Isoeugenol is a selective potentiator of camptothecin cytotoxicity in vertebrate cells lacking TDP1

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Camptothecin (CPT), a topoisomerase I (TOP1) inhibitor, exhibits anti-tumor activity against a wide range of tumors. Redundancy of TOP1-mediated repair mechanisms is a major challenge facing the efficiency of TOP1-targetting therapies. This study aims to uncover new TOP1 targeting approaches utilising a selection of natural compounds in the presence or absence of tyrosyl DNA phosphodiesterase I (TDP1); a key TOP1-mediated protein-linked DNA break (PDB) repair enzyme. We identify, isoeugenol, a phenolic ether found in plant essential oils, as a potentiator of CPT cytotoxicity in Tdp1 deficient but not proficient cells. Consistent with our cellular data, isoeugenol did not inhibit Tdp1 enzymatic activity in vitro nor it sensitized cells to the PARP1 inhibitor olaparib. However, biochemical analyses suggest that isoeugenol inhibits TDP2 catalytic activity; a pathway that can compensate for the absence of TDP1. Consistent with this, isoeugenol exacerbated etoposide-induced cytotoxicity, which generates TOP2-mediated PDBs for which TDP2 is required for processing. Together, these findings identify isoeugenol as a potential lead compound for developing TDP2 inhibitors and encourage structure-activity relationship studies to shed more light on its utility in drug discovery programs.

Camptothecin (CPT), a quinoline alkaloid isolated from the wood and bark of the Chinese tree Camptotheca acuminata, is a well-known topoisomerase I (TOP1) inhibitor that was first isolated and identified as an anti-cancer agent in 1966. TOP1 functions to relax supercoils by creating a nick in the DNA strand and forming a covalent bond between its tyrosyl residue and a 3’ DNA phosphate. An intermediate structure known as TOP1 cleavage complex (TOP1cc) is formed through cleavage, followed by religation of the DNA strand. CPT acts as a cellular poison by reversibly trapping TOP1ccs onto the DNA. TOP1ccs are normally transient; however collision of replication or transcription machineries with TOP1cc converts them to irreversible protein-linked DNA single-strand breaks (SSBs) or double-strand breaks (DSBs). Tyrosyl DNA phosphodiesterase I (TDP1) catalyzes the hydrolysis of the tyrosyl-3’ phosphate linkage formed between TOP1 and DNA, allowing the rejoining of the DNA strand break and hence help cells to overcome CPT-induced cytotoxicity. Mutations of TDP1 lead to the autosomal recessive disease SCAN-1. Cell lines derived from patients with SCAN-1 accumulate TOP1-mediated protein-linked DNA breaks (PDBs) and were found to be hypersensitive to CPT.

Owing to CPT’s severe toxicity, other semisynthetic analogues have been developed, namely irinotecan and topotecan, which are currently used alone or in combination with other drugs as chemotherapeutic agents. Irinotecan, also known as CPT-11, is approved for colon cancer treatment, whereas topotecan is used for the treatment of ovarian cancer. CPT-11 is mainly used for the treatment of metastatic colon cancer as part of a combination regimen alongside 5-fluorouracil. Several studies have demonstrated that combined therapy of two or more drugs against cancer cells promotes synergism, enhances therapeutic effectiveness, suppresses drug resistance, and reduces the drug side effects. Functional redundancy in repairing TOP1-mediated PDBs is a potential cause for resistance to TOP1-based chemotherapy strategies. Herein, we investigated the cytotoxic effects of 41

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natural compounds in combination with CPT in the presence or absence of TDP1, with the goal of unraveling novel approaches to target TOP1.

We assembled a library of natural derived chemicals that are isolated from either plant or animal origin. Natural products provide a vast resource for drug discovery research. For example, in 1971 and as a part of a National Cancer Institute program, paclitaxel was identified as the active constituent from a crude extract of the bark of *Taxus brevifolia*. Paclitaxel is the first taxane to enter clinical trials as a chemotherapeutic agent and is used against ovarian, breast, and lung cancers. "Ginkgo biloba extract 761 (EGb761) is another natural compound extracted from *Ginkgo biloba* leaves that was proposed to have anti-angiogenic and antioxidant activities."

