Identification of Small Molecule Synthetic Inhibitors of DNA Polymerase β by NMR Chemical Shift Mapping*§


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DNA polymerase β (β-pol) plays a central role in repair of damaged DNA bases by base excision repair (BER) pathways. A predominant phenotype of β-pol null mouse fibroblasts is hypersensitivity to the DNA-methylating agent methyl methanesulfonate. Residues in the 8-kDa domain of β-pol that seem to interact with a known natural product β-pol inhibitor, koetjapic acid, were identified by NMR chemical shift mapping. The data implicate the binding pocket as the hydrophobic cleft between helix-2 and helix-4, which provides the enzyme, nine structurally related synthetic compounds, containing aromatic or other hydrophobic groups in combination with two carboxylate groups, were then tested. They were found to bind to the same or a very similar region on the surface of the enzyme. The ability of these compounds to potentiate methyl methanesulfonate cytotoxicity, an indicator of cellular BER capacity, in wild-type and β-pol null mouse fibroblasts, was next ascertained. The most active and β-pol-specific of these agents, pamoic acid, was further characterized and found to be an inhibitor of the deoxyribosyl phosphate lyase activity and DNA polymerase activities of purified β-pol on a BER substrate. Our results illustrate that NMR-based binding techniques can be used in the design of small molecule enzyme inhibitors including those with potential use in a clinical setting.

Base lesions in DNA can arise endogenously from spontaneous base loss, base oxidation, or deamination, among other ways, and can also occur after exposure to alkylating agents, some of which are used for chemotherapy. DNA polymerase β (β-pol)1 is critical for protection of cells against the harmful effects of such single base lesions in DNA, most probably because of its roles in multiple DNA base excision repair (BER) pathways. The simplest and most well characterized ‘single nucleotide’ BER pathway comprises five sequential steps: excision of the damaged base by a specific glycosylase, strand cleavage by apurinic/apyrimidinic (AP) endonuclease, DNA synthesis and removal of the deoxyribose phosphate (dRP) by β-pol, and ligation of the nick by a DNA ligase (1). Controlled proteolysis and structural studies have revealed that β-pol is organized into two distinct domains. The 31-kDa domain has double stranded DNA binding and DNA polymerase activity, and the 8-kDa domain contributes single-stranded nucleic acid binding and dRP lyase activity (2). The solution structure of the 8-kDa domain (residues 1–87) has been determined by NMR (3, 4). The cellular requirement for β-pol, specifically the dRP lyase activity of β-pol, for repair of damaged bases was demonstrated earlier by studies of the hypersensitivity of β-pol null mouse embryonic fibroblasts to the monofunctional methylating agent methyl methanesulfonate (MMS) (5–7).

In recent years, there has been considerable effort toward isolating and identifying natural product inhibitors of mammalian DNA polymerases, in particular β-pol. Active agents, as ascertained by inhibition of β-pol DNA synthesis activity in in vitro assays, have been discovered in extracts from bacteria, marine organisms, fungi, and higher plants (8–14). Many of the “β-pol inhibitors” compounds (glycoglycerolipids, sulfolipids, triterpenoids, phenalenone derivatives) have been found to inhibit other polymerases as well (e.g. DNA polymerases α, γ, and λ and HIV-1 reverse transcriptase) (15–20); certain triterpenoid-derivative polymerase inhibitors were more recently found to also inhibit topoisomerasers (21).

In several previous studies, the long-term aim given for the identification of potent β-pol inhibitors is the possibility of inhibiting repair of DNA adducts formed after treatment with DNA-damaging anticancer drugs and thus potentiating chemotherapeutic treatments. However, β-pol null cells are not significantly hypersensitive to many clinically useful, DNA-alkylating antitumor agents, including the monofunctional and bifunctional chloroethylylation agents mitomycin and melphan and the nitrosourea 1,3-bis(2-chloroethyl)-1-nitrosourea (7), nor are they hypersensitive to ionizing radiation (5, 22). Although β-pol has been shown to be involved in the repair of bleomycin-induced DNA damage in vitro (23, 24), hypersensitivity to this agent is observed only in late passage, but not early passage, β-pol null mouse fibroblasts (25). Overexpression of MMS, methyl methanesulfonate; KJA, koetjapic acid; PA, pamoic acid; GA, glycyrrhizic acid; CBX, carbenoxolone; MB, mordant blue.

