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Synergistic Effects of an Irreversible DNA Polymerase Inhibitor and DNA Damaging Agents on HeLa Cells

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Abstract

DNA repair is vital to maintaining genome integrity, but thwarts the effects of cytotoxic agents that target nucleic acids. Consequently, repair enzymes are potential targets for molecules that modulate cell function and anti-cancer therapeutics. DNA polymerase β (Pol β) is an attractive target because it plays a key role in base excision repair (BER), a primary pathway that repairs the effects of many DNA damaging agents. We previously identified an irreversible inhibitor of Pol β whose design was based upon a DNA lesion that inactivates Pol β and its back up BER enzyme, DNA polymerase λ (Pol λ). Using this molecule as a starting point we characterized an irreversible inhibitor (**13**) of Pol β ($IC_{50} = 0.4 \mu M$) and Pol λ ($IC_{50} = 1.0 \mu M$) from a 130-member library of candidates that is ~50-fold more effective against Pol β . Pro-**13** ($5 \mu M$) is only slightly cytotoxic to human cervical cancer cells (HeLa), but potentiates the cytotoxicity of methyl methanesulfonate (MMS). DNA isolated from HeLa cells treated with MMS contain a ~3-fold greater amount of abasic sites when pro-**13** is present, consistent with inhibition of DNA repair. Proinhibitor pro-**13** continues to induce cytotoxicity in DNA damaged cells following MMS removal. HeLa cell cytotoxicity is increased ~100-fold following a 8 h incubation with pro-**13** after cells that were originally subjected to conditions under which 20% of the cells survive and reproduce. The potentiation of MMS cytotoxicity by pro-**13** is greater than any previously reported BER enzyme repair inhibitor.

TOC image

Keywords

DNA repair; enzyme inhibition; DNA polymerase β

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Supporting Information. Experimental procedures and spectroscopic data of new compounds. Charts showing carboxylic acids used to prepare inhibitor libraries and the preliminary hits, demonstration that **13** does not inhibit Ape1, DNA damage in HeLa cells by bleomycin, reaction of dRP with ARP, concentration dependence of inhibition of strand displacement synthesis by **13**, and effect of pro-**1** in HeLa cells. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

A variety of potentially cytotoxic endogenous and exogenous agents target DNA and modify its structure. The structural modifications include single strand breaks, modified nucleotides, and abasic sites.^{1,2} The base excision repair (BER) pathway mitigates the cytotoxicity of these agents by removing the damage and restoring the DNA to its original structure.^{3,4} While BER is critical for maintaining the genome integrity of healthy cells, it is a hindrance for anti-cancer modalities that target DNA.⁵ Consequently, the enzymes involved in BER are inhibition targets.^{6–12} There are an increasing number of examples of BER enzyme inhibitors that potentiate the effects of cytotoxic DNA damaging agents.^{13–17} We have identified an irreversible inhibitor of DNA polymerase β (Pol β) that works synergistically with methyl methanesulfonate (MMS) to kill HeLa cells.

Pol β , a member of the X-family of polymerases, is a vital component of the BER pathway (Scheme 1), and cells lacking functional enzyme are not viable.^{18, 19} Pol β is often over expressed in tumor cells and is mutated in ~30% of human cancers.²⁰ The enzyme is responsible for two steps during BER (Scheme 1); in addition to filling in nucleotide gaps produced during the repair process, Pol β excises the remnants of AP sites (dRP) following 5'-incision by apurinic endonuclease 1 (Ape1). The lyase activity on dRP protects cells against cytotoxic agents.^{21–25} The AP sites are themselves produced via glycosidic bond hydrolysis either spontaneously or by one of several BER glycosylases present in cells.^{19, 26} The single nucleotide gap produced in DNA by 5'-dRP excision is filled following transfer of the DNA to the polymerase active site. DNA polymerase λ (Pol λ) is also an X-family polymerase. It too possesses 5'-dRP lyase activity, albeit kinetically less efficient than Pol β , and is believed to act as a back up to Pol β .^{27–30}

