ABSTRACT: Specialized DNA polymerases participate in replication stress responses and in DNA repair pathways that function as barriers against cellular senescence and genomic instability. These events can be co-opted by tumor cells as a mechanism to survive chemotherapeutic and ionizing radiation treatments and as such, represent potential targets for adjuvant therapies. Previously, a high-throughput screen of \( \sim 16,000 \) compounds identified several first generation proof-of-principle inhibitors of human DNA polymerase kappa (hpol\( \kappa \)). The indole-derived inhibitor of 5-lipoxygenase activating protein (FLAP), MK886, was one of the most potent inhibitors of hpol\( \kappa \) discovered in that screen. However, the specificity and mechanism of inhibition remained largely undefined. In the current study, the specificity of MK886 against human Y-family DNA polymerases and a model B-family DNA polymerase was investigated. MK886 was found to inhibit the activity of all DNA polymerases tested with similar IC\( \text{50} \) values, the exception being a 6- to 8-fold increase in the potency of inhibition against human DNA polymerase iota (hpol\( \iota \)), a highly error-prone enzyme that uses Hoogsteen base-pairing modes during catalysis. The specificity against hpol\( \iota \) was partially abrogated by inclusion of the recently annotated 25 a.a. N-terminal extension. On the basis of Michaelis–Menten kinetic analyses and DNA binding assays, the mechanism of inhibition by MK886 appears to be mixed. In silico docking studies were used to produce a series of models for MK886 binding to Y-family polymerases, while a third pocket near the thumb domain appears to be unique to hpol\( \iota \). Overall, these results provide insight into the general mechanism of DNA polymerase inhibition by MK886.
at the transcript and protein levels in several tumor types.\textsuperscript{7–9} As such, they represent targets for sensitizing tumor cells to genotoxic agents, including cisplatin, doxorubicin, or Temozolomide.\textsuperscript{5,10–13}

The ability to target individual members of any class of enzymes requires sufficient structural and/or functional diversity to achieve specific inhibition. DNA polymerases present a unique opportunity for targeted inhibition. While the active site residues involved in nucleic acid synthesis are generally conserved in different DNA and RNA polymerases from a variety of organisms,\textsuperscript{14} considerable variation exists for both the structural and functional attributes of polymerase subfamilies. Of note here are the translesion DNA synthesis properties of Y-family DNA polymerases.\textsuperscript{5,16} The structure of Y-family DNA polymerases exhibit several general features that distinguish them from their high-fidelity counterparts (e.g., spacious active sites and the lack of proofreading exonuclease activity).\textsuperscript{16} Y-family DNA polymerases can accommodate bulky DNA adducts in their active site in a manner that remains conducive to polymerization.\textsuperscript{17–20} The finger domain of Y-family DNA polymerases is composed of shorter $\alpha$-helices than those present in A-, B-, or C-family polymerases, a trend that can be found in all Y-family members studied to date. Additionally, the polymerase-associated or "little finger" domain is only observed in Y-family DNA polymerases. Yet even within the Y-family there is substantial diversity of structure and function, especially near the extreme N-terminus of the four mammalian enzymes. On the basis of the structural and functional properties of the Y-family, it is not unreasonable to suppose that these specialized polymerases could be targets of specific inhibition by small molecules, yielding interesting biological results. However, finding small molecule inhibitors of specialized polymerases has proven challenging. Although, recent reports have identified natural product inhibitors of Y-family DNA polymerases.\textsuperscript{21,22} Synthetic routes to obtain these compounds remain to be determined, and specificity to individual Y-family members has yet to be achieved.