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1 The abbreviations used are: β-pol, DNA polymerase β; BER, base excision repair; AP, apurinic/apyrimidinic; dRP, deoxyribose phosphate; MMS, methyl methanesulfonate; KJA, koetjapic acid; PA, pamoic acid; GA, glycyrrhizic acid; CBX, carbenoxolone; MB, mordant blue.
ion of β-pol has been associated with resistance to cisplatin (26) and down-regulation of β-pol by antisense RNA was able to sensitize mammalian cells to this agent (27); however, in the β-pol null cells, no hypersensitivity is observed (25). The only clinical agent found to elicit significant hypersensitivity in β-pol null mouse fibroblasts is the methylating triazene derivative temozolomide (7).

Another valid reason for discovery of specific β-pol inhibitors that can penetrate cells is the potential for use of such agents as probes to dissect DNA polymerase functions in the various sub-pathways of repair. For example, inhibition of cellular β-pol-dependent repair by such a compound in an already repair-deficient background (e.g. x-ray cross-complementing factor 1 [XRC5]) –/– or xeroderma pigmentosum complementation group A [XPA] –/–) could provide information on the requirement for activity of other repair proteins within a repair pathway and associations between repair activities across specific repair pathways. As discussed above, a predominant phenotype of β-pol null mouse fibroblasts is hypersensitivity to monofunctional alkylating agents such as MMS. Thus, in mouse fibroblasts, an appropriate way to assay for a block of intracellular β-pol activity is to look for an increase in cellular sensitivity to MMS. Such an enhancement of MMS cytotoxicity has been reported previously for one β-pol inhibitor (15); however, in most studies in which cellular sensitivity was evaluated, the DNA-damaging agents used were bleomycin or cisplatin (8, 14, 28).

The interaction of bile acid and long-chain fatty acid β-pol inhibitors with the N-terminal 8-kDa domain of β-pol has been analyzed previously by NMR (29, 30). In the current study, the binding site for a known natural product β-pol inhibitor, koetjapic acid (KJA) (10), was localized by NMR chemical shift mapping of the β-pol 8-kDa domain. Large chemical shifts were observed upon binding, and this allowed us to make approximate assignments of the inhibitor and β-pol interfaces. The NMR binding study was then extended to include structurally similar synthetic terpenoids and finally other synthetic compounds containing aromatic or other hydrophobic groups in combination with two carboxylate groups. Dissociation constants for binding were estimated for these compounds, and the binding sites on the β-pol 8-kDa domain were mapped. The ability of the nine synthetic compounds with the highest binding affinities to potentiate MMS cytotoxicity in wild-type and β-pol null mouse embryonic fibroblasts was then ascertained. The most active and most β-pol-specific agent, paclitaxel acid (PA), was found to be inhibitory for activity in vitro assays for both the dRP lyase and DNA polymerase activities of β-pol.

Overall, these results illustrate the utility of NMR chemical shift mapping in small molecule enzyme inhibitor development, including those with potential for use in a clinical setting.

EXPERIMENTAL PROCEDURES

Materials—Synthetic oligodeoxynucleotides purified by high pressure liquid chromatography were obtained from Oligos Etc, Inc. (Wilsonville, OR). [α-32P]ddATP and [α-32P]dCTP (3000 Ci/mmole) were purchased from Amersham Biosciences. Recombinant human full-length β-pol, 8-kDa β-pol, uracil-DNA glycosylase with 84 amino acids deleted from the amino terminus, and AP endonuclease were overexpressed and purified as described previously (2, 31, 32).

The previously characterized β-pol inhibitor KJA was isolated directly from Sandicorum koetjape as described previously (10). The synthetic compounds, glycyrrhizaic acid (GA), carbonoxalone (CBX), PA, mardont blue (MB), biquinoline-dicarboxylic acid, naphthochrome green, 4,4'-hexafluoroisopropylidene-1,1'-bi(benzoic acid), and 4,4'-biphe-nyl dicarboxylic acid were obtained from Sigma-Aldrich, and 3-(4-carboxyphenyl) 2,3-dihydrotrimethyl 1-indene-5-carboxylic acid was from Acros Organics/Fisher Scientific. These agents were dissolved in high pH 25 mM sodium phosphate dibasic at a concentration of 10 mM; unless specified otherwise, the pH of the solutions was brought down or raised to pH 7.2–7.4 by addition of the appropriate volume of HCl or NaOH with constant stirring and monitoring of the pH. Solutions were stored at 4 °C.

