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Protein Domain Specific Covalent Inhibition of Human DNA Polymerase β

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DNA polymerase β (Pol β) is a frequently overexpressed and/or mutated bifunctional repair enzyme. Pol β possesses polymerase and lyase active sites, that are employed in two steps of base excision repair. Pol β is an attractive therapeutic target for which there is a need for inhibitors. Two mechanistically inspired covalent inhibitors (**1**, $IC_{50} = 21.0$ μM; **9**, $IC_{50} = 18.7$ μM) that modify lysine residues in different Pol β active sites are characterized. Despite modifying lysine residues in different active sites, **1** and **9** inactivate the polymerase and lyase activities of Pol β. Fluorescence anisotropy experiments indicate that they do so by preventing DNA binding. Inhibitors **1** and **9** provide the basis for a general approach to preparing domain selective inhibitors of bifunctional polymerases. Such molecules could prove to be useful tools for studying the role of wild type and mutant forms of Pol β and other polymerases in DNA repair.

Bifunctional DNA polymerase β (Pol β) is involved in single- and double-strand break repair. Pol β carries out two of the 5 steps during base excision repair of modified nucleotides (Scheme 1).[1] More recently, Pol β was found to be associated with the double-strand break repair pathway, alternative non-homologous end joining.[2] The attractiveness of Pol β as a target is further increased by the possibility that its inhibition is synthetic lethal in cells that are deficient in the tumor suppressor BRCA1.[3] Although a number of Pol β inhibitors have been described, there remains a need for additional tools for modulating the activity of this enzyme.[4] Previously reported Pol β inhibitors often lack selectivity and/or binding information. We have developed a mechanism-based approach for identifying covalent Pol β inhibitors.[5] Two molecules that inactivate Pol β by recognizing different structural domains but utilize a common mechanism, are described below.

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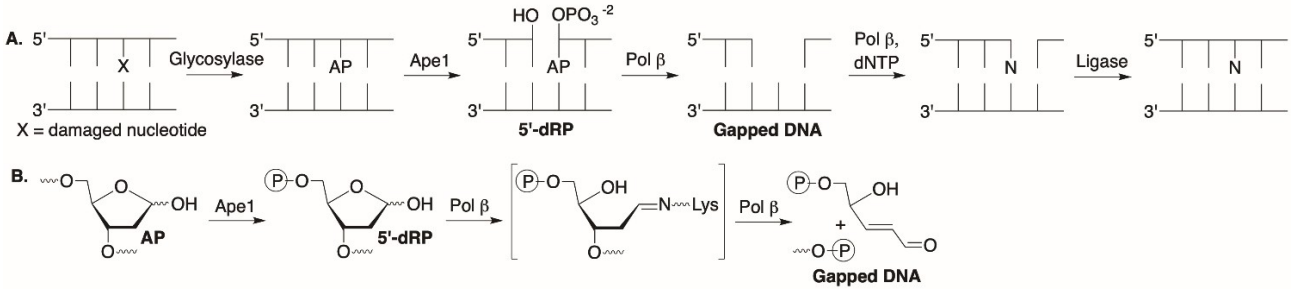
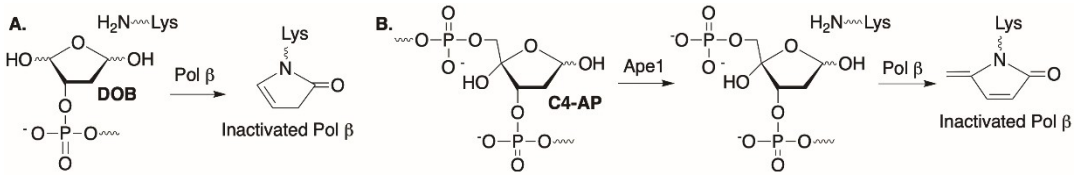
Pol β engages with damaged DNA during base excision repair following hydrolysis of the glycosidic bond of a damaged nucleotide by a glycosylase, and incision by apurinic endonuclease 1 (Ape1) (Scheme 1). Bifunctional Pol β contains DNA polymerase and lyase active sites in separate domains. Lyase activity resides within an 8 kDa N-terminal domain. Polymerization is catalyzed by a 31 kDa domain, whose structure includes the characteristic features similar to other polymerases.[6] Inhibitors that selectively target one domain over the other could be valuable tools. For instance, Pol β is mutated in a large number of cancers, including ~40% of colorectal cancers.[7] Targeting the domain that is not mutated would enable one molecule to inhibit wild type and mutant forms of Pol β. Alternatively, since Pol β is present in normal and cancerous cells, selectively targeting a mutated domain would be useful for inhibiting Pol β in the latter cell type, which would be attractive from a therapeutic standpoint.

