



Validating TDP1 as an Inhibition Target for the Development of Chemosensitizers for Camptothecin-Based Chemotherapy Drugs

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ABSTRACT

Cancer chemotherapy sensitizers hold the key to maximizing the potential of standard anti-cancer treatments. We have a long-standing interest in developing and validating inhibitors of the DNA repair enzyme tyrosyl-DNA phosphodiesterase 1 (TDP1) as chemosensitizers for topoisomerase I poisons such as topotecan.

Euphemia Leung and Jinal Patel contributed equally to this work.

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Herein, by using thieno[2,3-*b*]pyridines, a class of TDP1 inhibitors, we showed that the inhibition of TDP1 can restore sensitivity to topotecan, results that are supported by TDP1 knockout cell experiments using CRISPR/Cas9. However, we also found that the restored sensitivity towards topoisomerase I inhibitors is likely regulated by multiple complementary DNA repair pathways. Our results showed that one of these pathways is likely modulated by PARP1, although it is also possible that other redundant and partially overlapping pathways may be involved in the DNA repair process. Our work thus raises the prospect of targeting

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multiple DNA repair pathways to increase the sensitivity to topoisomerase I inhibitors.

Keywords: DNA repair; TDP1; Thieno[2,3-*b*]pyridines; Topotecan; Topoisomerase I

Key Summary Points

Why carry out this study?

Side effects and the development of chemoresistance are unresolved challenges in cancer chemotherapy.

Chemosensitizers may help solve these issues by making cancer cells more vulnerable to conventional chemotherapy drugs.

Our work aims to validate the DNA repair enzyme tyrosyl-DNA phosphodiesterase 1 (TDP1) as an inhibition target for the development of chemosensitizers for camptothecin-based chemotherapy drugs such as topotecan.

What was learned from the study?

Inhibition of TDP1 may restore sensitivity to topotecan.

TDP1 inhibitors also induced a potentiation effect in TDP1 knockout cells, indicating that multiple complementary DNA repair pathways may exist to repair the DNA damage caused by topotecan.

Our results highlight the prospect of targeting multiple DNA repair pathways to increase the sensitivity to topoisomerase I inhibitors.

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DIGITAL FEATURES

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INTRODUCTION

Although chemotherapy is now routinely applied to treat cancer patients, the unpleasant (and sometimes severe) side effects and the eventual development of chemoresistance remain unresolved challenges. In order to mitigate these issues, the use of chemosensitizers to enhance the efficacy of chemotherapy has been a focus of intense research in the last two decades [1, 2]. Chemosensitizers make cancer cells more vulnerable to conventional chemotherapy drugs, allowing lower dosages to be used [1, 2]. Hence, chemosensitizers hold the potential to delay or reverse chemoresistance, reduce dosage-related toxicity and minimize side effects.

Topotecan and irinotecan are among the Food and Drug Administration (FDA)-approved chemotherapy drugs that are structurally based on camptothecins [3–5]. Camptothecins are pentacyclic quinoline alkaloids that target DNA topoisomerase I, a DNA-cleaving enzyme that plays a key role in relaxing tangled DNA during its replication and transcription [6, 7]. Camptothecins inhibit topoisomerase I by trapping the covalent topoisomerase I-DNA complex, which in turn interferes with the cellular replicative and transcriptional machinery [8, 9]. This causes DNA damage and potent cytotoxicity, which is utilized to kill rapidly dividing cancer cells. However, the cytotoxicity of camptothecins is limited by intrinsic cancer cell resistance, which is thought to be mediated by DNA repair pathways [10–12]. One of these DNA pathways is modulated by the enzyme tyrosyl-DNA phosphodiesterase 1 (TDP1) [13–15]. TDP1 releases stalled topoisomerase I-DNA cleavage complexes by catalyzing the hydrolysis of the phosphodiester bond between the catalytic tyrosine residue of topoisomerase I

and the phosphate group of DNA [13–15]. The activity of TDP1 and topoisomerase I in tumor cells is positively correlated [16]. There is evidence that TDP1 expression may lessen the cytotoxicity of camptothecin-based drugs [17]. Preclinical data have also shown that the level of TDP1 activity may influence cellular sensitivity to camptothecin-based drugs and other topoisomerase I inhibitors [18, 19].

Our laboratories have a long-standing interest in the development and validation of sensitizers for camptothecin-based chemotherapy drugs [20–38]. Specifically, our recent work indicated that TDP1 inhibitors may potentiate topotecan both in vitro and in vivo [31]. Although this study provides an impetus for the development of selective TDP1 inhibitors as camptothecin sensitizers, the presence of multiple DNA repair pathways and enzymes other than TDP1 may present an obstacle to this drug development prospect [39–43]. Thus, it is important to illuminate the association between TDP1 activity and camptothecin sensitivity. Our previous work showed that compounds containing the thieno[2,3-*b*]pyridine scaffold are inhibitors of TDP1 [35, 38], and thus represent excellent model compounds for the investigation. We therefore set out to study the modulation of TDP1 activity with thieno[2,3-*b*]pyridines to potentiate the effects of topotecan.

