



RESEARCH PAPER



Discovery of a novel DNA polymerase inhibitor and characterization of its antiproliferative properties

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ABSTRACT

Chromosomal duplication is targeted by various chemotherapeutic agents for the treatment of cancer. However, there is no specific inhibitor of DNA polymerases that is viable for cancer management. Through structure-based *in silico* screening of the ZINC database, we identified a specific inhibitor of DNA polymerase δ . The discovered inhibitor, Zelpolib, is projected to bind to the active site of Pol δ when it is actively engaged in DNA replication through interactions with DNA template and primer. Zelpolib shows robust inhibition of Pol δ activity in reconstituted DNA replication assays. Under cellular conditions, Zelpolib is taken up readily by cancer cells and inhibits DNA replication in assays to assess global DNA synthesis or single-molecule bases by DNA fiber fluorography. In addition, we show that Zelpolib displays superior antiproliferative properties to methotrexate, 5-fluorouracil, and cisplatin in triple-negative breast cancer cell line, pancreatic cancer cell line and platinum-resistant pancreatic cancer cell line. Pol δ is not only involved in DNA replication, it is also a key component in many DNA repair pathways. Pol δ is the key enzyme responsible for D-loop extension during homologous recombination. Indeed, Zelpolib shows robust inhibition of homologous recombination repair of DNA double-strand breaks and induces “BRCAness” in HR-proficient cancer cells and enhances their sensitivity to PARP inhibitors.

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Introduction

The majority of successful chemotherapeutics are those that interfere with DNA replication^{1,2}. The success of these drugs is explicable given that the defining characteristic of neoplastic cells, regardless of histology, is unregulated and persistent cell division. Various components of DNA metabolism serve as targets for cancer therapy including chromosomal template for replication, required deoxynucleotides (dNTPs) for replicating the daughter strand, and necessary enzymes. The most diverse category of chemotherapeutic strategies act to directly damage the template³ to prevent its replication. These strategies involve various covalent modifications of DNA strands including crosslinking, alkylation, and lysis, as elicited by both chemical and physical means. Strategies that damage the DNA, however, have a narrow therapeutic window because of the propensity to damage all cells^{1,3}.


The second class of compounds, broadly termed antimetabolites⁴, target the synthesis of dNTPs. Thus, these compounds act to deplete the substrates for DNA replication. This strategy is – in general – less toxic than the aforementioned strategies that damage DNA because it has minimal impact on non-dividing cells. Consequently, the relevant compounds offer a better side-effect profile and can be taken for longer duration than the DNA damaging agents. Methotrexate and its analogs⁵, which competitively inhibit

dihydrofolate reductase, constitute important examples of antimetabolite drugs. Indeed, methotrexate is prescribed for numerous disorders that exhibit aberrant cell proliferation, such as arthritis and psoriasis. The long course of these diseases has meant that, in some cases, methotrexate has been prescribed and well-tolerated for decades. Even so, the targets of antimetabolites, such as dihydrofolate reductase⁵, thymidylate synthase², or ribonucleotide reductase⁶ lie at the intersection of numerous metabolic pathways, which increases the propensity for off-target side effects.

The required enzymes that can be targeted for chemotherapeutic intervention are topoisomerases^{7–9} which act to prepare chromosomes for replication, helicases which act to unwind the duplex DNA, and polymerases which synthesize the daughter strand during replication. Of these, topoisomerases⁵ have been successfully targeted. A number of topoisomerase inhibitors have gained FDA approval, such as the topotecan analogs and etoposide. Topoisomerases, however, are indispensable for the resolution of DNA topology issues during transcription¹⁰ and topoisomerase inhibitors also have the propensity to induce toxic single- and double-DNA strand breaks¹¹.

Ostensibly missing are chemical entities that target the most important enzymes in DNA replication – DNA polymerases, even though DNA or RNA polymerases are the targets for many antiviral therapeutics. Reversible inhibitors

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 Supplementary data can be accessed [here](#)

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of DNA polymerases should inhibit cancer cell proliferation and possess some advantages over DNA damaging agents and topoisomerase inhibitors. First, due to the specific function of replicative polymerases, reversible DNA polymerase inhibitors will inhibit DNA replication alone, and thus cell proliferation without affecting non-proliferating cells. Secondly, these inhibitors will not damage the chromosomes of non-dividing cells and should have minimal impact on transcription. Consequently, the side-effect profile of polymerase inhibitors should be at least as good as antimetabolites exemplified by methotrexate. There are three essential replicative DNA polymerases, Pol α , Pol δ and Pol ϵ . Cells will not replicate DNA without one of the three and inhibiting one is sufficient to disable chromosome duplication and cancer cell proliferation. On the other hand, the three replicative Pols share a common fold and near identical active site structures as shown by x-ray crystallography¹²⁻¹⁴. Inhibitors that bind to the active sites most likely will inhibit all three Pols simultaneously.

Here we report the discovery of a novel Pol δ inhibitor, Zelpolib through structural based *in silico* screen of the ZINC database¹⁵. We further show that Zelpolib causes robust inhibition of Pol δ *in vitro* and *in vivo* and exhibits superior antiproliferative activities as compared to a number of currently-used chemotherapeutics.

Results

Identification of Zelpolib through *in silico* screen

Through two cycles of screening (Figure 1) top 300 compounds (supplemental movie 1) were selected for detailed

analysis. We aimed to select compounds that will inhibit Pol δ when it is actively engaged in DNA replication. At the minimum, three key requirements are necessary: interaction with template strand, hydrophobic stacking with the preceding nucleotide base of the primer, and extensive interaction with Pol δ . We selected 10 compounds that satisfy our requirements. All selected compounds showed inhibition of Pol δ activity when assayed at 100 μ M with the poly(dA)/oligo(dT) assay (data not shown) and only one compound showed significant inhibition when assayed at 10 μ M. This compound, subsequently named Zelpolib (Figure 2(c), Zilch Polymerase activity), is predicted to have reasonable pharmacological properties (Figure 1). Zelpolib exhibits a unique binding mode in the model structure (Figure 2(a)). The catechol moiety occupies the base position of the incoming nucleotide (Figure 2(a,b), and supplemental movie 2), in a position to form hydrophobic stacking with previous base of the primer (cytosine). The catechol groups are in position to form two hydrogen bonds with guanine on the template strand (Figure 2(a)). In fact, half of the inhibitor molecule (from catechol to thiourea segment of the inhibitor, Figure 2(c)) almost overlaps with the incoming nucleotide in the crystal structure (3IAY.PDB, dCTP, Figure 2(a) vs 2B). In the final model, Zelpolib is also in a position to form 5 potential hydrogen bonds with Pol δ (Figure 2(d)). The three residues forming hydrogen bonds with Zelpolib, N698, D602 and D757, also play important roles in interaction with incoming nucleotide (3IAY). Potentially, more hydrogen bonds are formed between amide of Y607 and guanidine side chain of R667

