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Identification of novel PARP inhibitors using a cell-based TDP1 inhibitory assay in a quantitative high-throughput screening platform

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Abstract

Anti-cancer topoisomerase I (Top1) inhibitors (camptothecin and its clinical derivatives irinotecan and topotecan, and the indenoisoquinolines) induce lethal DNA lesions by stabilizing Top1-DNA cleavage complex (Top1cc). These lesions are repaired by parallel repair pathways including the tyrosyl-DNA phosphodiesterase 1 (TDP1)-related pathway and homologous recombination. As TDP1-deficient cells in vertebrates are hypersensitive to Top1 inhibitors, small molecules inhibiting TDP1 should augment the cytotoxicity of Top1 inhibitors. We developed a cell-based high-throughput screening assay for the discovery of inhibitors for human TDP1 using a TDP1-deficient chicken DT40 cell line (*TDP1-/-*) complemented with human TDP1 (*hTDP1*). Any compounds showing a synergistic effect with the Top1 inhibitor camptothecin (CPT) in hTDP1 cells should either be a TDP1 inhibitor or an inhibitor of alternate repair pathways for Top1cc. We screened the 400,000-compound Small Molecule Library Repository (SMLR, NIH Molecular Libraries) against hTDP1 cells in the absence or presence of CPT. After confirmation in a secondary screen using both hTDP1 and TDP1-/- cells in the absence or presence of CPT, five compounds were confirmed as potential TDP1 pathway inhibitors. All five compounds showed

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synergistic effect with CPT in hTDP1 cells, but not in *TDP1-/-* cells, indicating that the compounds inhibited a TDP1-related repair pathway. Yet, *in vitro* gel-based assay revealed that the five compounds did not inhibit TDP1 catalytic activity directly. We tested the compounds for their ability to inhibit poly(ADP-ribose)polymerase (PARP) because PARP inhibitors are known to potentiate the cytotoxicity of CPT by inhibiting the recruitment of TDP1 to Top1cc. Accordingly, we found that the five compounds inhibit PARP activity by ELISA and Western blotting. We identified the most potent compound (Cpd1) that offers characteristic close to veliparib, a leading clinical PARP inhibitor. Cpd1 may represent a new scaffold for the development of PARP inhibitors.

Keywords

TDP1; PARP; topoisomerases; drug discovery; combination therapy

1. Introduction

Tyrosyl-DNA phosphodiesterase 1 (TDP1) is a DNA repair enzyme (for review see [1, 2]) that removes topoisomerase I (Top1) cleavage complexes (Top1 cc) resulting from the trapping of Top1 on DNA. Reversible Top1 cc are generated during replication and transcription to relax DNA supercoiling. However, they can also be trapped by DNA lesions including abasic sites, oxidized bases and carcinogenic adducts [3-5]. Anticancer Top1 inhibitors such as topotecan, irinotecan and non-camptothecin indenoisoquinolines stabilize Top1cc and cause lethal DNA double-strand ends when Top1cc are collided by replication forks [6-10] and transcription complexes [11-14]. TDP1 repairs Top1cc by excising the covalent bond between the catalytic tyrosine residue of Top1 linked and the DNA 3'-phosphate group [15-17]. TDP1 can also remove a wide variety of 3'-DNA blocking lesions including 3'-phosphoglycolates [18, 19], 3'-deoxyribose phosphate [20, 21] and chain-terminating nucleotides [22]. In addition, TDP1 plays a role in the backup of the topoisomerase II (Top2) cleavage complexes repair pathway [23, 24]. TDP1 is part of the XRCC1 complex that interacts with poly(ADP-ribose)polymerase 1 (PARP1) in the repair of Top1cc [25-27]. PARP1 poly-ADP-ribosylates TDP1, which enhances TDP1 stability and its recruitment to DNA damage sites [25]. Hence, PARP1 is emerging as a key component driving the repair of Top1cc by TDP1, which, at least in part [28, 29], accounts for the synergistic effect of PARP inhibitors with Top1 inhibitors.