METHODS

Chemicals and Reagents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich, Merck, Life Technologies, Bio-Rad, Abcam and AK Scientific. Topotecan and olaparib were purchased from Sigma-Aldrich.

Cell Culture

Culture conditions have been described in detail previously [44, 45]; Hs578T, BT549, MDA-MB-231, SKBR3, MCF7, T47D, BT474, MDA-MB-453, MDA-MB-436, A172, SUM149T, HCF116,

MiaPaCa, PANC1, A549, HeLa and H460 cancer cell lines were purchased from the American Type Culture Collection (ATCC). SUM149PT and SUM150PT were grown in Ham's F-12 with 5% fetal calf serum (FCS) supplemented with 5 µg/mL insulin, 1 µg/mL hydrocortisone, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4) and penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively). All other cells were grown in α -MEM containing 5% fetal bovine serum (FBS), insulin/transferrin/selenium supplement, added according to the manufacturer's instructions (Roche), and penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively). Ethics committee approval was not required for the handling of commercial cell lines.

Cell Proliferation Assay

As described in detail previously [44], proliferation was measured using a thymidine incorporation assay. A total of 3000 cells were seeded in 96-well plates in the presence of varying concentrations of inhibitors for 3 days. ³H-thymidine was added to each well and incubated for 6 h; the cells were harvested on glass fiber filters using an automated Tomtec harvester. Filters were incubated with Betaplate Scint and thymidine incorporation measured in a TriLux/Betaplate counter. The effects of the inhibitors were determined relative to the incorporation of ³H-thymidine into DNA of control (non-drug-treated) cells.

Guide Ribonucleic Acid (gRNA) Design and Cloning

Optimal gRNA [46] with forward and reverse oligos 5'-CACC GACATCTCTGCTCCCAATGA-3' and 5'-AAAC TCATTGGGAGCAGAGATGTC-3' were ligated and cloned into the pSpCas9 (BB)-2A-GFP (PX458) vector by Golden Gate cloning [47]; pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138; <http://n2t.net/addgene:48138>; RRID:Addgene_48138). Cells were transfected by Lipofectamine 3000, and GFP-positive cells were isolated 48 h later by flow cytometric

sorting and seeded as single cells. Knockout clones were confirmed by PCR amplification of the targeted exon followed by sequence analysis (see Supporting Information, Figure S1) to confirm disruption of coding sequences at the targeted site. Cells are referred to as MCF7 TDP1_KO_2.8 and H460 TDP1_KO_1.3. Ethics committee approval was not required for the mutation of the cell lines.

Western Blotting

As described in detail previously [44], breast cancer cell lines were grown to log-phase, washed twice with ice-cold PBS, and lysed in SDS lysis buffer according to the manufacturer's protocol (Cell Signaling Technology, Danvers, MA, USA). Protein concentration was quantified using the bicinchoninic acid reagent (Sigma). Cell lysates containing 25 µg of protein were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Millipore). Membranes were subjected to immunoblotting with antibodies against TDP1 (Abcam AB4166), PARP1 (Cell Signaling Technology #9542), and tubulin (Sigma). Bound antibody was visualized using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and chemiluminescence detection by the ChemiDoc imaging system (Bio-Rad).

Flow Cytometric Analysis

Cells (5×10^5 cells for MCF7 or 5×10^5 cells for H460 per well) were grown in 6-well plates overnight. After 24 h, cells were incubated for 2 h with inhibitors. The concentration used for DJ009 was 5 µM, and the topotecan concentration was 0.5 µM for MCF7 or 1 µM for H460. Cells were harvested, washed and resuspended in 1 mL of blocking buffer (1% FCS/PBS), and incubated with antibody to γ -H2AX (phosphorylated Ser139) (Millipore, USA) in blocking buffer (1:500 dilution) at room temperature for 2 h. Cells were washed, incubated with goat anti-mouse Alex 488 Fab fragment secondary antibody (Invitrogen) (1:400 in blocking buffer for 1 h, at room temperature; dark), washed and

resuspended in 1 mL of blocking buffer containing RNase (1 µg/mL) and propidium iodide (PI) (10 µg/mL) for 30 min at room temperature. Cells were analyzed in a Becton Dickinson BD Accuri C6 flow cytometer.

Data analysis

The Bliss additivism model [48] was used to classify the effect of combining topotecan and other inhibitors as additive, synergistic, or antagonistic. A theoretical curve was calculated for combined inhibition using the equation $E_{\text{bliss}} = EA + EB - EA \times EB$, where EA and EB are the fractional inhibitions obtained by drug A alone and drug B alone at specific concentrations. E_{bliss} is the fractional inhibition that would be expected if the combination of the two drugs was exactly additive. The differences (Bliss) in the experimentally measured fractional inhibition (E_{xpt}) and E_{bliss} are defined as synergism ($E_{\text{xpt}} < E_{\text{bliss}}$), additivity ($E_{\text{xpt}} = E_{\text{bliss}}$), and antagonism ($E_{\text{xpt}} > E_{\text{bliss}}$). Bliss = 0 would indicate the combination is additive; Bliss > 0 would indicate the percentage increase in maximal inhibition above additivity (synergy); and Bliss < 0 would indicate the percentage decrease in maximal inhibition below additivity (antagonism).