Our screen yielded a hit compound that appears to be a promising lead as a tyrosyl DNA phosphodiesterase II (TDP2) inhibitor. Like TDP1, TDP2 is a tyrosyl DNA phosphodiesterase but that catalyzes the cleavage of 5′-tyrosyl bonds that are present between topoisomerase II (TOP2) and the DNA. It is known that both enzymes while structurally different, can serve as back up for each other in circumstances where one is lacking, but with much less efficiency than with their preferred substrate.

**Results**

Establishment of sub-lethal concentrations of the library compounds. A selection of 41 diverse natural products most of which occur in foods or dietary supplements were subjected to a series of cellular and biochemical experiments to identify potential hits to improve TOP1 targeting therapy (Fig. 1A). The large therapeutic index reported for these chemicals is encouraging in terms of developing future and safe drugs for treatment of cancer. The library is comprised of molecules belonging to different natural products classes viz. alkaloids, flavonoids, saponins, terpenes, and phenylpropanoids. To determine sub-lethal doses, the cytotoxic effect of each compound was examined at six different concentrations (0.01, 0.1, 1, 5, 10, and 50 μM) in Tdp1−/− DT40 cells (Supplementary Fig. 1). The viability assay used in this study utilizes a blue dye, resazurin, that when encountered with viable cells, is reduced to the highly fluorescent red dye resorufin. The cytotoxicity, therefore, is measured as fluorescence intensity quantified by a plate reader. The selected sub-lethal doses (Table 1) were then used for the primary screen in combination with a single lethal and three sub-lethal doses of CPT (4, 1, 0.5, 0.25 nM) in the same cellular model (Supplementary Figs 2 and 3). The combination of any of the compounds 4,
Examination of the TOP1-mediated cytotoxic effect in the presence or absence of human TDP1. Seven compounds including prunin, isoquercetin, 2,4,4′-trihydroxy chalcone, isoeugenol, xanthohumol, harmine, and thymol (4, 8, 10, 14, 21, 24, 33, resp.) that showed the highest synergistically lethal effect with CPT in Tdp1−/− cells were selected for further screening by comparing their effect on chicken DT40 Tdp1−/− and an isogeneic control stably expressing human TDP1 (hTDP1) (Fig. 1 and Supplementary Fig. 4). The intensity of fluorescence was normalized to a DMSO control (same volume as that of the highest concentration of combined drugs) and the results were obtained from three biological replicates. All tested compounds, except compound 14, gave the same effect on both cell lines (Tdp1−/− and Tdp1−/− complemented with hTDP1).

Table 1. A list of the compounds employed in the screen.

| Compound | Name | Class | CAS number | Selected sublethal dose [μM] |
|----------|------|-------|------------|-----------------------------|
| 1        | Caffeic acid | Phenolic acid | 331–39–5 | 10 |
| 2        | Khellin | Chromone | 82–02–0 | 10 |
| 3        | p-Coumaric acid | Phenolic acid | 7400–08–0 | 10 |
| 4        | Prunetin | Flavonoid | 552–59–0 | 10 |
| 5        | Allyl anthranilate | Terpenoid | 7493–63–2 | 50 |
| 6        | Cyanin | Phenolic acid | 1182–34–9 | 1 |
| 7        | Caffeine | Alkaloid | 58–08–2 | 10 |
| 8        | Isoquercitrin | Flavonoid | 482–35–9 | 10 |
| 9        | Umbelliferone | Coumarin | 93–35–6 | 10 |
| 10       | 2,4,4′-trihydroxy chalcone | Flavonoid | 961–29–5 | 5 |
| 11       | Emodin | Anthraquinone | 518–82–1 | 10 |
| 12       | Chrysin | Flavonoid | 480–40–0 | 10 |
| 13       | Sennoside A | Anthraquinone | 81–27–6 | 10 |
| 14       | Isoeugenol | Phenylpropanoid | 97–54–1 | 50 |
| 15       | Pseudoephedrine | Alkaloid | 90–82–4 | 10 |
| 16       | Glycyrrhizin | Saponin | 1405–86–3 | 1 |
| 17       | Epicatechin | Flavonoid | 490–46–0 | 10 |
| 18       | Ratin | Flavonoid | 153–18–4 | 10 |
| 19       | Vanillin | Phenylpropanoid | 121–33–5 | 10 |
| 20       | Gallic acid | Phenolic acid | 149–91–7 | 1 |
| 21       | Xanthohumol | Flavonoid | 6754–58–1 | 0.1 |
| 22       | Glycyrrhetinic acid | Terpenoid | 471–53–4 | 10 |
| 23       | Naringenin | Flavonoid | 67604–48–2 | 10 |
| 24       | Harmin | Alkaloid | 442–51–3 | 0.1 |
| 25       | Luteolin | Flavonoid | 491–70–3 | 10 |
| 26       | Kaempferol | Flavonoid | 520–18–3 | 10 |
| 27       | Hesperidin | Flavonoid | 520–26–3 | 10 |
| 28       | Bisabolene | Terpenoid | 17627–44–0 | 0.01 |
| 29       | α-ionone | Terpenoid | 127–41–3 | 0.01 |
| 30       | cis-3-Hexenyl acetate | Terpenoid | 3681–71–8 | 0.01 |
| 31       | α-Pinene | Terpenoid | 80–56–8 | 50 |
| 32       | Commaldehyde | Phenylpropanoid | 122–03–2 | 50 |
| 33       | Thymol | Phenylpropanoid | 89–83–8 | 50 |
| 34       | Caryophyllene oxide | Terpenoid | 17627–43–9 | 50 |
| 35       | Carvone | Terpenoid | 99–49–0 | 50 |
| 36       | Dimethyl Disulphide | Sulphur compound | 624–92–0 | 0.01 |
| 37       | Amentoflavone | Flavonoid | 1617–53–4 | 1 |
| 38       | Sarcophane | Terpenoid | 55038–27–2 | 0.01 |
| 39       | Chlorogenic acid | Phenolic acid | 327–97–9 | 10 |
| 40       | Vitexin | Flavonoid | 3681–93–4 | 10 |
| 41       | Quercetin | Flavonoid | 117–39–5 | 1 |