The rationale for developing TDP1 inhibitors is rooted in the hypersensitivity of TDP1-deficient cells to Top1 inhibitors [12, 13, 30], to monofunctional alkylating agents including temozolomide [31, 32] and to chain terminating nucleosides [22]. Therefore, TDP1 inhibitors should exhibit synergistic activity when administered in combination with Top1 inhibitors, temozolomide, and chain terminators. Furthermore, cancer cells are very often deficient in alternative DNA repair and/or checkpoint pathways [33]. For example, deficiency in XPF-ERCC1, which belong to the exonuclease pathway for Top1cc repair, selectively sensitizes cancer cells to the combination of PARP and Top1 inhibitors [27]. It is also possible that deficiency in BRCA1, a key player of homologous recombination (HR) might sensitize familial breast and ovarian cancers to combination therapy with Top1

inhibitors and TDP1 inhibitors, in addition to the established activity of PARP inhibitors in BRCA-deficient tumors [34, 35]. This is because both the HR and TDP1 pathways act in parallel (synthetic lethality paradigm) for the repair of Top1-induced lesions. Still, there is currently no TDP1 inhibitor with desirable properties for drug development [for review see [36]]. In this study, we developed a cell-based high throughput-screening method for TDP1 pathway inhibitors, screened 400,000-compounds, and report small molecules that potentiate the cytotoxicity of CPT, and which all turn out to be PARP inhibitors.

2. Material and Methods

2.1. Cell lines and Drugs

DT40 cells were cultured with RPMI 1640 medium (GIBCO 11875-093) supplemented with 10% fetal calf serum (Gemini Bio-Products 100-106), 1% chicken serum (Invitrogen 16110082), and 50 μM 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 [23]. Wild-type, PARP1-deficient (*PARP1-/-*), and BRCA2-trancate mutant (*BRCA2tr/-*) DT40 cells were obtained from Dr. Takeda, Laboratory of Radiation Genetics, Graduate School of Medicine, Kyoto University (Kyoto, Japan), and described before [37, 38]. CPT and veliparib were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, DCTD, NCI. Tetra-n-octylammonium bromide was obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Primary high-throughput screening assay

Conditions and details for our cell-based high-throughput screening assay can be found on PubChem under the AID# 686978 and 686979 (https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=686981&loc=ea_ras). Briefly, DT40 hTDP1 cells cultured without or with 20 nM CPT were dispensed at 400 cells in 5 μl per well in 1536-well white wall/solid bottom assay plates (Greiner Bio-One North America, NC) using a Multidrop Combi 8 channel dispenser (Thermo Fisher, Waltham, MA). Compounds at various concentrations were transferred in a volume of 23 nl to the assay plates using a pintool station (Kalypsys, San Diego, CA). Assay plates were then incubated at 37°C for 48 h, followed by addition of 3 μl/well of CellTiter-Glo reagent (cell viability assay, Promega, Madison, WI) for measurement of intracellular ATP level. After 30 min incubation at room temperature in the dark, the luminescence intensity of the plates was measured using a ViewLux plate reader (PerkinElmer, Shelton, CT).

2.3. Secondary screening assay

Drug cellular sensitivity was measured as previously described [39]. Briefly, cells were continuously exposed to various drug concentrations for 72 h 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 h by adding 20 μl of ATPlite solution (ATPlite 1-step kit, PerkinElmer). After 5 min incubation, luminescence was measured on an EnVision Plate Reader (PerkinElmer). The ATP level in untreated cells was defined as 100% percent and viability of treated cells was defined as (ATP level of treated cell/ ATP level of untreated cells) ×100.

2.4. PAR ELISA assay

Poly-ADP-ribosylation was measured by the previously reported PAR enzyme-linked immunosorbent assay (ELISA) [40, 41]. Detailed procedure can be viewed at http://dctd.cancer.gov/ResearchResources/biomarkers/PolyAdenosylRibose.htm.