NMR Chemical Shift Mapping—NMR titrations were performed using 180 μM β-pol 9-kDa domain. Inhibitor stock solutions were 5–20 mM and were titrated such that the final 600-μM β-pol sample was diluted by no more than 10%. All NMR HSQC spectra were acquired at 25 °C on a four-channel Varian Inova-500 spectrometer equipped with a pulse field gradient triple-resonance probe. NMR data were processed and peak picked using Felix (Biosym Inc.) with scripts generated from the web site sbtools.uchc.edu.

Measurement of Dissociation Binding Constants—When the inhibitor exchange rate is greater than the chemical shift difference between the free and bound states (as observed for the inhibitors in this study), the observed chemical shift, δobs, is related to the dissociation constant, [I]x, through its effect on the concentration of inhibitor-bound protein, [P]x, by the following equation (3, 33) given by

\[
\delta_{\text{obs}} = \delta_{\text{p}} + \left(1 - \frac{[P_x]}{[P_T]} \right) \delta_I
\]

where δp is the chemical shift of the protein in the absence of inhibitor, δI is the chemical shift of the protein bound to inhibitor, [P]T is the total concentration of protein. In the fast exchange regime, addition of inhibitor results in the gradual shifting of the protein resonances from the free to the bound states (see Supplemental Material). The dependence of the observed chemical shift, δobs, on the total inhibitor concentration, [SI], is given by

\[
[P_x] = 0.5 \left( K_C + [S_I] \right) + \left( \sqrt{K_C^2 + [S_I] [P_T]} - [S_I] \right) \frac{[P_T]}{4}
\]

(Eq. 2)

Dissociation constants of the order of the total protein concentration can therefore be determined quite accurately, although the accuracy decreases for very tight binders. Nevertheless, dissociation constants 1 order of magnitude lower than the protein concentration can readily be distinguished with this method (see Supplemental Material).

Cell Lines and Cytotoxicity Studies—Clones of the previously characterized wild-type and β-pol null mouse embryonic fibroblasts (MB16.3 and MB19.4, respectively) and cell culture conditions have been described previously (5, 34). Alternate isogenetically matched wild-type (MB36.3) and β-pol null (MB38.4) cell lines and β-pol null cells expressing Flag epitope-tagged, full-length, wild-type or 8-kDa domain β-pol proteins have also been described previously (6). Cells were grown

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kx</th>
<th>Wild-type</th>
<th>β-pol null</th>
<th>Enhancement ratio (x/y)</th>
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<tr>
<td>KJA</td>
<td>11 ± 10</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>PA</td>
<td>9 ± 3</td>
<td>11.3</td>
<td>1.3</td>
<td>8.7</td>
</tr>
<tr>
<td>BQD</td>
<td>243 ± 13</td>
<td>3.6</td>
<td>1.2</td>
<td>3.0</td>
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<tr>
<td>NCG</td>
<td>3 ± 4</td>
<td>3.2</td>
<td>1.2</td>
<td>2.7</td>
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<tr>
<td>MB</td>
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<td>2.9</td>
<td>1.1</td>
<td>2.6</td>
</tr>
<tr>
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<td>2.6</td>
<td>2.0</td>
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<tr>
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<td>1.5</td>
<td>1.7</td>
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<td>208 ± 19</td>
<td>4.5</td>
<td>2.7</td>
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<tr>
<td>CBX</td>
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<td>3.7</td>
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<tr>
<td>BPDC</td>
<td>856 ± 84</td>
<td>1.0</td>
<td>1.1</td>
<td>0.9</td>
</tr>
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</table>

Abbreviations are defined in Fig. 1.

**a** Dissociation binding constants (Kx) were determined from NMR titrations as described under “Experimental Procedures.” Standard error is quoted as the “goodness of fit” in fitting the data.