8, 10, 14, 21, 24, or 33 with CPT gave the highest levels of cytotoxicity (viability inhibition) ranged from 81% for compound 8 to 47% for compound 24, at the highest concentration of CPT.

Examination of the TOP1-mediated cytotoxic effect in the presence or absence of human TDP1.

Seven compounds including prunin, isoquercetin, 2,4,4′-trihydroxy chalcone, isoeugenol, xanthohumol, harmine, and thymol (4, 8, 10, 14, 21, 24, 33, resp.) that showed the highest synergistically lethal effect with CPT in Tdp1−/− cells were selected for further screening by comparing their effect on chicken DT40 Tdp1−/− and an isogenic control stably expressing human TDP1 (hTDP1) (Fig. 1 and Supplementary Fig. 4). The intensity of fluorescence was normalized to a DMSO control (same volume as that of the highest concentration of combined drugs) and the results were obtained from three biological replicates. All tested compounds, except compound 14, gave the same effect on both cell lines (Tdp1−/− and Tdp1−/− complemented with hTDP1). Isoeugenol annotated as compound 14 revealed the highest, reproducible cytotoxic effect (p < 0.001, 4 nM; p < 0.05, 1 nM) when combined with CPT on Tdp1−/− cells but not on cells complemented with human TDP1, wherein the viability dropped from 65.5% (CPT alone) to 34.6% (CPT plus isoeugenol) at 4 nM CPT, and a reduction of ~18.2% at 1 nM CPT (Fig. 1G and Supplementary Fig. 4F). These results indicate that isoeugenol
(4-propenyl-2-methoxyphenol), at 1 nM, does not exhibit a cytotoxic effect as a single agent, but rather holds a synergistic effect when combined with CPT in a TDP1-dependent manner. This was not an epiphenomenon of DT40 cells since isoeugenol also sensitized TDP1 depleted human MCF-7 cells to CPT, although the effect was much less apparent than DT40 cells (Supplementary Fig. 5).

