ARTHRITIS & RHEUMATOLOGY
Vol. 67, No. 7, July 2015, pp 1858–1867
DOI 10.1002/art.39119

The Topoisomerase I Inhibitor Irinotecan and the Tyrosyl-DNA Phosphodiesterase 1 Inhibitor Furamidine Synergistically Suppress Murine Lupus Nephritis

Andreas Keil,[1] Manuela Frese-Schaper,[1] Selina K. Steiner,[1] Meike Körner,[2] Ralph A. Schmid,[1] and Steffen Frese[1]

Objective. **The treatment of lupus nephritis is still an unmet medical need requiring new therapeutic approaches. Our group found recently that irinotecan, an inhibitor of topoisomerase I (topo I), reversed proteinuria and prolonged survival in mice with advanced lupus nephritis. While irinotecan is known to stabilize the complex of topo I and DNA, the enzyme tyrosyl-DNA phosphodiesterase 1 (TDP-1) functions in an opposing manner by releasing topo I from DNA. Therefore, we undertook this study to test whether the TDP-1 inhibitor furamidine has an additional effect on lupus nephritis when used in combination with irinotecan.**

Methods. **NZB/NZW mice were treated with low-dose irinotecan and furamidine either alone or in combination beginning at age 26 weeks. DNA relaxation was visualized using gel electrophoresis. Binding of anti–double-stranded DNA (anti-dsDNA) antibodies to DNA modified by topo I, TDP-1, and the topo I inhibitor camptothecin was determined by enzyme-linked immunosorbent assay.**

Results. **Compared to treatment with either agent alone, simultaneous treatment with low-dose irinotecan and furamidine significantly improved survival of NZB/NZW mice. Similar to what has been previously shown for irinotecan alone, the combination treatment did not change the levels of anti-dsDNA antibodies. In vitro, recombinant TDP-1 increased topo I–mediated DNA relaxation, resulting in enhanced binding of anti-dsDNA antibodies. In combination with topo I and camptothecin, TDP-1 reversed the inhibitory effects of camptothecin on DNA relaxation and anti-dsDNA binding.**

Conclusion. **Affecting DNA relaxation by the enzymes topo I and TDP-1 and their inhibitors may be a promising approach for the development of new targeted therapies for systemic lupus erythematosus.**

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease mainly affecting women of childbearing age. It is estimated that in the US up to 275,000 adult women have SLE (1). The disease involves different organs, but immune complex glomerulonephritis most strikingly influences the course of SLE. Between 10% and 30% of patients with lupus nephritis develop end-stage renal disease (ESRD) resulting in the requirement for hemodialysis or kidney transplantation (2). Due to the application of immunosuppressive drugs, the survival of patients with lupus-associated glomerulonephritis increased from a 5-year rate of 44% in the 1950s to a 10-year rate of 88% recently (3). Despite these advances in the treatment of SLE, the life expectancy of patients with lupus and renal damage was recently demonstrated to be 23.7 years shorter than that in the general population (4). Moreover, the incidence of ESRD associated with lupus nephritis did not decrease over recent years (5), indicating that current medication is insufficient to treat lupus nephritis.

Treatment with nonselective immunosuppressive drugs continues to be the central strategy for controlling lupus nephritis. Treatment consists of induction therapy with cyclophosphamide and prednisolone or mycophenolate mofetil followed by maintenance therapy with azathioprine or mycophenolate mofetil (6,7). Infections are

Supported by the Swiss National Science Foundation (grant 310030_140964 to Dr. Frese) and LUNIRI.

[1]Andreas Keil, Dipl Biochem, Manuela Frese-Schaper, PhD, Selina K. Steiner, Ralph A. Schmid, MD, Steffen Frese, MD: University Hospital Berne, Berne, Switzerland; [2]Meike Körner, MD: University of Berne, Berne, Switzerland.

Address correspondence to Steffen Frese, MD, Department of Clinical Research, University Hospital Berne, Murtenstrasse 50, PO Box 44, CH-3010 Berne, Switzerland. E-mail: steffen.frese@email.de.

Submitted for publication December 1, 2014; accepted in revised form March 12, 2015.

the major side effect of these medications and they confer a risk of malignancies, while cyclophosphamide also causes amenorrhea (8–10). Furthermore, none of the newly developed biologic agents such as belimumab, rituximab, ocrelizumab, atacicept, or abatacept demonstrated beneficial effects in patients with active lupus nephritis (11–17), suggesting that new treatment strategies are required.

We demonstrated previously that the topoisomerase I (topo I) inhibitor irinotecan efficiently suppressed lupus nephritis in NZB/NZW mice (18,19), an animal model of lupus nephritis (20). The initial finding was made serendipitously, and it was the first time that enzymatic activity of topo I was linked to lupus nephritis, where it might play a major role in pathogenesis, although the mechanism remains to be determined.

Topo I is ubiquitously expressed and highly conserved (21,22). Its major function is the relaxation of supercoiled DNA in order to release torsional stress from DNA that occurs during replication and transcription. To mediate DNA relaxation, topo I binds to DNA and cleaves one DNA strand, subsequently allowing the rotation of the cleaved strand around the other in a controlled reaction (23). Afterward, the nicked strand is religated by topo I, restoring intact double-stranded DNA (dsDNA) in a relaxed state.

Inhibitors of topo I stabilize a normally very transient catalytic intermediate in which topo I is bound to one strand of the DNA, known as the topo I cleavable complex. When the cleavable complex is stalled by topo I inhibitors, religation of the DNA is impossible (24). The consequences of prevented religation may vary; in proliferating cells, the stalled topo I cleavable complex can collide with replication forks, leading to irreparable DNA double-strand breaks and apoptosis (25,26). For this reason, topo I inhibitors such as camptothecin and its synthetic derivative irinotecan are widely used as anticancer drugs for several types of tumors (27). In nondividing cells, treatment with topo I inhibitors results in the production of single-stranded DNA (ssDNA) breaks. Single-stranded DNA breaks are believed to reduce a cell's replication capacity, which is not lethal (24). In addition to the induction of ssDNA and dsDNA breaks, topo I inhibitors were shown to inhibit DNA relaxation at least in vitro (28,29).

The stalled topo I cleavable complex can be resolved by tyrosyl-DNA phosphodiesterase 1 (TDP-1), an enzyme belonging to the phospholipase D superfamily (30). Cells bearing a loss-of-function mutation in TDP-1 show hypersensitivity to the topo I inhibitor camptothecin (31), whereas TDP-1 overexpression promotes camptothecin resistance (32). Therefore, it was suggested that concomitant inhibition of topo I and TDP-1 synergistically stabilizes the topo I cleavable complex (33).