Pol β is an interesting therapeutic target because its expression is up-regulated and/or it is mutated in many tumor cells. For instance, Pol β is mutated in as many as 40% of colon cancers.²⁰ Some mutated forms of Pol β retain activity, providing a possible tumor specific target. The potential benefit of Pol β inhibition has spurred the development of inhibitors, but there is room for improvement. The most effective inhibitors exhibit IC₅₀'s in the low micromolar range, and in some instances the selectivity for Pol β over other enzymes is not known and/or whether the molecule targets the polymerase or lyase activity.^{7–9} The approach described herein was inspired by the discovery that lesions produced by anti-tumor agents that oxidatively damage DNA irreversibly inhibit Pol β and Pol λ (Scheme 2).^{30–33} C4-AP and/or DOB are produced by C4'- and C5'-hydrogen atom abstraction, respectively. The polymerases recognize DOB directly and C4-AP (pC4-AP) following DNA incision by Ape1. The lesions contain a common 1,4-dicarbonyl motif, which results in the formation of a covalent adduct with one or more lysine residues within the lyase active sites of Pol β and Pol λ . The 1,4-dioxobutane moiety was incorporated into a library of small molecules that were screened for inhibition of Pol β , resulting in identification of **1** that irreversibly inhibits Pol β with an IC₅₀ = 21 μ M.³⁴ In addition, the proinhibitor (pro-**1**) potentiates the cytotoxicity of the DNA alkylating agent methyl methanesulfonate (MMS). When employed together, pro-**1** (20 μ M) and MMS (~0.3 mM) are at least 3-fold more potent against DU145 prostate cancer cells than expected based upon the cytotoxicity of the individual molecules. Irreversible inhibition of Pol β by targeting its lyase active site is a path less traveled in the search for inhibitors, and **1** served as the starting point for the current investigation.

RESULTS AND DISCUSSION

Design and Preparation of Second Generation Pol β Inhibitors

Inhibitor **1** and the corresponding proinhibitor (pro-**1**) were selected from a molecular library in which structural diversity was introduced at the 3'-terminus of the nucleotide. In the second-generation library of inhibitors described herein the methyl group of thymidine was functionalized with the goal of probing a different region of the protein-binding site. The library was prepared by synthesizing amides from amine **2** (Scheme 3). HBTU activation in the presence of HOBt was used to form the amide bond in pro-**3**. The acetate protecting groups were then removed using $\text{BF}_3 \cdot \text{Et}_2\text{O}$ in wet (2% H_2O) acetonitrile. Quenching the deprotection reaction with phosphate buffered saline was important for preventing inhibitor decomposition. An advantage of using the bis-acetate protecting group over the previously employed pentenyl acetal is that we anticipated using the immediate precursor (pro-**3**) as the proinhibitor in cells.³⁴

The requisite amine (**2**) was initially prepared from previously reported **4** (Scheme 4).^{34, 35} The azide group (**5**) was introduced in modest yield via the bromide, which was used crude.^{36, 37} The oxime containing a protected phenol was then introduced (**6**), followed by detritylation of the primary alcohol to yield **7**. The requisite aldehyde (**9**) used in the preparation of **7** was prepared from **8**. It was necessary to protect the phenol hydroxyl of **8** to prevent it from competing with the primary alcohol in **7** during phosphoramidite coupling with **11**. The latter was prepared from previously reported **10**.³⁴ The phosphoramidite was introduced via the protected dioxobutane component (**10**) to prevent intramolecular reaction with the azide. The successful coupling of phosphoramidite **11** with azide containing **7**, followed by in situ oxidation to the phosphate triester was consistent with previous reports in which these functional groups that could react with one another are successfully combined in oligonucleotide synthesis.³⁸ During the library synthesis the β -cyanoethyl and TBS groups in **12** were cleaved using Et_3N base. Azide **12** was the last intermediate that was purified prior to library synthesis, as the amine (**2**) used for preparing the amides obtained via hydrogenation was used crude. Following identification of a candidate inhibitor (**13**, below), the final sequence of steps for transforming into the bisacetate protected inhibitor

(pro-inhibitor, pro-**13**) were altered. Reducing the azide first, and cleaving the phosphate and phenol protecting groups after amide formation provided higher yield of more readily purified product that was used in subsequent experiments as a mixture of diastereomers.³⁹