TDP1 Biosensor Assay

The recombinant TDP1 was purified as described below. The TDP1-biosensor 5'-(5,6 FAM-aac gtc agg gtc ttc c-BHQ1)-3' was purchased from Integrated DNA Technologies; 5,6 FAM is fluorophore 5(6)-carboxyfluorescein, and BHQ1 is fluorescence quencher Black Hole Quencher 1. As described in detail previously [31], the TDP1-biosensor, with a final concentration of 50 nM, was incubated in a volume of 200 µL containing a TDP1 buffer (50 mM Tris–HCl pH 8.0, 50 mM NaCl, 7 mM β -mercaptoethanol) supplemented with a purified 3 nM TDP1. The reaction mixtures were incubated at a constant temperature of 24°C in an EnSpire 2300 Multimode Plate Reader (PerkinElmer, Singapore) to measure fluorescence every 1 min (Ex485/Em520 nm).

The relative TDP1 activity was measured at 7 min compared to that of DMSO control wells.

Molecular Modeling

The compounds were docked against the crystal structure of TDP1 (PDB ID: 6DIE, resolution 1.78 Å) [49] which was obtained from the Protein Data Bank (PDB) [50, 51]. The Scigress version FJ 2.6 program [52] was used to prepare the crystal structure for docking, i.e., the hydrogen atoms were added, the co-crystallised ligand benzene-1,2,4-tricarboxylic acid was removed as well as crystallographic water molecules except HOH 814, 821 and 1078. The water molecules were set on toggle—bound or displaced by the ligand during docking, and spin—automatic optimization of the orientation of the hydrogen atoms. The Scigress software suite was also used to build the inhibitors, and the MM2 force field [53] was applied to identify the global minimum using the CON-FLEX method [54] followed by structural optimization. The docking center was defined as the position of a carbon on the ring of the co-crystallized benzene-1,2,4-tricarboxylic acid ($x = -6.052$, $y = -14.428$, $z = 33.998$) with 10 Å radius. Fifty docking runs were allowed for each ligand with default search efficiency (100%). The basic amino acids lysine and arginine were defined as protonated. Furthermore, aspartic and glutamic acids were assumed to be deprotonated. The GoldScore (GS) [55] and ChemScore (CS) [56, 57] ChemPLP (Piecewise Linear Potential) [58] and ASP (Astex Statistical Potential) [59] scoring functions were implemented to predict the binding modes and relative energies of the ligands using the GOLD v5.4.1 software suite.

Recombinant TDP1 Production and Purification

Synthetic DNA encoding human TDP1 (residues 149–608) [14] was cloned into pET-28a(+) (GenScript), which was then transformed into *Escherichia coli* BL21(DE3) for recombinant protein production. Protein production was induced with 1 mM isopropyl β-D-1-

thiogalactopyranoside (IPTG) at 28 °C with overnight incubation. TDP1 was purified using affinity and size exclusion chromatography. The purified protein was aliquoted and stored at –80 °C until use.

Intrinsic protein fluorescence

Fluorescence was measured using a PerkinElmer EnSpire Multimode Reader. The TDP1 concentration was 10 μM and compound concentrations were 50 μM, 100 μM, 200 μM, 400 μM, 600 μM and 800 μM. The buffer was composed of 20 mM Tris and 250 mM NaCl (pH 8). The excitation wavelength was 280 nm and the intrinsic fluorescence was measured at 350 nm. Control experiments with TDP1 or compound on its own were also conducted. Background fluorescence arising from the compounds was subtracted from the final spectrum. The total volume per well was 30 μL. Dissociation constants (K_D) were calculated using the following formula, which takes nonspecific binding into account [60].

$$I = \frac{I_{\max} \times [L_T]}{K_D + [L_T]} + N_s[L_T],$$

where I indicates changes in fluorescence intensity from the titration, I_{\max} indicates the maximum fluorescence intensity change, $[L_T]$ is the titration ligand concentration, and N_s is the nonspecific term. Nonlinear curve fitting was conducted using SigmaPlot 13.0 (Systat Software, San Jose, CA, USA). Experiments were conducted in triplicate, and the errors shown are standard deviation.