Transferring these findings from cancer research experiments to the situation in SLE, we undertook to determine whether combined inhibition of topo I and TDP-1 has beneficial effects in murine lupus nephritis. For a TDP-1 inhibitor, we used the synthetic diamidine furamidine, which was previously shown to specifically inhibit TDP-1 at low micromolar concentrations (34). In addition to the observation of clinical effects in lupus-prone mice, we examined whether changes in DNA relaxation and alterations in anti-dsDNA binding were involved in the suppression of lupus nephritis.

MATERIALS AND METHODS

Mice. Six-week-old female NZB/NZW mice were purchased from The Jackson Laboratory and kept in isolated ventilated cages. All experimental protocols were approved by the Kantonale Tierkommission (Berne, Switzerland). Immediately after arrival, mice were randomly assigned to the respective groups (5 per cage).

Animal study of combination treatment with low-dose irinotecan and furamidine. Beginning at age 26 weeks, NZB/NZW mice were treated intraperitoneally with saline, 1 mg/kg irinotecan (Campto; Pfizer), 1 mg/kg furamidine (Toronto Research Chemicals) dissolved in sterile water at 2 mg/ml as stock solution (further dilutions were made with saline), or with the combination of irinotecan and furamidine using the indicated concentrations. The volume of each injection was 10 ml/kg. Treatment was performed 3 times a week and repeated every 4 weeks. Beginning at age 22 weeks, mice were screened for proteinuria and body weight once a week. Proteinuria was measured with Albustix (Siemens Healthcare Diagnostics) and quantified as grade 0 (negative), grade 1+ (≥30 mg/dl), grade 2+ (≥100 mg/dl), grade 3+ (≥300 mg/dl), and grade 4+ (≥2,000 mg/dl) according to the manufacturer's recommendations. The onset of disease was defined as 2 instances of grade 4+ proteinuria occurring 1 week apart. Mice were killed when disease became severe (proteinuria grade 4+ and a body weight loss of ≥25% from the onset of disease). The experiment was terminated when mice reached age 60 weeks.

Histopathologic assessment of kidney sections. Kidneys were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Hematoxylin and eosin, periodic acid–Schiff, and methenamine–silver staining were performed using standard protocols. IgG deposits were analyzed on cryosections. Sections (6-μm thick) were fixed in acetone for 10 minutes and incubated with Alexa Fluor 488–conjugated goat anti-mouse IgG (H+L chain specific; Invitrogen). The kidney score of glomerulonephritis was assessed by an independent pathologist (MK), who was blinded to the groups, using the International Society of Nephrology/Renal Pathology Society 2004 classification (35).

Isolation of splenocytes. Spleens were isolated, transferred into ice-cold 2% fetal calf serum (FCS)/phosphate buffered saline (PBS), and smashed on a sterile grid with a pestle. Cells were resuspended, and debris was allowed to settle out by centrifugation at 65*g* for 2 minutes at 4°C.

Subsequently, splenocytes were isolated by centrifugation at 290*g* for 5 minutes at 4°C. Cells were resuspended in RPMI 1640 supplemented with 5% FCS and adjusted to 10^7 cells/ml after checking viability by trypan blue exclusion.

Flow cytometry. Splenocytes (10^6 per sample) were incubated with Fc receptor–blocking monoclonal antibody (clone 2.4G2; BD Biosciences) for 30 minutes, followed by incubation with fluorochrome-labeled monoclonal antibodies for 30 minutes on ice. Cells were measured on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Anti-CD4 (clone RM4-5), anti-CD8a (clone 5H10), anti-CD45R/B220 (clone RA36B2), and anti-CD69 (clone H1.2F3) were obtained from Caltag.

Enzyme-linked immunospot (ELISpot) assay. Serial dilutions of splenocytes ($2 \times 10^5 - 1.25 \times 10^4$ per well) in RPMI 1640 supplemented with 5% FCS were added to 96-well Multiscreen HTS Immobilon-P flat-bottomed plates (Millipore) coated with goat anti-mouse IgG antibody (Fc specific; Sigma-Aldrich). After 4 hours at 37°C, plates were washed and incubated with alkaline phosphatase–conjugated goat anti-mouse IgG (H+L chain specific; SouthernBiotech) for 1 hour. Spots were developed with BCIP/nitroblue tetrazolium plus substrate (Sigma-Aldrich) and counted using an ELISpot reader (Autoimmun Diagnostika).

Enzyme-linked immunosorbent assay (ELISA). To measure total IgG in the plasma, 96-well Nunc MaxiSorp plates were coated with 5 μg/ml goat anti-mouse IgG (SouthernBiotech) and incubated overnight at 4°C. Plates were blocked with PBS containing 1% bovine serum albumin (BSA) for 1 hour at 37°C. Serum samples were diluted 1:100,000 in PBS and incubated for 1 hour at 37°C. Subsequently, plates were incubated with alkaline phosphatase–conjugated goat anti-mouse IgG for 1 hour at 37°C and developed with *p*-nitrophenyl phosphate (Sigma-Aldrich). Optical density was measured at 405 nm with a reference filter at 490 nm. Sample concentrations were calculated by using a standard curve of purified mouse IgG (Sigma-Aldrich).

To measure anti-dsDNA antibodies in the plasma, calf thymus DNA (Invitrogen) was passed through a Millex-HA 0.45 μm filter (Millipore) to remove any ssDNA fragments. Maxisorp plates were half-coated with 100 μg/ml calf thymus DNA in PBS overnight at 4°C. Plates were blocked with PBS containing 1% BSA for 1 hour at 37°C. Serum samples were diluted 1:500 in PBS and incubated for 1 hour at 37°C. Bound anti-dsDNA autoantibodies were detected as described above for total IgG ELISA.

Effect of DNA modification on anti-dsDNA binding. Calf thymus DNA (50 μg/ml) was incubated with 3.75 μg/ml recombinant human topo I (Creative Biomart) in 50 m*M* Tris HCl (pH 7.5), 50 m*M* KCl, 2 m*M* dithiothreitol (DTT), 1 m*M* EDTA, and 50% glycerol for 30 minutes at 37°C. Then, 30 μl of sample per well was used for coating a 384-well Nunc MaxiSorp plate overnight at 4°C. Plates were blocked with PBS containing 1% casein (Pierce) for 1 hour at 37°C, and plates were incubated with the monoclonal anti-dsDNA antibody HYB331-01 (ab27156; Abcam) at a concentration of 1 μg/ml for 1 hour at 37°C. Bound antibodies were detected as described above for anti-dsDNA ELISA.

To evaluate the effects of camptothecin and TDP-1 on topo I–changed anti-dsDNA binding, 50 μg/ml calf thymus DNA was incubated with topo I and camptothecin and/or recombinant human TDP-1 (Topogen), as indicated, for 30 minutes at 37°C. As a control for camptothecin and TDP-1, 10% DMSO or BSA, respectively, was used. Binding of monoclonal anti-dsDNA HYB331-01 to modified calf thymus DNA was assessed as described above.