Identification and Characterization of a Pol β Inhibitor (**13**)

Inhibitor candidates (**130**)³⁹ were screened using a previously reported assay that capitalizes on the ability of Pol β to carry out strand displacement synthesis (See Scheme S1).^{34, 40} Inhibition of strand displacement synthesis results in decreased fluorescence growth compared to the absence of inhibitor. The screening conditions were the same with the exception of the inhibitor concentration. The oxime library that yielded **1** was screened using 50 μM of candidates, whereas **3** were present at 10 μM . Of the 130 candidates screened, 5 completely shut down fluorescence growth at 10 μM when preincubated with Pol β (5 nM). The 5 most promising candidates bore little resemblance to one another other than that they were aromatic carboxylic acids.³⁹ Their inhibition of Pol β was examined at as low as 1 μM using the fluorescence assay, which identified **13** as the most promising candidate.³⁹ Candidate **13** was resynthesized, spectroscopically characterized, and inhibition by it was directly quantified.³⁹

Irreversible inhibition of Pol β lyase activity was determined using 3'-³²P-dRP substrate **14**. Preincubation of Pol β with **13** (3 μM) for 30 min resulted in 99% loss of lyase activity, which was not recovered following dialysis for 1 day (Figure 1A). Additional evidence for Pol β inactivation was obtained by examining the remaining lyase activity as a function of preincubation time and concentration of **13** (Figure 1B). A modest decrease in Pol β activity was observed when the enzyme was preincubated with 0.25 μM **13**, but much greater activity loss was observed upon less than 30 min preincubation times in the presence of 1.0 or 1.5 μM inhibitor (Figure 1B). The data in Figure 1B indicate that **13** inactivates Pol β

significantly more effectively than **1**.³⁴ Comparing the IC₅₀'s of the respective compounds reinforced this comparison. Upon preincubating **1** (21 μ M) or **13** for 30 min with Pol β , the IC₅₀ (0.4 μ M) of the latter was more than 50-times lower than the previously reported inhibitor (Figure 1C). In addition, to marking a significant improvement over **1**, the activity of **13** against Pol β compares favorably to other previously reported inhibitors, some of which also target the lyase domain.⁹ The IC₅₀ for Pol λ inactivation by **13** was even lower (0.25 μ M, Figure 1D). Although this was surprising, it would be helpful in cellular toxicity studies if **13** inactivated Pol β and its back-up repair enzyme.

Potentialiation of DNA Damaging Agent Cytotoxicity in HeLa Cells

Previous studies showed that proinhibitor pro-**1** was converted to **1** (presumably by cellular esterases), and was more effective at inhibiting lyase activity on **15** in prostate cancer cell (DU145) lysates. Furthermore, pro-**1** (20 μ M) yielded more than a 3-fold enhancement in MMS (0.2 mM) cytotoxicity.³⁴ Herein, we examined the effect of pro-**13** on bleomycin (BLM) and MMS cytotoxicity in HeLa cells using a clonogenic assay. BLM oxidatively damages DNA by abstracting hydrogen atoms from the C4'-position of pyrimidines.^{41–44} Oxidation results in direct strand breaks and the alkali labile C4-AP oxidized abasic site, a known irreversible inhibitor of Pol β and Pol λ .^{30, 31} BLM also produces double-strand breaks.^{45, 46} In contrast MMS is an alkylating agent, which largely reacts with the N7-position of dG.^{47–49} Although BLM and MMS react very differently with DNA, both activate base excision repair, and therefore Pol β is involved.

The proinhibitor (pro-**13**) exhibits modest cytotoxic effects on HeLa cells. Exposing cells to 5 μ M pro-**13** for 1 h results in 2% cell death and 93% of the cells survive when exposed for 2 h. No observable cell death is detected when HeLa cells are exposed to BLM (2 μ M) for 2 h, followed by a 2 h recovery time (Figure 2C), although a Comet assay confirms DNA damage.³⁹ In contrast, only 43% of the cells survive, a more than 2-fold decrease in fraction cell survival, when pro-**13** (5 μ M) and BLM (2 μ M) are co-incubated. The potentiation of MMS cytotoxicity by pro-**13** was greater in HeLa cells (Figure 2A,B) when compared to that of BLM.