Isoeugenol does not act through the PARP1/TDP1 axis. This data suggest that isoeugenol sensitizes cells to CPT only if TDP1 is absent, and that it may suppress TDP1 parallel pathways for repairing TOP1-induced PDBs. To further ascertain that the observed effects are independent of TDP1, we next examined the effect of isoeugenol on TDP1 catalytic activity using an in vitro biochemical assay employing a 13-mer oligonucleotide Cy5.5-labelled substrate containing a 3′-phosphotyrosine modification. TDP1, in the reaction mixture, cleaves tyrosine from the substrate allowing it to move faster and further through the gel, giving a slightly shifted band. As a positive control, total cell lysate from wild-type DT40 cells was used as a source of TDP1 while Tdp1−/− cell lysate was used as a negative control. Different concentrations of isoeugenol (200 μM, 500 μM, and 1000 μM) were incubated with the 3′-phosphotyrosine substrate along with the cell lysates and reaction products were fractionated by denaturing PAGE and analysed by fluorescence imaging. The addition of isoeugenol to the reaction did not inhibit the conversion of 3′-PY substrate to 3′-P (indicated by arrows) even at the highest concentration (1000 μM), confirming that isoeugenol does not inhibit TDP1 activity (Fig. 2A, B). We reasoned that if isoeugenol potentiated CPT cytotoxicity independently of TDP1 one would predict that co-addition of PARP1 inhibitors would be epistatic to isoeugenol, since PARP1 and TDP1 are working together in the same pathway for TOP-PDB repair. To test this hypothesis, we compared the viability of Tdp1−/− cells and controls following CPT treatment in presence and absence of isoeugenol and the FDA approved PARP inhibitor olaparib, either separately or in combination. Addition of olaparib to TDP1 proficient cells led to a marked increase in CPT cytotoxicity (Fig. 2C, p < 0.01). Importantly, co-addition of isoeugenol and olaparib did not further sensitize cells to CPT than addition of olaparib alone (Fig. 2C). In contrast to Tdp1 proficient cells, addition of olaparib to Tdp1−/− cells did not result in additional sensitisation, confirming that TDP1 and PARP1 operate in the same pathway (Fig. 2D). Notably, whilst addition of isoeugenol alone sensitized Tdp1−/− cells to CPT (p < 0.05), co-addition of isoeugenol did not. Together, we conclude from these findings that isoeugenol potentiates CPT cytotoxicity independently of the TDP1/PARP1 axis.
Isoeugenol does not increase single-strand break (SSB) accumulation in CPT-treated cells. It is known that TDP1 deficient cells accumulate higher levels of DNA single-strand breaks (SSBs) in the presence of TOP1 inhibitors than controls\(^1\)\(^-\)\(^^4\)\(^-\)\(^^7\). We therefore examined if isoeugenol would increase the level of DNA strand breaks observed in Tdp1\(^{-/-}\) DT40 cells, using alkaline single-cell gel electrophoresis (comet assays). As expected, the lack of TDP1 caused a CPT-dependent increase in SSB accumulation (P < 0.05); yet, isoeugenol did not potentiate such effect neither alone nor in combination with CPT (Fig. 3).

Isoeugenol inhibits TDP2 catalytic activity in vitro and potentiates the cytotoxic effect of etoposide in vivo. We previously reported that TDP2 could protect from TOP1-induced damage in the absence of TDP1\(^8\). Consequently, we examined whether or not isoeugenol would inhibit TDP2 activity, potentially providing a mechanistic insight explaining our data. Whole cell lysate from wild-type DT40 was employed.
as the source for TDP2. Incubation of 5′-phosphotyrosine substrate with increasing concentrations of isoeugenol revealed a dose-dependent reduction in the conversion of 5′-PY to 5′P, which is specific for TDP2 activity (Fig. 4A). Whilst incubation with extract alone led to ~50% processing, co-addition of 200 μM isoeugenol suppressed TDP2 activity resulting in ~34% processing (Fig. 4B). Increasing the concentration of isoeugenol to 500 μM and 1000 μM showed a corresponding increase in the inhibition of TDP2 activity reaching as little as 6% or no conversion, respectively. The putative TDP2 inhibitory effect of isoeugenol was not due to the vehicle in which it was dissolved (DMSO) since we kept the concentration of DMSO constant in all control and test conditions. These observations suggest that isoeugenol inhibits chicken TDP2 catalytic activity at high doses. To test if this is an epiphenomenon for chicken DT40 or is also true in human cells, we repeated the experiments using HeLa cell lysates (Fig. 4C). Consistent with its TDP2 inhibitory activity, isoeugenol also inhibited human TDP2 but less efficiently than chicken TDP2 with only 20% inhibition at the highest isoeugenol dose examined (1000 μM). If isoeugenol inhibits TDP2 activity we reasoned that it would sensitize human cells to TOP2 poisons, which specifically require TDP2 to liberate stalled TOP2 from DNA termini. Incubation with etoposide led to a dose dependent decline in viability of both HeLa and MCF7 cells and the co-addition of isoeugenol resulted in further sensitisation (Fig. 4E,F). At 50 μM etoposide, the viability of HeLa and MCF-7 cells dropped, upon the addition of isoeugenol, from 94.8% to 64.1% and from 91.1% to 72.1%, (P < 0.001, P < 0.05; resp.). At a higher dose of 100 μM etoposide, viability of HeLa cells decreased from 75.5% to 49.6%, and from 91.2% to 56.9% for MCF-7 cells (P < 0.01). Importantly, addition of low micromolar doses of isoeugenol (1 μM) sensitized HeLa cells to all concentrations of etoposide tested, as measured by clonogenic survival assays (Fig. 4G). Together, these findings identify isoeugenol as a promising lead compound with potential TDP2 inhibitory activity.

