SCAN1 mutant Tdp1 accumulates the enzyme–DNA intermediate and causes camptothecin hypersensitivity

Heidrun Interthal¹, Hong Jing Chen¹,
Thomas E Kehl-Fie¹, Jörg Zotzmann²,
John B Leppard¹ and James J Champoux^{1,*}

¹Department of Microbiology, School of Medicine, University of Washington, Seattle, Washington, USA and ²Chiracon GmbH, Biotechnologie Park, Luckenwalde, Germany

Tyrosyl-DNA phosphodiesterase (Tdp1) catalyzes the hydrolysis of the tyrosyl-3' phosphate linkage found in topoisomerase I–DNA covalent complexes. The inherited disorder, spinocerebellar ataxia with axonal neuropathy (SCAN1), is caused by a H493R mutation in Tdp1. Contrary to earlier proposals that this disease results from a loss-of-function mutation, we show here that this mutation reduces enzyme activity ~25-fold and importantly causes the accumulation of the Tdp1–DNA covalent reaction intermediate. Thus, the attempted repair of topoisomerase I–DNA complexes by Tdp1 unexpectedly generates a new protein–DNA complex with an apparent half-life of ~13 min that, in addition to the unrepaired topoisomerase I–DNA complex, may interfere with transcription and replication in human cells and contribute to the SCAN1 phenotype. The analysis of Tdp1 mutant cell lines derived from SCAN1 patients reveals that they are hypersensitive to the topoisomerase I-specific anticancer drug camptothecin (CPT), implicating Tdp1 in the repair of CPT-induced topoisomerase I damage in human cells. This finding suggests that inhibitors of Tdp1 could act synergistically with CPT in anticancer therapy.

The EMBO Journal (2005) **24**, 2224–2233. doi:10.1038/sj.emboj.7600694; Published online 26 May 2005

Subject Categories: genome stability & dynamics; molecular biology of disease

Keywords: DNA repair; phospholipase D; SCAN1; topoisomerase I; tyrosyl-DNA phosphodiesterase

Introduction

As a member of the type IB subfamily of topoisomerases, human DNA topoisomerase I catalyzes the cleavage and religation of one strand of duplex DNA by a mechanism that involves the formation of a nicked intermediate with the enzyme covalently attached to the 3' end of the broken strand. A nucleophilic attack by the O-4 atom of the active

site tyrosine on the scissile phosphate in the DNA generates the covalent enzyme–DNA complex (Champoux, 2001). By providing a temporary single-strand break in the DNA, topoisomerase I solves many of the topological problems associated with DNA replication, transcription, recombination, and chromatin remodeling (Wang, 1996, 2002; Nitiss, 1998; Champoux, 2001).

The anticancer drug camptothecin (CPT) specifically poisons type IB topoisomerases (Hsiang *et al*, 1985; Hsiang and Liu, 1988) by binding to the enzyme–DNA covalent complex and interfering with the religation step of the reaction (Porter and Champoux, 1989; Stewart *et al*, 1998; Staker *et al*, 2002). The predominant cytotoxic effect of the drug is S-phase dependent since DNA synthesis inhibitors protect cells from its lethal effects (Holm *et al*, 1989; D'Arpa *et al*, 1990; Ryan *et al*, 1994). It is generally believed that the cytotoxic lesions produced by the drug are double-strand breaks resulting from the collisions of replication forks with topoisomerase I–DNA stalled complexes.

In addition to CPT, a number of unusual DNA structures have been shown *in vitro* to trap topoisomerase I in a covalent complex on DNA. These structures include gaps, nicks, base mismatches, abasic sites, thymine dimers, and certain modified bases in the vicinity of the cleavage site (Pourquier and Pommier, 2001). Little is known about the frequency of this type of topoisomerase-related DNA damage *in vivo*, but if it occurs and is not repaired the consequences for the cell are dire.

Tyrosyl-DNA phosphodiesterase (Tdp1) was discovered by virtue of its ability to hydrolyze a phosphodiester bond between the O-4 of tyrosine and the 3' end of a DNA strand (Yang *et al*, 1996). The substrate specificity of Tdp1 suggests that it is involved in the repair of covalent topoisomerase I–DNA complexes. As CPT specifically traps the topoisomerase I covalent complex, cells lacking Tdp1 activity might be expected to be hypersensitive to CPT. Surprisingly, a *Saccharomyces cerevisiae* strain containing a null mutation in the *TDP1* gene is no more sensitive to CPT killing than the wild type (wt) strain (Pouliot *et al*, 2001; Liu *et al*, 2002, 2004; Vance and Wilson, 2002). However, when introduced into a *rad9* mutant background, the null strain becomes hypersensitive to CPT, suggesting that multiple pathways exist for the repair of CPT-induced DNA damage in yeast (Liu *et al*, 2002, 2004). Subsequent genetic studies in yeast have revealed at least three separate pathways that can contribute to the repair of topoisomerase I–DNA covalent complexes (Pouliot *et al*, 2001; Liu *et al*, 2002; Vance and Wilson, 2002). In addition to Tdp1, the structure-specific endonucleases Rad1/Rad10 and Mus81/Mms4 are involved in the repair of such complexes. Recently, a functional connection between human Tdp1 and DNA single-strand break repair of topoisomerase I covalent complexes has been shown (Plo *et al*, 2003; El-Khamisy *et al*, 2005). Besides an involvement in the repair of topoisomerase

*Corresponding author. Department of Microbiology, Box 357242, University of Washington, Seattle, WA 98195-7242, USA.
Tel.: +1 206 543 8574; Fax: +1 206 543 8297;
E-mail: champoux@u.washington.edu

Received: 14 January 2005; accepted: 3 May 2005; published online: 26 May 2005

I-related DNA damage, Tdp1 has also been reported to hydrolyze DNA 3' phosphoglycolate adducts (Inamdar *et al*, 2002; Liu *et al*, 2002). Such adducts occur as a result of naturally occurring reactive oxygen species *in vivo*, or after exposure of cells to ionizing radiation or the drug bleomycin (Friedberg *et al*, 1995; Povirk, 1996).

As a member of the phospholipase D superfamily, human Tdp1 is predicted to require a combination of two histidines (H263 and H493) and two lysines (K265 and K495) for catalytic activity (Interthal *et al*, 2001; Davies *et al*, 2002, 2003). Biochemical experiments and the crystal structure of human Tdp1 confirm this prediction and demonstrate that the reaction proceeds through an intermediate in which the active site H263 is covalently bound through a phosphoamide bond to the 3' end of the DNA moiety of the substrate (Interthal *et al*, 2001; Davies *et al*, 2003; Raymond *et al*, 2004). In the second step of the reaction, hydrolysis of the covalent intermediate yields the DNA product and frees the enzyme.

