Biochemical Assays for the Discovery of TDP1 Inhibitors

Christophe Marchand¹, Shar-yin N. Huang¹, Thomas S. Dexheimer², Wendy A. Lea², Bryan T. Mott², Adel Chergui¹, Alena Naumova¹, Andrew G. Stephen³, Andrew S. Rosenthal², Ganesha Rai², Junko Murai¹, Rui Gao¹, David J. Maloney², Ajit Jadhav², William L. Jorgensen⁴, Anton Simeonov², and Yves Pommier¹

Abstract

Drug screening against novel targets is warranted to generate biochemical probes and new therapeutic drug leads. TDP1 and TDP2 are two DNA repair enzymes that have yet to be successfully targeted. TDP1 repairs topoisomerase I-, alkylation-, and chain terminator–induced DNA damage, whereas TDP2 repairs topoisomerase II–induced DNA damage. Here, we report the quantitative high-throughput screening (qHTS) of the NIH Molecular Libraries Small Molecule Repository using recombinant human TDP1. We also developed a secondary screening method using a multiple loading gel-based assay where recombinant TDP1 is replaced by whole cell extract (WCE) from genetically engineered DT40 cells. While developing this assay, we determined the importance of buffer conditions for testing TDP1, and most notably the possible interference of phosphate-based buffers. The high specificity of endogenous TDP1 in WCE allowed the evaluation of a large number of hits with up to 600 samples analyzed per gel via multiple loadings. The increased stringency of the WCE assay eliminated a large fraction of the initial hits collected from the qHTS. Finally, inclusion of a TDP2 counter-screening assay allowed the identification of two novel series of selective TDP1 inhibitors. Mol Cancer Ther; 13(8); 2116–26. ©2014 AACR.
obtained from our qHTS campaign using libraries at the National Center for Advancing Translational Sciences (http://www.ncats.nih.gov/research/reengineering/ncgc/ncgc.html), and the use of TDP2 for counter-screening. We also discuss the importance of reaction conditions and counter screening for the characterization of TDP1-selective inhibitors.

Materials and Methods

Chemicals

JLTO48 (CAS# 664357; 4-(5-[[1-(2-fluorobenzyl)-2,5-dioxo-4-imidazolidinylidene]methyl][2-furyl]benzoic acid) was purchased from ChemBridge Corporation. CPT and veliparib were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, DCTD, NCI.

All reactions were performed under argon in oven-dried or flame-dried glassware. All commercially available reagents were purchased from Sigma Aldrich and used as received. All experiments were monitored by analytical thin layer chromatography performed on Silicycle silica gel 60 Å glass-supported plates with 0.25-mm thickness. Yields are not optimized. Low-resolution mass spectra (electrospray ionization) were acquired on an Agilent Technologies 6130 quadrupole spectrometer coupled to an Agilent Technologies 1200 series HPLC. High-resolution mass spectrum–electron ionization spray (HRMS-ESI) were obtained on an Agilent Technologies 6120 quadrupole spectrometer coupled to an Agilent Technologies 1200 series HPLC system equipped with a Phenomenex Luna 75/3 mm, C18, 3-μm column at 45°C [UV detection at 220 nm and 254 nm, band width (BW) 8 nm; flow rate: 0.8 mL/min (increasing); injection volume: 1.0 μL; sample solvent: 100% methanol; sample conc.: ~0.01 mg/mL; mobile phase A: water with 0.1% acetic acid; mobile phase B: acetonitrile with 0.1% acetic acid] coupled to an Agilent 6210 time-of-flight mass spectrometer [ion source: Dual ESI, minimum range: 115 m/z, maximum range: 1,400 m/z, scan rate: 0.9 seconds; gas temperature: 340°C; gas flow: 10 L/min, nebulizer: 50 PSI, ion polarity: positive, VCap: 3,500 V, fragmentor: 175 V, skimmer1: 65 V, OctopoleRFPeak: 250 V, reference mass: enabled (Agilent P/N G1969–85001)]. Data were analyzed using Agilent Masshunter Workstation Data Acquisition (v B.02.00, Patch 1,2,3) and Agilent Masshunter Qualitative Analysis (v B.02.00, Build 2.0.197.7, Patch 3).

Preparation steps for NCCG00183974 are described in Supplementary Materials and Methods.