**b** Ratio of enhancement in wild-type and β-pol null cells (x/y).

**c** Not determined.
Fig. 1. Molecular structures of the β-pol inhibitors. Shown is the known natural product β-pol inhibitor KJA and the nine other structurally related synthetic compounds characterized.
Fig. 2. NMR titrations using the β-pol 8-kDa domain. a, superposition of the HSQC spectra of β-pol 8-kDa domain without (black) and with (red) a 2-fold excess of GA, representing the second titration point. Labeled are residues that shift the most upon binding GA. b, the sum of the absolute chemical shift differences, $|\Delta \delta^1H| + |\Delta \delta^{15}N|$ (Hz), are plotted versus residue number for a 5-fold excess of GA, representing the fifth
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at 34 °C in a 10% CO₂ incubator in Dulbecco’s modified Eagle’s medium (HyClone Laboratories, Logan, UT) supplemented with GlutaMAX-1 (Invitrogen), 10% fetal bovine serum (HyClone), and hygromycin (80 μg/ml; Roche Molecular Biochemicals). Cells were routinely tested and found to be free of mycoplasma contamination.

Cytotoxicity was determined by growth inhibition assays as described previously (34). Wild-type and β-pol null mouse fibroblasts (MB16.3 and MB19.4, respectively, unless otherwise indicated) were seeded at a density of 40,000 cells/well in six-well dishes. The following day, cells were exposed for 1 h to a range of concentrations of MMS in balanced salt solution (Invitrogen), and fresh medium plus or minus inhibitor was added. Dishes were incubated for 4–5 days at 34 °C in a 10% CO₂ incubator until untreated control cells were ~80% confluent. Cells (triplicate wells for each drug concentration) were counted by a cell lysis procedure (35), and results were expressed as the number of cells in drug-treated wells relative to control wells.

Dephosphorylated 34-mer oligodeoxyribonucleotide (5′-CTGCGAGTGTGGGATCCGTCATCAGCTGCAG-3′) containing a uracil residue at position 16 was 3′-end-labeled with terminal deoxynucleotidyltransferase and [α-32P]ddATP. The complementary DNA strand (5′-GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG-3′) was annealed by heating the solution at 90 °C for 3 min, and the solution was allowed to slowly cool to 25 °C. 32P-labeled duplex oligonucleotide was separated from unincorporated [α-32P]ddATP using a Sephadex G-25 column according to the manufacturer’s suggested protocol. The radiolabeled oligonucleotide was stored at ~30 °C until use.

Uracil-DNA Glycosylase/AP Endonuclease Treatment of DNA Substrate—In general, 50 nM 32P-labeled duplex oligonucleotide substrate was pretreated with 10 nM human uracil-DNA glycosylase in 50 mM HEPES, pH 7.4, 20 mM KCl, 0.5 mM EDTA, and 2 mM dithiothreitol for 20 min at 37 °C. The reaction mixture was then supplemented with 5 mM MgCl₂ and 10 nM AP endonuclease and the incubation continued for 20 min at 37 °C. These reactions generated substrates for DNA synthesis (15-mer with 3′-OH) and dRP lyase assays (32P-labeled 19-mer with 5′-sugar phosphate) as shown in Fig. 6a. Because of the labile nature of the dRP-containing DNA, this DNA substrate was prepared just before performing the experiment.

Pretreatment of β-pol with PA—β-Pol (100 nM) and PA (50 to 2500 μM) were mixed (1:1, v/v) in enzyme dilution buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 100 μg/ml bovine serum albumin, and 20% glycerol) and incubated at 25 °C for 10 min. This mixture was used to initiate the dRP lyase/DNA gap-filling synthesis reaction resulting in a final concentration of 10 nM β-pol and 5–250 μM PA.

DNA Gap-Filling Synthesis and dRP Lyase Assay—The lyase and DNA gap-filling synthesis reactions were performed as described previously (1). The reaction mixture (10 μl) contained 50 mM HEPES, pH 7.5, 20 mM KCl, 2 mM dithiothreitol, 4 mM ATP, 5 mM MgCl₂, and 2.3 μM GTP and 2 μM c3′-pol and 1 μM PA.

This error is smaller than the data symbols.