Our research group was inspired by the discovery that an oxidized abasic site (DOB) and the structurally related C4-AP (following incision by Ape1), produced by antitumor agents that oxidatively damage DNA irreversibly inhibit Pol β (Scheme 2).[8] A methylene group was inserted between the dioxobutane and phosphate group to stabilize the inhibitor. Consequently, we synthesized and screened libraries of small molecules that were designed to form adducts with lysine but not undergo elimination. This resulted in the identification of covalent Pol β inhibitors (Scheme 3) such as **1** and **2**, the corresponding proinhibitors of which (*pro*-**1**, *pro*-**2**) work synergistically with DNA damaging agents to kill cells.[5a,d] Inhibitor **2** prevents DNA binding by modifying lysine 113 or 234 in the polymerase domain.[5d] However, the mechanism of action of **1** was not reported.[5a] For a number of reported Pol β inhibitors, the domain recognized and/or mechanism of action are unknown.

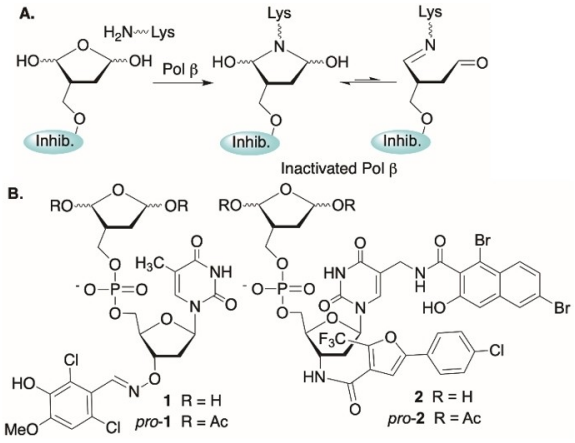
With the goal of applying the strategy employed for identifying covalent inhibitors **1** and **2**, we prepared a library of candidates in which the C5-pyrimidine position was the sole site of diversification. This position was used as a site for introducing structural diversity during the identification of **2**, and is desirable because C5-substitued pyrimidines are frequently compatible with polymerases. The library was synthesized (Scheme 4) by reducing **3** and protecting the amine as its trifluoroacetamide (**4**). The use of the trifluoroacetamide avoided azide reduction by the phosphoramidite in the subsequent steps. Following deprotection of the primary alcohol and phosphitylation, **5** was coupled with the bis-pentenyl acetal of the ultimate DOB electrophile (**6**) as a mixture of diastereomers.[5a,d] A stereoisomeric mixture of **6** was used because it was anticipated that the inhibitor candidates



Scheme 1. Base excision repair. A. BER is a five-step process. B. Pol β excises 5′-dRP.



Scheme 2. Irreversible inhibition of Pol β by oxidized abasic sites.



Scheme 3. Lysine modification by irreversible Pol β inhibitors. A. Reaction of 1,4-dioxobutane with lysine. B. Previously reported inhibitors and proinhibitors.