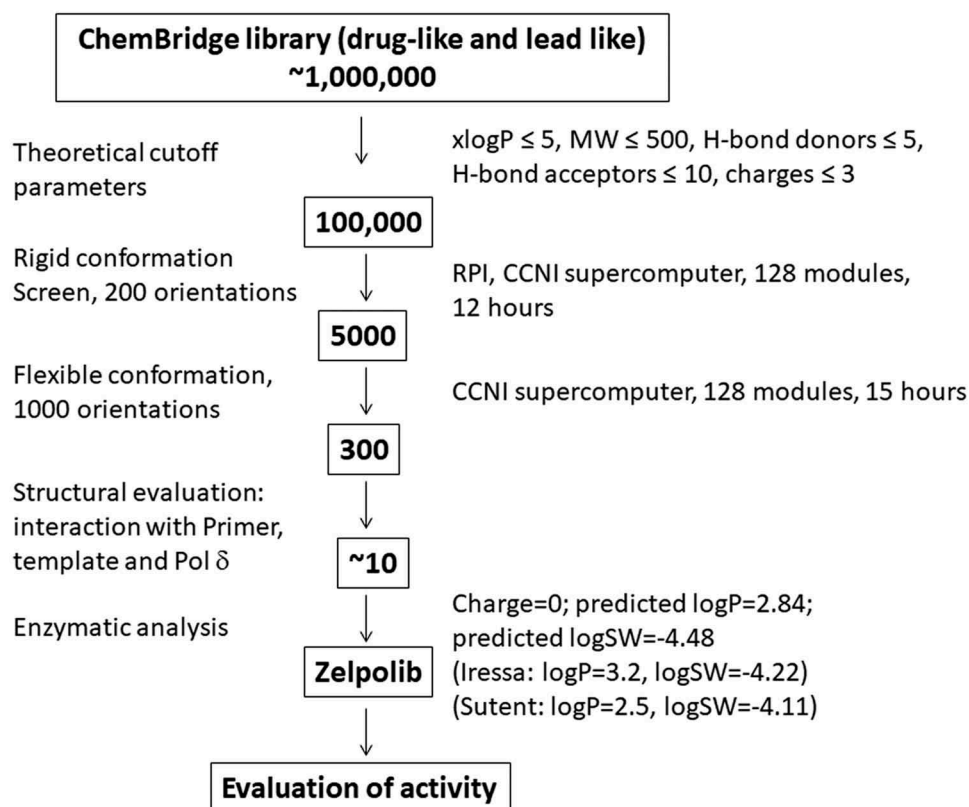


Figure 1. Flow-chart of the *in silico* screening protocol by Dock 6.4 of the ChemoBridge library.

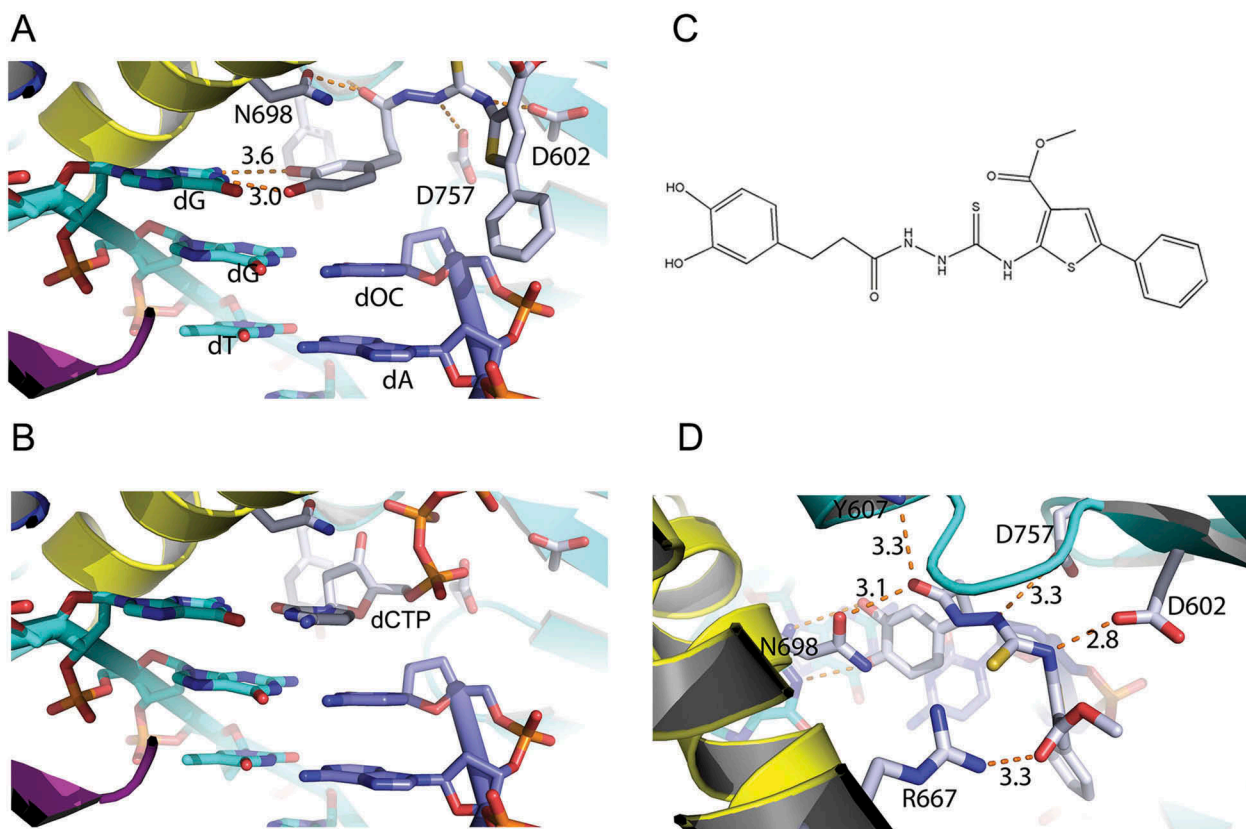


Figure 2. Interactions of Zelpolib at the active site of Pol δ . A, Zelpolib forms two hydrogen bonds with dG on the template strand. The catechol moiety stacks against dOC (dideoxycytosine) on the primer. B, comparative position of dCTP at the active site of Pol δ . C, chemical structure of Zelpolib. D, 5 potential hydrogen bonds can be formed between Zelpolib and Pol δ .