Recently, a recessive mutation in the human *TDP1* gene was found to be responsible for the human hereditary disorder spinocerebellar ataxia with axonal neuropathy (SCAN1) (Takashima *et al*, 2002). The ataxia first becomes noticeable in the teenage years and apparently results from an effect on nondividing, terminally differentiated neuronal cells. The mutation in SCAN1 individuals is not associated with an increased risk for cancer. The responsible mutation changes the key active site residue H493 to an arginine and was originally proposed to be a loss-of-function mutation (Takashima *et al*, 2002). Here we characterize the activity of Tdp1 containing the SCAN1 mutation and evaluate the sensitivity of the homozygous mutant SCAN1 cell lines to CPT. The results reveal unexpected properties of the mutant enzyme that provide new clues concerning the phenotype of patients with SCAN1.

Consistent with the biochemical results cited above, extracts of cell lines from SCAN1 patients were recently found to be deficient in the removal of 3' phosphoglycolates (Zhou *et al*, 2005). In addition, while this manuscript was under revision, a paper appeared showing that SCAN1 cells are defective in the repair of topoisomerase I-associated single-strand breaks (El-Khamisy *et al*, 2005).

Results

SCAN1 cell lines exhibit reduced Tdp1 activity

It was previously shown that replacing H493 in human Tdp1 with alanine or asparagine substantially reduced the activity of the enzyme (Interthal *et al*, 2001; Raymond *et al*, 2004). To test if a change to arginine at this position has a similar effect, enzyme activity was assayed in extracts from EBV-immortalized lymphoblastoid cell lines (LCLs) derived from patients in whom the H493R mutation was identified as the cause for SCAN1 (Takashima *et al*, 2002). Figure 1A shows serial dilution assays of Tdp1 activity with cell extracts from two wt cell lines, two cell lines from heterozygous individuals, and three cell lines that carry the homozygous H493R mutation. This highly specific assay depends on the fact that Tdp1 hydrolyzes the phosphodiester bond between tyrosine and the 3' end of the DNA oligonucleotide in the 12-Y substrate to produce free tyrosine and a labeled 12-mer oligonucleotide with a 3' phosphate (12-P). In a dilution series with extracts

from the wt (wt-2, wt-5) and heterozygous (het-6, het-7) cell lines, activity was still detectable with the 1/100 dilution, whereas only a trace amount of activity could be detected in the undiluted homozygous SCAN1 mutant cell extracts (mut-1, mut-3, mut-4) (Figure 1A, compare lanes 2, 10, and 14 with lanes 8, 20, 24, and 28). A similar reduction in activity was observed when the activity of recombinant H493R mutant protein was compared with the recombinant wt enzyme (Figure 1B) (see below).

To test whether the observed reduction in enzyme activity in the SCAN1 mutant cell lines could be explained by a reduced amount of Tdp1 protein in the cells, extracts from equal numbers of cells were subjected to an immunoblot analysis using anti-Tdp1 antiserum (Figure 1C). The mutant cell extracts contained approximately two- to three-fold less Tdp1 protein than the wt extracts, and therefore the 100-fold reduction in Tdp1 activity observed for the SCAN1 cell lines could not be explained by a similar reduction in the amount of Tdp1 protein in the cells.

Residual Tdp1 activity detected in SCAN1 cells is due to the activity of the mutant H493R protein

To determine whether the residual activity observed in the mutant SCAN1 cell extracts was indeed due to a low activity of the H493R Tdp1 enzyme or caused by another, yet unidentified, Tdp1-like activity in human cells, extracts from two wt and two mutant cell lines were treated with an inhibitory anti-Tdp1 antiserum prior to carrying out the enzyme assays using the substrate 20-Topep. Pretreatment of the wt cell extracts with rabbit anti-human Tdp1 antiserum blocked the activity, whereas preimmune serum had no effect (Figure 1D, lanes 3, 4, 7, 8, 11, and 12). Similarly, Tdp1-specific antiserum, but not preimmune serum, inhibited the residual activity in the SCAN1 mutant extracts (Figure 1D, lanes 5, 6, 9, 10, 13, and 14). These results established that the residual Tdp1 activity observed in SCAN1 cell extracts was due to the mutant form of Tdp1.

Tdp1 H493R accumulates the covalent reaction intermediate *in vitro*

Previously, we showed using a rapid SDS stop procedure that it is possible to trap the Tdp1–DNA covalent reaction intermediate (Interthal *et al*, 2001). Interestingly, we consistently observed an accumulation of labeled material in the wells of sequencing gels only when SCAN1 cell extracts were used in Tdp1 activity assays (data not shown). To further investigate the H493R enzyme with respect to the possible accumulation of the covalent intermediate, we studied the properties of the purified mutant enzyme.

The overall activity of purified H493R was reduced approximately ~25-fold when compared to purified wt Tdp1 in a dilution series experiment with substrate 12-Y (Figure 1B). When purified H493R enzyme was incubated with 5' end-labeled 20-Topep, a substantial amount of the label remained in or near the well of the sequencing gel (Figure 2A, lane 3). Upon trypsin digestion, most of the trapped material collapsed into one major band (20-Tdpep) with a slightly slower mobility than the substrate 20-Topep (Figure 2A, lane 4). The mobility of the 20-Tdpep band was consistent with the predicted size of a 20-mer DNA plus the 11-amino-acid Tdp1 tryptic peptide containing the H263 residue. Further digestion of the 20-Tdpep intermediate with proteinase K