2.5. PAR Immunoblotting assay

Poly-ADP-ribosylation was also measured by immunoblotting. Briefly, five million DT40 cells in 10 ml medium were treated without or with drug for 30 min Cells were collected and lysed with CelLytic™M lysis reagent (C2978, Sigma-Aldrich, St Louis, MO). After thorough mixing and incubation at 4°C for 30 min, lysates were centrifuged at 20,000 g (~15,000 rpm) at 4°C for 10 min, and supernatants were collected. Immunoblotting was carried out using standard procedures. Rabbit polyclonal anti-PAR polymer antibody (#4336-BPC-100) was from Trevigen (Gaithersburg, MD). Secondary antibodies were horseradish peroxidase (HRP)-conjugated antibodies to rabbit IgG (GE Healthcare, Pittsburgh, PA).

2.6. Gel-based TDP1 inhibition assay

TDP1 gel-based assays were performed as described [31]. Briefly, a 5'-[^{32}P]-labeled single-stranded 14 nt DNA oligonucleotide containing a 3'-phosphotyrosine (N14Y) was incubated with 5 pM recombinant human TDP1 in the absence or presence of inhibitor for 15 min at room temperature in a buffer containing 50 mM Tris HCl, pH 7.5, 80 mM KCl, 2 mM EDTA, 1 mM 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 mM EDTA, 0.01% (w/v) xylene cyanol, and 0.01% (w/v) bromophenol blue]. Samples were subjected to a 16% denaturing PAGE and gels were exposed after drying to a PhosphorImager screen (GE Healthcare). Gel images were scanned using a Typhoon 8600 (GE Healthcare).

3. Results and discussion

3.1. Screening strategy and experimental design

A cell-based assay was developed in a quantitative high throughput-screening (qHTS) format to discover novel TDP1 pathway inhibitors capable of synergizing with CPT. We used chicken DT40 B lymphoma cells that are widely used for reverse genetic studies [42]. DT40 cells have several advantages for drug screening, including efficient gene targeting, a stable phenotype, rapid proliferation, and easy handling [23, 39]. These advantages are suitable for qHTS implementation, which requires fast inhibition of growth within 48 h. Our overall screening strategy is summarized in Figure 1. The first assay (primary qHTS screen, Fig. 1 upper panel) consisted in a 1536-well plate format robotic cell-based high throughput-screening (HTS) assay measuring viability of *hTDP1* cells (*TDP1-/-* cells complemented with human TDP1) exposed to a range of concentrations for each compound of the library in the absence or presence of CPT. Since *hTDP1* cells are much more tolerant to CPT compared to *TDP1-/-* cells [23], TDP1 inhibitors were therefore expected to show a synergistic effect in the presence of CPT and to reduce cell viability to levels similar to *TDP1-/-* cells (Fig. 1A). This hypersensitivity should not be observed in the absence of CPT. Compounds identified in the primary qHTS screen for their synergistic effect in the presence

of CPT were then characterized in a cell-based assay secondary screen (Fig. 1B). In this secondary cell viability assay, both *hTDP* and *TDP1-/-* cells were exposed to the compound of interest in the absence or presence of CPT. Inhibitors of the TDP1 pathway are supposed to maintain their synergistic effect with CPT in *hTDP1* cells but not in *TDP1-/-* cells (Fig. 1B).

Because we recently demonstrated that PARP1 appears to drive the TDP1-related repair pathway [25, 27], we used veliparib (ABT-888) as a positive control in the screening assay. Tetra-n-octylammonium bromide, a highly cytotoxic compound, was used as a non-specific control (Supplemental Figure S1). Veliparib showed average IC_{50} values (Inhibitory concentration 50%) of 20.4 μM for untreated cells (No CPT) and 0.064 μM for the cells treated with 20 nM CPT, resulting in a 438-fold increase in potency, which recapitulates our recent data [25]. On the other hand, tetra-n-octylammonium bromide as a non-specific control showed average IC_{50} values of 1.3 and 2.4 μM for untreated cells and cells treated with 20 nM CPT, respectively.