DNA relaxation assay. Supercoiled plasmid DNA pBR322 (50 μg/ml; Inspiralis) was incubated with 150 ng/ml topo I and increasing concentrations of camptothecin, as indicated, for 30 minutes at 37°C. To investigate the effect of TDP-1,

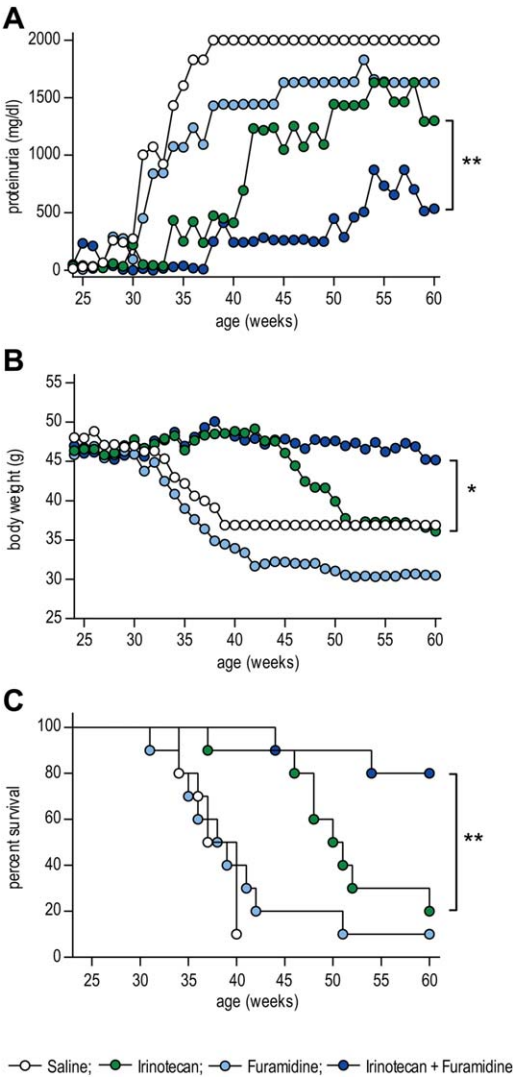

Figure 1. Simultaneous treatment with the topoisomerase I inhibitor irinotecan and the tyrosyl-DNA phosphodiesterase 1 inhibitor furamidine suppresses proteinuria and prolongs survival of lupus-prone NZB/NZW mice. Starting at age 26 weeks, groups of NZB/NZW mice (n = 10 per group) were treated with saline, 1 mg/kg irinotecan, 1 mg/kg furamidine, or a combination of irinotecan and furamidine. Treatment was performed 3 times a week and repeated every 4 weeks. **A** and **B,** Proteinuria (**A**) and body weight (**B**) were measured once a week starting at age 22 weeks. Values are the mean. **C,** Survival was measured. * = $P < 0.05$; ** = $P < 0.01$, by two-way analysis of variance (**A** and **B**) or Mantel-Cox log rank test (**C**). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.39119/abstract.

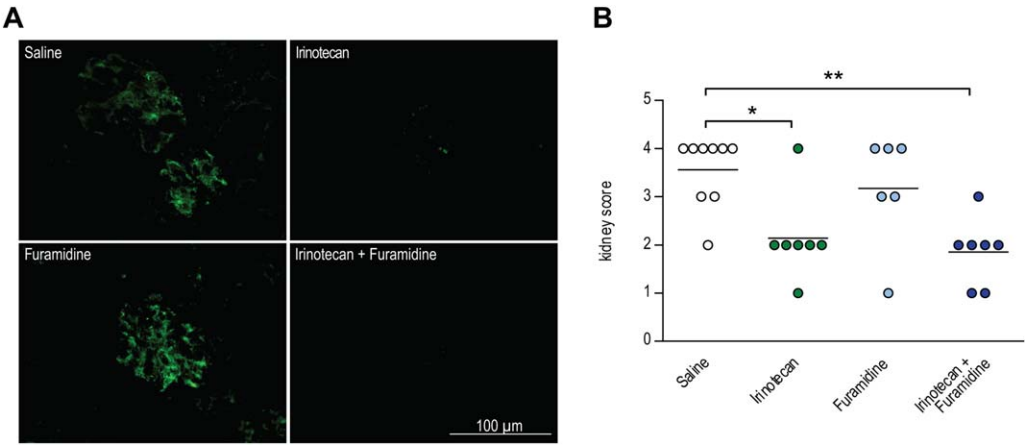

Figure 2. Improved kidney score of lupus-prone mice treated with low-dose irinotecan alone and in combination with furamidine. Starting at age 26 weeks, NZB/NZW mice were treated with irinotecan and/or furamidine as indicated in Figure 1. One week after the third treatment, mice were killed (at age 35 weeks). **A,** IgG deposits in the kidneys were determined on frozen sections. **B,** The kidney score was analyzed by staining paraffin sections with hematoxylin and eosin, periodic acid–Schiff, and methenamine–silver. Frozen sections were stained for IgG. The kidney score of glomerulonephritis was assessed by an independent pathologist, who was blinded with regard to the groups, using the International Society of Nephrology/Renal Pathology Society 2004 classification. Symbols represent individual mice; horizontal lines indicate the mean. $* = P < 0.05$; $** = P < 0.01$, by one-way analysis of variance.

50 μg/ml pBR322 was incubated with 150 ng/ml topo I, 100 μM camptothecin, and 7.25 μg/ml TDP-1 for 30 minutes at 37°C. In another experiment, 50 μg/ml pBR322 was incubated with 30 ng/ml topo I and 7.25 μg/ml TDP-1 for 15, 30, and 60 minutes at 37°C. The modification reaction was performed in a buffer containing 40 mM Tris (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, and 30 μg/ml BSA. Samples were run on a 1% agarose gel and stained with 1 μg/ml ethidium bromide (Sigma-Aldrich) for 30 minutes at room temperature.

Statistical analysis. Data were analyzed using either one-way or two-way analysis of variance followed by Bonferroni adjustment for multiple comparisons or Tukey's multiple comparison test, respectively. Some data were assessed by paired *t*-test. Survival data were analyzed using the Mantel-Cox log rank test. GraphPad Prism software, version 6.0 was used for all tests. *P* values less than 0.05 were considered significant.