Figure 1 Tdp1 activity and protein in SCAN1 cells. (A) Sequencing gel analysis of Tdp1 activity assays with cell extracts from SCAN1 cells and related cell lines. Serial dilutions (10-fold) of extracts derived from cells that are either wt (wt-2, wt-5), homozygous for the H493R mutation (mut-1, mut-3, mut-4), or heterozygous at this locus (het-6, het-7) were incubated with substrate 12-Y, a 12-mer DNA oligonucleotide with a 3' tyrosine moiety. The product of the reaction is 12-P, a 12-mer with a 3' phosphate. Substrate 12-Y alone was loaded in lane 1. (B) Tdp1 assays of five-fold serial dilutions of recombinant wt and H493R Tdp1 proteins using substrate 12-Y. Substrate alone is shown in lane 1. The gel shown is representative of two independent experiments. (C) Immunoblot analysis of whole-cell extracts of the seven cell lines shown in (A). Rabbit anti-human Tdp1 antiserum was used to detect Tdp1. Purified recombinant His-tagged wt Tdp1 was loaded as a control in the far-right lane. (D) Specific inhibition of Tdp1 activity by anti-Tdp1 antiserum. Substrate 20-Topep, a 20-mer single-stranded DNA oligonucleotide with a small topoisomerase I-derived peptide attached to the 3' end, was used as the substrate (lane 1). The 20-P product of the Tdp1 reaction is shown in lane 2. In lanes 3–14, 10 μ l of a 1:2 dilution of the indicated cell extract was tested for Tdp1 activity. Where indicated, the cell extracts were preincubated with anti-Tdp1 antiserum (lanes 7–10) or preimmune serum (lanes 11–14) prior to the addition of substrate. A typical experiment of more than three independent experiments is shown.

resulted in an even smaller fragment (Figure 2A, lane 5), showing that 20-Tdpep did indeed contain a protein component. The faint slower migrating bands in lane 4 are most likely the result of incomplete trypsin digestion. No intermediate was detected for the wt enzyme under these conditions since the reaction had gone to completion by the time the reaction was stopped with SDS. The previously described Tdp1 H493A mutant enzyme (Interthal *et al*, 2001) also produced some 20-Tdpep intermediate, albeit to a lesser extent (Figure 2A, lanes 6 and 7). Analysis of the wt, H493R, and H493A samples from Figure 2A by SDS-PAGE supported these data (Figure 2B). Importantly, the intact H493R protein became radioactively labeled due to the covalently bound end-labeled DNA, but as expected, no radioactive band was visible after trypsin treatment.

Comparison of Tdp1 H493R activity with wt, H493A, and H493N activities

Since the accumulation of the covalent intermediate by the H493R mutant could be important for the SCAN1 disease, it was of interest to determine whether this effect was unique to this particular amino-acid change, or whether Tdp1 variants with other amino-acid changes at position 493 also accumu-

lated substantial amount of the intermediate. As judged by the appearance of the product 20-P, the H493R mutant was approximately 25-fold less active than the wt enzyme (Figure 3, compare lanes 11 and 6), while the H493A and H493N enzymes showed ~125- and ~625-fold reductions in activity, respectively (Figure 3, compare lanes 15 and 19 with lane 6). Clearly, H493R accumulated the most covalent reaction intermediate, but 20-Tdpep was also detectable in reactions with H493A. The least active mutant, H493N, also forms a very small amount of intermediate, which can only be visualized upon extended gel exposure. In contrast, no trapped intermediate could be detected in reactions with the wt enzyme (Figure 3, lanes 1–7). The covalent H493R–DNA complex (20-Tdpep) represents a true intermediate in the reaction, since it appeared as the product was being formed and disappeared when the substrate had been consumed (data not shown).

Determination of the apparent half-life of the Tdp1 H493R–DNA covalent intermediate

The discovery that H493R can accumulate large amounts of the enzyme–DNA covalent intermediate (Figure 3, lanes 8–10) and the potential that such lesions could be harmful

Figure 2 H493R mutant accumulates the covalent Tdp1-DNA reaction intermediate. **(A)** The indicated Tdp1 enzyme was incubated with substrate 20-Topep. The conditions for the wt enzyme shown in lanes 1 and 2 were the same as shown in Figure 3, lane 6, the conditions for the H493R mutant shown in lanes 3–5 were the same as shown in Figure 3, lane 10, and the conditions for the H493A mutant shown in lanes 6 and 7 were the same as shown in Figure 3, lane 14. Reactions were stopped with SDS and a portion of each was digested with trypsin as indicated. The product resulting from trypsin treatment of the Tdp1–DNA covalent intermediate is 20-Tdpep, and is predicted to consist of the 20-mer DNA with an 11-mer trypsin-resistant Tdp1 fragment attached to its 3' end. The sample in lane 5 was treated with proteinase K after trypsin treatment, which results in a 20-mer with a smaller peptide (labeled with a star). **(B)** A subset of the reactions shown in (A) was analyzed by SDS–PAGE. Tdp1 proteins that are trapped in the reaction intermediate (20-Tdp) are radioactively labeled via the 5' end-labeled 20-mer DNA. Lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6 correspond to lanes 1 and 2, lanes 3 and 4, and lanes 6 and 7 of (A), respectively. The mobility of two molecular weight markers is indicated on the right side of the gel. All experiments shown were repeated several times with identical results.

to cells prompted us to ask how long the Tdp1 H493R–DNA complexes persist *in vitro*. To measure the kinetics of decay of the H493R enzyme–DNA covalent complex, excess H493R was incubated with substrate 20-Topep for 2 min to ensure that essentially all of the substrate had been consumed and therefore all of the radioactivity was either in the product 20-P or the covalent intermediate 20-Tdp. Treatment of a portion of the reaction with trypsin at this time point and analysis on a sequencing gel confirmed that the substrate was exhausted and that more than 70% of the label was associated with the covalent intermediate 20-Tdpep (Figure 4A, lane 2). Additional portions were removed and analyzed in the same way to follow the disappearance of the covalent intermediate with time (Figure 4A, lanes 3–10). The percentage of intermediate remaining was plotted against time and the apparent half-life of the H493R–DNA complex was determined to be ~13 min (Figure 4B). The half-life of the wt Tdp1–DNA intermediate is extremely short and cannot be determined under these experimental conditions (Interthal *et al*, 2001).

SCAN1 cell lines are hypersensitive to CPT

Although mutations in the yeast *TDP1* gene when combined with mutations in other genes render cells hypersensitive to CPT, none of the *TDP1* mutations when tested alone, including mutants in which the *TDP1* gene was completely deleted, has been found to render cells CPT sensitive (Pouliot *et al*, 2001; Liu *et al*, 2002, 2004; Vance and Wilson, 2002). To test whether the SCAN1 mutant cell lines are defective for the repair of CPT-induced topoisomerase I DNA damage, the viability of two mutant, two wt, and one heterozygous cell line from the same family was tested in the presence of the drug. Actively dividing cells (average doubling times were 32 ± 1 h) were cultured in the continuous presence of different concentrations of CPT and live cells were counted every 24 h for 3 days. The CPT sensitivity profiles after 72 h are shown in Figure 5A. Both mutant cell lines showed markedly increased CPT sensitivities when compared to the heterozygous cell line and the two wt cell lines. Moreover, the differences in CPT sensitivity between the SCAN1 and wt cell lines were observed at all CPT concentrations tested and became more pronounced over time (Figure 5B).