3.3. Primary Screen

The 400,000-compound Small Molecule Library Repository (NIH Molecular Libraries) was screened on the robotic platform of the NIH Chemical Genomics Center (NCGC, now is part of the National Center for Advancing Translational Sciences, NCATS). The entire results were deposited into PubChem (https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=686981&loc=ea_ras) under AID# 686978 and AID# 686979. Both Pubchem sites list the most cytotoxic compounds identified in the absence (AID# 686978) and in the presence (AID# 686979) of CPT and do not report the positive hits selected for confirmation and characterization. Positive hits were selected based on their IC_{50} value and inhibition curve quality (curve class) [43]. Compounds showing more than 2-fold decreased in IC_{50} value for the 20 nM CPT-treated cells (CPT20) compared to untreated cells were selected as positive hits. Compounds that exhibited a class 4 curve (non responsive class) in the absence of CPT and a curve in the presence of CPT categorized as class 1, 2 or 3 (responsive class with various degrees), were selected as primary hits because some compounds may only exhibit their cytotoxicity when combined with CPT. Compounds meeting the above criterions but showing an IC_{50} value greater than 20 μM in the presence of CPT were not retained based on their lack of potency. Based on these criterions, 500 best compounds were selected and retested in quadruplicate using the primary qHTS assay in the absence and the presence of CPT using *hTDP1* cells (See Fig. 1B). Five positive hits were selected for further characterization and the mean of their IC_{50} values in the absence or presence of CPT are reported in Table 1.

3.3. Secondary Screen

The five selected compounds from the primary screen were tested in the secondary screen (Fig. 1B) in the absence and the presence of CPT in *hTDP1* and *TDP1-/-* cells (15 and 20 nM CPT for *hTDP1* cells and 5 and 10 nM CPT for *TDP1-/-* cells). The cell viability curves for the five compounds are presented in Table 1, Figure 2A and Supplemental Figure S2. Cpd1 showed the greatest potentiating effect in the presence of CPT in *hTDP* cells (Fig. 2A) without showing any apparent potentiating effect with CPT in *TDP1-/-* cells, which, as

expected were exquisitely hypersensitive to CPT compared to the *hTDP1* cells [23, 39] (Fig. 2B). The synergistic effect observed in *hTDP1* cells for MLS002706582 (Cpd1) is very similar that for veliparib (Fig. 2C) with IC_{50} values of 150 nM and 6 nM for Cpd1 and veliparib, respectively (Table 1). Cpd1 also behaves similarly to veliparib regarding its lack of synergistic effect with CPT in *TDP1-/-* cells (Fig. 2D). All five compounds were not cytotoxic as single agents at concentrations below 25 μM (Table 1) but in the presence of CPT, Cpd1 exhibited a 166-fold potentiation of cytotoxicity (466-fold for veliparib). These results suggested that Cpd1 was either a direct inhibitor of TDP1 or a TDP1 repair pathway inhibitor (acting indirectly on TDP1 in cells).

3.4. TDP1 catalytic inhibition

To determine whether the five selected compounds from both the primary and secondary screens were direct TDP1 inhibitors, we ran them against recombinant TDP1 in our gel-based biochemical assays [23, 26]. When tested in comparison with a published TDP1 inhibitor [44, 45], none of the five compounds could inhibit efficiently recombinant TDP1 (Supplemental Figure S3). Although, we found a weak TDP1 inhibition for Cpd5 at a dose above 300 μM, these results suggested that the five compounds did not directly target TDP1.

3.5. PARP catalytic inhibition

Because these five compounds contained nicotinamide mimicking moieties (see structures in Table 1) and behave like veliparib (see Fig. 2), we next evaluated their potency as PARP inhibitors. All five compounds inhibited PARP. Moreover, Cpd1 showed PARP catalytic inhibition comparable to veliparib in ELISA assay [40, 41] (Fig. 2E) with sub-nanomolar IC_{50} values for both compounds (Table 1). These results were confirmed by immunoblotting assay [37]. Thus, all five compounds derived from the cell-based TDP1 screen turned out to be PARP inhibitors (Fig. 2F).