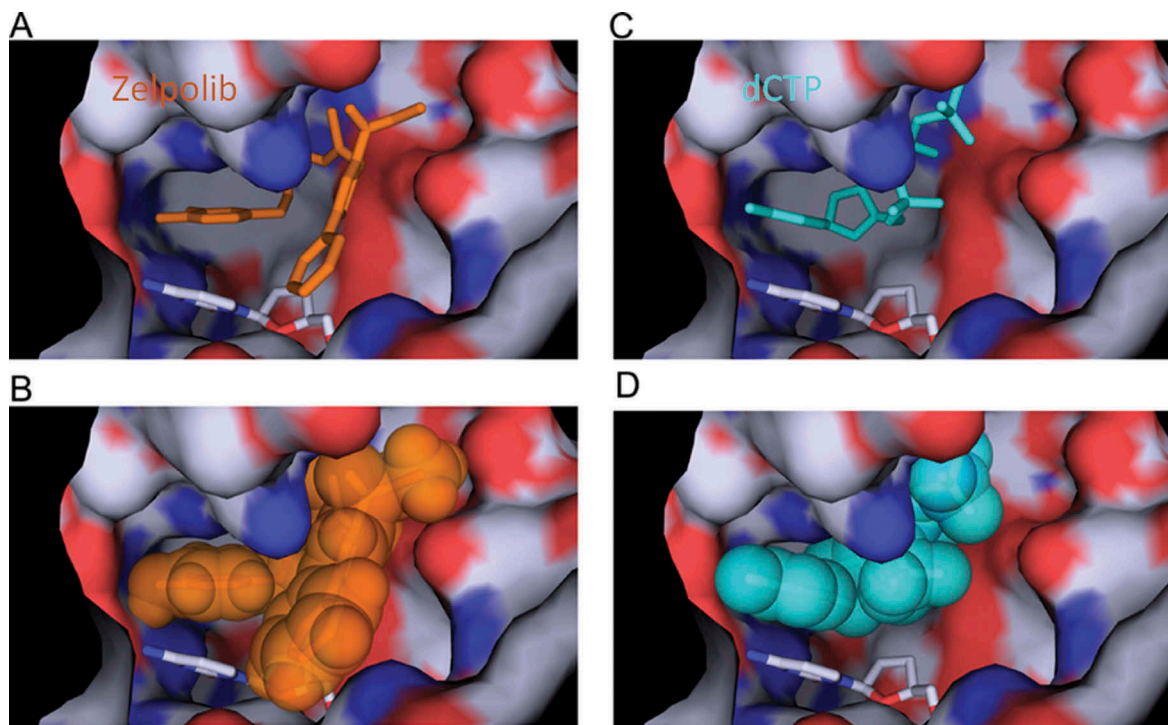


Figure 3. Comparison of Zelpolib with dCTP in binding to the active site of Pol δ . Zelpolib in stick (A) and ball (B) representation with dCTP (C and D) in comparison.

with Zelpolib. Overall, Zelpolib occupies significantly more space within the Pol δ active site (Figure 3) than the incoming nucleotide and is projected to be a strong

inhibitor that binds to Pol δ active site when it is in complex with template/primer, i.e. when actively synthesizing DNA during replication or repair.

Inhibition of Pol δ activity by Zelpolib in enzymatic assays

In order to validate the predicted inhibitory properties of Zelpolib, we used the well-established DNA polymerase assay, poly(dA)/oligo(dT) method^{1,16}, to quantify the inhibition constants. In this assay, a poly(dA) template (up to 4 kilobases) was randomly primed with oligo(dT) primers (40mer). In the presence of proliferating cell nuclear antigen (PCNA), Pol δ will initiate the elongation of oligo dT primer with the incorporation of radio-labeled deoxythymidine ³H³H. The polymerase activity can then be precisely quantified by counting the isotope incorporated. In this assay, Zelpolib showed robust inhibition of Pol δ activity in a non-competitive manner (Figure 4(a)) against dTTP with an inhibition constant K_i of $4.3 \pm 0.3 \mu\text{M}$ ($R^2 = 0.99$).

Next, we investigated the inhibitory properties of Zelpolib in a primer extension assay^{17,18} with a template that consists of all four deoxynucleotide, which is more reflective of cellular DNA replication processes than the poly(dA) template. The substrate consisted of a 25mer primer hybridized to a 40mer template. In the presence of PCNA and dNTPs, the primers were extended upon addition of Pol δ and a full extension would generate a 40mer copy of the template. The primers were labeled at the 5'-end with ³²P, and the products were separated on a sequencing gel for visualization and

quantification. Consistent with results from poly(dA)/oligo(dT) assays, Zelpolib showed concentration dependent inhibition of the primer extension activities of Pol δ (Supplemental Figure S1). In the second assay, we aimed to investigate whether inhibition of DNA replication is exhibited by other existing cancer drugs, especially kinase inhibitors. The idea originated from the fact that most kinase inhibitors compete for binding with ATP. It is conceivable that they might compete with dATP for binding to polymerases. In addition, there are some structural similarities between Zelpolib and many kinase inhibitors currently in clinical application. We randomly selected 10 small-molecule-cancer drugs with nine being kinase inhibitors²⁰ and one parp inhibitor (olaparib)²¹. At 20 μM , Zelpolib was the only molecule shown to exhibit robust inhibition of Pol δ activity (Figure 4(b)), with 94% of the primer unextended (Figure 4(d)). In the presence of any other inhibitors, at least 30% of the primers were extended. If we analyze the full-length product formed, it is barely detectible in the presence of 20 μM Zelpolib, whereas in the presence of any other inhibitors, the full-length products are clearly detectible (Figure 4(c)).

In our *in silico* screening, the active site cavity was created by the removal of dCTP in the original crystal structure. One of the questions we asked was if Zelpolib displays any preference in the inhibition of a specific dNTP among the four

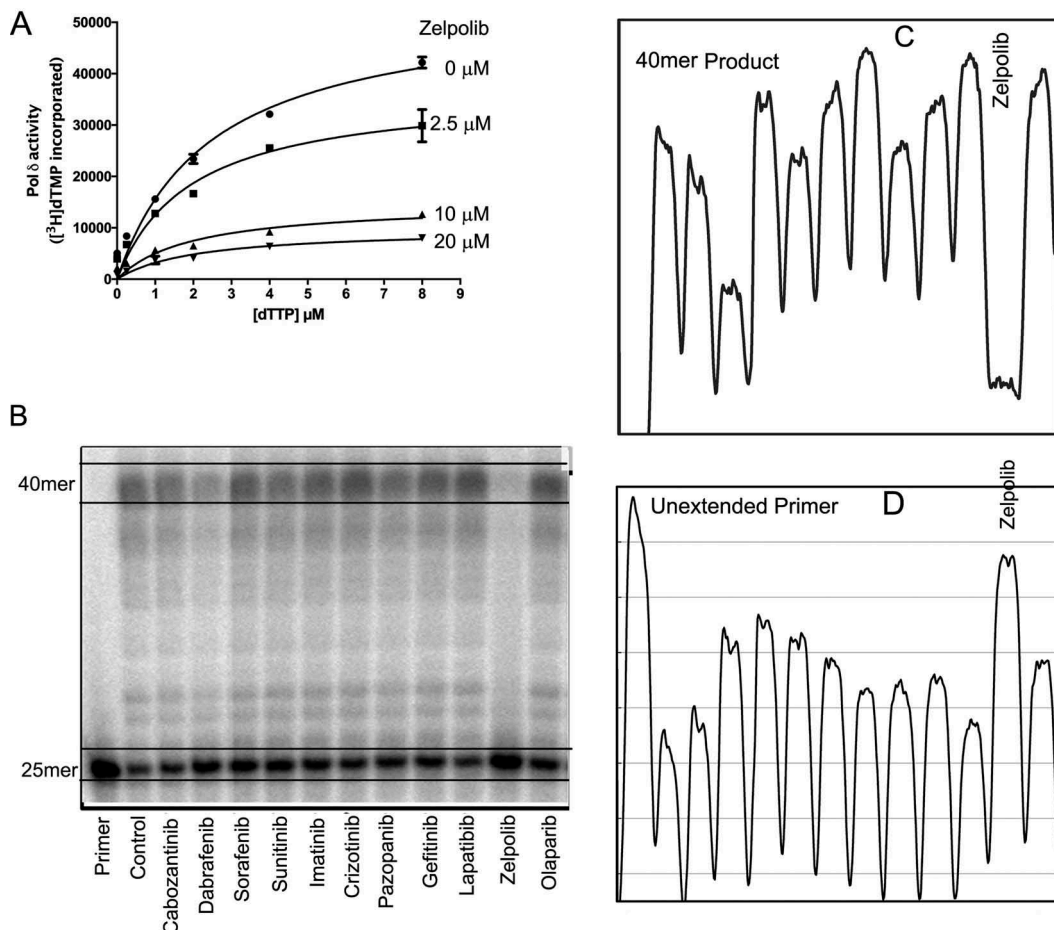


Figure 4. Zelpolib inhibits Pol δ activity. A, enzymatic assay by poly(dA)/oligo(dT) method demonstrates noncompetitive inhibition with K_i of $4.3 \mu\text{M}$. B, Zelpolib is likely unique in inhibiting Pol δ activity in comparison to current FDA approved small molecule oncology drugs. C, quantification of the full-length products (40mer, integration by ImageJ). Lower peak height indicates less product. D, quantification of unextended primer. Higher peaks correspond to higher amounts of primer left.

dNTPs. Therefore, we assayed the inhibition patterns of all four individual dNTP molecules in a single nucleotide incorporation assay. In this assay, only one nucleotide is present for the primer extension for the next appropriate base pairing to the template. Interestingly, Zelpolib inhibited the incorporation of all four nucleotides to similar extents in a noncompetitive manner (Supplemental Figure S2).