Discussion

Drug combination therapy is a promising strategy used in treating complex diseases such as cancer, cardiovascular diseases, and infectious diseases19,20. Synergistic drug combinations are recognized as effective and therapeutically specific20. Overcoming toxicity, side effects linked to high doses of single drugs, and drug resistance can be achieved by synergistic combinations of two or more agents. Consequently, this study aimed to achieve a synergistic therapeutic effect and minimize dose through combination between the TOP1 inhibitor CPT and natural compounds. Screening of 41 compounds from natural origin belonging to diverse classes of natural products viz. alkaloids, phenylpropanoids, saponins and terpenes in combination with CPT revealed a synergistic effect between the phenolic ether isoeugenol and CPT against Tdp1−/− DT40 cells. Such a synergistic effect encourages further research and may lead to a new anti-cancer strategy that target a specific class of tumors, such as those that develop resistance to camptothecins, for example colorectal cancers (CRCs). Our screen identified isoeugenol, a phenylpropanoid, as a compound that potentiates CPT cytotoxic effect on cells that lack TDP1. Several studies have shown that phenylpropanoids exhibit different biological activities that include analgesic, anti-inflammatory, and anti-tumor activities21–23. Isoeugenol (4-propenyl-2-methoxyphenol) is abundantly present in several plant essential oils and is regularly used in spices, perfumes, and detergents, thus is unlikely to be toxic to humans24. It is a structural isomer of eugenol found in clove and cinnamon oil and is mostly used in toothpastes and mouthwashes. Because of its presence in various foods, it is likely to be ingested by the general population. Isoeugenol has been found to inhibit TDP2 catalytic activity in vitro25,26. Isoeugenol was found to display genotoxic activity via TOP2 inhibition, halting cells in the S-phase of the cell cycle27. However, the mechanism of isoeugenol-mediated TOP2 inhibition is not well understood. In planta studies with tumorigenic mouse mammary gland tumor cells have shown that isoeugenol inhibits TOP2 when incubated with cell lysates from Chicken DT40 or HeLa cells. These observations were further supported by a significant potentiation of cytotoxicity inflicted by the TOP2 poison etoposide, which specifically generates TOP2-mediated PDBs for which TDP2 is required for processing. TOP2’s discovery as a 5′-tyrosyl DNA phosphodiesterase in 200928 has since impelled scientists to search for an inhibitor. It has been proposed that drug resistance to TOP2 inhibitors, such as etoposide, may stem from an acquired or intrinsic overexpression in TOP2, which as discussed earlier guards against the abortive activity of TOP2 during replication and transcription. In 2013, Oglivie et al. have managed to uncover two classes of compounds, toxoflavins and deazaflavins, as selective TDP2 inhibitors following a high-throughput screening29. More recently, isoquinoline-1,3-diones was also found to selectively inhibit TDP2 at a low micromolar concentration30. Isoeugenol exhibits a dose dependent inhibitory activity on TOP2, but not TOP1, albeit at higher concentrations. Notably, vanillin (4-hydroxy-3-methoxybenzaldehyde) a structural analogue for isoeugenol (Supplementary Fig. 5) that only differs in an aldehyde group instead of a prop-1-ene moiety displayed no activity in our assays, suggesting that the presence of an alkenyl group is crucial for biological activity. In planta, there exists several other isoeugenol analogues particularly found in spices and medicinal herbs such as eugenol anethole, estragole, safrole, myristicin, and methyl isoeugenol, all of which have yet to be assessed and might provide more potent