3.6. Hypersensitivity of BRCA2-deficient cells

Because PARP inhibitors are known to be selectively cytotoxic to homologous recombination-deficient cells [34, 35], MLS002706582 (Cpd1) was tested for its cytotoxic effect in *BRCA2tr/-* cells (homologous recombination-deficient cells) in comparison to *PARP1-/-* and *hTDP1* cells. Cpd1, similar to veliparib [37], was selectively cytotoxic to *BRCA2tr/-* cells but not to *PARP1-/-* and *hTDP1* cells (Fig. 2G).

4. Conclusions

Altogether, our results demonstrate that screening of the 400,000-compound Small Molecule Library Repository (NIH Molecular Libraries) allowed the identification of five compounds as inhibitors of the TDP1-related DNA repair pathway. These compounds do not inhibit TDP1 directly but act indirectly on the TDP1 pathway by inhibiting PARP1. These finding are in agreement with recent mechanism-based molecular studies showing that PARP is critical to stabilize and recruit TDP1 to Top1-induced DNA lesions [25]. Among the five compounds identified, one of them, Cpd1 offers characteristic close to veliparib and may represent a new scaffold for the development of PARP inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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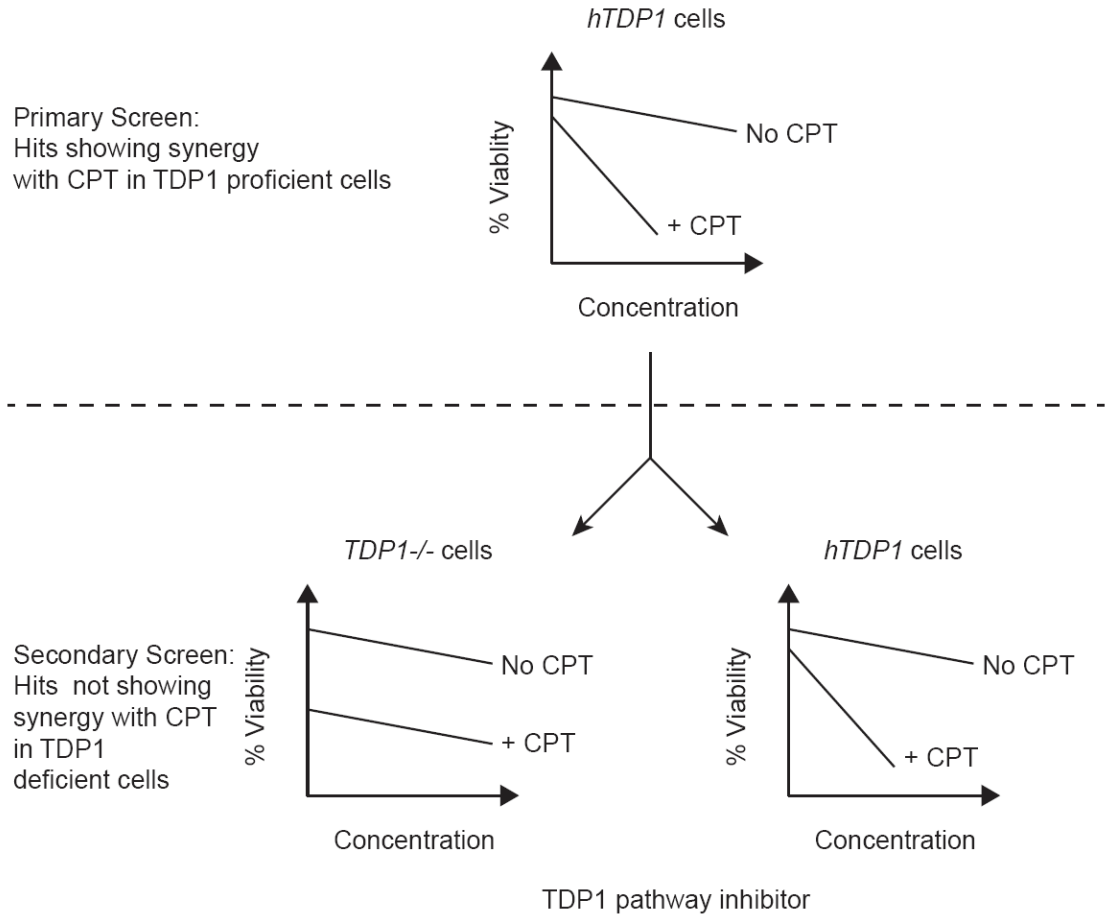