Cells

DT40 knockout cells (TDP1−/−) and complemented with human TDP1 (hTDP1) were established and authenticated by Southern blot, RT–PCR, and Western blot in 2012 in the Developmental Therapeutics Branch, CCR NCI (17). DT40 cells were cultured with RPMI 1640 medium (GIBCO 11875-093) supplemented with 10% FCS (Gemini Bio-Products 100–106), 1% chicken serum (Invitrogen 16110082), and 50 μmol/L β-mercaptoethanol at 37°C. TDP1-deficient (Tdp1−/−) cells, and TDP1−/− cells complemented with human TDP1 (hTDP1) in chicken DT40 B cell line, have previously been reported and described here (17).

qHTS assay

TDP1 enzyme in the HTS buffer containing 1× PBS, pH 7.4, 80 mmol/L KCl, and 0.01% Tween-20 (31) was dispensed at 3 μL into 1536-well Kalypsys black solid bottom plates (Kalypsys). Compounds and controls (23 mL) were transferred via a Pin Tool station (Kalypsys) at 23 mL. The plates were incubated for 15 minutes at room temperature, and then 1 μL of DNA substrate (Supplementary Fig. S1) was added to start the reaction. After 5 minutes incubation at room temperature, 1 μL of AlphaScreen donor/acceptor bead mix (PerkinElmer Lifesciences) was added, and the plates were further incubated for 10 minutes at room temperature. The detection was then performed on a PerkinElmer Envision reader (PerkinElmer Lifesciences). Compounds were first classified as having full titration curves, partial modulation, partial curve (weaker actives), single-point activity (at highest concentration only), or inactive (32). For all active compounds, a score range was given for each curve class type given above. Active compounds received a Pubchem Activity_Score between 40 and 100. Inconclusive compounds received Pubchem Activity_Score between 1 and 39. All inactive compounds received a Pubchem Activity_Score of 0.

Whole cell extract TDP1 assay

DT40 hTDP1 were collected, washed, and centrifuged. Cell pellets were then resuspended in 100 μL of Celllytic M cell lysis reagent (Sigma-Aldrich C2978). After 15 minutes on ice, lysates were centrifuged at 12,000 g for 10 minutes, and supernatants were transferred to a new tube. Protein concentrations were determined using a Nanodrop spectrophotometer (Invitrogen), and whole cell extracts (WCE) were stored at −80°C. This method of protein concentration determination is acceptable in this case because cellular nucleic acids have been precipitated and discarded during the precipitation step described above and because the amount of WCE to be used is determined by the dilution of WCE required to achieve 30% to 40% of TDP1 cleavage in the assay. A 5'-[32P]-labeled single-stranded DNA oligonucleotide containing a 3'-phosphothymosine (N14Y; ref. 31) was incubated at 1 nmol/L with 4 μg/mL of WCE (WCE quantity to achieve 30%–40% of TDP1 cleavage) in the absence or presence of inhibitor for 15 minutes at room temperature in the WCE buffer containing 50 mmol/L Tris HCl, pH 7.5, 80 mmol/L KCl, 2 mmol/L EDTA, 1 mmol/L DTT, 40 μg/mL BSA, and 0.01% Tween-20. Reactions were terminated by the addition of 1 volume of gel loading buffer [99.5% (v/v) formamide, 5 mmol/L EDTA, 0.01% (w/v) xylene cyanol, and 0.01% (w/v) bromophenol blue]. Samples were subjected to a 16% denaturing PAGE (Accueil 19:1, National Diagnostics) in 1× Tris–borate EDTA with multiple loadings at 2117 Mol Cancer Ther; 13(8) August 2014
12-minute intervals. Gels were run at 70 Watts for a total time of 2.5 hours, dried, and exposed to a PhosphorImager screen (GE Healthcare). Gel images were scanned using a Typhoon 8600 (GE Healthcare), and densitometry analyses were performed using the ImageQuant software (GE Healthcare).

**Recombinant TDP1 and TDP2 assays**

The N14Y DNA substrate was incubated at 1 nmol/L with 10 pmol/L recombinant TDP1 in the absence or presence of inhibitor for 15 minutes at room temperature in WCE buffer (see above and Fig. 3A). When indicated, parallel reactions were performed in the HTS assay buffer containing 1× PBS, pH 7.4, 80 mmol/L KCl, and 0.01% Tween-20 (31; Fig. 3A). Samples were then analyzed similarly to the WCE TDP1 assay (see above).