Our enzymatic analyses of Zelpolib on Pol δ activity have validated the *in silico* screen results. Zelpolib show strong inhibition of Pol δ activity in two different assays and inhibited the incorporation of all four nucleotide in a noncompetitive manner with K_i in the μM range. In addition, the mechanism of inhibiting DNA replication by Zelpolib is not likely shared by other small molecule drugs currently in clinical application in oncology.

Zelpolib inhibits DNA replication directly under cellular conditions

Upon establishing that Zelpolib inhibits Pol δ activities in enzymatic assays, we conducted experiments to establish that Zelpolib inhibits DNA synthesis *in vivo*. Extensive cellular studies have shown that kinase inhibitors can arrest cell cycle progression and induce apoptosis²² upon prolonged exposure of 24 to 96 hours. The effects of prolonged exposure could be a combination of many cellular pathway disturbances that eventually results in halting DNA synthesis. In order to assess the direct effect on DNA replication, we avoided prolonged cellular exposure to Zelpolib so that any effects on DNA replication are not due to cell cycle regulation. In our first assay, we incubated cells with Zelpolib for 1 to 2 hours to ensure uptake. Then the cells were pulse labeled with 5-ethynyl-2'-deoxyuridine (EdU)²³ so that the majority of incorporation was due to ongoing DNA replication processes. The uridine analog, EdU, is incorporated into DNA as dTTP analogs and can be detected by "Click" chemistry with fluorescent azide^{23,24}. We tested Zelpolib on a pancreatic cancer cell line, BxPC-3 cells, followed by EdU incorporation analysis using laser scanning cytometry. Zelpolib showed concentration dependent inhibition of EdU incorporation. The integrated fluorescence signal is shown in Figure 5(a), with significant reduction of EdU incorporation at 10 and 20 μM in comparison with sham treatment.

In our search for Pol δ inhibitors, we focused on the identification of compounds that can inhibit Pol δ when it is actively replicating DNA. DNA fiber fluorography with dual labeling technique²⁵ provides a possibility that we can analyze the effect of Zelpolib on ongoing DNA replication at a single-molecule level. Both IdU and CldU are halogenated thymidine analogs that can be incorporated into DNA. Subsequent DNA fiber spreading and antibody detection are used to visualize the replicated DNA fiber. The incorporation of IdU gives rise to red fibers and CldU produces green fibers with antibody detection²⁵. Therefore, in this assay, the cells were first incubated with IdU for 20 minutes in the absence of Zelpolib (Figure 5(b)) to establish ongoing DNA replication and the basal replication rates of each individual DNA fibers. Subsequently, CldU were added to cell culture either with or without simultaneous addition of Zelpolib. DNA replication

were allowed for an additional hour. For any ongoing replication, the green fiber (60 minutes of replication) should be collectively about 3X the length of the red fiber (20 minutes of replication, Figure 5(c)) either in the absence of Zelpolib or even in the presence of Zelpolib if the cells failed to take up Zelpolib fast enough in that short duration. Under this stringent condition, Zelpolib showed concentration dependent inhibition of ongoing cellular DNA replication (Figure 5(d), BxPC-3 cells). In order to be certain that this inhibition was not cell line dependent, we conducted an identical experiment on a triple-negative breast cancer cell line as well. All three concentrations of Zelpolib showed significant inhibition of ongoing DNA replication (Figure 5(c,e)). The DNA fibers (Figure 5(c)) from a single field of view (Supplemental Figure S3) were realigned for visual comparison.

These cellular studies demonstrated that Zelpolib inhibits cellular DNA replication significantly with concentrations that inhibited Pol δ activities in enzymatic assays. The DNA fiber analysis also demonstrates that, as intended in the screening protocol, Zelpolib can be taken up readily by cancer cells and inhibits ongoing DNA replication. At 20 μM , Zelpolib inhibited the DNA fiber extension by about 40%, indicating that cellular uptake took less than 30 minutes as the total exposure time was 60 minutes.

Zelpolib displayed superior antiproliferative properties to widely used anti-cancer drugs

DNA replication precedes cell division. Thus, cancer cells cannot divide and proliferate without successful chromosomal duplication. A DNA polymerase inhibitor should inhibit cancer cell proliferation. We characterized the antiproliferative properties of Zelpolib on cancer cell lines originated from pancreatic cancer (BxPC-3) and breast cancer (MCF7 for ER + breast cancer). In addition, we developed a cisplatin resistant subline of a pancreatic cancer cell line, R-BxPC3 for our studies. We used the MTT method²⁶ for analyzing cell proliferation. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by live cells to an insoluble formazon, which were subsequently dissolved in DMSO and quantified by UV absorbance. Therefore, the cell number is proportional to the optical density in the established linear region. Based on this assay, Zelpolib showed robust antiproliferative properties against BxPC-3, R-BxPC-3, and MCF7 cells (Figure 6), all reaching a plateau at about 5 μM . The plateau phenomena indicate inhibition of proliferation without cell killing, similar to these observed from hydroxyurea²⁷, aphidicolin²⁸, and to a certain extent, methotrexate treatments²⁹. Direct inhibition of DNA polymerase activity provides a unique mechanism that is not employed by any of the current cancer therapeutics. We selected a number of most widely prescribed chemotherapeutics with distinct mechanisms to compare their antiproliferative potential in the two cancer cell lines. We included alkylating agent cisplatin, antimetabolites methotrexate and 5-flourouracil (5FU)³⁰⁻³². When investigated on pancreatic cancer BxPC-3 cells, Zelpolib is superior to methotrexate and 5-FU at all concentrations; is superior to cisplatin up to 10 μM (Figure 6). With MCF7 cells, Zelpolib is superior to