RESULTS

Improved kidney function and prolonged survival of lupus-prone mice treated with the combination of irinotecan and furamidine. Our previous work established the topo I inhibitor irinotecan as a suppressor of murine lupus nephritis (18,19). To determine whether the combination of irinotecan and the TDP-1 inhibitor furamidine has additional positive effects against lupus nephritis, NZB/NZW mice were treated beginning at age 26 weeks. At this time point, mice already show signs of lupus such as elevated anti-dsDNA titers (19,36). Three groups of mice (10 per group) were treated with low-dose irinotecan (1 mg/kg), furamidine (1 mg/kg), or the combination of both drugs; 10 control mice received a similar volume of saline. Treatment was performed 3 times a week and repeated every 4 weeks. The dosage of irinotecan used (3 mg/kg per week) was the lowest effective dosage in previous experiments (19). A dosage of 3 mg/kg per week was chosen for furamidine, since this was the lowest effective dosage in a mouse model of African trypanosomiasis (37).

Compared to treatment with saline, treatment with the TDP-1 inhibitor furamidine alone had no beneficial effect on proteinuria, body weight, or survival (Figure 1) or on the kidney score (Figure 2). Compared to saline, irinotecan alone at the indicated low-dose concentration reduced proteinuria until age 40 weeks, stabilized body weight until age 45 weeks, and prolonged survival (Figure 1). Moreover, irinotecan alone significantly improved the kidney score at age 35 weeks (Figure 2).

Further improved effectiveness against lupus nephritis was shown by combination treatment with irinotecan and furamidine. The combination of both drugs significantly reduced proteinuria beyond week 40 and stabilized body weight beyond week 45 compared to irinotecan alone (Figures 1A and B). It can be assumed that the beneficial effects of combination treatment beyond week 40 might have been reflected by the kidney score as well. However, given their reduced lifespan, NZB/NZW control mice were killed at week 35 for functional analyses, making impossible the assessment of the kidney score beyond week 40. Most important, combination treatment demonstrated improved survival compared to all other treatments (Figure 1C), suggesting that the combination of irinotecan and furamidine may represent a new treatment option for SLE.

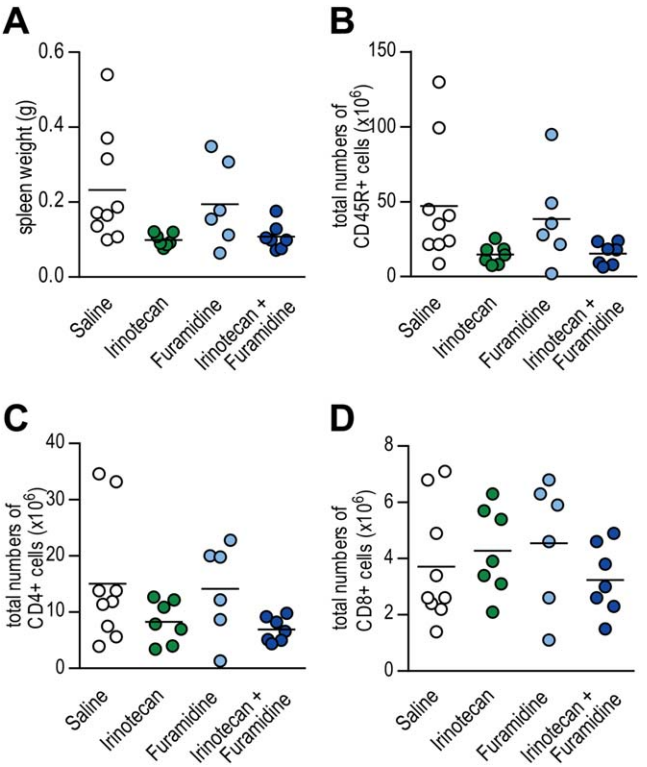

Figure 3. Effects of treatment with irinotecan and furamidine on the total number of splenocyte populations. NZB/NZW mice were treated with saline (n = 9), with 1 mg/kg irinotecan alone (n = 7), with 1 mg/kg furamidine alone (n = 6), or with the combination of irinotecan and furamidine (n = 7) beginning at age 26 weeks. Treatment was performed 3 times a week and repeated every 4 weeks. Mice were killed at age 35 weeks. **A,** Spleen weight. **B–D,** Flow cytometric analysis of numbers of splenic CD45R+ cells (**B**), CD4+ cells (**C**), and CD8+ cells (**D**). Symbols represent individual mice; horizontal lines indicate the mean. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.39119/abstract.

Immunomodulatory effects of treatment with irinotecan and furamidine. Previous data from our group demonstrated that low-dose irinotecan alone yielded moderate immunosuppression, which we interpreted as the correction of enhanced immune activation under autoimmune conditions (19). We therefore examined whether the effects of combined treatment with irinotecan and furamidine were accompanied by more pronounced immunosuppression. However, as already mentioned regarding the kidney score, these data are of limited significance since mice had to be killed for analysis at week 35, while clinical effects of combination treatment were observed much later.

We first examined treatment effects on splenocyte populations. Both irinotecan and combination treatment decreased spleen weight somewhat, although this effect was not significant (Figure 3A). Similarly, we observed some reduction in the numbers of CD45R+ B cells and CD4+ T cells, but not in the numbers of CD8+ T cells (Figures 3B–D); however, none of the effects reached significance.

Regarding T and B cell activity, treatment either with irinotecan alone or with the combination significantly reduced the frequency of the activation marker CD69 on CD4+ cells (Figure 4A). Moreover, some decline in the numbers of antibody-secreting cells was observed in groups treated either with irinotecan alone or with the combination; however, this effect did not differ significantly from saline treatment (Figure 4B). Importantly, analysis of