HeLa cells were treated with 0.1 mM (Figure 2A) or 0.2 mM (Figure 2B) MMS for 1 or 2 h with and without pro-**13** (5 μ M). The potentiation of MMS cytotoxicity was greater under all conditions than what was observed using BLM. The cytotoxic effects of MMS and BLM with and without pro-**13** were most similar under the mildest conditions (0.1 mM MMS, 1 h, Figure 2A) examined. Increasing the MMS exposure time to 2 h increased the overall cytotoxicity, but potentiation by pro-**13** was greater as well (Figures 2A,B). Individual treatment with pro-**13** (5 μ M) or MMS (0.1 mM) for 2 h resulted in 7% and 15% cell death, respectively (Figure 2A). The synergistic effects of pro-**13** and MMS lead to average fraction survival of 0.23, which is ~3.4-fold lower than that expected based upon the cytotoxic effects of the agents used separately. An even greater potentiation of MMS cytotoxicity by pro-**13** was observed at longer reaction time when using 0.2 mM of the alkylating agent (Figure 2B). Fewer than 2% of the HeLa cells survived when pro-**13** was included compared to 25% when MMS was used without the proinhibitor, accounting for an almost 13-fold enhancement in cytotoxicity compared to the individual agents. To directly

compare the efficacy of pro-**13** (5 μ M) with the effects of pro-**1** (5 μ M) on MMS cytotoxicity in HeLa cells, comparable experiments were carried out with the latter.³⁹ Pro-**1** was significantly more cytotoxic, killing 40% of the cells after 2 h incubation. In addition, no synergism was observed with MMS under any of the above concentration and time conditions, indicating that pro-**13** is a superior adjuvant to MMS in HeLa cells.

Co-administration of pro-**13** and MMS Increases AP/dRP Levels in HeLa Cells

Evidence that pro-**13** leads to BER inhibition in HeLa cells was obtained by determining its effect on the level of aldehyde reactive probe (ARP) sites (Table 1). ARP is routinely used to quantify AP sites in DNA, and has been employed to validate Ape1 inhibition, but we were unaware of dRP detection via this method.^{15, 16, 50, 51} Inhibitor **13** does not affect Ape1 activity on a duplex containing AP.³⁹ Hence, any increase in ARP reactive sites would presumably result from reaction with dRP. Inhibition of Pol β (and/or Pol λ) should lead to a build-up of dRP sites instead of AP, which would be indistinguishable from AP using ARP (Scheme 5). Although one would assume that the more labile dRP reacts with ARP, we demonstrated that this is indeed the case using a ternary complex containing the former (**14**).³⁹ We subsequently measured the level of AP/dRP sites in the genomic DNA isolated from HeLa cells treated with MMS, pro-**13** or a combination. Following treatment with the DNA damaging agent (1 h), the medium was replaced with fresh medium with or without pro-**13** (5 μ M), and the cells were incubated an additional 23 h. The level of ARP reactive sites increased ~3-fold when cells were treated with a combination of MMS and pro-**13** compared to just the alkylating agent (Table 1), consistent with the inhibition of BER in cells treated with the proinhibitor.

DNA Repair Inhibition After Removing MMS Increases Cell Death

The AP/dRP detection in genomic DNA isolated from HeLa cells (Table 1) suggests that the inhibitor significantly prolongs the lifetime of these lesions in cells. We postulated that unrepaired lesions would induce cell death in the absence of DNA damaging agent. Consequently, HeLa cells were treated with MMS (0.1 mM) for 2 h, with or without pro-**13** (5 μ M) (Figure 2D). These conditions produce modest levels of cell death (Figure 2A); even with inhibitor the survival fraction is 0.21 ± 0.04 . After 2 h, the media from cells treated with MMS was replaced with media lacking the alkylating agent. Samples containing cells treated with MMS and pro-**13** were treated similarly. However, the replacement media for a third set of cells that were treated with the above mixture of MMS and proinhibitor contained pro-**13** (5 μ M). The 3 sets of cells were then incubated for up to 8 h before plating for the clonogenic assay (Figure 2D). No significant additional cell death occurs if the replacement media lacks pro-**13**, regardless of whether the cells were initially treated with MMS or proinhibitor and alkylating agent. However, the fraction of surviving cells continually decreases as the incubation period increases when the replacement media contains pro-**13** (5 μ M). After 4 h additional incubation with the proinhibitor, the fraction cell survival is $1/7^{\text{th}}$ as much as those incubated in the absence of pro-**13**. Finally, following 8 h of additional incubation, only 0.20 ± 0.02 % of the cells survive when incubated in the presence of pro-**13**. This is a 100-fold smaller fraction of cells that survive following initial co-treatment with MMS and pro-**13**.