Recently, along with additional collaborators, we identified several inhibitors of human DNA polymerase kappa (hpol $\kappa$).\textsuperscript{23} Among the top hits for inhibition of hpol $\kappa$ was the indole derivative 3-(1-(4-chlorobenzyl)-3-buty-thio-5-isoproylindol-2-y1)-2,2-dimethylpropanoic acid, MK886. MK886 was originally discovered as an inhibitor of leukotriene biosynthesis.\textsuperscript{24,25} MK886 was identified as a small molecule inhibitor of DNA polymerase inhibition by this indole-derived compound.\textsuperscript{24,25} The pBG101 plasmid was used to prepare constructs encoding human DNA polymerases $\eta$ (a.a. 1–437), $\iota$ (a.a. 1–446 and 26–446), and $\kappa$ (a.a. 19–526). The pBG101 vector encodes a 6X-histidine tag and a glutathione transferase (GST) fusion protein upstream of the polymerase-encoding region. A protease recognition sequence (LEVLFQGP) just upstream of the polymerase insert allows cleavage of the N-terminal affinity tags during purification. All the human polymerases used in the study were expressed in E. coli cells (BL21 DE3) and purified in an identical manner. Briefly, the pBG101 vector encoding the polymersases just downstream of 6X-Histidine and GST-tags was transformed into E. coli cells (BL21 (DE3) strain). Cells were grown at 37 °C and 250 rpm for three hours (OD$\textsubscript{600}$ = 0.5–0.6), followed by induction for three hours (37 °C and 250 rpm) by the addition of isopropyl $\beta$-D-1-thiogalactopyranoside (1 mM), and finally harvested by centrifugation. Buffer containing 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 10% glycerol (v/v), 5 mM $\beta$-mercaptoethanol ($\beta$-ME), lysozyme (1 mg/ml), and a protease inhibitor cocktail (Roche, Basel, Switzerland) was added to the harvested pellet. The suspension was sonicated and supernatant recovered from an ultracentrifugation step (35,000g, 1 h, 4 °C). After the removal of cellular debris by ultracentrifugation, the resulting clear lysate was loaded onto a 5 ml HisTrap column (GE Healthcare Life Sciences), followed by washing the column sequentially with 50 mM Tris-HCl (pH 7.3 at 22 °C) buffer containing 0.5 M NaCl, 5 mM $\beta$-ME, 10% glycerol, and 20 mM imidazole to remove nonspecifically bound proteins. The remaining bound proteins were then eluted using a linear gradient from 60 mM to 400 mM imidazole. The eluted proteins were loaded onto a 2 ml GSTrap column (GE Healthcare Life Sciences) in 25 mM HEPES (pH 7.5) buffer containing 0.1 M NaCl, 5 mM $\beta$-ME, and 10% glycerol. Cleavage of the GST tag was performed on the bound proteins by injecting a solution containing the PreScission protease (GE Healthcare Life Sciences) onto the column and allowing it to incubate overnight at 4 °C. The GST-tag-free proteins were eluted in the GSTrap running buffer and concentrated using an Amicon spin concentrator (MilliPore). The purity of each polymerase was analyzed by SDS–polyacrylamide gel electrophoresis. The highly pure proteins were stored at −80 °C in HEPES buffer (pH 7.5) containing 0.1 M NaCl, 5 mM $\beta$-ME, and 30% glycerol. The model B-family DNA polymerase, Dpo1, from Sulfolobus solfataricus was expressed and purified as described previously.\textsuperscript{31}

Determination of IC$_{50}$ Values Using Polymerase Assays. To obtain an estimate of the IC$_{50}$ for the inhibition of DNA polymerase activity by MK886, polymerase-catalyzed DNA synthesis was measured in the presence of all four dNTPs at a single-time point with increasing concentrations of inhibitor. The MK886 concentration range used for the enzymes hpol $\eta$, hpol $\iota$, and hpol $\kappa$ was 0.1–500 M, while for hpol $\kappa$ was 0.1–100 M. For DNA substrate preparation, the FAM-labeled primer was annealed to a complementary template oligonucleotide (1:2, primer/template molar ratio for the increased potency against this highly error-prone enzyme was examined biochemically and using molecular modeling tools. The results provide a framework for understanding DNA polymerase inhibition by MK886.

### EXPERIMENTAL PROCEDURES

All chemicals were of molecular biology grade or better. All dNTPs were purchased from GE Healthcare Life Sciences (Piscataway, NJ). The oligonucleotides used in this work were synthesized by either Integrated DNA Technologies (Coralville, IA) or Biosearch Technologies, Inc. (Novato, CA) and purified by the manufacturer using HPLC, with analysis by matrix-assisted laser desorption time-of-flight MS. The primer sequence used in the extension assays, inhibition assays, single-nucleotide kinetics assays, and the binding assays was 5'-FAM-TTGGG GGGAAGGATTCC-3'. The template DNA sequence used in the extension assays, inhibition assays, and the binding assays was 5'-TCACGGAATCTCCCTCCCGG-3'. MK886 was purchased from either Sigma-Aldrich (St. Louis, MO) or EMD Millipore Corp. (Billerica, MA).