X-Ray Crystallography

Crystallization

Crystallography screening was performed in crystallization plates in a 96-well format. The highly purified human TDP1₁₄₈₋₆₀₈ was buffer-exchanged into a buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 50 mM NaCl at pH 7.8 and was then concentrated to 6.5 mg/mL. After 6–8 days of incubation at 18 °C, the formation of large

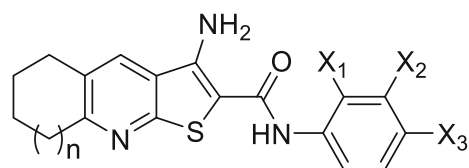
needle-shaped crystals was observed in the fine-screened liquid containing 200 mM ammonium chloride, 100 mM HEPES, pH 7 and 20% PEG 6000. The crystals were supplemented with 20% glycerol and then flash-frozen in liquid nitrogen for data collection.

Data Collection and Processing

The data for TDP1₁₄₈₋₆₀₈ were collected remotely at the School of Biological Sciences, The University of Auckland, using the Australian Synchrotron MX2 beamline. The data were collected using MX2 beamline with the help of Blu-Ice software with a resolution of 2.13 Å [61]. The TDP1₁₄₉₋₆₀₈ data were indexed and integrated using the autoprocessing software XDS [62]. The data were scaled using the AIMLESS software package [63].

Crystallographic Refinement

The structure was solved by molecular replacement using the PHASER MR program [64] using the available published crystal structure from the PDB [65]. The structure was solved in P212121, which is expected as per published PDB (1JY1) (detail numbers are described in Supporting Information, Table S1). The final structure was then refined using the REFMAC software package [66], and coordinates and electron density were observed using COOT modeling software [67]. The water molecules were identified by their spherical electron density and appropriate hydrogen bond geometry with the surrounding structure. The preliminary coordinates were visualized, and figures were produced using Pymol [68].



DJ009: $n = 3$, $X_1 = X_2 = \text{H}$, $X_3 = \text{Cl}$

DJ016: $n = 2$, $X_1 = \text{H}$, $X_2 = X_3 = \text{Cl}$

DJ282: $n = 5$, $X_1 = \text{Cl}$, $X_2 = X_3 = \text{H}$

Fig. 1 Structures of DJ009, DJ016 and DJ282

RESULTS

As our initial work with thieno[2,3-*b*]pyridines and TDP1 was conducted with a relatively small library [35, 38], we first carried out *in vitro* studies to validate thieno[2,3-*b*]pyridines as inhibitors of TDP1. One of our laboratories has synthesized and acquired > 100 thieno[2,3-*b*]pyridines derivatives [35, 38, 69–77]. By using this extensive library, we screened for inhibition of TDP1 activity using a fluorescence-based inhibition assay with a synthetic oligonucleotide biosensor as substrate [78]. At 25 μM ligand concentration, 28 (22%) out of the 128 derivatives showed > 50% TDP1 inhibition (see Supporting Information, Figure S2). For the purpose of this study, three of the 28 compounds were chosen for further evaluations (DJ009, DJ016 and DJ282; Fig. 1). They were chosen because of their strong inhibitory effect in the biosensor assay and low antiproliferative activity at 500 nM. Binding studies using intrinsic fluorescence spectroscopy showed that these three compounds bind to TDP1 with affinity in the micromolar range (see Supporting Information, Figures S3–S5). In agreement with previous studies [35, 38], molecular modeling showed that these three compounds have a plausible binding conformation in the active site (see Supporting Information, Figures S6–S8 and Table S2). Protein X-ray crystallography was also attempted. However, despite numerous attempts using both the soaking and co-crystallization methods, we were only able to obtain the structure of apo-TDP1, which is almost identical to the published TDP1 structure (see Supporting Information Figure S9 and Table S1) [65].

Having confirmed that thieno[2,3-*b*]pyridines inhibit TDP1 *in vitro*, we set out to examine the synergistic effects of thieno[2,3-*b*]pyridines and topotecan on the proliferation of cancer cell lines. DJ009 was used against a panel of 16 cancer cell lines as an initial screen. These cell lines were chosen to reflect the cancers that topotecan is currently used to treat in the clinic, as well as a number of other cell lines to evaluate whether TDP1 inhibitors could broaden the spectrum of cancers that could be targeted