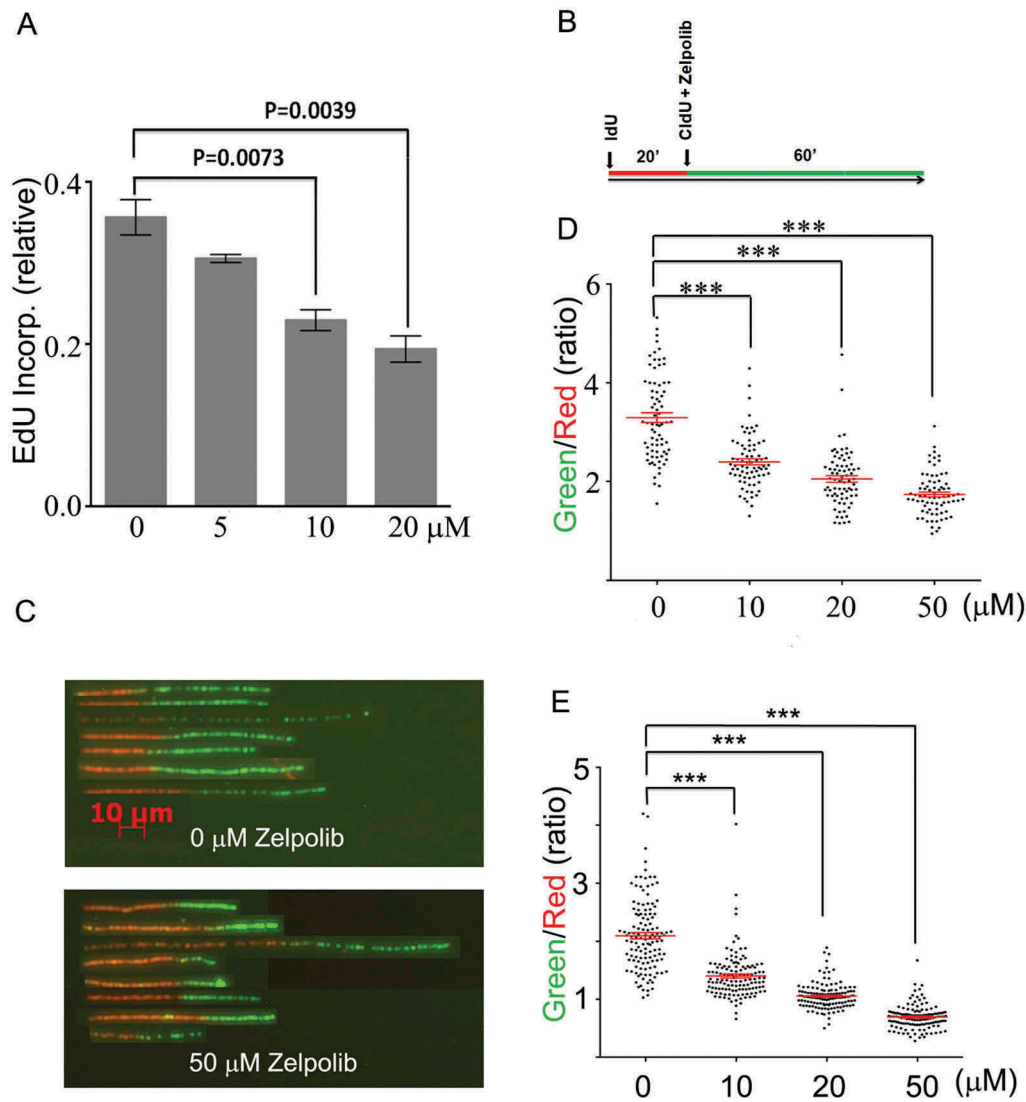


Figure 5. Zelpolib inhibits cellular DNA replication. **A**, EdU incorporation by whole cell population is inhibited by Zelpolib. Exponentially growing HCC1395 (TNBC) cells were treated with Zelpolib for 2 hours prior to pulse labeling with EdU for 30 minutes. Amount of EdU quantified with “click” chemistry and measured by laser scanning cytometry (LSC). Error bar shows mean value with SEM (triplicates) and P values were calculated using unpaired Student’s t-test. **B**, treatment scheme of DNA fiber fluorography assay. **C**, DNA fiber-length comparison between untreated and treated with 50 μM Zelpolib (HCC1395 cells, see Figure S4 for original images). **D**, quantification of DNA fiber length for BxPC-3 cells. **E**, quantification of DNA fiber length for HCC1395 cells. 75 fibers were analyzed per sample. Scattered dot plot shows ratio of green/red fiber lengths (ratios) with SEM. P values were calculated using unpaired T test. *** indicates $p < 0.0001$.

methotrexate and 5-FU across the concentration range. Whereas the comparison with cisplatin is biphasic. Zelpolib is at least as effective as cisplatin in inhibiting MCF7 cell growth up to 5 μM, whereas cisplatin is more effective beyond 5 μM, as the cytotoxic effects of cisplatin start to dominate (Figure 6). Similar advantages of Zelpolib were also observed with cisplatin-resistant Pancreatic cancer cells (Figure 6). Within the time frames of our study of 48 to 72 hours, treatment with Zelpolib alone does not lead to significant increase in apoptosis or senescence (data not shown), consistent with its mechanism of action. Aphidicolin, a Pol δ inhibitor, is commonly used for cell cycle synchronization in laboratory practice without induction of cell apoptosis or senescence.

Indeed, Zelpolib provides effective antiproliferative activities with a unique mechanism of action, which has not been successfully employed in current cancer therapeutics.

Zelpolib inhibits DNA double strand break repair by homologous recombination (HR)

In addition to being indispensable for the duplication of chromosomes, Pol δ also plays critical roles in various DNA repair pathways¹⁹. It has been shown that Pol δ is the preferred polymerase for the D-loop extension during homologous recombination repair³³. Thus, we investigated the effect of Zelpolib on HR. We used the two plasmid DR-GFP assay developed by Nakanishi et al³⁴, which is a modified version of the initial method³⁵. The first plasmid contains a mutant GFP that has lost the ability to fluoresce. The second plasmid contains the coding region of a restriction enzyme, I-SceI. The co-transfection of the two plasmids will result in the expression of I-SceI, which can digest the DR-GFP plasmid at the mutation site leading to the formation of a double-