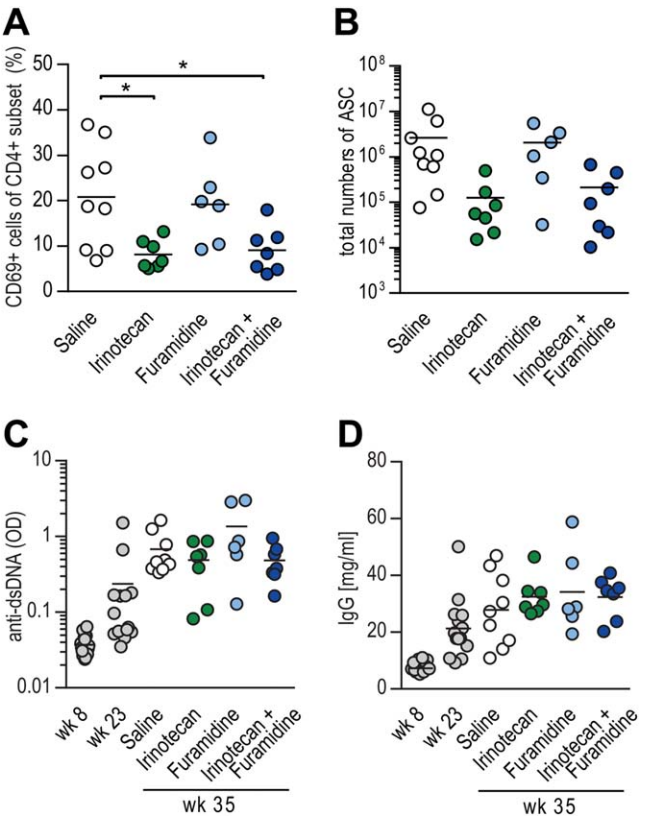

Figure 4. Effects of treatment with irinotecan and furamidine on the activity of splenocyte populations. NZB/NZW mice were treated with saline (n = 9), with 1 mg/kg irinotecan alone (n = 7), with 1 mg/kg furamidine alone (n = 6), or with the combination of irinotecan and furamidine (n = 7) beginning at age 26 weeks. Treatment was performed 3 times a week and repeated every 4 weeks. Mice were killed at age 35 weeks. **A,** The frequency of splenic CD4+CD69+ cells was measured by flow cytometry. **B,** The number of antibody-secreting cells (ASCs) was analyzed by enzyme-linked immunospot assay. **C** and **D,** Levels of anti–double-stranded DNA (anti-dsDNA) (**C**) and total IgG (**D**) in the plasma of NZB/NZW mice were measured by enzyme-linked immunosorbent assay. Plasma from mice killed at weeks 8 and 23 served as a control. Symbols represent individual mice; horizontal lines indicate the mean. $* = P < 0.05$ by one-way analysis of variance. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.39119/abstract.

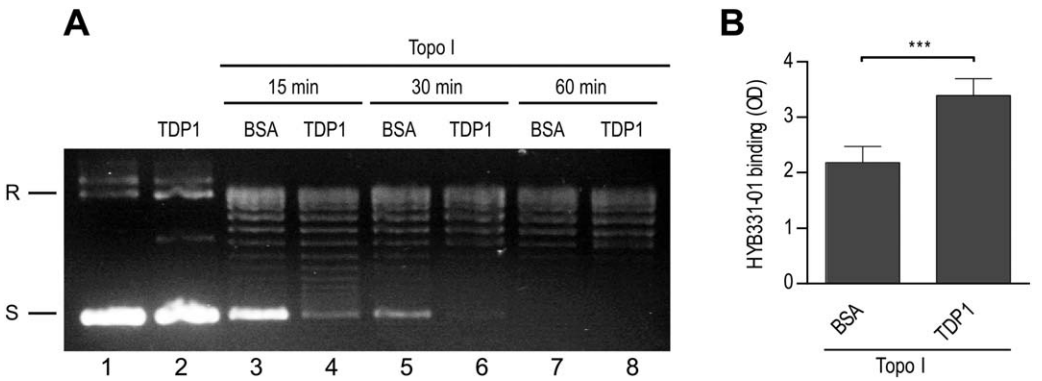

Figure 5. Tyrosyl-DNA phosphodiesterase 1 (TDP-1) accelerates topoisomerase I (topo I)–mediated DNA relaxation and increases anti–double-stranded DNA (anti-dsDNA) binding. **A,** Supercoiled plasmid DNA pBR322 (50 μg/ml) was incubated with 30 ng/ml topo I and 7.25 μg/ml TDP-1 or bovine serum albumin (BSA) for 15, 30, or 60 minutes at 37°C. DNA relaxation was visualized using gel electrophoresis. R = relaxed DNA; S = supercoiled DNA. **B,** Calf thymus DNA (50 μg/ml) was incubated with 3.75 μg/ml topo I and 4.35 μg/ml TDP-1 or BSA for 30 minutes. Binding of the anti-dsDNA antibody HYB331-01 was determined by enzyme-linked immunosorbent assay. Values are the mean ± SEM of 3 independent experiments performed in duplicate. *** = $P < 0.001$ by paired t-test.

plasma anti-dsDNA and total IgG levels revealed no differences among the 4 groups of mice (Figures 4C and D). In conclusion, irinotecan induced rather moderate immunomodulatory effects insufficient to influence levels of anti-dsDNA antibodies. When irinotecan was combined with furamidine, no additional effects on the immune system were observed.

Enhancement of anti-dsDNA binding by TDP-1 in combination with topo I. TDP-1 is known to interfere with the stalled topo I cleavable complex and to catalyze the release of topo I from DNA. It is not clear whether TDP-1 influences the rate of topo I–mediated DNA relaxation when the cleavable complex is not stalled. Because our previous data showed an important role of DNA relaxation in anti-dsDNA binding (19), we examined whether TDP-1 affects the rate of DNA relaxation. Supercoiled plasmid DNA pBR322 was incubated with recombinant topo I in the presence or absence of recombinant TDP-1 for different periods of time. The extent of DNA relaxation was visualized using gel electrophoresis. The results demonstrated that topo I alone was able to relax DNA completely within 60 minutes (Figure 5A). When TDP-1 was added, we observed higher amounts of relaxed DNA and accordingly less supercoiled DNA after 15 and 30 minutes of incubation (Figure 5A), suggesting that TDP-1 increased DNA relaxation in cooperation with topo I.

Next, we examined whether enhanced DNA relaxation mediated by topo I and TDP-1 has an effect on anti-dsDNA binding. DNA was modified by topo I and TDP-1 under the same conditions described above. Anti-dsDNA binding was measured by ELISA. The analysis demonstrated that the addition of TDP-1 was accompanied by increased anti-dsDNA binding (Figure 5B). Summarizing these experiments, we concluded that TDP-1 enhanced topo I–mediated DNA relaxation. Furthermore, consistent with previous data, this change in DNA conformation produced increased anti-dsDNA binding.

TDP-1 reverses the effects of topo I inhibition on DNA relaxation and anti-dsDNA binding. Since NZB/NZW mice had an improved course of lupus nephritis when treated with TDP-1 and inhibitors of topo I, we were further interested in how TDP-1 influences the topo I cleavable complex under conditions of its stabilization through a topo I inhibitor. In previous experiments, we demonstrated that inhibition of topo I diminished DNA relaxation and subsequent binding of anti-dsDNA antibodies derived from plasma of lupus-prone mice or from human SLE serum (19). Using the monoclonal anti-dsDNA antibody HYB331-01, we first determined whether increased anti-dsDNA binding following topo I–mediated DNA relaxation (19) could be reversed by inhibition of topo I. To this end, supercoiled plasmid DNA pBR322 was incubated with recombinant topo I and increasing amounts of camptothecin, a water-insoluble analog of irinotecan that is widely used for in vitro experiments. Visualization of DNA relaxation using agarose gel electrophoresis revealed a higher amount of supercoiled DNA with rising concentrations of camptothecin (Figure 6A). This effect was accompanied by decreased binding of HYB331-01 to DNA modified by topo I and camptothecin (Figure 6B), demonstrating that the extent of DNA relaxation remains crucial for anti-dsDNA binding.