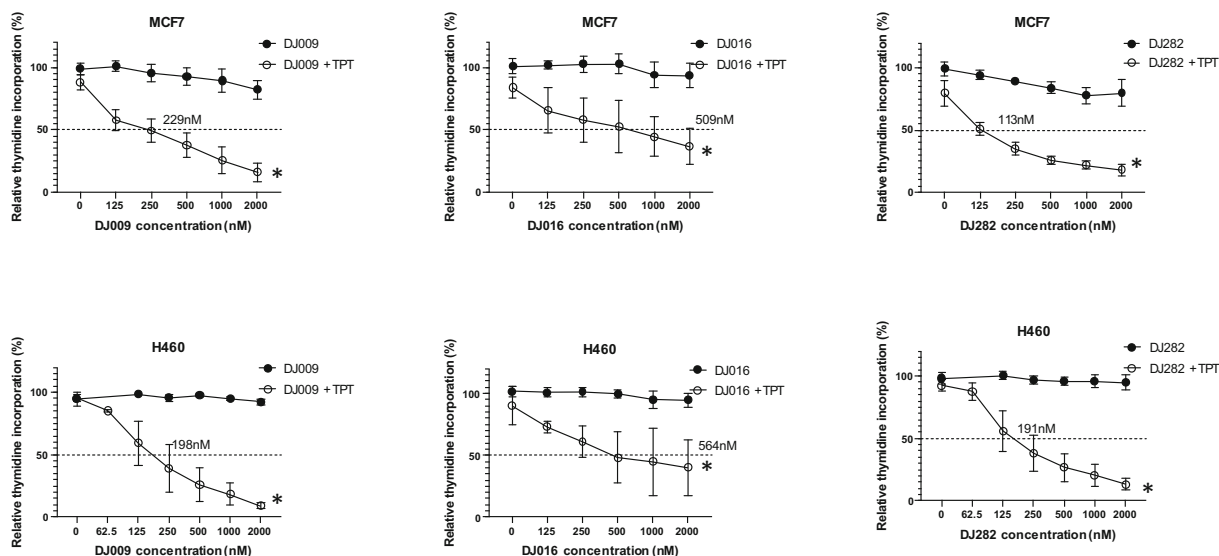


Fig. 2 The potentiating effect of DJ009, DJ016 and DJ282 with topotecan (abbreviated as TPT in the figure). The topotecan concentration is 12.5 nM for MCF7 and 25 nM for H460. IC₅₀ values for the combination treatments (by identification of the 50% value, dashed line) are shown on each graph. Data are mean ± SE of three independent experiments. DJ009 showed the strongest synergistic effect as calculated using the Bliss additivism model [48]. The average Bliss value (where Bliss > 1, indicates synergy) for the combination of the

thieno[2,3-*b*]pyridine compounds and topotecan were 44.2 ± 11.4, 31.8 ± 8.1 and 37.4 ± 8.0 for DJ009, DJ016, and DJ282, respectively in MCF7 cells. The combined treatment for H460 cells also showed a synergistic effect with Bliss values of 60.9 ± 17.8, 31.1 ± 8.9 and 58.6 ± 14.8 for DJ009, DJ016 and DJ282, respectively. These results clearly demonstrate a strong synergistic effect between the three thieno[2,3-*b*]pyridines and topotecan

by topotecan. The IC₅₀ (concentration that reduces cell proliferation by 50%) values of topotecan alone or in combination with DJ009 were determined using thymidine incorporation assay after 72 h of drug exposure. Interestingly, sensitivity to topotecan varied between the cell lines. At 500 nM, DJ009 significantly sensitized the inhibitory effect of topotecan in the MCF7 breast cancer cell line, as well as the lung cancer cell lines A549 and H460 (Fig. 2, and see Supporting Information, Figure S10). Similarly, DJ016 and DJ282 showed a synergistic effect to topotecan when tested against MCF7 and H460 cells (Fig. 2). As control, DJ009, DJ016 or DJ282 on their own at 1 μM concentration had no or little effect on the proliferation of the cancer cell lines (Fig. 2), indicating that the inhibition observed in the combination experiments was likely due to topotecan. To further verify that topotecan is the compound that reduces the observed growth, we tested for

DNA damage in H460 and MCF7 cells by measuring γ-phosphorylation of the histone H2AX, which is a marker for DNA damage (see Supporting Information, Figure S11) [79]. A comparison of flow cytometry profiles following treatment for 2 h showed an S-phase-specific DNA damage profile with a significant γ-H2AX increase in combination treatment with DJ009 and topotecan compared with topotecan alone (see Supporting Information, Figure S11). As control, DJ009 induced no DNA damage response (see Supporting Information, Figure S11). This result showed that the growth inhibitory effect and increased DNA damage are likely due to impaired DNA repair mechanisms in H460 and MCF7 cells, consistent with our hypothesis that the thieno[2,3-*b*]pyridines act as potentiators of topotecan.

To confirm the involvement of TDP1 blockade in potentiating topotecan, we used CRISPR/Cas9 (clustered regularly interspaced short