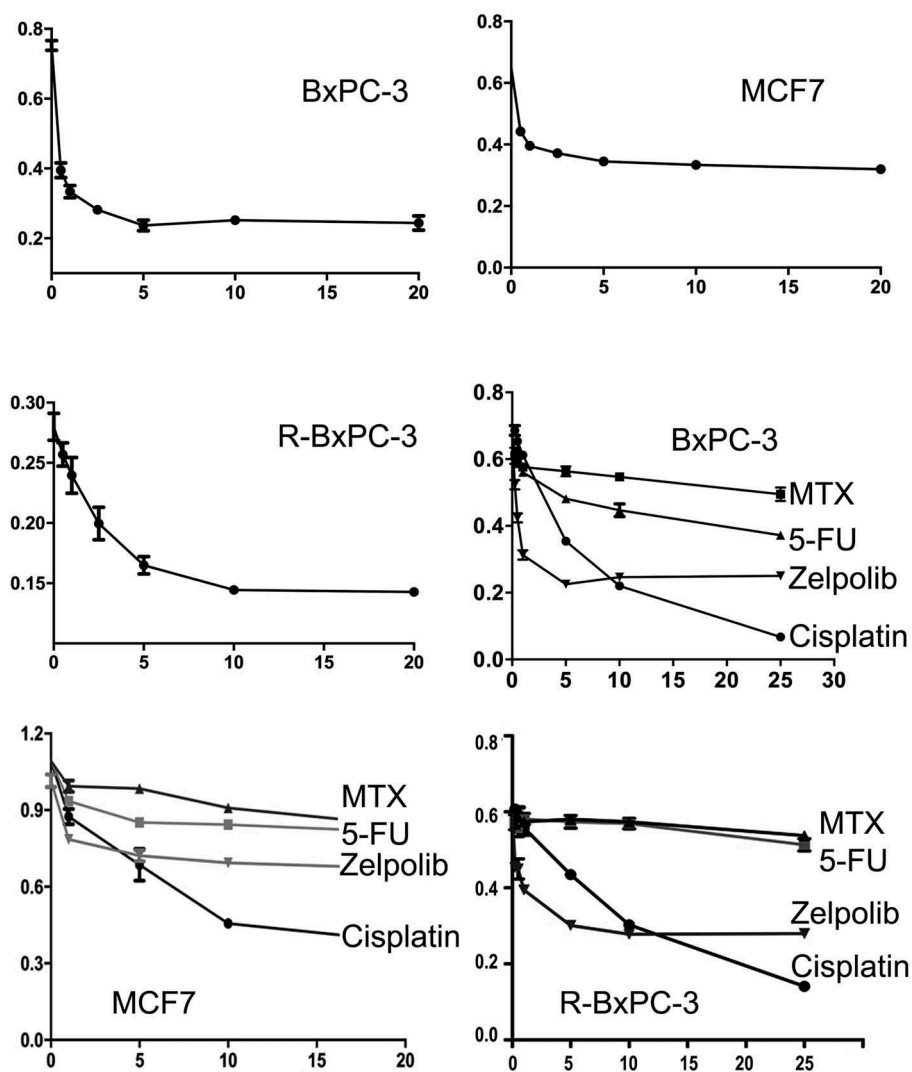


Figure 6. Antiproliferative activities of Zelpolib. Concentration dependent inhibition of cell proliferation by Zelpolib on three different cell lines and comparison with methotrexate, 5-FU, and cisplatin by MTT assays. All samples were in triplicates and presented as averages with standard deviation.

strand break. Successful homologous recombination repair of the double strand break will restore the GFP fluorescent signal. Therefore, the percentage of fluorescent cells is the readout of the efficacy of HR. To have conditions for valid comparison, HEK 293T cells were transfected with both plasmids under identical conditions. The transfected cells were allowed to recover for 4 to 5 hours and then replaced with fresh media. Subsequently, Zelpolib was added at two different concentrations. The efficacy of HR was analyzed about 40 hours later by flow cytometry. Zelpolib showed dose-dependent inhibition of HR (Figure 7A). The reduction in HR efficacy at 20 μ M Zelpolib is comparable to those induced by siRNA knockdown of BRCA1 or BRCA2 individually as reported³⁶.

Zelpolib increases the sensitivity of HR proficient cells to PARP inhibitors

PARP inhibitors have been a breakthrough therapy for HR-deficient ovarian cancers due to BRCA1/2 mutations³⁷⁻⁴¹.

However, there have been minimal clinical benefits for HR-proficient cancers³⁷⁻⁴¹. Since Zelpolib can inhibit HR, we explored the effect of Zelpolib in combination with PARP inhibitors on HR-proficient cancer cells. The hypothesis is that Zelpolib will lead to an HR-defect phenotype through inhibition of Pol δ and render HR-proficient cells sensitive to PARP inhibition. We tested the combination on a triple-negative breast cancer cell line, HCC1395, which is insensitive to PARP inhibitors Niraparib⁴² or Rucaparib⁴³. The cells were treated with increasing concentrations of PARP inhibitor Niraparib (Figure 7(b)) or Rucaparib (Figure 7(c)) in the absence or presence of 2.5 μ M Zelpolib for 48hrs. The number of cells were then analyzed by MTT assay. Zelpolib increased the sensitivity of HCC1395 cells to Niraparib across the whole range of concentrations, with the differences being significant up to 20 μ M. The effect of 25 μ M of Niraparib on the proliferation of HCC1395 cells can be achieved by 5 μ M in the presence of 2.5 μ M Zelpolib. The combination of Zelpolib with Rucaparib behaved similarly to Zelpolib and Niraparib combination, with the difference being statistically significant up to 25 μ M.

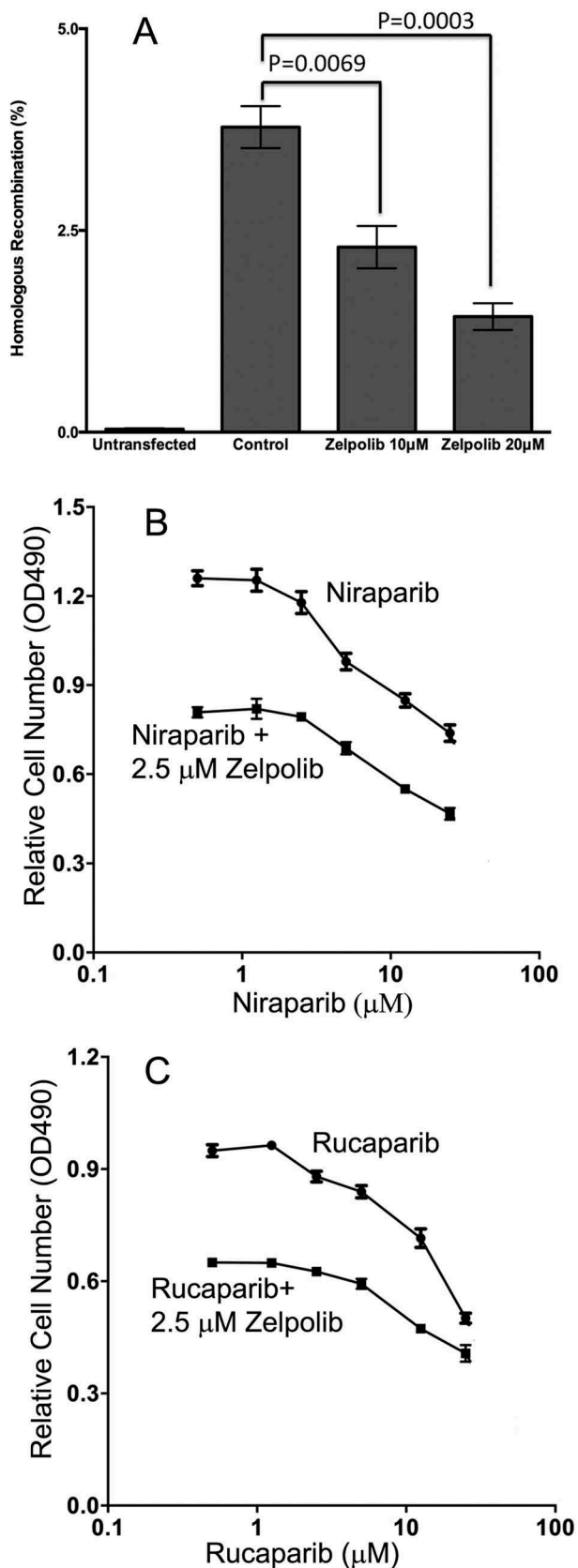


Figure 7. Zelpolib inhibits homologous recombination in cell-based assay. a, Dual plasmids (DR-GFP and I-SceI) reporter assay was used to measure the effect of Zelpolib on DSB repair in 293T cells as reported³⁴. The error bar represents mean values of four repeats with SEM. P values were calculated using unpaired T test. b, Zelpolib enhances the sensitivity of triple negative breast cancer cells (HCC1395) to niraparib by MTT assay. c, Zelpolib enhanced the sensitivity of TNB cells (HCC1395) towards Rucaparib.