Expression and Purification of Recombinant Proteins. The pBG101 plasmid was used to prepare constructs encoding human DNA polymerases $\eta$ (a.a. 1–437), $\iota$ (a.a. 1–446 and 26–446), and $\kappa$ (a.a. 19–526). The pBG101 vector encodes a 6X-histidine tag and a glutathione transferase (GST) fusion protein upstream of the polymerase-encoding region. A protease recognition sequence (LEVLFQGP) just upstream of the polymerase insert allows cleavage of the N-terminal affinity tags during purification. All the human polymerases used in the study were expressed in E. coli cells (BL21 DE3) and purified in an identical manner. Briefly, the pBG101 vector encoding the polymersases just downstream of 6X-Histidine and GST-tags was transformed into E. coli cells (BL21 (DE3) strain). Cells were grown at 37 °C and 250 rpm for three hours (OD$\textsubscript{600}$ = 0.5–0.6), followed by induction for three hours (37 °C and 250 rpm) by the addition of isopropyl $\beta$-D-1-thiogalactopyranoside (1 mM), and finally harvested by centrifugation. Buffer containing 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 10% glycerol (v/v), 5 mM $\beta$-mercaptoethanol ($\beta$-ME), lysozyme (1 mg/ml), and a protease inhibitor cocktail (Roche, Basel, Switzerland) was added to the harvested pellet. The suspension was sonicated and supernatant recovered from an ultracentrifugation step (35,000g, 1 h, 4 °C). After the removal of cellular debris by ultracentrifugation, the resulting clear lysate was loaded onto a 5 ml HisTrap column (GE Healthcare Life Sciences), followed by washing the column sequentially with 50 mM Tris-HCl (pH 7.3 at 22 °C) buffer containing 0.5 M NaCl, 5 mM $\beta$-ME, 10% glycerol, and 20 mM imidazole to remove nonspecifically bound proteins. The remaining bound proteins were then eluted using a linear gradient from 60 mM to 400 mM imidazole. The eluted proteins were loaded onto a 2 ml GSTrap column (GE Healthcare Life Sciences) in 25 mM HEPES (pH 7.5) buffer containing 0.1 M NaCl, 5 mM $\beta$-ME, and 10% glycerol. Cleavage of the GST tag was performed on the bound proteins by injecting a solution containing the PreScission protease (GE Healthcare Life Sciences) onto the column and allowing it to incubate overnight at 4 °C. The GST-tag-free proteins were eluted in the GSTrap running buffer and concentrated using an Amicon spin concentrator (MilliPore). The purity of each polymerase was analyzed by SDS–polyacrylamide gel electrophoresis. The highly pure proteins were stored at −80 °C in HEPES buffer (pH 7.5) containing 0.1 M NaCl, 5 mM $\beta$-ME, and 30% glycerol. The model B-family DNA polymerase, Dpo1, from Sulfolobus solfataricus was expressed and purified as described previously.\textsuperscript{31}
(dCTP/Mg2+ (Mg2+ at 2 mM; dCTP at concentrations between 1 and 1500 μM, HEPES (pH 7.5) buffer containing 50 mM NaCl, 1 mM DTT, 0.1 mg/mL BSA, and 10% (v/v) glycerol). The DNA was preincubated with the enzyme for five min prior to starting the polymerase reaction. Next, inhibitor was added and allowed to incubate with the polymerase-DNA solution for five min. In the control reaction, DMSO was added instead of the inhibitor solution. Each reaction was initiated by the addition of the dNTP-Mg2+ (0.25 mM of each dNTP and 5 mM MgCl2) solution to a preincubated enzyme-DNA complex (20 nM enzyme and 200 nM DNA). The reactions were performed in a final volume of 15 μL at 37 °C. Reactions were terminated by adding quench solution (20 mM EDTA and 95% (v/v) formamide) to each reaction at different time intervals and then heating the samples at 95 °C for five min. The samples were separated using a 15% (w/v) polyacrylamide/7 M urea gel, and the products were analyzed using a Typhoon imager and ImageQuant software (GE Healthcare Life Sciences). The total product formed in each reaction was calculated by adding the band intensities of the +1, +2, +3, +4, and +5 products. The fraction of substrate converted to product was calculated by dividing the above by the total amount in each reaction (summation of +0, +1, +2, +3, +4, and +5 product bands). The percent product formed at each MK886 concentration was calculated by normalizing to the DMSO control, which was considered as 100%. This percent product was plotted as a function of MK886 concentration and fit to a four-parameter logistic model (eq 1) using Prism software (GraphPad, San Diego, CA):