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Figure 4. Isoeugenol inhibits TDP2 catalytic activity in vitro and potentiates etoposide cytotoxic effect in vivo. (A) Wild-type DT40 cell lysate was incubated with DMSO or with the indicated concentrations of isoeugenol. Reaction products were resolved by denaturing PAGE and analysed by fluorescence imaging. Arrows point at the position of substrate (PY) and cleaved product (P). (B) The bands from A were quantified using ChemiDoc™ MP Imaging System software and the average % substrate remaining of three independent experiments ± s.e.m is shown. Asterisks denote statistical difference (p < 0.01, 1000 μM and 500 μM; p < 0.05, 200 μM) using t-test between DMSO and each concentration. (C) HeLa cell lysate was incubated with DMSO or with the indicated concentrations of isoeugenol and reaction products analysed as described in A,B. (D) The bands were quantified using ChemiDoc™ MP Imaging System software and the average of % substrate remaining for three independent experiments ± s.e.m is shown. Asterisks denote statistical difference (p < 0.01, 1000 μM; p < 0.05, 500 μM and 200 μM) using t-test between DMSO and each concentration. Viability of HeLa (E) and MCF-7 (F) cells was compared using the indicated doses of etoposide (sub-lethal: 50, 25, 10, and 5 μM; lethal: 100 μM) in the presence and absence of sub-lethal doses of isoeugenol (200 μM and 400 μM for Hela and MCF-7, resp.). Results are represented on a semi-log graph and present the average of 3 biological replicates ± s.e.m. Asterisks denote statistically significant difference between etoposide alone and etoposide in combination with isoeugenol (P < 0.01, 0.001, 0.01, 0.05 and 0.001 for doses 1, 0.7, 0.5, 0.3, 0.2 μM; resp.).
drug candidates. Finally, we noted that the effect of isoeugenol on Tdp1−/− DT40 cells was more prominent compared to TDP1 depleted human cells. This may reflect residual TDP1 in human cells, due to siRNA knock-down versus genetic disruption in DT40 cells, which is sufficient to mask the requirement for alternative pathways (i.e. TDP2). Alternatively, it could reflect the differential inhibition of chicken and human TDP2 by isoeugenol.

In support of the latter possibility, incubation of 500 μM isoeugenol with DT40 cell extract nearly reached full inhibition with ~100% substrate remaining (Fig. 4A,B) versus ~80% for HeLa cell extract (Fig. 4C,D). These observations point at cross species differences, which may be illuminating in future studies aiming at improving the potency of isoeugenol as a candidate for drug discovery.

In summary, we identify isoeugenol as a potentiator of CPT cytotoxicity in a TDP1 dependent manner and suggest that it acts by inhibiting TDP2 activity. Isoeugenol therefore may act as a potential lead compound for developing TDP2 inhibitors. Future structure-activity relationship studies might be warranted to improve potency and shed light on its utility in drug discovery programs.

Materials and Methods

Chemicals. Caffeic acid, p-Coumaric acid, naringinen, chlorogenic acid, quercetin, isouqueretin, umbelliferone, harmine, luteolin, caffeine, 2,4,4′-trihydroxy chalcone, naringinin-7-glucoside, pseudophedrine, glycyrrhizin, epicatechin, vanillic acid, gallic acid, β-glycyrrhetenic acid, xanthohumol and amentoflavone were purchased from Sigma Aldrich (St. Louis, MO, USA). Bisabolone, α-ionone, (Z)-3-hexenyl acetate, allyl anthranilate, isoeugenol, α-pinene, cuminaldehyde, thymol, carophyllene oxide, carvone, and methyl disulphide were provided from Bedoukian Research (Danbury, CT, USA). Cynarin, emodin, rutin, and sennoside A were purchased from Chromadex (Wesel, Germany). Khellin was a gift from Prof. Dieter Treutter (Tech Univ. of Munich, Germany). Sarcophine was isolated from Sarcophytum ehrenbergii soft coral whereas vitexin and chrysin were isolated from Passiflora edulis leaf in Dr. Farag laboratory, Cairo University (Supplementary Fig. 6).

Cell culture. Tyrosyl-1-DNA phosphodiesterase 1-knockout DT40 cells (Tdp1−/−) and those complemented with human TDP1 (hTDP1) were cultured at 37 °C and 5% CO2 (NuAire) in RPMI 1640 medium (Lonza) supplemented with 7% FBS (Sigma), 3% chicken serum (Sigma), 1% L-glutamine (Lonza), and 1% penicillin/streptomycin (Lonza) at 37 °C and 5% CO2.