Inhibition of the polymerase activity required for homologous recombination by Zelpolib can render HR proficient cells more sensitive to PARP inhibition.

Discussion

Advantages of a reversible polymerase inhibitor

Current cancer therapies take advantage of the quantitative differences in biological pathways between cancer and non-cancer cells. These differences have been summarized by the landmark publication of “Hallmarks of Cancer”⁴⁴ by Hanahan and Weinberg and a subsequent update⁴⁵. Among these, the most prominent difference is the rate of proliferation. The high proliferating rates of cancer cells demand expedited nutritional supply through accelerated neoangiogenesis, and rapid chromosome duplication at the sacrifice of genomic stability through inactivation of checkpoint and DNA repair pathways. In fact, these differences and vulnerabilities have been the targets of majority of current therapeutic regimen. Kinase inhibitors, especially VEGFR inhibitors, disrupt neoangiogenesis^{46,47} and inhibit cancer cell proliferation. These inhibitors are an essential part of our current anticancer arsenal.

On the disruption of chromosome duplication front, the most successful chemotherapy reagents target DNA replication by direct DNA damage. These drugs include platinum derivatives that form DNA interstrand crosslinks, and alkylating agents that modify DNA bases. These reagents can modify DNA of all cell types and impact normal cellular function on many fronts. One of the unintended consequences of direct DNA damage is the effect on gene transcription inhibition^{10,48}, a necessary cellular activity shared by all cells, proliferating or quiescent. Some of the side effects and toxicities of DNA damaging agents likely originate from inhibition of transcription in normal tissues⁴⁹. The consequences of transcription inhibition are somewhat under-appreciated, even though the toxicity from α -amanitin, the toxin from some poisonous mushrooms, is well known^{50,51}. The initial effects of α -amanitin exposure share significant similarities with those from chemotherapy, such as abdominal pain, vomiting, and diarrhea^{51,52}. Evidently, for cancer treatment, the challenge is to inhibit DNA replication with minimal impact on other nucleotide metabolism pathways such as transcription.

The three replicative polymerases, Pol α , δ , and ϵ , share a relatively conserved active-site structure and an inhibitor of one most likely will inhibit the others as well. A reversible inhibitor of replicative DNA polymerases could accomplish the goal of inhibiting DNA replication without significant impact on other cellular processes such as transcription or translation. This is partially confirmed by results from this study, as Zelpolib alone showed minimal cell cytotoxicity with pronounced antiproliferative activities. Additional support is from studies on one of the well-characterized replicative polymerase inhibitor, aphidicolin. A number of publications have shown the robust anticancer activities of aphidicolin in cell line and animal models⁵³⁻⁵⁶. More importantly, the safety profile in mice⁵⁵ and phase 1 clinical study⁵⁷ further validated the tolerability of polymerase inhibitors. However, aphidicolin

progressed no further in clinical development due to limitations in biological availability and stability^{55,57}.

Inhibition of cellular polymerases requires much lower concentrations of inhibitors in comparison with tyrosine kinase inhibitors, if the inhibition constants were comparable. There are over 30 kinase inhibitors which have been widely used to treat various cancers. The difficulty in inhibiting kinases arises from the fact that cellular ATP concentrations are relatively high (2–10 mM) and its K_m values of ATP towards kinases are generally in the 10 to 50 μ M range. Therefore, it requires a high [inhibitor]/ K_i ratio for the inhibitors to overcome the ATP saturation of kinase active sites. On the other hand, cellular deoxyribonucleotide concentrations (dNTPs) are much lower and generally in the micromolar range^{58,59}, therefore it requires lower inhibitor [inhibitor]/ K_i ratio to compete with dNTPs for the polymerase active sites. These analyses are supported by the fact that nucleoside analogs such as cytosine arabinoside^{60,61} (ara-C), which has similar affinities towards polymerases after conversion to triphosphate to those of dNTPs, is effective in cancer therapy.

The antiproliferative potential and unique mechanism of action Zelpolib displays could make it a component of cancer therapy to minimize the toxicities associated with current cancer chemotherapeutics.

Inhibition of DNA repair

The majority of current cancer therapeutics has been based on the quantitative differences in biochemical pathways between cancer and non-cancer cells. More recent advances take advantage of the qualitative differences between cancer and non-cancer cells to minimize the side effects or to induce more durable responses. The breakthroughs come from investigations on the deficiencies in cancer cells, notably DNA repair pathways⁶². The universally increased genomic instability of cancers most likely originates from loss of certain DNA repair functions, some have been identified and others remain to be characterized. Two identified pathways, homologous repair deficiency and mismatch repair defect, have been characterized across many cancer types, with HR deficiency due to BRCA1/2 mutations mainly in female reproductive cancers⁶³ and MMR deficiency⁶⁴ in colorectal cancers⁶⁵.

The first major advance in a long time in ovarian cancer treatment comes from the discovery that nearly half of epithelial ovarian cancers (EOCs) are HR deficient due to genetic or epigenetic alterations and roughly 40 percent of these are due to BRCA1/2 mutations⁶⁶. Cancers with HR defects have exquisite sensitivity to PARP inhibition as a consequence of synthetic lethality. In 2012, results from a pivotal clinical trial with olaparib for the maintenance therapy in patients with platinum-sensitive relapsed disease attained a hazard ratio of 0.18²¹ (82% reduction in the risk of progression or death) in progression-free survival for the BRCA1/2 mutant population. Subsequent clinical trials with other PARP inhibitors, niraparib⁶⁷ and rucaparib⁴³, showed similar efficacy to olaparib³⁸. These results are unprecedented, considering the challenges throughout the years in treating ovarian cancers. In addition, PARP inhibitors have been well tolerated and

patients have been on olaparib continuously for many years under maintenance settings.

The loss of MMR function, which results in microsatellite instability⁶⁵ (MSI), was first observed in colorectal cancer. Later, it was found in a small percentage of cancers of all types. Clinical trials have shown that cancers with MSI, regardless of histology, respond favorably to immunotherapy with pembrolizumab, a monoclonal antibody that blocks PD-1 induced immune suppression^{68,69}. Sensitivity to immunotherapy represents a breakthrough in cancer therapy, as these responses are generally durable and significantly prolong patient survival.

However, both breakthrough treatments are limited by the facts that only a small percentage of all cancers have either HR or MMR defects. The question is whether these defects can be induced to cancers without genetic mutations in HR or MMR proteins. Both HR and MMR pathways involve many proteins that coordinate the repair process and could provide targets for pharmaceutical interventions. One common enzymatic activity required for both pathways is DNA polymerization, through a polymerase. In HR, the required DNA polymerase has been suggested to be either Pol δ or Pol ϵ , with reported preference for Pol δ ³³. siRNA knockdown of either protein reduces the HR efficacy by about 50%. Other polymerases, such as Pol η and Pol ξ , have been implicated by a limited number of publications⁷⁰. In MMR, a polymerase is required to refill the gap post excision and most studies suggest the role is filled by Pol δ ⁷¹.

Zelpolib displays robust inhibition of the homologous recombination process and renders HR proficient triple negative breast cancer cells more sensitive to PARP inhibition. These results suggest possible clinical applications to widen the scope of applicability of PARP inhibitors. More broadly, the participation of Pol δ in a wide range of DNA repair pathways implies that Zelpolib will impact many DNA repair pathways in cancer cells. The disruption of these DNA repair pathways could render various cancers more responsive to advanced immune therapies available today and tomorrow.