\[
P = P_0 + \left( P_{\text{max}} - P_0 \right) \frac{[\text{DNA}_{\text{total}}] + [K_d] + \sqrt{[\text{DNA}_{\text{total}}] + [K_d]^2 - 4[\text{DNA}_{\text{total}}][K_d]}}{2[\text{DNA}_{\text{total}}]}
\]

Where \(P\) = the measured change in fluorescence polarization, \(P_{\text{total}}\) = the concentration of enzyme, \(\text{DNA}_{\text{total}}\) = the concentration of fluorescein-labeled DNA, and \(K_d\) = the measured equilibrium dissociation constant for enzyme binding to DNA. The binding curves were fit such that \(\text{DNA}_{\text{total}}\) was fixed to be constant between curves.

**Kinetic Analysis of Polymerase Activity in the Presence of MK886.** In order to determine the mechanism of inhibition of Y-family DNA polymerases by MK886, kinetic analyses were performed using hpol ϕ232-446 under steady-state conditions using two approaches.

In the first approach, single nucleotide insertion by hpol ϕ232-446 was studied on a fluorescein-labeled primer–template, in the presence of varying concentrations of MK886. The dsDNA substrate, in which the incoming correct nucleotide is dCTP, was generated as described earlier. Polymerase assay conditions were also identical to those described previously. The full-length extension assays were performed in a 200 μL reaction mixture in a 96-well plate. Initially, 170 μL of a master mix containing the enzyme, inhibitor at appropriate concentration (or DMSO in control), and assay buffer was pipetted into each well of the 96-well plate. This was followed by the addition of 10 μL of stock solution of dTTP to each well to obtain final concentrations of dTTP between 1 and 200 μM. Reactions were initiated by the addition of 20 μL of the DNA substrate to each well with a multichannel pipet. After mixing well, the fluorescence was continuously measured in a Biotek SynergyH4 plate reader using the appropriate filter sets (λex = 485 ± 20 nm and λem = 525 ± 20 nm). All titrations were performed at 25 °C in 50 mM HEPES buffer (pH 7.5) containing 10 mM KCl, 10 mM MgCl2, 0.1 mM MnCl2, 0.1 mM EDTA, 2 mM β-ME, 10% (v/v) DMSO, and 0.1 mg/mL BSA. The experiments were performed in the presence of 0, 1, 10, 25, 50, 100, 150, and 250 μM MK886. DMSO was substituted in the no MK886 control experiment. Polarization was determined using eq 2:

\[
P = \frac{[F_0 - F_L]}{[F_0 + F_L]}
\]

where \(F_0\) equals fluorescence intensity parallel to the excitation plane, and \(F_L\) equals the fluorescence intensity perpendicular to the excitation plane. The resulting change in polarization units was plotted against protein concentration and fit to a quadratic equation (eq 3).

\[
y = \text{bottom} + \left( \text{top} - \text{bottom} \right) \frac{1}{1 + \left( x/K_{\text{IC}50} \right)^2}
\]

The experiments were performed using at least 11 concentrations of MK886 and were performed at different time points to verify the results.