TDP2 reactions were carried out as described previously (33) with the following modifications. The 18-mer single-stranded oligonucleotide DNA substrate (α32P-cordycepin-3′-labeled) was incubated at 1 nmol/L with 25 pmol/L recombinant human TDP2 in the absence or presence of inhibitor for 15 minutes at room temperature in a buffer containing 50 mmol/L Tris-HCl, pH 7.5, 80 mmol/L KCl, 5 mmol/L MgCl2, 0.1 mmol/L EDTA, 1 mmol/L/DTT, 40 μg/mL BSA, and 0.01% Tween 20. Reactions were terminated and treated similarly to WCE and recombinant TDP1 reactions (see above).

**Kinetics experiments**

To determine the kinetic parameters for the 3′-tyrosyl-DNA phosphodiesterase activity of TDP1, 10 pmol/L and 100 pmol/L of TDP1 was incubated at room temperature with various amounts of substrate (N14Y) in excess in WCE and HTS buffer respectively. All reactions were spiked with 1 nmol/L of 32P-labeled N14Y. The extent of reaction progression was followed in a time-dependent manner and terminated at different times by adding 1 volume of gel loading buffer. Samples were analyzed by 16% denaturing PAGE, and the initial portions of the reaction curves were fitted to a linear equation to approximate the pre–steady-state reaction velocities using Prism (Graphpad software). The Line-weaver–Burk plot was then generated with the pre–steady-state reaction velocities and the corresponding substrate concentrations.

**Surface plasmon resonance analysis**

Binding experiments were performed on a Biacore T100 instrument (GE Healthcare). TDP1 was amine coupled to a CM5 sensor chip (GE Healthcare). Coupling reagents (N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide), EDC, N-hydroxysuccinimide (NHS), and ethanolamine were purchased from GE Heathcare, Neutravidin was obtained from Pierce. To protect the amine groups with the active site from modification, TDP1 was bound with a 14-base oligonucleotide before coupling to the surface. Specifically, 1-μmol/L TDP1 was incubated with 2 μmol/L of a 14-base oligonucleotide containing at phosphate group at the 3′ end (GATCTAAAAGACTT) in 10 mmol/L sodium acetate pH 4.5 for 20 minutes. The CM5 chip surface was activated for 7 minutes with 0.1 mol/L NHS and 0.4 mol/L EDC at a flow rate of 20 μL/min, and TDP1–oligonucleotide mixture was injected until approximately 4,000 response units (RU) was attached. Activated amine groups were quenched with an injection of 1 mol/L solution of ethanolamine pH 8.0 for 7 minutes. Any bound oligonucleotide was removed by washing the surface with 1 mol/L NaCl. A reference surface was prepared in the same manner without coupling of TDP1. Compound 70 was diluted into running buffer [10 mmol/L Hepes, 150 mmol/L NaCl, 0.01% tween 20 (v/v), 5% DMSO (v/v) pH 7.5] and injected over all flow cells at 30 μL/min at 25°C. Following compound injections, the surface was regenerated with a 30-second injection of 1 mol/L NaCl, a 30-second injection of 50% DMSO (v/v), and a 30-second running buffer injection. Each cycle of compound injection was followed by buffer cycle for referencing purposes. A DMSO calibration curve was included to correct for refractive index mismatches between the running buffer and compound dilution series.

**Drug combination experiments**

Drug cellular sensitivity was measured as previously described (23). Briefly, cells were continuously exposed to various drug concentrations for 72 hours in triplicate. DT40 cells were seeded at 200 cells per well into 384-well white plate (PerkinElmer) in 40 μL of medium. Cell viability was determined at 72 hours by adding 20 μL of ATPlite solution (ATPlite 1-step kit; PerkinElmer). After 5-minute incubation, luminescence was measured on an EnVision Plate Reader (PerkinElmer). The ATP level in untreated cells was defined as 100%, and viability of treated cells was defined as (ATP level of treated cell/ATP level of untreated cells) ×100.