Materials and methods

In silico screening of compound library

A model structure of the catalytic domain of human Pol δ was generated based on the structure of yeast pol δ ⁷² by SWISSPDB⁷³. The primary sequences of both proteins are conserved, and the active sites structures were virtually identical between the model structure and that of yeast pol δ . The bound primer/template was kept in the complex whereas the incoming nucleotide was removed in order to identify molecules that occupy the active site. Residues within a radius of 10 Å of the ϵ -amino group of residue 694K were defined as the active site to construct a grid for the virtual screening. The compound library from ChemBridge (~1,000,000 compounds) from ZINC database was used for screening. Initial cutoff loosely based on Lipinski's Rule of Five⁷⁴ (xlogP<5, MW<500, H-bond donors<5, H-bond acceptors<10, and charges<3) reduced the library size to 100,000. A three-step protocol (Figure 1) was used with DOCK (6.4)⁷⁵ to complete

the screening process. The most probable conformation of the compound was initially used as a rigid molecule and maximum orientation of 200 was used in the first step. The 5,000 compounds with the highest scores were selected for a second cycle of screening. In this cycle, the conformations of these compounds were regarded as flexible and the maximum number of orientations was increased to 1,000. Subsequently, the 300 compounds (supplemental movie 1) with the highest scores were selected and analyzed by Pymol. The individual structural analysis with PyMol to select compounds that could inhibit Pol δ when it is actively engaged in DNA replication. We set three key requirements for the selection process: 1) the compound can interact with the template strand, especially the nucleoside base that incoming nucleotide will pair with; 2) the compound can form meaningful hydrophobic interactions with the preceding nucleotide base of the primer, preferably through π - π electron interactions; 3) extensive interaction with Pol δ . 10 compounds satisfied our initial requirements and were purchased for initial enzymatic analysis by poly(dA)/oligo(dT) with inhibitor concentrations of 100 μ M. Further assay with 10 μ M concentration narrowed the choice to a single compound.

Cell culture

MCF7, BxPC-3, HCC1395, and 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in media recommended by ATCC. Cisplatin resistant pancreatic cancer cell line, R-BxPC-3, was derived from parental BxPC-3 cells with sequential increases in the concentration of cisplatin with the final concentration of 1.25 μ M according to protocol published previously^{76,77}.

Protein expression and purification

Recombinant Pol δ was expressed in insect cells and purified as reported previously¹⁸. PCNA was expressed in E coli and purified as reported¹⁸.

DNA polymerase assay

Three different assays are used to analyze DNA polymerase activities and the impact of Zelpolib. The poly(dA)/oligo(dT) assay^{1,16} was used for precise quantification as reported. Briefly, the standard reaction mixture for DNA polymerase assay contained 0.375 μ M sparsely primed poly(dA)/oligo(dT) (Supertechs, MD), 150 μ g/ml BSA, 7.5% glycerol, 7.5 mM MgCl₂, 75 mM HEPES (pH 6.5), 0.75 μ M ³HdTTP (100 cpm/pmol). The reaction was initiated by the addition of pol δ and PCNA. After 10 to 30 minutes, the reaction was terminated by the addition of 20 mM EDTA. Aliquots of reaction mixtures were spotted on DE81 ion exchange membranes followed by washing three times with 0.3 M ammonium formate (pH 7.8) and once with 95% ethanol. ³HdTMP incorporation was quantified on liquid scintillation counter. Experiment was performed in triplicates.

The primer extension assays were conducted as previously reported¹⁸. Sequences for 25-mer primer oligonucleotide and four different 40-mer oligonucleotide templates are as follows:

25-mer: 5'-GCC ACT ACA GCA CCT TGA CAG CCA G-3'
40-mer (G): 5'-TCA TCG GTC G CA TCG CTG GCT GTC AAG GTG CTG TAG TGG C-3'

40-mer (C): 5'-TCA TCG GTC G CA TCC CTG GCT GTC AAG GTG CTG TAG TGG C-3'

40-mer (A): 5'-TCA TCG GTC G CA TCA CTG GCT GTC AAG GTG CTG TAG TGG C-3'

40-mer (T): 5'-TCA TCG GTC G CA TCT CTG GCT GTC AAG GTG CTG TAG TGG C-3'

Briefly, the standard reaction mixture contained 5 μ M dNTP, 5 mM MgCl₂, 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 100 mg/ml BSA, 50 mM NaCl, 20 nM primer/template, 50 nM PCNA, 10 nM Pol δ and variable concentrations of inhibitors. The reactions were initiated by addition of dNTPs and MgCl₂. Reaction was allowed to proceed at 25°C for 2 min before addition of stopping buffer (95% formamide/25 mM EDTA). The products were separated on a 20% polyacrylamide urea gel. Products were visualized on phosphor-imager and quantitated using Image-J software. For single nucleotide primer extension assay¹⁸, the protocol is identical to primer extension assay except a single dNTP is used.

Cell proliferation assay-MTT method

Human cancer cells ($5 \times 10^3 - 7.5 \times 10^3$) were initially seeded in 96-well plates (Cellstar) containing 0.2 ml media per well. After 24 hrs, these cells were treated with variable concentrations of drug combinations for 48-72 hrs. Subsequently, 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) (20 μ l of 5 mg/ml) was added to each well for 3 hrs. Formazan crystals were then dissolved in 150 μ l DMSO and optical density was read at 490 nm²⁶. All experiments were performed in triplicates. Results were analyzed by GraphPad Prism. Data represent mean value \pm SEM for each concentration of inhibitor combinations.

DNA fiber fluorography dual labeling

To analyze the effect of Zelpolib, DNA fiber fluorography was used with dual labeling technique as reported. Cells were seeded in 6-well plates (Cellstar) and allowed to attach for 24 hours before pulse labeled with 25 μ M IdU for 20 min. Subsequently, cells were washed three times with PBS and pulse labeled with 250 μ M of CldU (Sigma) for 60 min in the presence or absence of Zelpolib. Cell lysis and fiber spreading were conducted according to Schwab & Niedzwiedz²⁵. IdU and CldU were detected by primary mouse anti-BrdU antibody (BD biosciences, 1:25) and rat anti-BrdU antibody (Abcam, 1:200) and secondary fluorescent antibodies Alexa anti-mouse 594 (ThermoFisher scientific, 1:625) and Alexa anti-rat 488 (ThermoFisher scientific, 1:200). DNA fibers were imaged on fluorescence microscope and fiber lengths were measured using Image-J software. Statistics were calculated using GraphPad prism software.

Fluorescence based double strand break repair assay

The efficacy of homologous recombination was analyzed by a two-plasmids system according to published protocol^{34,35}. HEK

293T cells (0.15×10^6 cells/ml) were seeded in 6-well plate allowed to recover for 24 hours before being transfected with 1.5 μg of pDRGFP and pCBASceI (1:1) plasmids using Lipofectamine 3000 (Invitrogen). After 4 hours, separate wells were treated with different concentrations of Zepolib or DMSO alone for 44 hours. Resulting cells were washed three times in ice cold 1X PBS and trypsinized. Cellular fluorescence for GFP was measured by flow cytometry (Beckman coulter, MoFlo XDP). About 32,000 cells were analyzed per sample. Transfection efficacy was estimated with a GFP plasmid (Invitrogen) under identical conditions. The experiment was repeated three more times and statistics were calculated using GraphPad prism software.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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