3′-End Labeling—Dephosphorylated 34-mer oligodeoxyribonucleotide (5′-CTGCGAGTGTGGGATCCGTCATCAGCTGCAG-3′) containing a uracil residue at position 16 was 3′-end-labeled with terminal deoxynucleotidyltransferase and [α-32P]ddATP. The complementary DNA strand (5′-GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG-3′) was annealed by heating the solution at 90 °C for 3 min, and the solution was allowed to slowly cool to 25 °C. 32P-labeled duplex oligonucleotide was separated from unincorporated [α-32P]ddATP using a Sephadex G-25 column according to the manufacturer’s suggested protocol. The radiolabeled oligonucleotide was stored at ~30 °C until use.

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This error is smaller than the data symbols.
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RESULTS

Chemical Shift Mapping of the Interaction Interface of Inhibitors and β-pol—15N-labeled recombinant 8-kDa domain of human β-pol (2) was first titrated with the known natural product β-pol inhibitor, KJA (10). The compound bound with high affinity (KD = 11 μM; Table I), and residues that seemed to interact with the inhibitor were identified by NMR chemical shift mapping. Thirty-four structurally related and soluble synthetic compounds were similarly analyzed. Of these, nine compounds were selected that contained aromatic or other hydrophobic groups in combination with two carboxylate groups and had binding constants in the micromolar range estimated from NMR titrations. The molecular structures of KJA, and the nine compounds chosen for more detailed analysis are shown in Fig. 1. 15N-labeled 8-kDa domain of human β-pol was titrated with each of these synthetic compounds, and the backbone amide chemical shifts were monitored in 15N-HSQC spectra. For each of the agents studied, the amide proton and nitrogen resonances of affected residues shifted gradually as the concentration of inhibitor was increased, indicating that the binary complexes were in fast exchange on the NMR timescale (kex > 150 s−1). Changes in chemical shift were analyzed to ascertain both the structural interface as well as the dissociation constant of interaction, as shown for KJA, GA, CBX, and PA in Figs. 2 and 3.

To identify the interaction interface, the absolute chemical shift changes, |Δδ1H| + |Δδ15N| (Hz), between the free and bound state were plotted versus β-pol residue number for the final point in each titration (Figs. 2b and 3b). From each plot, the mean chemical shift change was determined. Residues with significantly large chemical shift changes (/>1.5 standard deviations) upon addition of each inhibitor were mapped onto the known structure of β-pol (4). The binding site was found to be the same or very similar for each compound in this group. Taken together, the data implicate as the binding pocket the hydrophobic cleft formed between helix-2 and the N-terminal portion of helix-4, which provides the DNA binding and dRP lyase activities of the enzyme. Inhibitors seem to require two carboxylate groups and aromatic components that could form favorable hydrophobic interactions with Ala-38, Tyr-39, Gly-66, Ile-69, and Leu-85.

Binding constant values were estimated by monitoring the absolute change in individual resonance chemical shifts, |Δδ1H| or |Δδ15N| (Hz), as a function of inhibitor concentration. In each case, the chemical shift titration curves were fit to a two-state equilibrium model, assuming a single binding site for the inhibitor, as was described previously for single-stranded DNA binding (3). Chemical shift titrations with PA for various residues in the β-pol 8-kDa domain were analyzed (see Supplemental Material). The results for each of these residues were similar, yet the magnitude of the chemical shift for Ile-69 was greater. The measured KD of a single inhibitor differed by no more than 1 order of magnitude for the various resonances found to shift upon binding. Because the accuracy of chemical shift measurements is the same for all resonances, measurement of the change in chemical shift is most accurate for resonances that shift the greatest amount. In addition, the resonances that shift the most are expected to be most representative of direct interaction, rather than indirect effects. For this reason, the estimates for the equilibrium dissociation constants for binding of the compounds to the β-pol 8-kDa domain listed in Table I are those arising from the H1 of Ile-69, the resonance found to shift the most throughout the titration experiment. Likewise, the titration curves for KJA, GA, CBX, and PA shown in Figs. 2, c, e, and g and 3c, respectively, were determined by monitoring the absolute change in the proton chemical shift, |Δδ1H|, of Ile-69. About half of the compounds tested were found to bind with high affinity (KD <10 μM), whereas the remaining compounds were moderately weak binders (KD >10 μM). The error in the measured KD is quoted as the standard error of the fit. For accurate measurements of this type, it is assumed that a good estimation of the chemical shift of the bound state is made (requiring the KD to be no more than 1 order of magnitude larger than the protein concentration) as well as several titration points during the inflected part of the curve (in our case, requiring the KD to be no less than 1 order of magnitude smaller than the protein concentration). Therefore, for our study, a KD between 10 μM and 1 mM is expected to be accurate, whereas a KD less than 10 μM is assumed to be approximate and an upper estimate.