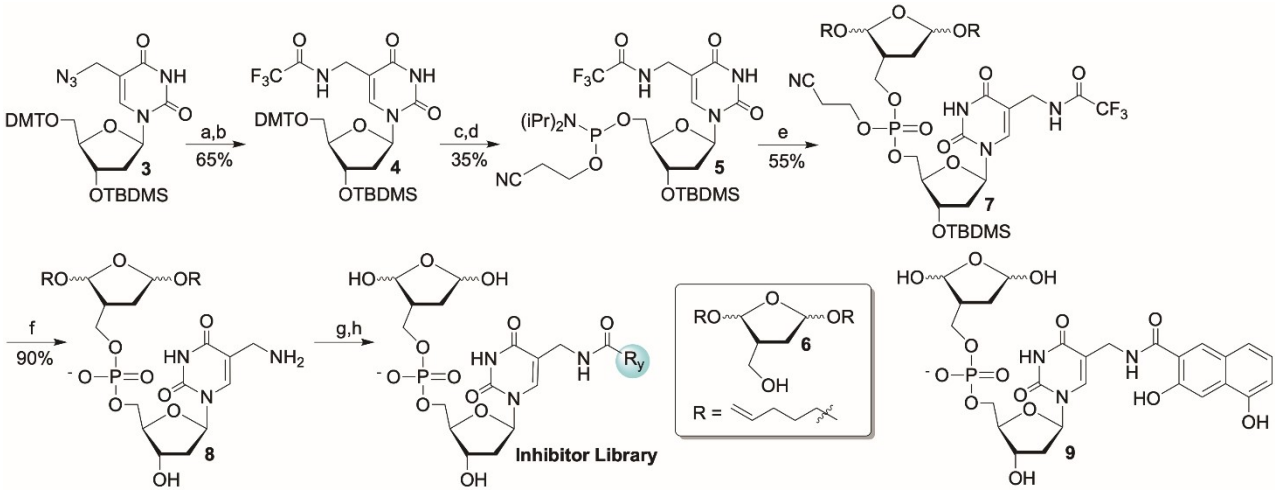
would readily epimerize in aqueous buffer. The substrate for introducing structural diversity (**8**) was obtained by desilylating the secondary alcohol, followed by cleavage of the phosphate and amine protecting groups.

Individual library candidates were prepared by coupling the carboxylic acids (Chart S1) in microtiter plates. A portion of each unpurified bis-acetal protected amide was transferred to a second microtiter plate and treated with aqueous *N*-bromosuccinimide to release the inhibitor candidate.[9] The ability of crude, freshly prepared candidates to inhibit strand displacement synthesis by Pol β was screened using a fluorescent assay (Figure S1).[10] Two preliminary hits were independently synthesized and their inhibition of strand displacement synthesis was examined as a function of concentration (Figure S2a). Their effects on dRP lyase activity was also examined (Figure S2b). Compound **9** was the superior candidate in both assays, and Pol β inhibition by it was characterized more rigorously.

Confirmation that **9** irreversibly inhibits Pol β was obtained by examining Pol β activity before and after dialysis (Figure S3). No Pol β lyase activity was recovered following inactivation by **9**. The IC_{50} for lyase inhibition of Pol β by **9** (preincubation = 30 min) was 18.7 μM (Figure 1A), which was very similar to that reported by **1** (21 μM) under the same conditions.[5a] The IC_{50} for **9** is considerably greater than that of **2** (< 1 μM), but this was to be expected as the latter is the product of 2 rounds of library selection during which the structure at the 3′-terminus and C5-pyrimidine regions were sequentially diversified. A difference between **1** and **9** was revealed when the inhibitors' effects on individual Pol β domains were compared to those on the intact wild-type enzyme (Figure 1B, C). Consistent with the IC_{50} measurements, treating Pol β with **1** and **9** under the same conditions (30 μM inhibitor, 30 min preincubation) time, had comparable effects on lyase activity.

Compound **9** demonstrated a similar effect when the 8 kDa domain of Pol β was examined (Figure 1B). However, **1** had no effect on the isolated lyase domain (Figure 1C). Furthermore, while **1** and **9** inhibited strand displacement synthesis by Pol β, only the former inhibited this activity by the 31 kDa domain of Pol β (Figure S4).