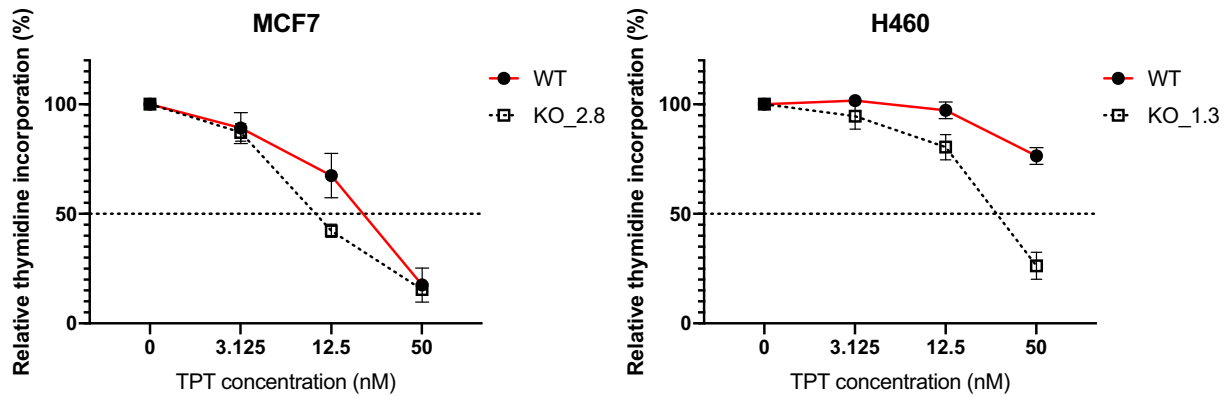


Fig. 3 The effect of TDP1 KO on the antiproliferative activity of topotecan (abbreviated as TPT in figure axis) as compared to WT cells. TDP1 KO increases the sensitivity

of cells to topotecan, with the greatest effect observed in H460 cells. Data are mean \pm SE of three independent experiments

palindromic repeats/CRISPR associated protein 9) to knock out TDP1. We introduced frameshift mutations in the TDP1 coding sequence using gRNAs against TDP1 to generate TDP1 knockout (KO) clones in MCF7 and H460 cancer cells. Frameshift mutations in knockout cell lines were validated by Sanger sequencing, and complete TDP1 ablation was verified by immunoblotting (see Supporting Information, Figure S12). We then tested the sensitivity of topotecan in the TDP1 KO clones (MCF7 KO_2.8 and H460 KO_1.3) and compared our results with the wild-type cells (MCF7 and H460). Our results showed that TDP1-deficient H460 and MCF7 cells proliferated less when exposed to topotecan (Fig. 3). The IC_{50} values for topotecan were 25.2 ± 1.6 nM in wild-type MCF7 cells and 10.5 ± 1.1 μ M in TDP1 KO_2.8 MCF7 cells. Similarly, the IC_{50} values for topotecan were 32.4 ± 1.8 nM in TDP1 KO_1.3 cells, and IC_{50} was not reached at 50 nM in wild-type H460 cells. Our results showing that cells lacking TDP1 have hypersensitivity to topotecan are indicative that TDP1 is critical for the repair of DNA damage (measured by increased H2AX), which is consistent with previous studies [18, 19].

One of the curious findings in our cell proliferation experiments was that different cell lines have different sensitivity towards topotecan on its own or in combination with DJ009. We reasoned that TDP1 abundance may

influence the sensitivity of the cell lines to topotecan tested in this study. To test this hypothesis, we investigated the expression level of TDP1 protein in the panel of cell lines. Immunoblotting showed that TDP1 is expressed in all the cell lines tested, although the level is relatively low in the pancreatic cancer cell lines MiaPaCa and PANC1, and lung cancer cell line A549 (see Supporting Information, Figure S12). We also computed Pearson correlation coefficients between the sensitivity of 358 diverse cancer cell lines to topoisomerase I inhibitors irinotecan/topotecan and their TDP1 mRNA expression using data downloaded from the Cancer Dependency Map portal (see Supporting Information, Figure S13). By applying network correlation analysis, we found that the correlation between TDP1 mRNA expression and the potency of topoisomerase I inhibitors is very weak (median $r = -0.3$). This analysis suggests that elevated TDP1 expression cannot predict the sensitivity of cancer cells towards topoisomerase I inhibitors, an observation that is in agreement with previous studies [80–82]. The finding from the network correlation analysis is also consistent with our experimental results showing that the sensitivity to topotecan or the combination effect of DJ009 and topotecan was not correlated with the protein expression. For example, SKBR3 showed similar TDP1 expression level to H460 cells (see Supporting Information, Figure S12), but no significant