Using similar conditions, we further examined how TDP-1 affected these experiments. Notably, recombinant TDP-1 alone had no effect on DNA relaxation (Figure 6C). When TDP-1 was added to DNA modified by

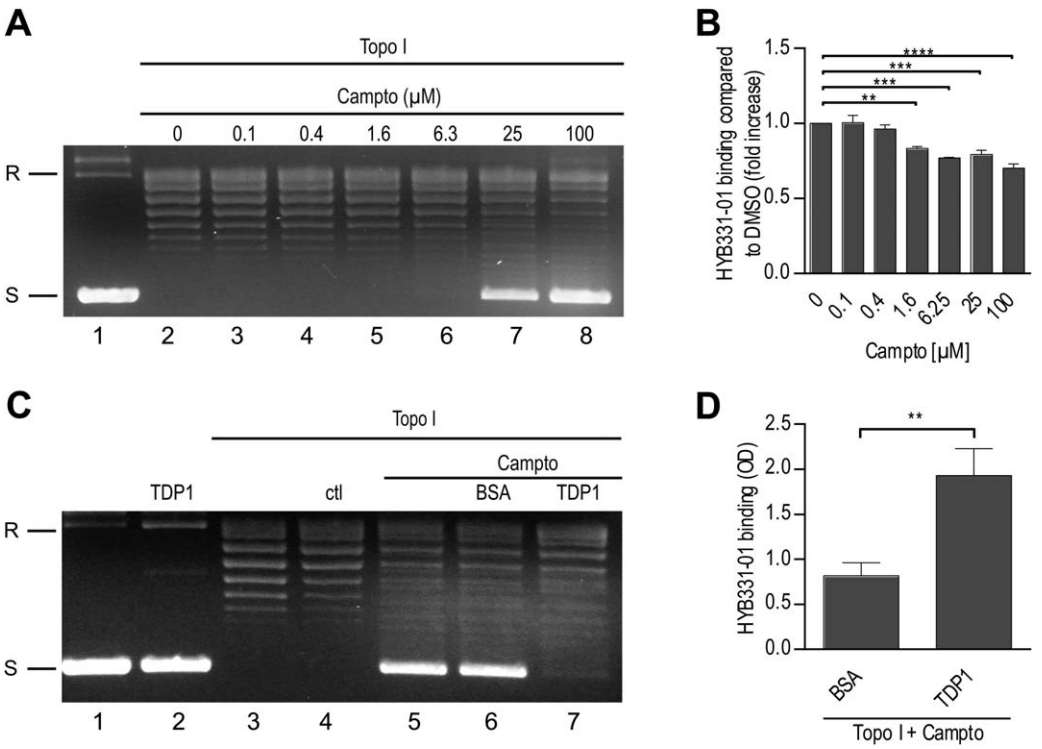

Figure 6. Tyrosyl-DNA phosphodiesterase 1 (TDP-1) reverses the inhibitory effect of camptothecin (campto) on DNA relaxation and anti–double-stranded DNA (anti-dsDNA) binding. **A,** Inhibition of DNA relaxation by camptothecin. Supercoiled plasmid DNA pBR322 (50 μg/ml) was incubated with 150 ng/ml topoisomerase I (topo I) alone or in combination with the indicated concentrations of camptothecin for 30 minutes at 37°C. DNA relaxation was visualized using gel electrophoresis. R = relaxed DNA; S = supercoiled DNA. **B,** Effect of camptothecin on anti-dsDNA binding. Calf thymus DNA (50 μg/ml) was incubated with 3.75 μg/ml topo I alone or in combination with the indicated concentrations of camptothecin. Binding of the monoclonal anti-dsDNA antibody HYB331-01 to modified DNA was measured by enzyme-linked immunosorbent assay (ELISA). Values are expressed as the fold increase relative to probes incubated with DMSO, which was used as solvent for camptothecin. **C,** Inhibitory effects of camptothecin on DNA relaxation reversed by TDP-1. Supercoiled plasmid DNA pBR322 (50 μg/ml) was incubated with 150 ng/ml topo I and 100 μM camptothecin in the presence or absence of 7.25 μg/ml TDP-1 (5 units). Relaxation of supercoiled DNA was analyzed by gel electrophoresis. ctl = control; BSA = bovine serum albumin. **D,** Influence of TDP-1 on the effects of topo I and camptothecin on anti-dsDNA binding. Calf thymus DNA (50 μg/ml) was incubated with 3.75 μg/ml topo I and 100 μM camptothecin in the presence or absence of 4.35 μg/ml TDP-1 (3 units). Binding of HYB331-01 to modified calf thymus DNA was measured by ELISA. BSA was used as control for TDP-1. In **B** and **D,** values are the mean ± SEM of 3–4 independent experiments performed in duplicate. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by one-way analysis of variance (**B**) or paired *t*-test (**D**).

topo I and camptothecin, TDP-1 reversed the effect of camptothecin by reinforcing the production of relaxed DNA (Figure 6C). Next, we examined whether TDP-1–mediated recovery of DNA relaxation changed anti-dsDNA binding. We found a significant increase in binding of HYB331-01 to DNA incubated with topo I, camptothecin, and TDP-1 compared to DNA incubated with topo I, camptothecin, and BSA (Figure 6D). Incubation of DNA with TDP-1 alone had no effect on HYB331-01 binding (data not shown).

DISCUSSION

Treatment of lupus nephritis remains an unsolved medical problem. Our previous work established the topo I inhibitor irinotecan as a potential new treatment option for this disease. The data presented here extend these findings while using irinotecan in combination with furamidine. Both substances have been used to treat diseases other than SLE. While the topo I inhibitor irinotecan has been used to treat metastatic malignancies for many years, the furamidine analog pafuramidine (DB289) was evaluated for the treatment of human African trypanosomiasis, also called sleeping sickness (38).

As anticancer or as antiparasite agents, both inhibitors seem to use mechanisms different from those used for the manipulation of SLE. While the anticancer drug irinotecan is known to induce apoptosis in cancer cells (39), the same drug inhibited apoptosis in the kidneys of lupus-prone mice (18). The situation with furamidine is similar; its trypanocidal action is attributed to its binding to the minor groove of dsDNA independently of TDP-1

(33). If this was the leading mechanism for the suppression of lupus nephritis as well, furamidine should have shown effects in lupus-prone mice when used alone. In contrast, our data demonstrated that furamidine alone was ineffective for the treatment of murine SLE. Furamidine affected the course of SLE only when combined with irinotecan, implying that mechanisms other than binding to DNA were involved. Inhibition of TDP-1 is suggested to be an alternative mechanism of furamidine action in SLE.