These data suggested that although inhibitors **1** and **9** inactivate the polymerase and lyase functions of Pol β, they were doing so by binding to different domains of the protein. This hypothesis was examined by preincubating Pol β separately with **1** and **9**, and analyzing the trypsin digests by LC-MS/MS (Figure 2, and Figures S5, S6). Indeed, these experiments revealed that although each inhibitor modified two lysines, the residues resided in different protein domains. The proposed lysine adducts (Scheme 3A) are expected to exist as an equilibrium mixutre of ring opened and closed isomers. In



Scheme 4. Synthesis of a library of inhibitor candidates. a) H_2, Pd/C. b) Ethyl trifluoroacetate. c) Trichloroacetic acid. d) 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA. e) i. *S*-Ethyl tetrazole, **6**; ii. *t*-BuOOH. f) i. $Et_3N \cdot 3HF$; ii. NH_4OH. g) R_yCO_2H (y = 1-325), HBTU, HOBt. h) NBS, H_2O.

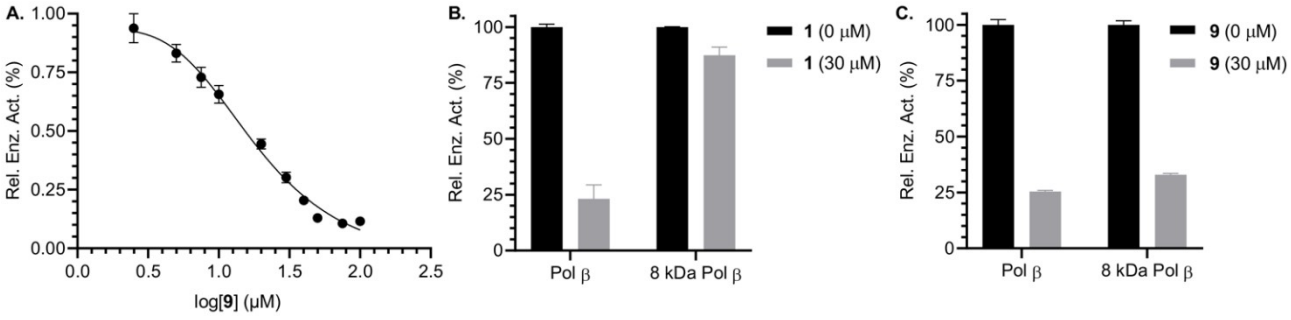


Figure 1. Irreversible inhibition of Pol β lyase activity. A) IC_{50} of Pol β inactivation by **9**. B) Lyase inactivation of Pol β and the 8 kDa domain of Pol β by **1**. C) Lyase inactivation of Pol β and the 8 kDa domain of Pol β by **9**.

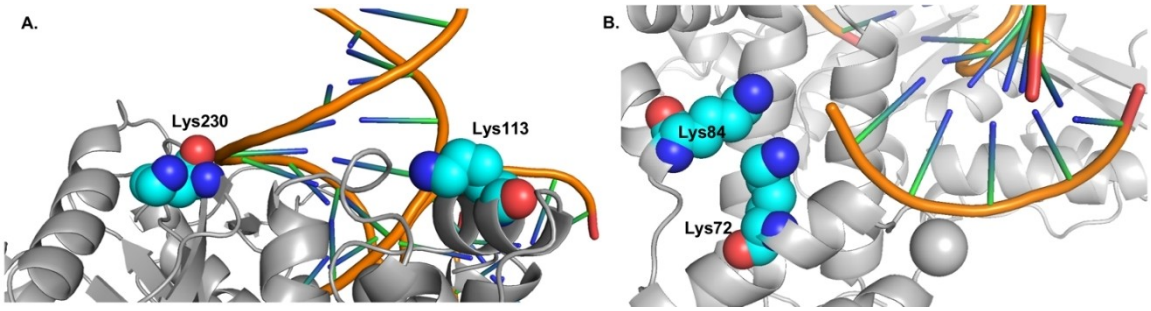


Figure 2. Covalently modified amino acid residues in Pol β (PDB: 1BPX) by A) **1** and B) **9**.

support of this, modified peptides containing either the ring closed bis-hemiaminal form or the ring opened dehydrated isomer were detected (Figures S5, S6). Compound **1** modified two residues, Lys113 and Lys230, proximal to where Pol β interacts with DNA in the polymerase active site. In contrast, **9** modified two residues, Lys72 and Lys84, in the lyase binding site. Lys72 is believed to be the primary nucleophile responsible for Schiff base formation during 5′-dRP excision (Scheme 1) by Pol β.[11] Studies utilizing mutant forms of Pol β indicate that Lys84 plays a secondary role in this process.[11] Although each inhibitor was found to modify two lysines within one Pol β active site, we believe that modification of only one lysine is sufficient for inactivation. This proposal is in agreement with the concentration dependence (Figure 1A) of inhibition.[5a] The locations of the modified lysines suggested that the mechanism of action for **1** and **9** was to prevent DNA binding by Pol β.