Viability assays and clonogenic survival. The compounds were screened on both DT40 Tdp1−/− and DT40 Tdp1−/− complemented with hTDP1 cells. Cells were seeded into 96-well plates at a density of 3000 cells/well on day 0. The compounds, CPT (Sigma), and other inhibitors were added at varying concentrations in triplicate. On day 2, 20 μl cell-titer blue (Promega) was added and the plate incubated at 37 °C. After 4 hours, the viability was quantified as fluorescence intensity using a microplate reader, FLUOstar Omega (BMG LABTECH). Olaparib (MedKoo Biosciences) and isoeugenol (0.1 and 50 μM, resp.) were co-added along with increasing concentrations of CPT to DT40 cells (2000 cell/well). After a 72-hour incubation, viability was assessed using the cell-titer blue kit as above. HeLa and MCF-7 cells (5000 cell/well) were treated with five doses of etoposide (Sigma) one lethal (100 μM) and four sub-lethal doses (50, 25, 10, and 5 μM) in the presence or absence of sub-lethal doses (200 and 400 μM for HeLa, MCF-7, resp.) of isoeugenol in triplicate. Treated cells were incubated at 37 °C for 48 h and cell viability measured using the cell-titer blue. Two hundred HeLa cells were seeded in 6 cm dishes at 37 °C overnight. Cells were treated with DMSO (Sigma) or indicated doses of etoposide (1, 0.7, 0.5, 0.3, 0.2 μM) in the presence or absence of isoeugenol (1 μM). Cells were allowed to grow for 10 days. Visible colonies were stained and survival calculated a described below. MCF-7 cells were transfected using siPORT™ NeoFX™ (Invitrogen) transfection reagent with siRNA at a final concentration of 100 nM. The siRNA sequences that were used to target TDP1 are 5′-CUAGACAGUUUCAAGAGUA-3′, 5′-UCACUUUGAAACUGUACG-3′, 5′-GACCAUAUCUGAUAUGGUC-3′, 5′-UCAGGUACUUAGUCCGUAU-3′, 5′-UUAGGCCAUAGAUAACGUA-3′, and 5′-GAAGCUUAAAGCAGAAGUA-3′, 5′-UUAACCUUUAGGCUCUAAC-3′, and for scrambled siRNA control, 5′-GGGCGCUUUGAAGGUAUAGG-3′ and 5′-GGAUCCAAAUCAAAAAGCCGC-3′ was used. Cells were incubated at 37 °C for 24 h then the media replaced with complete media and incubated for an additional 24 h. One hundred MCF-7 cells were seeded in 6 cm dishes at 37 °C overnight. Then cells were treated with DMSO (Sigma) or indicated doses of CPT in the presence or absence of isoeugenol (4 μM) in triplicate. Cells were allowed to grow for 14 days. Colonies were fixed with 75% methanol and stained with 1% Giemsa stain. The surviving fraction was calculated as ((colonies counted/total cells seeded)treated)÷(colonies counted/total cells seeded)untreated).

Western Blot. Cells were lysed in lysis buffer (10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 50 mmol/L NaF, 1 mmol/L phenylmethylsulfon fluoride, and 1 mmol/L Na3 VO4). Total protein concentration was determined using Bradford assay, and equal amounts of total protein were then boiled at 95 °C for 5 min with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (25% glycerol, 31.2 ml 0.25 M Tris-HCl (pH 6.8), 7.5 ml 10% SDS, 0.3 M DTT and a dash of bromophenol blue/100 ml) and separated by 11% SDS-PAGE. Separated proteins were then transferred onto apolyvinylidene difluoride (PVDF) membranes (Millipore) following standard methods. The membranes were incubated in blocking solution (2% non-fat milk in PBS) for 1 h, further incubated with primary antibodies (mouse monoclonal anti-TDP1 (Santa Cruz; cat no. SC-365674) at a dilution of 1:500 and anti-human beta actin at a dilution of 1:1000) overnight at 4 °C, and then incubated with a secondary antibody (anti-mouse horseradish peroxidase, (HRP)-linked IgG antibody at a dilution of 1:1000 for TDP1 and beta actin) (Invitrogen) for 1 h at room temperature. After 3 × 10-min washes...
in T-PBS (0.1% Tween-20 in PBS) the membranes were developed with enhanced chemiluminescence detection reagent, clarity™ western ECL blotting substrate (Biorad). Signal on membranes was obtained using ChemiDoc™ MP Imaging System (Biorad).