CONCLUSIONS

Disrupting the base excision repair pathway sensitizes cells to DNA damaging agents. DNA repair enzyme inhibitors that can be used as adjuvants to nucleic acid damaging molecules are potentially useful as therapeutic agents.^{23–25} DNA polymerase β is an attractive target because this enzyme is required for cell viability. A structurally diverse group of Pol β inhibitors have been identified.^{7, 9} Some of these molecules target the polymerase active site, while others such as the molecule examined here inhibit the enzyme's lyase activity. Comparison of inhibitors from independent studies is challenging due to different cell types, DNA damaging agents employed, and inhibition mechanism.^{9, 13, 17} Nucleic acid lesions produced by highly cytotoxic DNA damaging agents that inactivate Pol β (and Pol λ) have provided the inspiration for the design of the small libraries of irreversible inhibitors used in our research. Inhibitors that target the lyase activity of Pol β are less common than those that affect the polymerase site.⁹ Irreversible inhibitors are even more rare. Inhibiting Pol β lyase activity results in concomitant build-up of cytotoxic dRP, increasing the attractiveness of such inhibitors.¹⁹ Inhibitor **13**, which is 50-fold more effective at inactivating Pol β than a first generation molecule (**1**) based on this approach, was identified in the current study.³⁴ Using previous studies comparing the effects of **1** and pro-**1** in cells as a guide, experiments in HeLa cells were carried out using a proinhibitor of **13** (pro-**13**). Pro-**13** (5 μ M) is itself only weakly cytotoxic, but exerts synergistic effects on MMS cytotoxicity. Pro-**13** potentiated MMS cytotoxicity in HeLa cells as much as ~13-fold. The previously reported proinhibitor (pro-**1**) showed no synergistic effect with MMS in HeLa cells under identical conditions. Furthermore, administration of pro-**13** in fresh media to cells previously co-treated with MMS and the proinhibitor under conditions that result in modest toxicity increases cell death ~100-fold, yielding 0.2% HeLa cell survival. The extent of this potentiation of MMS cytotoxicity by pro-**13** is greater than any previously reported BER enzyme repair inhibitor that we are aware of. Furthermore, the concentration at which pro-**13** is effective in HeLa cells is comparable to or superior to even the most recent reported Pol β inhibitors.^{7, 9, 13} These observations indicate that Pol β inactivation is a promising approach for potentiating DNA damaging agents.

METHODS

General methods

All reactions were run in Pol β reaction buffer: 50 mM HEPES (pH = 7.5), 5 mM MgCl₂, 0.2 mM EDTA, 50 mM KCl and 0.01% Tween 20 and analyzed by 20% denaturing PAGE. Quantitative analysis was carried out using a Storm 840 phosphorimager and ImageQuant TL software.

Library Synthesis (pro-3)

A solution of **12** in water (0.1 mL, 45 mM, 4.5 μ mol) was diluted with 1:1 methanol-ethanol (2 mL). Pd/C (2.2 mg) was added and the mixture was stirred under a blanket of H₂ for 3 h. The suspension was filtered through a pad of celite (1 $\times$ 2 cm) and washed with methanol. The combined filtrate and washings were concentrated under reduced pressure and the amine (**2**) was dissolved in 0.1 mL H₂O; HRMS (ESI) *m/z* calculated for C₂₇H₃₂N₄O₁₅PCl₂