The SCAN1 mutant cell lines were no more sensitive to bleomycin or the topoisomerase II poison, etoposide, than the wt cells (data not shown).

Flow-cytometric analysis of CPT-treated cell lines

Flow cytometry was used to perform a more detailed analysis of the consequences of CPT treatment of SCAN1 cells. To determine the relative numbers of live, dead, and apoptotic cells, we followed a protocol established by Poot *et al* (1999) using LCLs. The wt and mutant cell lines used in the viability assays described above were treated with CPT and, every 24 h, cells were harvested and stained simultaneously with Hoechst 33342, SYTO11, and propidium iodide (PI). Live cells with intact membranes stain with Hoechst 33342, but not with PI, which is only taken up by dead cells. Early apoptotic cells are characterized by low SYTO 11 fluorescence (Poot *et al*, 1997). As before, SCAN1 cell lines mut-3 and mut-4 proved to be much more CPT sensitive than the wt control cell lines, as evidenced by fewer relative numbers of live cells in the SCAN1 cultures (Figure 6A) and increasing amounts of

Figure 3 Activity assays of wt and mutant forms of Tdp1. Five-fold serial dilutions of the indicated proteins (starting at 1.4 μ M) were incubated with substrate 20-Topep for 10 min, treated with trypsin, and analyzed on a sequencing gel. 20-Tdpep denotes the covalent reaction intermediate after trypsin treatment and 20-P the product of the cleavage reaction. One representative experiment of three is shown here.

Figure 4 Half-life of the covalent Tdp1 H493R-DNA intermediate. (A) Tdp1 H493R and 20-Topep were incubated for 2 min (zero time point, lane 2) before samples were taken at the indicated time points (lanes 2–10). Samples were treated with trypsin and analyzed in a sequencing gel. Lane 1 contains the substrate 20-Topep alone. (B) The decay of the Tdp1-DNA covalent intermediate with time is plotted for the time-course samples shown in (A). Averages of three independent experiments are shown, with error bars representing standard deviations. Some error bars are too small to be seen.

dead cells in SCAN1 cell lines as compared to wt cells (Figure 6B). These results showed that the reduced growth potential of the CPT-treated cells seen in Figure 5 results from cell death and not simply from the cessation of cell division.

Low SYTO11 fluorescence is indicative of early apoptotic cells (Poot *et al*, 1997). As expected, the percentages of early apoptotic cells were relatively low in all the cell lines, but SCAN1 cell lines accumulated early apoptotic cells to levels that were four times higher than the wt cell lines in response to the drug (Figure 6C). These data suggest that at least some of the CPT-induced cell death in SCAN1 cells occurred through an apoptotic pathway.

Figure 5 CPT sensitivity of SCAN1 cell lines. (A) Two wt cell lines (wt-2, ■; wt-5, ▲), two homozygous mutant SCAN1 cell lines (mut-3, □; mut-4, △), and one heterozygous cell line (het-6, ○) were grown in the presence of the indicated amounts of CPT and live cells were counted at 72 h. The percentage of live cells compared to the untreated control was determined as a function of the CPT concentration. This particular analysis was performed in triplicate and the results are shown with error bars representing the standard deviation. (B) The percentage of live cells is plotted versus time at 2.5 nM CPT for the experiment shown in (A).

SCAN1 cells accumulate in S phase upon CPT treatment

Since the double-strand breaks that arise during DNA replication are considered to be the major cytotoxic lesions caused by CPT (Holm *et al*, 1989; D'Arpa *et al*, 1990; Ryan *et al*, 1994), it was of interest to determine the effects of CPT on the cell cycle distribution of SCAN1 cells. Using the data derived from the flow cytometry experiment described above, the

DNA content of all live cells was determined using the fluorescence emitted by DNA-bound Hoechst 33342. Figure 7A shows a representative cell cycle distribution for wt-2 and the SCAN1 mut-3 cell lines after 24 h of CPT treatment. When treated with 5 nM CPT, ~70% of the living

cells in both mutant cell lines were in S phase (Figure 7A and C), compared to only ~30% of the cells in the untreated controls. This result is in stark contrast to the wt cell lines

Figure 7 Cell cycle distribution of live and early apoptotic wt and SCAN1 mutant cell lines. (A) Cells were either untreated or treated with 5 nM CPT for 24 h. The abscissas represent the DNA content per cell as measured by the fluorescence intensity of Hoechst 33342, and reflect the G1, S, and G2 states of the cell cycle as indicated. The ordinates represent cell numbers. (B) Analysis is the same as described for the 5 nM CPT treatment in (A), except that cell numbers indicate those cells with low SYTO11 staining reflective of early apoptosis. (C) Percentage of cells in S phase after a 24-h treatment with the indicated concentrations of CPT (average of three experiments).

Figure 6 Multiparameter flow cytometry analysis of cell survival, cell death, and early apoptosis. (A–C) Two wt cell lines (wt-2, ■; wt-5, ▲) and two SCAN1 cell lines (mut-3, □; mut-4, △) were grown in the presence of the indicated amounts of CPT. The relative numbers of live, dead, and early apoptotic cells were determined at 48 h. Each data point shows the mean and standard deviation of triplicate samples. (A) The percentage of live treated cells as compared to live untreated cells at each data point is plotted as a function of the CPT concentration. (B) The difference between the percentage of dead cells in the treated cell population and the percentage of dead cells in the untreated control population is plotted as a function of CPT concentration. (C) The difference between the percentage of early apoptotic cells in the treated cell population and the percentage of apoptotic cells in the untreated control population is plotted as a function of CPT concentration.

which also accumulated some cells in late S/early G2 upon CPT treatment, but far less than the SCAN1 mutant cells (Figure 7A and C). Cell cycle analysis of early apoptotic cells revealed that in fact the majority of early apoptotic cells were in S phase (Figure 7B).