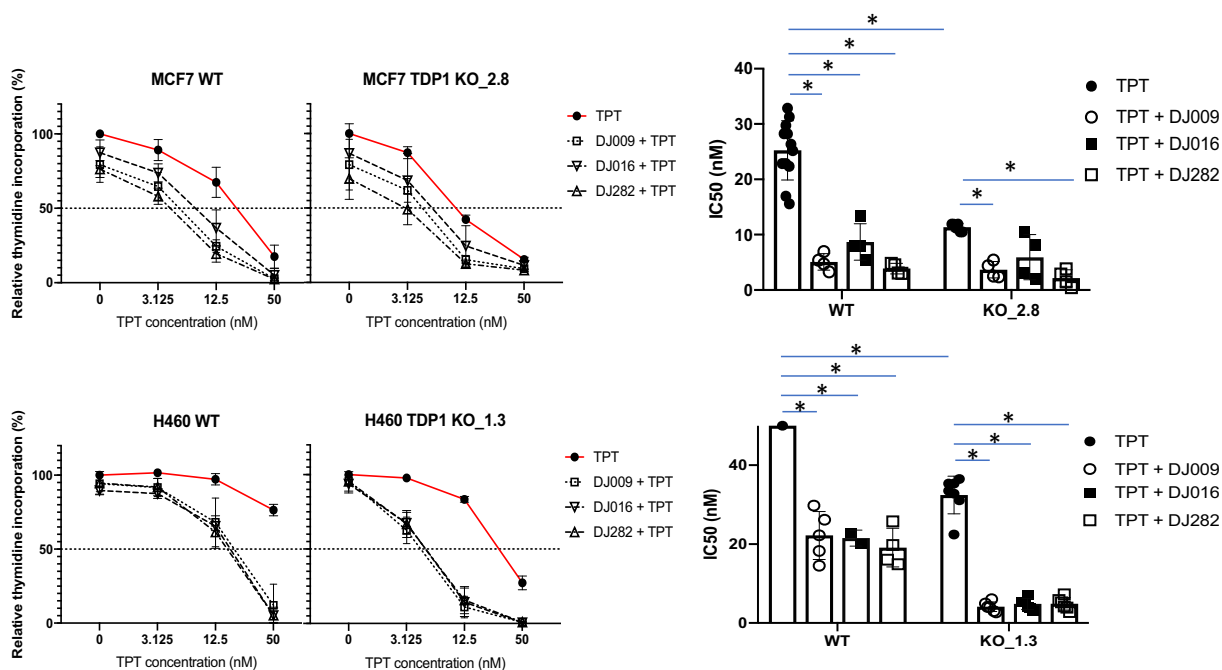


Fig. 4 The dose response plots and corresponding bar graphs with the IC₅₀ values of the different potency of each compound in combination with topotecan the WT and the corresponding TDP1 knockout (KO) in

(a) MCF7 breast cancer cell line and (b) H460 lung cancer cell line. The concentration of each TDP1 inhibitor was 430 nM for all the experiments. **p* < 0.05 and data are mean ± SE of three independent experiments

difference was observed in the treatment of topotecan alone or in combination with DJ009 in the SKBR3 cell line (as opposed to H460, which showed a significant potentiation effect) (see Supporting Information, Figure S10).

Thieno[2,3-*b*]pyridines have many different targets [35, 38, 69, 73, 76, 77], and off-target toxicity is also commonly reported in cancer drug candidates. We therefore exposed MCF7 and H460 KO clones to TDP1 inhibitors and topotecan concomitantly to examine potential off-target effects. Unexpectedly, all three inhibitors tested also sensitized the TDP1 KO cells to topotecan antiproliferative effects apart from DJ016 in the MCF7 knockout KO_2.8 cell line (Fig. 4), indicating that these compounds are modulating other pathways that may contribute to the observed potentiation effect of topotecan. Previous studies have proposed that TDP2 and poly(ADP-ribose)polymerase 1 (PARP1) may aid the repair of DNA strand breaks caused by topoisomerase I inhibitors [39, 40, 83]. To determine whether the

potentiation effects observed with the KO cell lines were due to thieno[2,3-*b*]pyridines targeting TDP2, we tested the potentiation effect of DJ009, DJ016 or DJ282 with topoisomerase II inhibitor (doxorubicin). This is because TDP2 has been reported to be involved in the repair of topoisomerase II inhibitor-induced damage [84, 85]. However, our results showed that there are no synergistic effects between DJ009/DJ016 and doxorubicin (see Supporting Information, Figure S14). The only exception is DJ282 in MCF7 cells. However, as DJ282 showed some inhibitory effect towards MCF7 cell growth in the absence of doxorubicin, the additional reduced proliferation by DJ282 is not likely due to enhancement of doxorubicin inhibition. Hence, our data indicate that DJ009, DJ016 and DJ282 can selectively enhance the efficacy of topoisomerase I inhibitor topotecan but not topoisomerase II inhibitor doxorubicin; i.e., thieno[2,3-*b*]pyridines are unlikely to target TDP2. In contrast, we found that PARP1 inhibitor olaparib can further sensitize the cancer

cells to topotecan (see Supporting Information, Figure S15). Proliferation assay showed a potentiating effect in MCF7 and H460 cells treated with olaparib and topotecan. The synergistic interaction between olaparib, DJ009 and topotecan was most notable in the H460 cell line (see Supporting Information, Figure S15). Although it is not clear whether our thieno[2,3-*b*]pyridine compounds are directly targeting PARP1 or its downstream/upstream pathway(s) (in addition to targeting TDP1), our results collectively showed that sensitivity to topoisomerase I inhibitors is likely to be modulated by multiple DNA repair enzymes, including TDP1 and PARP1.

