine-DNA methyltransferase-transduced marrow progenitors repopulate nonmyeloablated mice after drug selection. Blood 95:3078

Fairbairn DW, Olive PL, O'Neill KL (1995) The comet assay: a comprehensive review. Mutat Res 339:37

Fantz CR, Shaw D, Moore JG, Spencer HT (1998) Retroviral coexpression of thymidylate synthase and dihydrofolate reductase confers fluoropyrimidine and antifolate resistance. Biochem Biophys Res Commun 243:6

Froelich-Ammon SJ, Osheroff N (1995) Topoisomerase poisons: harnessing the dark side of enzyme mechanism. J Biol Chem 270:21429

Galipeau J, Li H, Paquin A, Sicilia F, Karpati G, Nalbantoglu J (1999) Vesicular stomatitis virus G pseudotyped retrovector mediates effective in vivo suicide gene delivery in experimental brain cancer. Cancer Res 59:2384

Hsiang YH, Lihou MG, Liu LF (1989a) Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. Cancer Res 49:5077

Hsiang YH, Liu LF, Wall ME, Wani MC, Nicholas AW, Manikumar G, Kirschenbaum S, Silber R, Potmesil M (1989b) DNA topoisomerase I-mediated DNA cleavage and cytotoxicity of camptothecin analogues. Cancer Res 49:4385

Interthal H, Pouliot JJ, Champoux JJ (2001) The tyrosyl-DNA phosphodiesterase Tdp1 is a member of the phospholipase D superfamily. Proc Natl Acad Sci U S A 98:12009

Knab AM, Fertala J, Bjornsti MA (1993) Mechanisms of camptothecin resistance in yeast DNA topoisomerase I mutants. J Biol Chem 268:22322

Koc ON, Allay JA, Lee K, Davis BM, Reese JS, Gerson SL (1996) Transfer of drug resistance genes into hematopoietic progenitors to improve chemotherapy tolerance. Semin Oncol 23:46

Lansiaux A, Facompre M, Wattez N, Hildebrand MP, Bal C, Demarquay D, Lavergne O, Bigg DC, Bailly C (2001) Apoptosis induced by the homocamptothecin anticancer drug BN80915 in HL-60 cells. Mol Pharmacol 60:450

Lanza A, Tornaletti S, Rodolfo C, Scanavini MC, Pedrini AM (1996) Human DNA topoisomerase I-mediated cleavages stimulated by ultraviolet light-induced DNA damage. J Biol Chem 271:6978

Licht T, Goldenberg SK, Vieira WD, Gottesman MM, Pastan I (2000) Drug selection of MDR1-transduced hematopoietic cells ex vivo increases transgene expression and chemoresistance in reconstituted bone marrow in mice. Gene Ther 4:348

McCarthy PJ, Sweetman SF, McKenna PG, McKelvey-Martin VJ (1997) Evaluation of manual and image analysis quantification of DNA damage in the alkaline comet assay. Mutagenesis 12:209

Nitiss JL, Wang JC (1996) Mechanisms of cell killing by drugs that trap covalent complexes between DNA topoisomerases and DNA. Mol Pharmacol 50:1095

Pouliot JJ, Yao KC, Robertson CA, Nash HA (1999) Yeast gene for a Tyr-DNA phosphodiesterase that repairs topoisomerase I complexes. Science 286:552

Pouliot JJ, Robertson CA, Nash HA (2001) Pathways for repair of topoisomerase I covalent complexes in Saccharomyces cerevisiae. Genes Cells 6:677

Pourquier P, Pommier Y (2001) Topoisomerase I-mediated DNA damage. Adv Cancer Res 80:189

Roffler SR, Chan J, Yeh MY (1994) Potentiation of radioimmunotherapy by inhibition of topoisomerase I. Cancer Res 54:1276

Shaw D, Berger FG, Spencer HT (2001) Retroviral expression of Escherichia coli thymidylate synthase cDNA confers high-level antifolate resistance to hematopoietic cells. Hum Gene Ther 12:51

Sorrentino BP (2002) Gene therapy to protect haematopoietic cells from cytotoxic cancer drugs. Nat Rev Cancer 2:431

Spencer HT, Sleep SE, Rehg JE, Blakley RL, Sorrentino BP (1996) A gene transfer strategy for making bone marrow cells resistant to trimetrexate. Blood 87:2579

Spencer HT, Villafranca JE, Appleman JR (1997) Kinetic scheme for thymidylate synthase from Escherichia coli: determination from measurements of ligand binding, primary and secondary isotope effects, and pre-steady state catalysis. Biochemistry 36:4212

Wu J, Yin MB, Hapke G, Toth K, Rustum YM (2002) Induction of biphasic DNA double strand breaks and activation of multiple repair protein complexes by DNA topoisomerase I drug 7-ethyl-10-hydroxy-camptothecin. Mol Pharmacol 61:742

Yang SW, Burgin AB Jr, Huizenga BN, Robertson CA, Yao KC, Nash HA (1996) A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. Proc Natl Acad Sci U S A 93:11534

Ychou M, Raoul JL, Desseigne F, Borel C, Caroli-Bosc FX, Jacob JH, Seitz JF, Kramar A, Hua A, Lefebvre P, Couteau C, Merrouche Y (2002) High-dose, single-agent irinotecan as first-line therapy in the treatment of metastatic colorectal cancer. Cancer Chemother Pharmacol 50:383