Figure 1.

Screening strategy. **A**: A quantitative robotic high throughput screening (qHTS) assay was run as a primary screen using DT40 chicken B lymphoma cells genetically modified to express human TDP1 (*hTDP1*) in a knockout background for the chicken TDP1 gene (*TDP1-/-*) [23]. The compound library was the NIH Roadmap Molecular Libraries 400,000 compound repository. Positive hits were selected based on cellular hypersensitivity in the presence of the Top1 inhibitor camptothecin (CPT). **B**: Positive hits identified during the HTS assay were confirmed in a secondary screen against both *hTDP1* and *TDP1-/-* cells. Inhibitors of the TDP1 pathway were selected for further characterization based on supra-additive cytotoxicity in the presence of CPT in *hTDP1* cells but not in *TDP1-/-* cells.

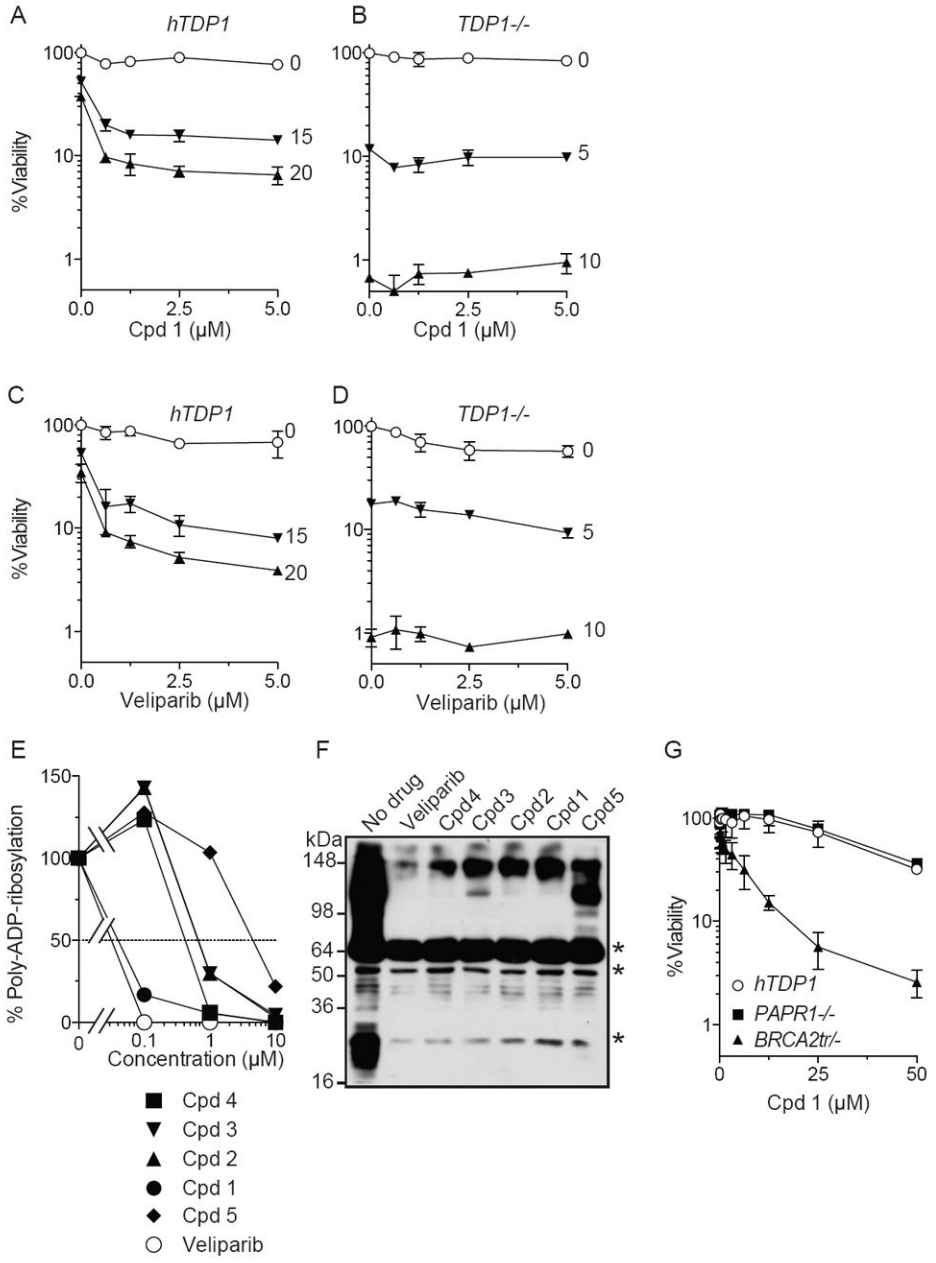
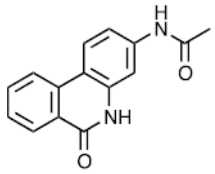
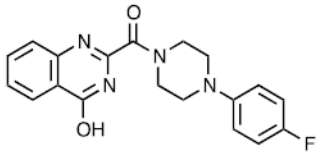
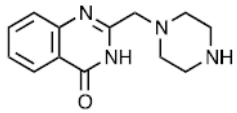
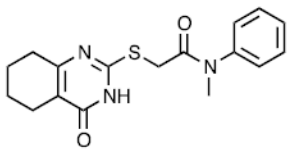
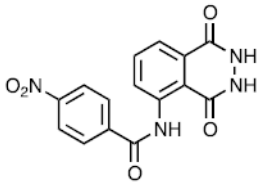