Normally, TDP-1 acts as an enzyme involved in DNA repair. TDP-1 is needed to separate topo I from DNA when the topo I cleavable complex is stalled. When the topo I cleavable complex is locked, religation of the DNA is impossible. This situation can occur under natural conditions when the cleavable complex is in proximity to DNA base mismatches or modified DNA bases (40,41) To avoid unligated DNA strands, TDP-1 mediates the release of topo I from DNA and thereby enables other DNA enzymes to repair the single-strand break (42). Other compounds that stall the topo I cleavable complex are topo I inhibitors that bind to the complex and inhibit DNA religation. Subsequently, topo I–mediated DNA relaxation is impaired (28,29).

According to this, TDP-1 acts if the topo I cleavable complex is stalled. However, what are the effects of topo I and TDP-1 on DNA structure (e.g., DNA relaxation) when the topo I cleavable complex is not stalled? This question has relevance to lupus nephritis, since we found in previous experiments that the degree of DNA relaxation is crucial for the binding of lupus-derived anti-dsDNA antibodies (19). Coincubation of recombinant topo I with TDP-1 increased the rate of DNA relaxation, which is consistent with previous data obtained by our group; DNA with a greater level of relaxation was more susceptible to anti-dsDNA binding. However, although these effects illustrate that DNA relaxation is important for anti-dsDNA binding, it is unlikely that this explains the beneficial effects of furamidine in lupus nephritis, because if TDP-1 together with topo I increased in vivo DNA relaxation and anti-dsDNA binding, treatment with furamidine alone should have improved SLE. Thus, the missing in vivo effects of furamidine alone suggested that neither binding of furamidine to DNA nor increased rates of DNA relaxation induced by the combination of topo I and TDP-1 was involved in the beneficial effects of irinotecan and furamidine in lupus nephritis.

Since furamidine exerted its effects only in combination with irinotecan, we further looked at the effects of TDP-1 treatment on the topo I cleavable complex stalled by the topo I inhibitor camptothecin. We demonstrated that TDP-1 resolved the stalled topo I cleavable complex, resulting in increased rates of relaxed DNA and enhanced anti-dsDNA binding. From these in vitro data, we concluded that the TDP-1 inhibitor furamidine impaired the effects of TDP-1 in vivo, which explains the effect of furamidine in lupus nephritis. Unfortunately, it was not possible to obtain more direct evidence for these hypotheses, since furamidine is not suitable for relaxation assays both because of its binding to DNA and because of its autofluorescence (data not shown).

In summary, although low-dose irinotecan was previously suggested to be an entirely new and feasible treatment option for SLE (19), the combination of low-dose irinotecan and the TDP-1 inhibitor furamidine seemed to be superior to irinotecan alone. However, a number of questions need to be answered in upcoming experiments. First, was the dose of furamidine too high? The furamidine dose was chosen as the lowest dose that was still effective as an antiparasite agent against trypanosomiasis (37). As outlined above, the antiparasite and antilupus mechanisms seem to be different; therefore, it should be determined whether decreased doses of furamidine are applicable for the treatment of lupus nephritis. Lower doses would most likely overcome the undesirable side effects that were observed in a phase III clinical trial of the furamidine analog pafuramidine (38). Second, do the in vitro–demonstrated effects on DNA relaxation and anti-dsDNA binding account for the effects of irinotecan and furamidine in murine lupus nephritis? Third, are the recently discovered substances that simultaneously inhibit topo I and TDP-1 (43) more potent suppressors of lupus nephritis than the combination of irinotecan and furamidine? If the answer is yes, further dose reductions would be possible to lower the risk of side effects. However, despite these open questions, affecting DNA relaxation by inhibition of topo I and TDP-1 may be a promising new approach to treat lupus nephritis. If the efficacy of the new treatment is shown in human lupus nephritis (a first clinical trial using low-dose irinotecan for refractory lupus nephritis is currently planned; the protocol can be seen at www.luniri.com), a broader application of irinotecan and furamidine in patients with less severe lupus symptoms might be considered in the future.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Frese had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Keil, Frese-Schaper, Schmid, Frese.

Acquisition of data. Keil, Frese-Schaper, Steiner, Frese.

Analysis and interpretation of data. Keil, Frese-Schaper, Steiner, Körner, Schmid, Frese.