**Results**

**qHTS assay**

A qHTS was used to screen the 352,260-compound NIH Molecular Libraries Small Molecule Repository (MLSMR) at 8 concentrations against TDP1. This small molecule repository constitutes the NCATS (formerly NCGC) library and therefore screening results can be crossed-compared with other assays run by NCATS. The optimization and validation of the TDP1 qHTS assay has previously been reported on the Sigma-Aldrich LOPAC1280 library of 1,280 known bioactive small molecules (31). This qHTS, based on the AlphaScreen technology (Supplementary Fig. S1; ref. 31), was run in 1536-well robotic plate format, and led to the identification of 986 positive hits, which have been deposited into PubChem under the AID# 485290 (http://pubchem.ncbi.nlm.nih.gov). A summary of the assay flowchart and selected chemical structures are included in Fig. 1.
Novel WCE TDP1 assay

The 986 positive hits from the primary qHTS screen were tested in a novel secondary biochemical assay using WCE in place of recombinant TDP1. Our recent finding revealed that WCE can selectively process TDP1 substrates (17). WCEs were generated from DT40 chicken lymphoma cells that have been genetically modified to express human TDP1 (hTDP1) in a knockout background for the chicken TDP1 gene (TDP1^−/−; ref. 17). As shown in Fig. 2A and B, endogenous TDP1 from hTDP1 WCE efficiently excised the 3'-phosphotyrosine from its DNA substrate to generate a 3'-phosphate product. This reaction was totally abolished when TDP1^−/− WCE was used (Fig. 2B), demonstrating the selectivity of the TDP1 reaction in a cellular extract environment.

The secondary WCE screening assay has the advantage to use native human TDP1 in a physiologically relevant (cellular extract) environment. In contrast to a screen carried out solely with recombinant TDP1, a screen carried out with WCE uses endogenous native TDP1 with its
posttranslational modifications and cofactors (18, 34), as well as a vast number of other cellular components that can also affect the drug and the substrate. In addition, nonspecific drug targets present in the WCE may selectively decrease the potency of compounds with a tendency to adsorb on different surfaces. This screening strategy should result in an increased stringency of the assay and allow the elimination of promiscuous inhibitors. The increased reactional complexity in the hTDP1 WCE assay still maintained a high specificity for the TDP1 reaction, as we did not detect any nonspecific nucleolytic degradation of the DNA substrate even at high concentrations of WCE (Fig. 2B).

Because the phosphotyrosine catalytic excision by TDP1 produces a single product (N14P, see Fig. 2A), we were also able to perform multiple loadings. With 12-minute intervals between each loading, up to 600 samples could be analyzed on a single sequencing gel (see representative image in Fig. 2C). WCE screening of the 986 qHTS positive hits led to the confirmation of 10 lead compounds with IC_{50} values below 111 μmol/L (Figs. 1C and 2C), indicating that our biochemical assay based on WCE can serve as a robust and efficient secondary screen for the large number of positive hits selected from qHTS assays.

**Importance of reaction buffer for TDP1 assays**

Our original qHTS assay was run in a buffer required for an optimal signal by the AlphaScreen technology and compatibility with robotic liquid handling (see buffer components in Table 1; ref. 31). On the other hand, WCE conditions could be adapted to more physiologic and stringent buffer conditions, including the use of serum albumin, metal chelating agents, and reducing agents. Table 1 outlines the differences in TDP1 kinetics between these two buffer conditions (the qHTS and WCE buffers). Figure 3A and B shows representative Lineweaver–Burk double-reciprocal plots...
allowing the determination of \( K_M \) values of 3,936 nmol/L in the HTS buffer versus 80 nmol/L in the WCE buffer (Table 1). This approximately 50-fold difference in \( K_M \) indicates that TDP1 recognizes its substrate distinctly more efficiently in the WCE buffer than in the HTS buffer. On the other hand, the turnover constants did not vary significantly for the two conditions. TDP1 had a \( k_{cat} \) value of 11 and 7 per second in the HTS and WCE buffers, respectively (Table 1). The resulting \( k_{cat}/K_M \) values of \( 2.8 \times 10^6 \) in the HTS buffer and \( 87.5 \times 10^6 \) L/mol per second in the WCE buffer suggest that TDP1 performs approximately 30-fold better in the WCE buffer than in the HTS buffer (Table 1).

The enhanced catalytic activity of TDP1 in the WCE buffer probably explains, at least in part, why some compounds tested in the WCE buffer failed to inhibit TDP1 below 100 \( \mu \)mol/L drug concentration, as recombinant TDP1 gave a similar difference when it was used under these buffer conditions. This is illustrated in Fig. 3C, which shows that compound NCGC00183964 inhibits recombinant TDP1 with an \( IC_{50} \) of 3.2 \( \pm \) 0.4 \( \mu \)mol/L in the HTS buffer, whereas its \( IC_{50} \) was 81 \( \mu \)mol/L in the WCE buffer, a 25-fold reduction in potency. Together, these experiments demonstrate the enhanced stringency of the TDP1 assays in WCE buffer over the HTS buffer.