This was verified using fluorescence anisotropy in which a fluorescently labeled ternary DNA substrate (0.25 nM) contain-

ing a stable abasic site analogue (See Supporting Information for the structure of the DNA complex) was incubated with various concentrations of intact Pol β, the 8 kDa lyase domain, or the 31 kDa polymerase domain. Both inhibitors prevented intact Pol β from binding DNA (Figure 3A, D). After preincubating the enzyme with **1** or **9**, it was not until intact Pol β was in several hundred-fold excess relative to DNA that fluorescence anisotropy indicated that the nucleic acid was completely bound. Equivalent treatment of the 31 kDa Pol β domain with **1** showed similar behavior (Figure 3C), consistent with modification of lysine residues in this region of the protein (Figure 2A). The magnitude of the change in fluorescence anisotropy when the 8 kDa domain of Pol β bound DNA was less than that observed upon binding the polymerase domain or intact Pol β. This was attributed to the relatively smaller mass of the protein fragment. Most importantly, **1** had no effect on DNA binding by the 8 kDa domain of Pol β (Figure 3B). This was in marked contrast to the effects of **9** on the fluorescence anisotropy induced by separate Pol β domains. Following preincubation with **9**, DNA binding by the 31 kDa domain was unaffected (Figure 3F). Yet, the same concentration of **9** exhibited an effect on DNA binding by the 8 kDa domain that was comparable to that imparted upon by intact Pol β (Figure 3E).

Covalent inhibitors **1** and **9** inactivate Pol β by modifying lysine residues that prevent DNA binding. However, they do so by recognizing different domains of this bifunctional enzyme. These and other molecules can be useful tools for probing the roles of this enzyme. Inhibitors that selectively bind one active site over another could also be invaluable for the design and identification of molecules that target cancer-associated Pol β mutants. One can target the active site that is not mutated. Alternatively, one could potentially discriminate between wild type and mutated Pol β by pursuing inhibitors that bind in the domain that is mutated. Furthermore, the approach or platform described could be a general one for inhibiting other bifunctional polymerases, including DNA polymerases ι,[12] λ[13] and θ.[14]

Experimental Section

General procedure for the preparation of inhibitor library: Amine scaffold **8** (100 nmol) was azeotropically dried with carboxylic acid (140 nmol, 1.4 eq) in pyridine (1×15 μL) using a Speed Vac concentrator in a 384-well microtiter plate. To each well, a 2× activating solution (5 μL; 28 mM HBTU and 28 mM HOBt in DMF), DIPEA (2 μL), and DMF (3 μL) were added. The final concentrations during reaction were: [**8**] = 10 mM, [acid] = 14 mM, [HBTU] = 14 mM, [HOBt] = 14 mM, 20% DIPEA in DMF. The well plate was shaken at 25 °C overnight. Random wells were analyzed by ESI-MS to confirm coupling efficiency. The solutions were concentrated using a Speed Vac concentrator and the well plate was covered and stored at −80 °C. Immediately before an assay, the amide was thawed, dissolved in DMF (4 μL, 25 mM). An aliquot (2 μL, 50 nmol) was mixed with NBS (8 μL, 15 mM, 2.4 eq, 40% H_2O in MeCN) at 0 °C for 9 min. The concentrations during reaction were: [SM] = 5 mM, [NBS] = 12 mM, 20% DMF, 3% H_2O in MeCN. After 4 min, sat. $Na_2S_2O_3$ (5 μL) was added and reaction quenched on ice for 10 min. Samples were concentrated with a Speed Vac concentrator. Random samples were analyzed by ESI-MS to confirm product formation.