Discussion

In this study, we characterized biochemically the H493R mutation in Tdp1 that causes SCAN1 and investigated the phenotype of SCAN1 cell lines. Contrary to earlier suggestions that the SCAN1 phenotype results from a loss-of-function mutation (Takashima *et al*, 2002; El-Khamisy *et al*, 2005; Zhou *et al*, 2005), we estimate that the H493R mutation in human Tdp1 reduces the activity of the enzyme only ~25-fold based on the rate of product formation by the purified enzyme. This value is consistent with the reduction in activity observed in crude extracts of the SCAN1 cell lines when the amount of enzyme per cell (see Figure 1C) is taken into account. This small amount of residual activity may be sufficient to provide most of the repair capacity required throughout the life of an individual and thus explain the late onset of the disease in SCAN1 individuals (Takashima *et al*, 2002). Alternatively, similar to the situation described in yeast (Pouliot *et al*, 2001; Liu *et al*, 2002; Vance and Wilson, 2002), there may be a functional overlap of other repair systems so that, in the absence of sufficient Tdp1 activity, other pathways capable of repairing topoisomerase I–DNA covalent complexes fulfill the needs of the organism.

Effect of mutations in H493 on Tdp1 catalysis

The first step of the Tdp1 reaction involves nucleophilic attack by H263 on the tyrosyl–DNA 3' phosphate bond to form a covalent intermediate with the histidine linked to the 3' phosphate of the DNA. In the second step of the reaction, this covalent intermediate is hydrolyzed to release the DNA. Mechanistically, it appears that H493 is involved in both steps of the Tdp1 reaction; in the first step it acts as a general acid catalyst to protonate the leaving phenoxide anion (Interthal *et al*, 2001; Davies *et al*, 2003, 2004; Raymond *et al*, 2004), and in the second step it has been suggested to act as a general base to increase the nucleophilicity of a water molecule (Davies *et al*, 2004; Raymond *et al*, 2004). The accumulation of the covalent reaction intermediate by H493R means that replacement of H493 with arginine affects the hydrolysis step to a greater extent than the initial cleavage step. It is conceivable that arginine retains the general acid catalytic function required in the first step of the reaction, but perhaps is unable to activate a water molecule for the second step, or possibly for steric reasons excludes a water molecule from the active site (D Davies, personal communication). Although the H493A mutant enzyme accumulates a very small amount of covalent intermediate, it appears that both the H493A and H493N mutant enzymes are nearly equally defective for both cleavage and hydrolysis, consistent with the fact that neither alanine nor asparagine can provide general acid–base catalytic functions.

Accumulation of the Tdp1–DNA covalent intermediate may provide important clues to the SCAN1 phenotype

We have considered the possibility that the two- to three-fold reduction in the amount of mutant protein relative to wt

protein observed in the immunoblot analysis shown in Figure 1C might be explained by the formation of persistent H493R Tdp1–DNA covalent complexes prior to lysis of the cells. However, it has not proven possible to detect the mutant enzyme covalently associated with cellular DNA by an *in vivo* complex of enzyme (ICE) assay (Subramanian *et al*, 2001) (data not shown), and therefore it seems likely that there is simply less Tdp1 protein in the SCAN1 cells. The lack of detectable covalent complexes *in vivo* is consistent with the fact that, unlike topoisomerase I which becomes stably associated with the DNA after CPT treatment, Tdp1–DNA complexes decay with a half-time of 13 min. The absence of detectable covalent complexes is also consistent with the lack of symptoms early in life in SCAN1 patients and the absence of a growth defect in cell lines in culture.

Ironically, attempted repair of a topoisomerase I-induced covalent lesion by Tdp1 H493R replaces the covalently bound topoisomerase I protein on the DNA with the mutant Tdp1 protein. The net result of the reduced activity of the H493R enzyme and the relatively long half-life of the mutant Tdp1–DNA covalent complex (~13 min) is the persistence of either topoisomerase I–DNA or Tdp1–DNA covalent complexes. Such long-lived protein–DNA complexes at DNA strand breaks would be expected to significantly impact transcription in the nondividing neuronal cells that are believed to be the affected target cells in the disease. For instance, it is conceivable that the ultimate effect on motor neurons leading to the relatively late onset of the disease results from the cumulative effects of these persistent covalent complexes on transcription, eventually leading to neuronal apoptosis, as first suggested by Takashima *et al* (2002). Intriguingly, the persistent DNA strand breaks observed after CPT treatment of SCAN1 cell lines described in a recent report (El-Khamisy *et al*, 2005) may well be covalently linked to mutant Tdp1.

The fact that heterozygous individuals are apparently unaffected and that the cell lines derived from these individuals are not hypersensitive to CPT could be explained by the ability of wt Tdp1 to remove covalently trapped mutant Tdp1. Indeed, we found that wt Tdp1 can cleave the phosphoamide bond between DNA and a tryptic peptide derived from the H493R mutant enzyme (H Interthal, HJ Chen and JJ Champoux, not yet submitted).

It remains unclear why neurons are specifically affected by the Tdp1 defect in SCAN1 individuals. It is possible that terminally differentiated neurons have an enhanced need for Tdp1-mediated repair or are hypersensitive to the effects of persistent topoisomerase I–DNA or Tdp1–DNA covalent complexes, perhaps because alternative repair pathways may be attenuated in differentiated neurons (Nospikel and Hanawalt, 2002). Interestingly, CPT was shown to induce apoptotic cell death in postmitotic cortical neurons, indicating an important replication-independent role for topoisomerase I in this cell type (Morris and Geller, 1996). To our knowledge, SCAN1 is the first example of a human genetic disorder that results from a failure to repair DNA–protein covalent complexes. However, a number of genetic disorders are caused by defects in the response to DNA damage (Friedberg *et al*, 1995; Hoeijmakers, 2001), including such neurodegenerative disorders as ataxia telangiectasia, Nijmegen breakage syndrome, Xeroderma pigmentosum, Cockayne syndrome, and trichothiodystrophy (Rolig and McKinnon, 2000). The SCAN1 phenotype is remarkable

in that the clinical phenotype, similar to AOA1 (ataxia ocular-motor apraxia) (Date *et al*, 2001; Moreira *et al*, 2001), appears to be restricted to terminally differentiated neuronal cells (Takashima *et al*, 2002; Caldecott, 2003, 2004).