β-pol Inhibitors Enhance Cellular Sensitivity to MMS—The sensitivity of mouse embryonic fibroblasts to MMS is dependent on the activity, in particular the dRP lyase activity, of β-pol within the cell (5, 6). If the β-pol inhibitors can be taken up into β-pol-expressing cells and bind to the 8-kDa domain of β-pol, as described above, and inhibit activity, then these agents should be able to enhance cellular sensitivity to MMS. If the mechanism of sensitization is β-pol-specific, sensitization will be seen in wild-type but not in β-pol null cells, and the resulting sensitivity to MMS in the wild-type cells will mimic that observed in β-pol null cells. In the initial study, the cytotoxicity of nine synthetic β-pol inhibitors was checked in wild-type mouse fibroblasts. For a 24-h exposure, toxicity at a concentration of 100 μM was seen only with CBX (data not shown). In the next series of experiments, the effect of 100 μM of the inhibitors (50 μM for CBX) on MMS cellular sensitivity was ascertained in both wild-type and β-pol null cells. In each case, cells were treated for 1 h with a range of concentrations of MMS in the presence or absence of inhibitor, and then the incubation with
The schedule and concentration dependence for the effectiveness of PA was first investigated. Wild-type cells were treated with MMS for 1 h in the presence of 300 μM PA. The incubation with PA was for a total of 24 h, as previously, but with varied incubation times before and after the 1-h MMS. A schedule of 7-h PA before MMS and 16-h PA after the 1-h MMS was shown to be the most effective at sensitizing wild-type cells to MMS (Fig. 5a). Negatively charged compounds such as PA enter cells slowly, and a lengthy incubation may be necessary to allow build up of an intracellular PA concentration sufficient to bind enough β-pol

**Fig. 5. Sensitization of mouse fibroblasts to MMS by co-treatment with PA.** Growth inhibition assays were conducted as described under “Experimental Procedures.” a, wild-type mouse fibroblasts (MB16.3) were exposed for 1 h to MMS in the absence (circles) or presence of 300 μM PA. Cells were treated with PA for 24 h (closed symbols) or 7 h (triangles), or 16 h (squares) before dosing with MMS; the total PA incubation time was always 24 h. b, wild-type (MB16.3; closed symbols) and β-pol null (MB19.4; open symbols) mouse fibroblasts were pretreated for 7 h with 200 μM (triangles), 300 μM (squares), or 400 μM (diamonds) PA, exposed to MMS for 1 h in the presence of the same concentration of PA, and then the PA incubation continued for a total of 24 h. c, wild-type (MB36.3; closed symbols) and β-pol null (MB38Δ4; open symbols) mouse fibroblasts were exposed for 1 h to MMS in the absence (circles) or presence (squares) of 300 μM PA. Pretreatment with PA was for 7 h, and the PA incubation continued after MMS for a total of 24 h. d, β-pol null cells (MB38Δ4) expressing wild-type β-pol protein (closed symbols) or 8-kDa domain β-pol protein (open symbols) were exposed for 1 h to MMS in the absence (triangles) or presence (diamonds) of PA. The schedule for PA co-treatment was as described for c. The sensitivity of control non-transfected β-pol null cells is also shown (open circles). Data are the mean ± S.E. of three to four independent experiments or are from representative experiments in which the values are the mean of triplicate determinations.

inhibitor was continued for a total of 24 h.