**TDPs activity assay: Gel-based assay.** The *in vitro* 3'-tyrosyl-DNA phosphodiesterase 1 and 2 inhibitory activity of compound 14 (200 μM, 500 μM, and 1000 μM) was determined using a gel-based 3'-tyrosyl-DNA phosphodiesterase activity assay. Biochemical assays were performed in 10 μL reaction volumes containing TDP1 reaction buffer (50 mM Tris HCL (pH 7.5), 7.5 mM KC1, 1 mM DTT, 10 μM EDTA, and 100 μg/ml BSA) or TDP2 reaction buffer (50 mM Tris HCL (pH 7.5) 50 mM KCl, 1 mM DTT, 100 μg/ml BSA, and 1 mM MgCl2), cell lysate (14 μg TDP1; 14 μg TDP2), and 50 nM Cy5.5-labelled substrate oligomer 5'- (Cy5.5)GATCTAAAAGACT(pY)-3' (Midland Certified Reagent Company, Texas, USA) for TDP1 or 5'- (pY) CATCGTGGCTTACCAT(Cy5)-3' for TDP2. The reactions progressed at 37°C for 2 h (TDP1) and 1 h (TDP2) and were quenched with 10 μL loading buffer (44% deionized formamide, 2.25 mM Tris-borate, 0.05 mM EDTA, 0.01% xylene cyanol, and 1% bromophenol blue). Samples were then heated at 90°C for 10 min prior to separation on a 20% urea SequaGel by gel electrophoresis at 100 V for 30 min. Reaction products were visualized by gel imaging using ChemiDoc™ MP Imaging System (Biorad)²².

**Alkaline single-cell agarose gel electrophoresis assay.** Cells were plated and incubated at a density of 60,000 cell/well with 50 μM CPT for 30 min, 50 μM of compound 14, or CPT plus compound 14 at 37°C for 48 h. DNA strand breakage, primarily (SSBs) and alkali-labile sites, were quantified by alkaline comet assays as described in³³. Briefly, cells were suspended in pre-chilled phosphate buffered saline (PBS) and mixed with equal volume of 1.3% low-gelling-temperature agarose (Sigma, Type VII) maintained at 42°C. Cell suspension was immediately layered onto pre-chilled frosted glass slides (Fisher), pre-coated with 0.6% agarose. The slides were maintained in the dark at 4°C until set, and for all further steps. Slides were immersed in pre-chilled lysis buffer (2.5M NaCl, 10 mM Tris-base, 100 mM EDTA (pH 8.0), 1% Triton X-100, and 10% DMSO; (pH 10.0)) for 1 h, washed with pre-chilled distilled water (2–10 min), and placed for 30 min in pre-chilled alkaline electrophoresis buffer (50 mM NaOH and 1 mM EDTA). Electrophoresis was then conducted at 1 V/cm for 30 min, followed by neutralization in 400 mM Tris- HCl (pH 7.0) for 1 h. Finally, DNA was stained with Sybr Green I nucleic (Sigma) (1:10000, in PBS) for 30 min. Average tail moments from 150 cells per sample were measured using Comet Assay IV software (Perceptive Instruments, UK)⁵⁰.

**Statistical analysis.** All statistical parameters were performed using excel version 2013 (Microsoft office). Viability was calculated by dividing the mean of treated cells by the untreated cells. Average tail moments from 150 cells per sample were measured using Comet Assay IV software (Perceptive Instruments, UK). All data are presented as means with conforming SEM of three biological triplicates. Student’s t-test was used to compare cell viability and tail moment between compounds, CPT alone, and combination with different concentrations of CPT. In all tests, the level of significance was set at p < 0.05.

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Author Contributions

W.E. and L.E. conducted and analysed the survival experiments. W.E. conducted the gel-based assays and the comet assays. M.H. helped with the knockdown and survival assays. M.F. assembled the compound library. All authors analysed the data and reviewed the manuscript. S.F.E-K conceived the study and coordinated the project.

Additional Information

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