Tdp1 is involved in the repair of topoisomerase I-DNA damage in human cells

We also show here that homozygous mutant SCAN1 cells are hypersensitive to the killing effects of CPT and that the treated cells arrest growth in the S phase, as expected for a drug that induces replication-dependent double-strand breaks (Hsiang *et al*, 1985; D'Arpa *et al*, 1990; Wang *et al*, 1997; Shao *et al*, 1999; Strumberg *et al*, 2000). These results clearly implicate Tdp1 in the repair of CPT-induced DNA damage in the LCLs and provide the first demonstration that a mutation in Tdp1 alone is sufficient to render cells CPT hypersensitive. This situation contrasts with what has been observed in yeast where hypersensitivity to CPT requires that the cells are also mutant in at least one other repair pathway (Pouliot *et al*, 1999; Liu *et al*, 2002, 2004). It appears that the relative importance of different pathways for the repair of topoisomerase I-related damage caused by CPT may be different in yeast and humans. A recent report suggested that Tdp1 might also indirectly be involved in the repair of topoisomerase II-DNA damage (Barthelmes *et al*, 2004), but we did not observe any increased sensitivity of the SCAN1 cell lines to etoposide, a potent topoisomerase II poison.

In studies that are complementary to those we report here, it has recently been found that overexpression of a human or yeast Tdp1 fusion protein alleviates some of the effects of CPT treatment (Barthelmes *et al*, 2004; Nivens *et al*, 2004). Importantly, these data and our observation that the SCAN1 cells are hypersensitive to CPT suggest that inhibitors of Tdp1 could act synergistically with CPT in a combined anticancer therapeutic regimen.

Materials and methods

Cell culture

The human EBV-transformed LCLs BAB1635 (Tdp1^{-/-}, mut-1), BAB1646 (Tdp1^{+/-}, wt-2), BAB1662 (Tdp1^{-/-}, mut-3), BAB1664 (Tdp1^{-/-}, mut-4), BAB1668 (Tdp1^{+/+}, wt-5), BAB1669 (Tdp1^{+/-}, het-6), and BAB1670 (Tdp1^{+/-}, het-7) were generous gifts from James Lupski (Takashima *et al*, 2002). Cells were grown in RPMI medium 1640 with 25 mM HEPES and L-glutamine (Gibco), 15% fetal bovine serum (Gibco), and 1% penicillin/streptomycin.

Immunoblot analysis

Purified His-tagged Tdp1 and equal numbers of cells from each cell line were boiled in SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.001% bromophenol blue) for 10 min, and the samples were subjected to 10% SDS-PAGE. The gels were either stained with Coomassie Blue or transferred to nitrocellulose membranes. The membranes were blocked in 5% milk in TBS (10 mM Tris-HCl (pH 8), 150 mM NaCl), followed by incubation with a 1:750 dilution of rabbit anti-human Tdp1 antiserum in 5% milk in TBS. The anti-Tdp1 antiserum was generated at Pocono Rabbit Farm and Laboratory Inc., Canadensis, PA, against recombinant purified native His-tagged full-length human Tdp1 (Interthal *et al*, 2001). Goat anti-rabbit IgG antibody coupled to horseradish peroxidase (PIERCE) was used as the secondary antibody and chemiluminescence detection was carried out using the SuperSignal West Pico Chemiluminescent substrate from PIERCE. Very similar results were obtained when cell extracts were normalized based on total protein content in the extracts (data not shown).

Preparation of cell extracts

Exponentially growing LCLs were harvested by centrifugation, washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄ (pH 7.2)), and resuspended in lysis buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, 2 mM MgCl₂, 1% Triton X-100, 15 mM DTT, 0.2 mg/ml PMSF, 1/1000 volume of protease inhibitor mixes Pic-D (5 mg/ml Pepstatin A, 1 mg/ml chymostatin in DMSO), and Pic-W (208 mg/ml benzamidine, 5 mg/ml aprotinin, and 1 mg/ml leupeptin in H₂O)). The cells were lysed by vortexing for 1 min and the cell extracts were clarified by centrifugation. For the experiments shown in Figure 1A, the cells were resuspended to a density of 2x10⁷ cells/ml in lysis buffer and for all other experiments to a density of 8x10⁷ cells/ml.

Mutagenesis, expression, and purification of Tdp1 proteins

The H493R mutant allele of human TDP1 was introduced into bacterial expression constructs using plasmid pHN1894S as described previously (Interthal *et al*, 2001). The generation of the other mutant alleles has been described previously (Interthal *et al*, 2001). The N-terminally His-tagged recombinant Tdp1 proteins (H493R, H493A, H493N, and wt Tdp1) were expressed in *Escherichia coli* BL21 (DE3) and purified as described previously (Interthal *et al*, 2001).

Preparation of Tdp1 substrates

The 12-Y Tdp1 substrate, consisting of a 12-mer DNA oligonucleotide (5'-GAAAAAAGAGTT) with a 3' phosphotyrosine, was purchased from Midland Certified Reagent Company (Midland, TX). Substrate 20-Topep, a 20-mer DNA oligonucleotide (5'-GTAGAG GATCTAAAAGACTT-3') with a small trypsin-resistant human topoisomerase I-derived peptide covalently bound to the 3' end via the active site tyrosine, was prepared as described previously (Interthal *et al*, 2001). Briefly, a duplex 30-mer suicide substrate for topoisomerase I containing a 5'-bridging phosphorothiolate linkage (Burgin *et al*, 1995) at the site of cleavage was incubated with human topoisomerase I, followed by trypsin treatment and gel purification. For activity assays, 12-Y and 20-Topep were ³²P-end-labeled with T4 polynucleotide kinase (New England Biolabs).

Tdp1 activity assays with cell extracts

Cell extracts were first diluted 1:2 with 2x reaction buffer (200 mM KCl, 40 mM Tris-HCl (pH 7.5), 40 mM EDTA, 2 mM DTT) and then 10-fold serially diluted in reaction buffer. For the experiment shown in Figure 1A, 10 μ l of the extract dilutions was added to 5 μ l of reaction buffer containing 0.01 pmol of 5' end-labeled substrate 12-Y and the reactions were incubated at 37°C for 30 min and stopped with an equal volume of formamide loading dye. All activity assays were analyzed on a 15% sequencing gel. Image retrieval and quantitation were carried out using a PhosphorImager and ImageQuant software (Amersham Biosciences).

In the experiments shown in Figure 1D, the reactions (final volume 15 μ l) contained 0.01 pmol of substrate 20-Topep and 10 μ l of a 1:2 dilution of the cell extracts. After a 30-min incubation at 37°C, reactions were stopped with an equal volume of formamide loading dye and analyzed on a sequencing gel. For the indicated reactions in Figure 1D, the cell extracts (10 μ l) were preincubated with 0.25 μ l of antiserum or preimmune serum for 4 min before the addition of substrate.