To compare the WCE and recombinant TDP1 assays, \( IC_{50} \) values for the 10 compounds presented in Fig. 1 were determined in both assays (Supplementary Table S1). A correlation can be established between the \( IC_{50} \) values determined in the WCE assay and in the recombinant TDP1 assay (Fig. 3D; \( P \) value = 0.0063 and Pearson and Spearman coefficients = 0.79 and 0.68, respectively). \( IC_{50} \) values determined in the WCE assay were approximately 5-fold higher than those determined in the recombinant TDP1 assay (Fig. 3D) reflecting the higher stringency of the hTDP1 WCE assay over the recombinant TDP1 assay.

### TDP2 counter-screening assay

To test the selectivity of TDP1 inhibitors active in the WCE assay, we set up a counter-screening assay with TDP2. TDP2 (encoded by the TTRAP/TDP2 gene) was recently discovered as a key enzyme involved in the repair of Top2-mediated DNA lesions as it excises the Top2 catalytic tyrosine residue from a trapped Top2-DNA complex (35–39). Similarly to TDP1, TDP2 cleaves a phosphotyrosine bond to generate a phosphate product, but this cleavage occurs preferentially with an opposite polarity compared with TDP1 (Fig. 4A and B; refs. 33, 35, 40, 41). Therefore, both enzymes are phosphotyrosine-processing enzymes with opposite preferential polarities (3’-Y for TDP1 and 5’-Y for TDP2; Fig. 4A). In addition, both enzymes preferentially process the same type of single-stranded DNA substrates (8, 33), which makes TDP2 a relevant counter-screening target for TDP1 inhibitors. Moreover, TDP2 is structurally unrelated to TDP1 (10, 40–42). TDP2 requires magnesium for its catalytic activity (33, 35), which is not the case for TDP1. Therefore, TDP2 was chosen as an appropriate counter-screening enzyme for testing the specificity of our TDP1 inhibitors.

The 10 compounds active in the hTDP1 WCE assays can be structurally categorized in two groups (Fig. 1C). Two analogs derived from these two groups, NCGC00183974 (Fig. 1D) and JLT048 (CAS# 664357-58-8; Fig. 1D), both inhibited recombinant TDP1 at low micromolar concentrations (Table 2 and Fig. 4C), and their potency was maintained in the WCE assay (Table 2). When tested in parallel against TDP1 and TDP2, JLT048 also inhibited TDP2, albeit with higher \( IC_{50} \) values (Fig. 4C and D, Table 2). NCGC00183974 was more selective for TDP1 (10, 40–42). TDP2 requires magnesium for its catalytic activity (33, 35), which is not the case for TDP1. Therefore, TDP2 was chosen as an appropriate counter-screening enzyme for testing the specificity of our TDP1 inhibitors.

### Cellular combination treatment with CPT

To determine whether the two compounds could potentiate the cytotoxic effect of a Top1 inhibitor, NCGC00183974 (Fig. 5A) and JLT048 (Fig. 5B) were tested in combination with CPT in DT40 hTDP1 cells for cytotoxicity. We observed no synergistic effect, in contrast to the PARP inhibitor, veliparib, which showed the expected strong synergism with CPT (26, 27; Fig. 5C).

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The two compounds also did not exhibit any cytotoxicity, suggesting that they do not enter cells efficiently and/or are inactivated. Therefore, further structural optimization is warranted to improve their cellular profile.

Discussion

TDP1 and TDP2 are two relatively new DNA repair enzymes, which are rational pharmacologic targets (see Introduction). Here, we report our screening approach including the development of a novel WCE gel-based assay and counter-screening with TDP2, which led to the identification of two novel TDP1 inhibitors that could serve as the basis for further development.

The new WCE assay has the advantage of using native endogenous human TDP1 enzyme in a cellular environment with its cofactors, binding partners (11, 34) and posttranslational modifications (43, 44). It is therefore likely to be more biologically relevant than assays based on recombinant TDP1, as exemplified by the fact that the protein kinase inhibitor, 7-hydroxystaurosporine (UCN-01), was found to inhibit Chk2 purified from cell extract by immunoprecipitation while being ineffective against the recombinant Chk2 enzyme (45). WCE also incorporates a complex cellular mixture, which promotes the adsorption
of nonspecific small molecules inhibitors to different cellular proteins and components, providing a more biologically relevant model of inhibitor distribution. The WCE assay is simpler and cheaper than assays using recombinant enzymes that require purification steps. In the present study, WCEs were generated from DT40 chicken lymphoma cells because these cells have a short doubling time and can be easily grown in large quantity in suspension. They are frequently used to generate genetic knockout cell lines (17, 23), and we previously engineered DT40 cells to express functional human TDP1 in a TDP1 knockout background (17). WCE from human cells can also be used in place of the DT40 WCE (46), which should render the WCE assays applicable to other platforms and reference cell lines.