(M-H)⁻ 753.0979, observed 753.0943). The crude amine was used to prepare the library. Carboxylic acids (10 μ L, 0.1 M in DMF, 1.0 μ mol) were activated in 96 well plates by adding HOBt (2.9 μ L, 0.4 M in DMF, 1.16 μ mol), HBTU (4.5 μ L, 0.2 M in DMF, 0.9 μ mol) and 20% DIPEA in DMF (2.7 μ L) to a well. The wells were capped and the plate was incubated at room temperature for 3 h. Aliquots (2.5 μ L, 50 mM, 0.12 μ mol) from the activated acid solutions were then added to a different 96 well plate **2** (7.5 μ L, 13.5 mM in DMF, 0.1 μ mol) in each well and mixed. The plate was covered with an aluminum lid and incubated at room temperature overnight. The reaction mixtures were then quenched by adding 0.1 mL/well H₂O and evaporated to dryness *in vacuo* to obtain pro-**3**, which was used directly in the next step without purification.

Deprotection to Produce **3** for Library Screening

The plate containing the library of pro-**3** was treated with ACN containing 2% H₂O (30 μ L/well) and a solution of BF₃·Et₂O in ACN (3 μ L, 0.56 M, 1.7 μ mol) and contents of each well were mixed. The plate was covered with an aluminum lid and incubated at room temperature for 1.5 h. The reaction mixture in each well was diluted to 150 μ L with phosphate buffer (20 mM, pH 7.2) containing NaCl (0.2 M). These solutions (0.675 mM) were used in the fluorescence screening assay and stored at -20 °C.

Screening of the inhibitors

A working solution of Pol β (125 nM, 200 μ L) was prepared in 1 $\times$ Pol β reaction buffer (50 mM HEPES buffer pH = 7.5, 5 mM MgCl₂, 0.2 mM EDTA, 50 mM KCl and 0.01 % Tween 20), containing BSA (0.1 mg/mL), and 30% glycerol (in autoclaved water). Pol β (8.0 μ L, 125 nM) was added to each well of a 96 well fluorescence spectrometer plate containing a solution of a different inhibitor (11.6 μ M, 172 μ L) in 1 $\times$ Pol β reaction buffer and the mixtures were pre-incubated for 25 min at room temperature. The pre-incubation mixtures were subsequently diluted with a solution (20 μ L) containing **14** (500 nM) and dTTP (1 mM) in 1 $\times$ Pol β reaction buffer and fluorescence was monitored for ~100 min.

Time dependent inactivation of Pol β by **13**

A working solution of Pol β (12.5 nM) was prepared in 1 $\times$ Pol β reaction buffer containing 30% glycerol and kept at 0 °C during the experiment. Pol β (2 nM) was pre-incubated with **13** (0, 0.25, 1.0, 1.5 μ M) in 1 $\times$ Pol β reaction buffer at room temperature. Aliquots (24 μ L) were withdrawn at the appropriate time intervals depending on the concentration of **13**, and added to **15** (2 μ L, 2.5 μ M) in 1 $\times$ Pol β reaction buffer to start the lyase reactions. Aliquots (4 μ L) were removed from the individual reactions at the indicated time points (2, 5, 10, 15, 20, 25 min) and stabilized by reducing unreacted **15** with NaBH₄ solution (2 μ L, 0.5 M). The aliquots were kept at 4 °C for 1 h and at room temperature for 3 h before mixing with loading buffer (6 μ L) and separating by 20 % denaturing PAGE.

Effect of dialysis on Pol β inactivated by **13**

Pol β (3 nM) in 1 $\times$ reaction buffer was pre-incubated in the absence or presence of **13** (3 μ M) for 30 min at room temperature. The Pol β lyase activity of each sample was immediately measured using **15** as described above. Three aliquots (500 μ L) were dialyzed

in reaction buffer (2×1 L; buffer was exchanged after 12 h) containing 10% glycerol for 24 h. The remaining lyase activity of the enzyme was measured (24 μ L aliquots, after 0 and 1 day) using **15** (2 μ L, 2.5 μ M) as described above. Aliquots (4 μ L) were removed at indicated time points (2, 5, 10, 15, 20, 25 min) and stabilized with NaBH₄ solution (2 μ L, 0.5 M). The aliquots were kept at 4 °C for 1 h and at room temperature for 3 h before mixing with loading buffer (6 μ L) and separating by 20 % denaturing PAGE.