Tdp1 activity assays with purified enzyme

Purified wt and mutant forms of Tdp1 were serially diluted 10-fold starting at 1.4 μ M protein, and 10 μ l aliquots were incubated with 5 nM 12-Y substrate in assay buffer (100 mM KCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT) for 10 min at 37°C. The reactions were stopped with formamide loading dye and analyzed by sequencing gel electrophoresis. When 20-Topep was used as a substrate, the reactions were stopped with SDS (0.5%), diluted 10-fold with TE, and one half of the sample treated with 1 μ g/ μ l trypsin at 37°C for 2 h prior to sequencing gel analysis. Where indicated, the samples were treated with proteinase K (70 μ g/ml) for 1 h at 37°C after the trypsin digestion. In some cases, SDS sample buffer was added to the reactions and the samples were analyzed by 10% SDS-PAGE.

Half-life of H493R-DNA intermediate

A 50-fold molar excess of H493R (0.1 μ M) was incubated with 20-Topep (2 nM) for 2 min at 37°C in assay buffer (final volume 25 μ l) to allow for the accumulation of the covalent enzyme-DNA

intermediate. At the indicated time points, samples were stopped by the addition of SDS to 0.5%. Subsequent trypsin digestion followed by sequencing gel analysis was carried out as described above.

Growth curves and drug sensitivity assays

Exponentially growing cell lines were diluted to 3×10^5 cells/ml and 300 μ l of each were distributed into the wells of a 48-well plate. An additional 300 μ l of medium containing the appropriate amounts of CPT (Sigma, 4 mg/ml in DMSO) was added and the cultures were incubated at 37°C in an atmosphere of 5% CO₂. Every 24 h, samples were taken and cells were mixed with an equal volume of 0.4% Trypan Blue in PBS. Live cells, which are refractory to Trypan Blue staining, were counted in a hemocytometer. Growth assays were performed with bleomycin (Sigma, 10 mg/ml stock) and etoposide (Sigma, 20 mM in DMSO stock) using the same procedure.

Flow cytometry

Every 24 h, the CPT-treated or control cells were stained with 20 μ M Hoechst 33342 (Sigma), 0.1 μ M SYTO11 (Molecular Probes) and

5 μ g/ml PI (Sigma) by incubation for 30 min at 37°C in the dark (Poot *et al*, 1999). The suspensions of stained cells were analyzed by flow cytometry using an Influx flow cytometer (Cytospea, Seattle, WA). Fluorescence emission was collected after passing through select bandpass filters: 525 $\pm$ 30 nm (SYTO 11), 615 $\pm$ 40 nm (PI), and 450 $\pm$ 40 nm (Hoechst 33342). Data analysis was performed using Cytomation Summit Offline Analysis software (DakoCytomation Colorado Inc., Fort Collins, CO). Cell cycle analysis was performed by using the MultiCycle software package (Phoenix Flow Systems, San Diego, CA).

Acknowledgements

We thank James Lupski for generously providing the SCAN1 cell lines and Achim Schnauffer and Sharon Schultz for their critical comments during the preparation of the manuscript. This work was supported by Grant GM49156 from the National Institutes of Health.