DISCUSSION

Topoisomerase I inhibitors such as topotecan and irinotecan are clinically used chemotherapeutic agents that have shown to be highly effective in the treatments of a number of cancers including ovarian cancer, colorectal cancer, small cell lung cancer and cervical cancer. The development of resistance against topoisomerase I inhibitors is a major obstacle for the successful treatments of these cancers. Extensive work has been conducted in the last two decades to develop chemosensitizers for topoisomerase I poisons to help delay or reverse their chemoresistance. Several DNA repair pathways, including (but not limited to) those mediated by TDP1, TDP2 and PARP1, have been proposed to be involved in repairing topoisomerase I-mediated DNA damage, and hence they are potential inhibition targets to improve the efficiency of topoisomerase I poisons. In particular, TDP1 is a potentially important target as it is directly involved in the release of the stalled topoisomerase I-DNA cleavage complexes that is caused by topoisomerase I inhibitors. Hence, in this study, we set out to validate TDP1 as an inhibition target for the development of new chemosensitizers against topoisomerase I poisons.

By using thieno[2,3-*b*]pyridines, a class of compounds that we have shown to be highly potent against TDP1, as model compounds, we showed that inhibition of TDP1 can restore

sensitivity to topoisomerase I inhibitors such as topotecan in some cancer cell lines. This is supported by the CRISPR/Cas9 knockout cell experiments. Hence, our results validate TDP1 as a target for the development of new chemosensitizers against topoisomerase I poisons. However, our results also revealed a more complex picture. Firstly, we found that sensitivity towards topotecan and the combination of topotecan and thieno[2,3-*b*]pyridines differs between different cell lines, and is not necessarily linked to the expression of TDP1. Secondly, we found that thieno[2,3-*b*]pyridines also induced a potentiation effect of topotecan in TDP1 knockout cells, showing that our thieno[2,3-*b*]pyridines are not specific to TDP1. These implied that the sensitivity towards topoisomerase I inhibitors that we observed is likely being modulated by multiple pathways, of which TDP1 is only one. Our results using topoisomerase II poisons and PARP1 inhibitors showed that pathway(s) modulated by PARP1 are likely candidate(s), although it is also highly possible that other redundant and partially overlapping pathways may be involved in the DNA repair process.

Our work also exposes challenges and opportunities in the development of chemosensitizers for topoisomerase I inhibitors. Firstly, our results help establish TDP1 as a desirable inhibition target for the development of chemosensitizers against topoisomerase I poisons. It also opens the possibility of targeting multiple DNA repair pathways to further increase the sensitivity towards topoisomerase I inhibitors. This is an area that warrants further research, as it may significantly improve clinical outcomes and maximize the potential for topoisomerase I inhibitor-based treatments. It is important to note, however, that thieno[2,3-*b*]pyridines only showed synergistic effects with topotecan in some cell lines. Further work is therefore needed to fully understand the reasons behind this observation. For example, it is possible that the intrinsic responsiveness and/or activity of DNA repair pathways between cell lines are different. It is also possible that there may exist other DNA repair pathway(s) (that do not respond to thieno[2,3-*b*]pyridines) that are more dominant in some cancer cells. Secondly,

our results showed that a better biomarker (or set of biomarkers) than TDP1 is needed to aid the prediction of sensitivity towards topoisomerase I inhibitors. The elucidation of the complete network of pathways that is involved in the repair of DNA damage caused by topoisomerase I inhibitors would be very helpful for biomarker identification. A more complete understanding of the intricate human DNA damage response and repair network would also help in the identification of further inhibition targets for the development of new chemosensitizers. One possible strategy to map the possible DNA repair pathways that are involved in repairing damage caused by topoisomerase I inhibitors is to design photocrosslinking chemical probes based on our thieno[2,3-*b*]pyridines. This may enable the identification of proteins and enzymes (other than TDP1) that interact with our thieno[2,3-*b*]pyridine compounds.

CONCLUSIONS

Overall, by using thieno[2,3-*b*]pyridines, a class of TDP1 inhibitors that we had previously developed, we showed that the inhibition of TDP1 can restore sensitivity to topotecan in some cell lines. However, experiments with TDP1 knockout cells showed that thieno[2,3-*b*]pyridines are not specific to TDP1, as we found that thieno[2,3-*b*]pyridines could also potentiate the effect of topoisomerase I poisons in TDP1 knockout cells. To further investigate this redundancy, we probed whether TDP2 and PARP1 are involved in repairing topoisomerase I inhibitor-induced DNA damage. We found that PARP1 inhibitor olaparib can further sensitize the effect of topoisomerase I poisons in the presence of thieno[2,3-*b*]pyridines, indicating that the PARP1 pathway is complementary to the TDP1 pathway in repairing damage caused by topoisomerase I inhibitors. Our work therefore opens up the avenue of targeting and inhibiting enzymes from multiple DNA repair pathways simultaneously to increase the sensitivity to topoisomerase I inhibitors.

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Compliance with Ethics Guidelines. Ethical committee approval was not required as no human, animal or human embryonic stem cells were used in the study. Our gene editing experiments were operated under the New Zealand HSNO approval (GM005-UA0022). The use of genetically modified *E. coli* for recombinant protein production was operated under the University of Auckland-wide approval (APP202708) by the New Zealand Environmental Protection Authority.

Data Availability. All data generated or analyzed during this study are included in this published article/as supplementary information files.

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