The WCE gel-based assay is convenient for drug screening because the TDP1 substrate is processed in a single product (Figs. 2 and 4), allowing multiple loading on a single gel (Fig. 2). The novel WCE assay was run in a more physiologically relevant buffer than the qHTS assay (31, 47, 48). When these two buffers were tested side-by-side, a more efficient TDP1 catalytic activity was observed in the WCE buffer than in the qHTS buffer. We observed a large difference in the $K_M$ of TDP1 and only a slight change (within experimental error) in its $k_{cat}$ values. This likely reflects the presence of phosphate salts in the HTS buffer acting as an inhibitor for TDP1. Indeed, we have observed that phosphate likely inhibits TDP1 by competing with its tyrosine-phosphodiester-DNA substrate (10). The other key difference between the two buffer systems is the presence of BSA. After investigating the impact of BSA on the kinetics of TDP1, we found that the removal of BSA from the WCE buffer resulted in a lower $k_{cat}$ but with little impact on the $K_M$ value, which may be the result of higher protein adhesion to the tube walls (data not shown). The specific example of TDP1 sheds light on the general importance of reaction buffers when developing screening assays, especially when robotic platforms require specific screening conditions.
TDP2 is TDP1’s counterpart for the repair of Top2-mediated DNA lesions with the cleavage of a 5'-phosphotyrosine bond. Although both enzymes process single-stranded substrates, they are structurally unrelated and differ in their biochemical mechanisms. TDP1 belongs to the phospholipase D family and its catalytic mechanism involves two histidine-lysine-asparagine (HKN) motifs and a covalent intermediate (9, 10). On the other hand, TDP2 is a magnesium-dependent phosphodiesterase that hydrolyzes the 5'-phosphotyrosyl bonds without covalent intermediate (33, 40, 41). Dual TDP1–TDP2 inhibitors are therefore likely to be promiscuous (49).

From the 10 TDP1 hits identified by qHTS and confirmed in the WCE assay, two analogs showed selectivity for TDP1 versus TDP2. Surface plasmon resonance experiments showed that the two compounds interacted with TDP1 directly without interacting with the DNA substrate (Supplementary Fig. S2). Yet, these inhibitors have some potential liabilities. JLT048 incorporates a methyleneimidazolinedione substructure that gives concerns for potential reactivity as a Michael acceptor (49). NCGC00183974 exhibits a higher selectivity for TDP1, but also inhibit other DNA-processing enzymes including DNA polymerase κ (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=49852749). Also, cellular cytotoxicity assays indicate that further studies are warranted to optimize the cellular activity of these series.

In summary, our WCE-based screening approach allowed stringent hit confirmation from qHTS, reducing the number of original hits and markedly enhancing the prospect of discovering selective and relevant inhibitors of TDP1. These results suggest the value of using WCE for the screening of TDP1 inhibitors, and the value of recombinant TDP1 and TDP2 for second-line screening assays and mechanism of action studies.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Authors' Contributions
Conception and design: C. Marchand, T.S. Dexheimer, A.S. Rosenthal, G. Rai, D.J. Maloney, W.L. Jorgensen, A. Simeonov, Y. Pommier
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Marchand, S.-y.N. Huang, B.T. Mott, A. Chergui, A. Naumova, A.S. Rosenthal, J. Murai, R. Gao, Y. Pommier
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Marchand, S.-y.N. Huang, T.S. Dexheimer, A. Chergui, A. Naumova, A.G. Stephen, A.S. Rosenthal, A. Jadhav, A. Simeonov, Y. Pommier

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Writing, review, and/or revision of the manuscript: C. Marchand, S.-y.N. Huang, T.S. Dexheimer, B.T. Mott, G. Rai, R. Gao, D.J. Maloney, W.L. Jorgensen, A. Simeonov, Y. Pommier
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Marchand, A.S. Rosenthal, J. Murai, Y. Pommier
Study supervision: C. Marchand, W.L. Jorgensen, Y. Pommier

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