References

- Barthelme HU, Habermeyer M, Christensen MO, Mielke C, Interthal H, Pouliot JJ, Boege F, Marko D (2004) TDP1 overexpression in human cells counteracts DNA damage mediated by topoisomerases I and II. *J Biol Chem* **279**: 55618–55625
- Burgin Jr AB, Huizenga BN, Nash HA (1995) A novel suicide substrate for DNA topoisomerases and site-specific recombinases. *Nucleic Acids Res* **23**: 2973–2979
- Caldecott KW (2003) DNA single-strand break repair and spinocerebellar ataxia. *Cell* **112**: 7–10
- Caldecott KW (2004) DNA single-strand breaks and neurodegeneration. *DNA Repair (Amst)* **3**: 875–882
- Champoux JJ (2001) DNA topoisomerases: structure, function, and mechanism. *Annu Rev Biochem* **70**: 369–413
- D'Arpa P, Beardmore C, Liu LF (1990) Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. *Cancer Res* **50**: 6919–6924
- Date H, Onodera O, Tanaka H, Iwabuchi K, Uekawa K, Igarashi S, Koike R, Hiroi T, Yuasa T, Awaya Y, Sakai T, Takahashi T, Nagatomo H, Sekijima Y, Kawachi I, Takiyama Y, Nishizawa M, Fukuhara N, Saito K, Sugano S, Tsuji S (2001) Early-onset ataxia with ocular motor apraxia and hypalbuminemia is caused by mutations in a new HIT superfamily gene. *Nat Genet* **29**: 184–188
- Davies DR, Interthal H, Champoux JJ, Hol WG (2002) The crystal structure of human tyrosyl-DNA phosphodiesterase, Tdp1. *Structure (Camb)* **10**: 237–248
- Davies DR, Interthal H, Champoux JJ, Hol WG (2003) Crystal structure of a transition state mimic for Tdp1 assembled from vanadate, DNA, and a topoisomerase I-derived peptide. *Chem Biol* **10**: 139–147
- Davies DR, Interthal H, Champoux JJ, Hol WG (2004) Explorations of peptide and oligonucleotide binding sites of tyrosyl-DNA phosphodiesterase using vanadate complexes. *J Med Chem* **47**: 829–837
- El-Khamisy SF, Saifi GM, Weinfeld M, Johansson F, Helleday T, Lupski JR, Caldecott KW (2005) Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. *Nature* **434**: 108–113
- Friedberg EC, Walker GC, Siede W (1995) *DNA Repair and Mutagenesis*. Washington, DC: ASM Press
- Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. *Nature* **411**: 366–374
- Holm C, Covey JM, Kerrigan D, Pommier Y (1989) Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells. *Cancer Res* **49**: 6365–6368
- Hsiang YH, Hertzberg R, Hecht S, Liu LF (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* **260**: 14873–14878
- Hsiang YH, Liu LF (1988) Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res* **48**: 1722–1726
- Inamdar KV, Pouliot JJ, Zhou T, Lees-Miller SP, Rasouli-Nia A, Povirk LF (2002) Conversion of phosphoglycolate to phosphate termini on 3' overhangs of DNA double strand breaks by the human tyrosyl-DNA phosphodiesterase hTdp1. *J Biol Chem* **277**: 27162–27168
- Interthal H, Pouliot JJ, Champoux JJ (2001) The tyrosyl-DNA phosphodiesterase Tdp1 is a member of the phospholipase D superfamily. *Proc Natl Acad Sci USA* **98**: 12009–12014
- Liu C, Pouliot JJ, Nash HA (2002) Repair of topoisomerase I covalent complexes in the absence of the tyrosyl-DNA phosphodiesterase Tdp1. *Proc Natl Acad Sci USA* **99**: 14970–14975
- Liu C, Pouliot JJ, Nash HA (2004) The role of TDP1 from budding yeast in the repair of DNA damage. *DNA Repair (Amst)* **3**: 593–601
- Moreira MC, Barbot C, Tachi N, Kozuka N, Uchida E, Gibson T, Mendonca P, Costa M, Barros J, Yanagisawa T, Watanabe M, Ikeda Y, Aoki M, Nagata T, Coutinho P, Sequeiros J, Koenig M (2001) The gene mutated in ataxia-ocular apraxia 1 encodes the new HIT/Zn-finger protein aprataxin. *Nat Genet* **29**: 189–193
- Morris EJ, Geller HM (1996) Induction of neuronal apoptosis by camptothecin, an inhibitor of DNA topoisomerase-I: evidence for cell cycle-independent toxicity. *J Cell Biol* **134**: 757–770
- Nitiss JL (1998) Investigating the biological functions of DNA topoisomerases in eukaryotic cells. *Biochim Biophys Acta* **1400**: 63–81
- Nivens MC, Felder T, Galloway AH, Pena MM, Pouliot JJ, Spencer HT (2004) Engineered resistance to camptothecin and antifolates by retroviral coexpression of tyrosyl DNA phosphodiesterase-I and thymidylate synthase. *Cancer Chemother Pharmacol* **53**: 107–115
- Nouspikel T, Hanawalt PC (2002) DNA repair in terminally differentiated cells. *DNA Repair (Amst)* **1**: 59–75
- Plo I, Liao ZY, Barcelo JM, Kohlhaagen G, Caldecott KW, Weinfeld M, Pommier Y (2003) Association of XRCC1 and tyrosyl DNA phosphodiesterase (Tdp1) for the repair of topoisomerase I-mediated DNA lesions. *DNA Repair (Amst)* **2**: 1087–1100
- Poot M, Gibson LL, Singer VL (1997) Detection of apoptosis in live cells by MitoTracker red CMXRos and SYTO dye flow cytometry. *Cytometry* **27**: 358–364
- Poot M, Gollahan KA, Rabinovitch PS (1999) Werner syndrome lymphoblastoid cells are sensitive to camptothecin-induced apoptosis in S-phase. *Hum Genet* **104**: 10–14
- Porter SE, Champoux JJ (1989) The basis for camptothecin enhancement of DNA breakage by eukaryotic topoisomerase I. *Nucleic Acids Res* **17**: 8521–8532
- Pouliot JJ, Robertson CA, Nash HA (2001) Pathways for repair of topoisomerase I covalent complexes in *Saccharomyces cerevisiae*. *Genes Cells* **6**: 677–687
- Pouliot JJ, Yao KC, Robertson CA, Nash HA (1999) Yeast gene for a Tyr-DNA phosphodiesterase that repairs topoisomerase I complexes. *Science* **286**: 552–555
- Pourquier P, Pommier Y (2001) Topoisomerase I-mediated DNA damage. *Adv Cancer Res* **80**: 189–216
- Povirk LF (1996) DNA damage and mutagenesis by radiomimetic DNA-cleaving agents: bleomycin, neocarzinostatin and other enediynes. *Mutat Res* **355**: 71–89

- Raymond AC, Rideout MC, Staker B, Hjerrild K, Burgin Jr AB (2004) Analysis of human tyrosyl-DNA phosphodiesterase I catalytic residues. *J Mol Biol* **338**: 895–906
- Rolig RL, McKinnon PJ (2000) Linking DNA damage and neurodegeneration. *Trends Neurosci* **23**: 417–424
- Ryan AJ, Squires S, Strutt HL, Evans A, Johnson RT (1994) Different fates of camptothecin-induced replication fork-associated double-strand DNA breaks in mammalian cells. *Carcinogenesis* **15**: 823–828
- Shao RG, Cao CX, Zhang H, Kohn KW, Wold MS, Pommier Y (1999) Replication-mediated DNA damage by camptothecin induces phosphorylation of RPA by DNA-dependent protein kinase and dissociates RPA:DNA-PK complexes. *EMBO J* **18**: 1397–1406
- Staker BL, Hjerrild K, Feese MD, Behnke CA, Burgin Jr AB, Stewart L (2002) The mechanism of topoisomerase I poisoning by a camptothecin analog. *Proc Natl Acad Sci USA* **99**: 15387–15392
- Stewart L, Redinbo MR, Qiu X, Hol WG, Champoux JJ (1998) A model for the mechanism of human topoisomerase I. *Science* **279**: 1534–1541
- Strumberg D, Pilon AA, Smith M, Hickey R, Malkas L, Pommier Y (2000) 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* **20**: 3977–3987
- Subramanian D, Furbee CS, Muller MT (2001) ICE bioassay. Isolating *in vivo* complexes of enzyme to DNA. *Methods Mol Biol* **95**: 137–147
- Takashima H, Boerkoel CF, John J, Saifi GM, Salih MA, Armstrong D, Mao Y, Quijcho FA, Roa BB, Nakagawa M, Stockton DW, Lupski JR (2002) Mutation of TDP1, encoding a topoisomerase I-dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. *Nat Genet* **32**: 267–272
- Vance JR, Wilson TE (2002) Yeast Tdp1 and Rad1–Rad10 function as redundant pathways for repairing Top1 replicative damage. *Proc Natl Acad Sci USA* **99**: 13669–13674
- Wang JC (1996) DNA topoisomerases. *Annu Rev Biochem* **65**: 635–692
- Wang JC (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* **3**: 430–440
- Wang Y, Perrault AR, Iliakis G (1997) Down-regulation of DNA replication in extracts of camptothecin-treated cells: activation of an S-phase checkpoint? *Cancer Res* **57**: 1654–1659
- Yang SW, Burgin Jr AB, 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 USA* **93**: 11534–11539
- Zhou T, Lee JW, Tatavarthi H, Lupski JR, Valerie K, Povirk LF (2005) Deficiency in 3'-phosphoglycolate processing in human cells with a hereditary mutation in tyrosyl-DNA phosphodiesterase (TDP1). *Nucleic Acids Res* **33**: 289–297