The data obtained on the enhancement of MMS-induced growth inhibition at an MMS concentration of 2 mM in wild-type cells and 1 mM in β-pol null cells is compiled in Table I. The values were calculated as represented diagrammatically in Fig. 4. This method for presenting survival data was chosen rather than the more usual comparison of the concentration required for equivalent growth inhibition (e.g. IC50 or IC90). It allowed a more clear distinction between active and inactive inhibitors, particularly in β-pol null cells in which these values were extremely close. All of the compounds except 4′,4″-biphenyl dicarboxylic acid were able to sensitize wild-type cells to MMS; 4′,4″-biphenyl dicarboxylic acid also had no effect in β-pol null cells. Co-treatment with GA, 2,3-dihydrotrimethyl 1-indene-5-carboxylic acid, and CBX resulted in 5.2-, 4.5- and 4.6-fold sensitizations, respectively, to MMS in wild-type cells. However, these agents also sensitized β-pol null cells (2.6-, 2.7-, and 3.7-fold, respectively), suggesting that the observed sensitization is not the result of a β-pol inhibitory mechanism alone. Another agent, 4,4″-(hexafluoroisopropylidene) bis(benzoic acid), induced a 2.6-fold sensitization in wild-type cells but also a 1.5-fold sensitization in β-pol null cells. The remaining compounds, PA, biquinoline-dicarboxylic acid, naphthochrome green, and MB, were able to sensitize wild-type cells to 2 mM MMS (2.9–11.3-fold), but had very little effect (<1.3-fold) in β-pol null cells, suggesting that for these agents, the sensitization mechanism is β-pol-specific. By far the most active (11.3-fold sensitization in wild-type cells) and the most specific compound (only 1.3-fold sensitization in β-pol null cells) was PA (Table I), which was further characterized in the experiments described below. PA was also found to bind with fairly high affinity to the 8-kDa domain of β-pol (9 μM; Table I).

**Pamoic Acid Can Sensitize Wild-type but Not β-Pol Null Mouse Fibroblasts to MMS**—The schedule and concentration dependence for the effectiveness of PA was first investigated. Wild-type cells were treated with MMS for 1 h in the presence of 300 μM PA. The incubation with PA was for a total of 24 h, as previously, but with varied incubation times before and after the 1-h MMS. A schedule of 7-h PA before MMS and 16-h PA after the 1-h MMS was shown to be the most effective at sensitizing wild-type cells to MMS (Fig. 5a). Negatively charged compounds such as PA enter cells slowly, and a lengthy incubation may be necessary to allow build up of an intracellular PA concentration sufficient to bind enough β-pol
protein to result in inhibition of intracellular β-pol activity.

Next, using this schedule, a range of concentrations of PA (200, 300 and 400 μM) was tested. A concentration of 300 μM PA was considerably more effective than 200 μM at sensitizing wild-type cells to MMS and produced only a minimal shift in the β-pol null cell survival curve (Fig. 5b), suggesting a β-pol-specific mechanism for the sensitization. A concentration of 400 μM PA was slightly more effective still in wild-type cells, but now there was a larger effect in the β-pol null cells, especially evident at low concentration MMS (0.5 mM). This suggests that, at this higher concentration, PA has a β-pol targeted but also a nonspecific mechanism.

Using the optimal treatment schedule and the concentration (300 μM) as determined above, the effect of PA was tested in an alternate set of wild-type and β-pol null mouse fibroblasts. Consistent with the results obtained in the first set of cell lines, co-treatment with PA resulted in a substantial sensitization to MMS in wild-type cells but was totally without effect in the β-pol null cells (Fig. 5c). In the next experiments, we used β-pol null mouse fibroblasts expressing either full-length wild-type β-pol protein or the 8-kDa dRP lyase domain of β-pol (6). As expected, because it is the dRP lyase activity of β-pol that has been shown to be required to protect against MMS-induced cytotoxicity (6), both of these cell lines were considerably more resistant to MMS than the control nontransfected β-pol null cells (Fig. 5d). In addition, PA was able to sensitize both types of transfected cells to MMS. In the 8-kDa-expressing cells, the MMS resistance gained after transfection with the dRP lyase domain was completely reversed by co-treatment with PA (Fig. 5d), suggesting that PA is able to quantitatively inhibit the dRP lyase activity of β-pol.