Figure 2.

Inhibition of PARP1 by compound **1 (Cpd1). A & B**: Cellular viability in the presence of **Cpd1** and various concentrations of CPT (indicated beside each curve in nanomolar unit) in *hTDP1* (**A**) and *TDP1-/-* cells (**B**). **C & D**: Cellular viability curves in the presence of veliparib and various concentrations of CPT (indicated beside each curve in nanomolar unit) in *hTDP1* (**C**) and *TDP1-/-* cells (**D**). **E & F**: Inhibition of PARP1 by compounds **1-5** in comparison to the PARP inhibitor veliparib measured by ELISA (**E**) and by Western blotting (**F**). Asterisks in (F) indicate non-specific bands. **G: Cpd1**-dependent viability curves in *PARP1-/-*, *BRCA2tr/-* and *hTDP1* cells.

Table 1

Cpd	Name	Structure	IC_{50} (μM) HTS NO CPT	IC_{50} (μM) HTS CPT	IC_{50} (μM) Poly (ADP-ribose) lation
1	MLS002706582		25.42 ± 6.07	0.15 ± 0.18	<0.02
2	NCGC00345095		25.72 ± 2.90	1.45 ± 0.62	0.68
3	MLS000552049		>42	3.96 ± 0.48	0.68
4	NCGC00119675		>42	5.19 ± 1.03	0.42
5	MLS003171605		>42	11.67 ± 2.65	4.6

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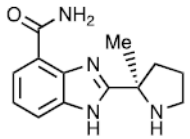
Cpd	Name	Structure	IC50 (μM) HTS NO CPT	IC50 (μM) HTS CPT	IC50 (μM) Poly (ADP-ribose) lation
	Veliparib		28.42 ± 8.58	0.06 ± 0.06	<0.02