IC₅₀ value for **13** against Pol β

A working solution of Pol β (62.5 nM) was prepared in $1 \times$ Pol β reaction buffer containing 30% glycerol and kept at 0 °C during the experiment. Pol β (2.5 nM) was preincubated with **13** (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0 μ M) for 30 min in $1 \times$ Pol β reaction buffer. All of the reaction mixtures contained 0.75% acetonitrile by volume. Aliquots (24 μ L) were mixed with **15** (2 μ L, 2.5 μ M) in $1 \times$ Pol β reaction buffer to start the lyase reactions. Aliquots (4 μ L) were removed from the individual reactions at the indicated time points (2, 5, 10, 15, 20, 25 min) and stabilized by reducing unreacted **15** with NaBH₄ solution (2 μ L, 0.5 M). The aliquots were kept at 4 °C for 1 h and at room temperature for 3 h before mixing with loading buffer (6 μ L) and separating by 20 % denaturing PAGE. The relative activities were fit to the following sigmoidal equation: Relative Activity = $A_2 + [(A_1 - A_2)/(1 + (x/x_0)^p)]$, where x = concentration of inhibitor, A_2 = minimum enzyme activity, A_1 = maximum enzyme activity, x_0 = IC₅₀, and p — Hill slope, which characterizes the slope of the curve at its midpoint. The data were fit iteratively using Origin 6.1.

IC₅₀ value for **13** against Pol λ

A working solution of Pol λ (125 nM) was prepared in $1 \times$ Pol β reaction buffer containing 30% glycerol and kept at 0 °C during the experiment. Pol λ (15 nM) was pre-incubated with **13** (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.2 μ M) for 30 min in $1 \times$ Pol β reaction buffer. All of the reaction mixtures contained 0.25% acetonitrile by volume. Aliquots (24 μ L) were mixed with **15** (2 μ L, 2.5 μ M) and reactions and analysis were carried out as described above for Pol β .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Irreversible inhibition by **13**. (A.) Normalized lyase activity of Pol β prior to and after dialysis (24 h) following incubation with or without **13** (3 μM). (B.) Relative Pol β lyase activity as a function of [13] and preincubation time of inhibitor with enzyme. (C.) IC_{50} of **13** on Pol β (D.) IC_{50} of **13** on Pol λ .

Figure 2.

Potentiation of DNA damaging agent cytotoxicity in HeLa cells by pro-13 (5 μ M). (A.) MMS (0.1 mM) (B.) MMS (0.2 mM) (C.) BLM (2 μ M) (D.) Effect of pro-13 on HeLa cell cytotoxicity during recovery time following removal of the MMS. Black bars: MMS (0.1 mM), 2 h; Light gray bars: MMS (0.1 mM) + pro-13, 2 h; Dark gray bars: MMS (0.1 mM) + pro-13 for 2 h, followed by additional incubation with pro-13 for the recovery time shown. Values shown are the ave. $\pm$ std. dev. of 3 replicates.

Scheme 1.
Base excision repair.

Scheme 2.
Pol β inactivation by oxidized abasic sites.

Scheme 3.

General library synthesis.

Key: a) HBTU, HOBT, RCO₂H b) BF₃·Et₂O, CH₃CN/H₂O

Scheme 4.

Synthesis of inhibitor library precursor (2).

- a) NBS, AIBN b) NaN_3 c) Hydrazine d) **9** e) $\text{AcOH}/\text{H}_2\text{O}$ f) $t\text{-BuMe}_2\text{SiCl}$ g) Phosphitylation
h) i. *S*-Ethyl tetrazole ii. $t\text{-BuOOH}$ i) Et_3N j) H_2 , Pd/C

Scheme 5.
AP and dRP detection by ARP.

Table 1

Increased AP/dRP content in DNA from HeLa cells treated with MMS and pro-13.

MMS (mM)	pro-13 (μ M)	AP/dRP sites/ 10^5 bp ^a
–	–	3.8 ± 0.9
–	5	4.6 ± 1.0
0.3	–	9.2 ± 1.2
0.3	5	28.0 ± 1.4
0.4	–	11.0 ± 1.5
0.4	5	33.0 ± 1.5

^a Average $\pm$ std. dev. of 3 independent experiments