Inhibition of β-pol dRP Lyase and DNA Synthesis Activities by PA—In view of the results described above, we next investigated the effect of PA on the in vitro dRP lyase and DNA synthesis activities of full-length β-pol protein. With the BER substrate shown in Fig. 6a, we used both [α-32P]dCTP and 3′-32P-end labeled DNA to separately analyze dRP lyase and DNA synthesis activities in the same reaction mixture. A gel showing the positions of the substrate and the anticipated repair products is presented in Fig. 6b. The products obtained in the absence of PA are shown in lane 2, and those obtained in the presence of increasing concentrations of PA (5–250 μM) are shown in lanes 3–8. A graphical representation of the data in Fig. 6b is presented in Fig. 6c. A PA concentration-dependent equivalent inhibition of both dRP lyase and DNA synthesis activities of β-pol was observed. Inhibition was seen only when PA was preincubated with β-pol before initiation of the BER reactions, perhaps implying that the binding of PA to the β-pol 8-kDa domain is not a rapid process; PA was not an effective inhibitor when preincubated with DNA alone. These findings are consistent with the kinetic behavior of KJA determined previously (10). The results suggest that binding of PA to the 8-kDa domain of β-pol results in inhibition of both the DNA synthesis and dRP lyase activities associated with the protein.

**DISCUSSION**

Even though natural product inhibitors of β-pol activity in vitro are well known, this is the first report characterizing the biological effects of a series of synthetic small molecule β-pol inhibitory compounds. Here, we correlated binding affinity to the β-pol 8-kDa domain and NMR mapping of the approximate inhibitor-enzyme interfaces to the effects of blocking β-pol activity in vivo in cells. Further, we showed that the most active and β-pol-specific of these agents, PA, can inhibit both dRP lyase and DNA polymerase activities of β-pol on a BER substrate (Fig. 6). As a result of this inhibitory activity in cells, PA is able to sensitize wild-type, but not β-pol null, mouse fibroblasts to the DNA-methylating agent MMS (Fig. 5). Most importantly, our results illustrate that NMR chemical shift mapping techniques can be used to select small molecule enzyme inhibitors, including those with potential for use in a clinical setting.

The natural product KJA has been described previously as a β-pol inhibitor (10). This triterpenoid compound was shown to inhibit rat β-pol-mediated DNA synthesis on activated calf thymus DNA. Now, we demonstrate that this compound binds with fairly high affinity (Kd = 11 μM; Table I) to the β-pol N-terminal domain. NMR chemical shift mapping suggests that surface residues that include Gly-66 and Ile-69 form the binding site (Fig. 2). These residues are part of the 8-kDa helix-hairpin-helix motif of β-pol (residues 55–79) that interacts directly with the sugar phosphate backbone downstream of gapped DNA (36). The two structurally similar triterpenoid compounds GA and CBX, and seven other synthetic compounds containing aromatic or other hydrophobic groups in combination with two carboxylate groups (Fig. 1) analyzed in the same
way, were found to bind to the same or similar sites on the β-pol N-terminal domain. The compounds bound with varying degrees of affinity, ranging from a KD of 2 μM for MB to 970 μM for 4,4'-(hexafluoropropylidene) bis(benzoic) acid (Table 1).

The residues of β-pol interacting with the β-pol inhibitors include Lys-35, Gly-66, Ile-69, Arg-83, and Leu-85 that overlap the DNA binding and dRP lyase active sites of the enzyme (Figs. 2 and 3). However, we note that the precision of our backbone chemical shift measurements for identifying ligand and individual side chain interactions should be considered suggestive only. Remote conformational effects can confound interpretations, as noted recently with MutT (37), and side-chain interactions are not directly measured through backbone chemical shift changes. Nevertheless, these residues are in the dRP lyase active site, and Lys-35 is known to be involved in both single-stranded DNA binding activity and in recognition of a 5'-phosphate in gapped DNA structures (38, 39).

Previous NMR mapping studies have analyzed binding of the fatty acids linoleic and nervonic acid and the bile acid lithocholic acid to the N-terminal 8-kDa domain of β-pol (29, 30). For nervonic acid, the major shifted amino acids were Leu-11, Lys-35, His-51, and Thr-79; for lithocholic acid, they were Lys-35, Gly-66, Ile-69, Arg-83, and Leu-85 that overlap in vivo by guest on November 15, 2017 http://www.jbc.org/ Downloaded from

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Identification of Small Molecule Synthetic Inhibitors of DNA Polymerase β by NMR Chemical Shift Mapping
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