General procedure for library screening: A 1 mM stock solution of each inhibitor is prepared using 50% MeCN in H_2O. A solution of Pol β (100 nM) was preincubated with library compounds (30 μM) in 1×reaction buffer (total volume: 50 μL; 50 mM HEPES buffer pH = 7.4, 5 mM $MgCl_2$, 0.2 mM EDTA, 50 mM KCl, 0.01% Tween 20, 0.01 mg/mL BSA, and 4% glycerol by volume) in a 384-well plate at 25 °C for 30 min. In control experiments, an equal volume of a control solution (containing all coupling and deprotection reagents but lacking inhibitor) was added to keep the percentage of solvents and reagents consistent. An aliquot (3 μL) was diluted with a 2× solution (15 μL) containing **TC2** (100 nM, Table S1) and dTTP

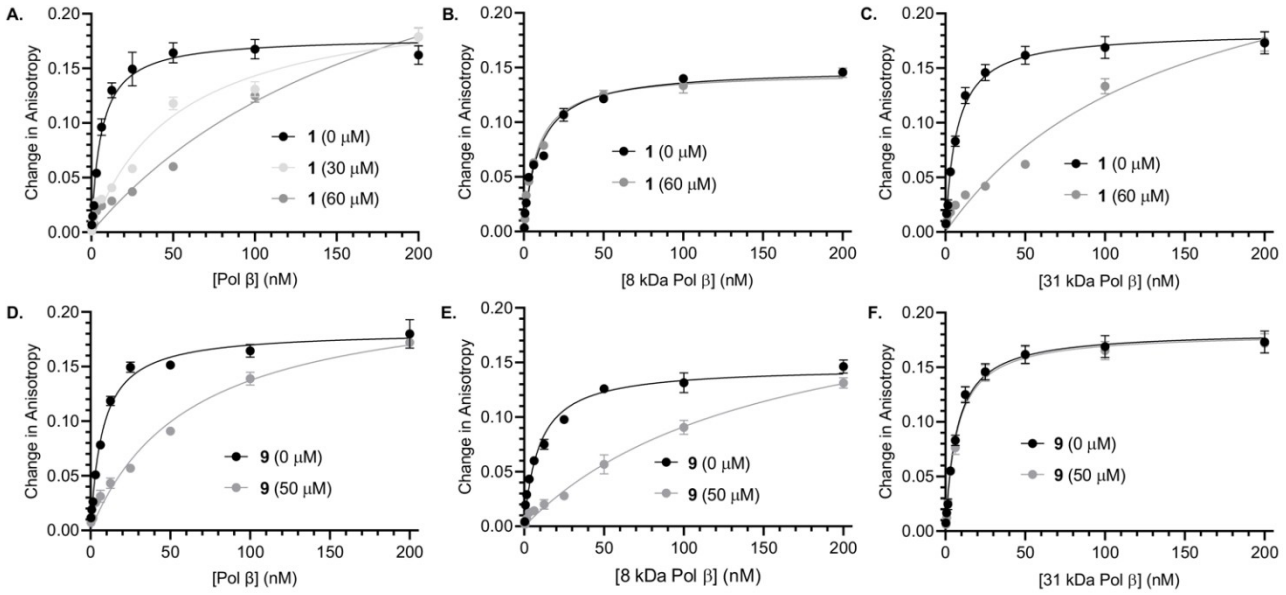


Figure 3. The effect of inhibitors **1** and **9** on DNA binding by Pol β determined via fluorescent anisotropy. **1**, A–C; **9**, D–F; intact Pol β, A, D; 8 kDa domain of Pol β, B, E; 31 kDa domain of Pol β, C, F.

(200 μM) in 1×reaction buffer (total volume: 30 μL) in a different 384-well plate. The final reaction mixture contained 10 nM Pol β, 3 μM inhibitor, 50 nM DNA, 100 μM dTTP, 1×reaction buffer, and 0.25% MeCN. The solution in each well was mixed thoroughly, and fluorescence measurements were immediately commenced.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: covalent inhibitors · DNA damage · DNA polymerases · DNA repair · enzyme inhibition

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