REFERENCES

1. Hochberg MC, Perlmutter DL, Medsger TA, Steen V, Weisman MH, White B, et al. Prevalence of self-reported physician-diagnosed systemic lupus erythematosus in the USA. Lupus 1995;4:454–6.
2. Lionaki S, Skalioti C, Boletis JN. Kidney transplantation in patients with systemic lupus erythematosus. World J Transplant 2014;4:176–82.
3. Cervera R, Khamashta MA, Font J, Sebastiani GD, Gil A, Lavilla P, et al. Morbidity and mortality in systemic lupus erythematosus during a 10-year period: a comparison of early and late manifestations in a cohort of 1,000 patients. Medicine (Baltimore) 2003;82:299–308.
4. Mok CC, Kwok RC, Yip PS. Effect of renal disease on the standardized mortality ratio and life expectancy of patients with systemic lupus erythematosus. Arthritis Rheum 2013;65:2154–60.
5. Ward MM. Changes in the incidence of endstage renal disease due to lupus nephritis in the United States, 1996-2004. J Rheumatol 2009;36:63–7.
6. Clark WF, Sontrop JM. What have we learned about optimal induction therapy for lupus nephritis (III through V) from randomized, controlled trials? Clin J Am Soc Nephrol 2008;3:895–8.
7. Hahn BH, McMahon MA, Wilkinson A, Wallace WD, Daikh DI, FitzGerald JD, et al. American College of Rheumatology guidelines for screening, treatment, and management of lupus nephritis. Arthritis Care Res (Hoboken) 2012;64:797–808.
8. Contreras G, Pardo V, Leclercq B, Lenz O, Tozman E, O'Nan P, et al. Sequential therapies for proliferative lupus nephritis. N Engl J Med 2004;350:971–80.
9. Casey TP. Azathioprine administration to NZB X NZW hybrid mice with lupus nephritis: beneficial effect complicated by development of malignant lymphomas. N Z Med J 1973;79:290–5.
10. Bernatsky S, Ramsey-Goldman R, Labrecque J, Joseph L, Boivin JF, Petri M, et al. Cancer risk in systemic lupus: an updated international multi-centre cohort study. J Autoimmun 2013;42:130–5.
11. Navarra SV, Guzman RM, Gallacher AE, Hall S, Levy RA, Jimenez RE, et al. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. Lancet 2011;377:721–31.
12. Furie R, Petri M, Zamani O, Cervera R, Wallace DJ, Tegzova D, et al, for the BLISS-76 Study Group. A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. Arthritis Rheum 2011;63:3918–30.
13. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, et al, for the LUNAR Investigator Group. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum 2012;64:1215–26.
14. Mysler EF, Spindler AJ, Guzman R, Bijl M, Jayne D, Furie RA, et al. Efficacy and safety of ocrelizumab in active proliferative lupus nephritis: results from a randomized, double-blind, phase III study. Arthritis Rheum 2013;65:2368–79.
15. Isenberg D, Gordon C, Licu D, Copt S, Rossi CP, Wofsy D. Efficacy and safety of atacicept for prevention of flares in patients with moderate-to-severe systemic lupus erythematosus (SLE): 52-week data (APRIL-SLE randomised trial). Ann Rheum Dis 2014. E-pub ahead of print.
16. Furie R, Nicholls K, Cheng TT, Houssiau F, Burgos-Vargas R, Chen SL, et al. Efficacy and safety of abatacept in lupus nephritis: a twelve-month, randomized, double-blind study. Arthritis Rheumatol 2014;66:379–89.
17. The ACCESS Trial Group. Treatment of lupus nephritis with abatacept: the Abatacept and Cyclophosphamide Combination Efficacy and Safety Study. Arthritis Rheumatol 2014;66:3096–104.
18. Frese-Schaper M, Zbaeren J, Gugger M, Monestier M, Frese S. Reversal of established lupus nephritis and prolonged survival of New Zealand Black × New Zealand White mice treated with the topoisomerase I inhibitor irinotecan. J Immunol 2010;184:2175–82.
19. Frese-Schaper M, Keil A, Steiner SK, Gugger M, Korner M, Kocher GJ, et al. Low-dose irinotecan improves advanced lupus nephritis in mice potentially by changing DNA relaxation and anti–double-stranded DNA binding. Arthritis Rheumatol 2014; 66:2259–69.
20. Hahn BH. Lessons in lupus: the mighty mouse. Lupus 2001;10: 589–93.
21. Koiwai O, Yasui Y, Sakai Y, Watanabe T, Ishii K, Yanagihara S, et al. Cloning of the mouse cDNA encoding DNA topoisomerase I and chromosomal location of the gene. Gene 1993;125:211–6.
22. Cheng C, Kussie P, Pavletich N, Shuman S. Conservation of structure and mechanism between eukaryotic topoisomerase I and site-specific recombinases. Cell 1998;92:841–50.
23. Koster DA, Croquette V, Dekker C, Shuman S, Dekker NH. Friction and torque govern the relaxation of DNA supercoils by eukaryotic topoisomerase IB. Nature 2005;434:671–4.
24. Hsiang YH, Hertzberg R, Hecht S, Liu LF. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J Biol Chem 1985;260:14873–8.
25. Hsiang YH, Lihou MG, Liu LF. Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. Cancer Res 1989;49: 5077–82.
26. Wu J, Liu LF. Processing of topoisomerase I cleavable complexes into DNA damage by transcription. Nucleic Acids Res 1997;25:4181–6.
27. Pommier Y. Drugging topoisomerases: lessons and challenges. ACS Chem Biol 2013;8:82–95.
28. Stewart L, Ireton GC, Champoux JJ. A functional linker in human topoisomerase I is required for maximum sensitivity to camptothecin in a DNA relaxation assay. J Biol Chem 1999;274:32950–60.
29. Lisby M, Olesen JR, Skouboe C, Krogh BO, Straub T, Boege F, et al. Residues within the N-terminal domain of human topoisomerase I play a direct role in relaxation. J Biol Chem 2001;276: 20220–7.
30. Yang SW, Burgin AB Jr, Huizenga BN, Robertson CA, Yao KC, Nash HA. A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. Proc Natl Acad Sci U S A 1996;93:11534–9.
31. Interthal H, Chen HJ, Kehl-Fie TE, Zotzmann J, Leppard JB, Champoux JJ. SCAN1 mutant Tdp1 accumulates the enzyme-DNA intermediate and causes camptothecin hypersensitivity. EMBO J 2005;24:2224–33.
32. Barthelmes HU, Habermeyer M, Christensen MO, Mielke C, Interthal H, Pouliot JJ, et al. TDP1 overexpression in human cells counteracts DNA damage mediated by topoisomerases I and II. J Biol Chem 2004;279:55618–25.
33. Huang SN, Pommier Y, Marchand C. Tyrosyl-DNA phosphodiesterase 1 (Tdp1) inhibitors. Expert Opin Ther Pat 2011;21:1285–92.
34. Antony S, Marchand C, Stephen AG, Thibaut L, Agama KK, Fisher RJ, et al. Novel high-throughput electrochemiluminescent assay for identification of human tyrosyl-DNA phosphodiesterase (Tdp1) inhibitors and characterization of furamidine (NSC 305831) as an inhibitor of Tdp1. Nucleic Acids Res 2007;35:4474–84.
35. Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, et al, on behalf of the International Society of Nephrology and Renal Pathology Society Working Group on the Classification of Lupus Nephritis. The classification of glomerulonephritis in systemic lupus erythematosus revisited [published erratum appears in Kidney Int 2004;65:1132]. Kidney Int 2004;65: 521–30.
36. Neubert K, Meister S, Moser K, Weisel F, Maseda D, Amann K, et al. The proteasome inhibitor bortezomib depletes plasma cells and protects mice with lupus-like disease from nephritis. Nat Med 2008;14:748–55.

37. Thuita JK, Karanja SM, Wenzler T, Mdachi RE, Ngotho JM, Kagira JM, et al. Efficacy of the diamidine DB75 and its prodrug DB289, against murine models of human African trypanosomiasis. Acta Trop 2008;108:6–10.
38. Burri C. Chemotherapy against human African trypanosomiasis: is there a road to success? Parasitology 2010;137:1987–94.
39. Frese S, Diamond B. Structural modification of DNA: a therapeutic option in SLE? Nat Rev Rheumatol 2011;7:733–8.
40. Pourquier P, Pilon AA, Kohlhagen G, Mazumder A, Sharma A, Pommier Y. Trapping of mammalian topoisomerase I and recombinations induced by damaged DNA containing nicks or gaps: importance of DNA end phosphorylation and camptothecin effects. J Biol Chem 1997;272:26441–7.
41. Pourquier P, Ueng LM, Kohlhagen G, Mazumder A, Gupta M, Kohn KW, et al. Effects of uracil incorporation, DNA mismatches, and abasic sites on cleavage and religation activities of mammalian topoisomerase I. J Biol Chem 1997;272:7792–6.
42. Nguyen TX, Morrell A, Conda-Sheridan M, Marchand C, Agama K, Bermingham A, et al. Synthesis and biological evaluation of the first dual tyrosyl-DNA phosphodiesterase I (Tdp1)-topoisomerase I (Top1) inhibitors. J Med Chem 2012;55:4457–78.
43. Conda-Sheridan M, Reddy PV, Morrell A, Cobb BT, Marchand C, Agama K, et al. Synthesis and biological evaluation of indenoisoquinolines that inhibit both tyrosyl-DNA phosphodiesterase I (Tdp1) and topoisomerase I (Top1). J Med Chem 2013;56:182–200.