**Fluorescence Anisotropy Experiments to Determine the Effect of MK886 upon Polymerase Binding to DNA.** Fluorescein-labeled DNA substrate (2 nM) was incubated with varying concentrations of protein, and fluorescence polarization was measured in a Biotek SynergyH4 plate reader using the appropriate filter sets (λex = 485 ± 20 nm and λem = 525 ± 20 nm). All titrations were performed at 25 °C in 50 mM HEPES buffer (pH 7.5) containing 10 mM KCl, 10 mM MgCl2, 0.1 mM MnCl2, 0.1 mM EDTA, 2 mM β-ME, 10% (v/v) DMSO, and 0.1 mg/mL BSA. The experiments were performed in the presence of 0, 1, 10, 25, 50, 100, 150, and 250 μM MK886. DMSO was substituted in the no MK886 control experiment. Polarization was determined using eq 2.
partial charges (using the AMBER99 force field). Additionally, some extra molecules/ligands such as DNA, dNTP, and metal ions, if present, were removed manually from the coordinate file. The 3-dimensional coordinates for the MK886 molecule were generated using the Marvin Sketch free software tool in the ChemAxon package. Automated in silico molecular docking was performed using the web-based docking server SwissDock (http://www.swissdock.ch/) that is based on the docking algorithm EADock DSS.34 The processed coordinates file (as described above) for each of the proteins and for the ligand MK886 were uploaded, and docking runs were performed using the “Accurate” parameters option, which is the most exhaustive in terms of the number of binding modes sampled. Docking runs were performed as blind, covering the entire protein surface, and not defining any specific region of the protein as the binding pocket in order to avoid sampling bias. Output clusters were obtained after each docking run and were ranked according to the FullFitness (FF) scoring function specified by the SwissDock algorithm (cluster 0 being the cluster with the best FullFitness score). A greater negative FF score indicates a more favorable binding mode with a better fit. Within each cluster, the individual binding poses were further arranged and ranked based on their FF score.

Docking runs were performed using the PDB files 4EBC (hpol ι), 3MR2 (hpol η), and 2OH2 (hpol κ) downloaded from the RCSB public database, for target Y-family DNA polymerases, either with the DNA coordinates (binary) in place or after removing the DNA atoms (apoenzyme). Docking with each protein PDB target was repeated five times. The best binding mode hits obtained after the five docking runs (based on FF score) were displayed using UCSF Chimera. All figures with molecular representations of the docking results were generated using UCSF Chimera and PyMOL. A series of control docking runs were also performed using the structure coordinates of two other proteins, viz. HIV-1 protease (PDB file 1HPV) and bovine serum albumin (BSA; PDB file 4F5S), to test the validity of our docking results with the Y-family DNA polymerases and MK886. The PDB file 1HPV, which is a crystal structure of the HIV-1 protease dimer in complex with a small-molecule inhibitor VX478, was processed as described above. The atoms of the inhibitor VX478 were also removed. Five independent docking runs were performed by uploading the coordinates of the protease dimer and VX478 separately in SwissDock. Similar multiple docking runs were performed with the BSA crystal structure coordinates, with both MK886 and VX478. Results of docking runs of MK886 with BSA were compared to similar results with the Y-family DNA polymerases, while those with VX478 and BSA were compared with those of HIV-1 protease.

■ RESULTS

Inhibition of Human Y-Family DNA Polymerases by MK886. The small molecule MK886 was previously identified as an inhibitor of hpol κ utilizing a high-throughput fluorescent-based screen.25 Our first goal was to determine the specificity of MK886 against hpol κ. DNA synthesis was measured in the
presence of increasing concentrations of MK886. To calculate polymerase activity, the total amount of product was first quantified for the DMSO control. Then the product formed in the presence of MK886 was divided by the control to obtain a percent activity. Under the conditions tested here, which used a five-min time point, hpol κ was inhibited by MK886 with an IC50 value of 63.8 ± 1.7 µM (Figure 1). Similar to hpol κ, the core polymerase domain of hpol η was inhibited with an IC50 value of 45.8 ± 8.4 µM, indicating that catalysis by hpol η and hpol κ is perturbed to a similar extent by MK886. Similar results were obtained when these experiments were repeated using different time points (Supporting Information, Figure S1). The IC50 values reported here are slightly different from those reported by our groups previously, likely due to variations in the experimental design (e.g., enzyme concentrations and DNA substrates).

Additionally, the inhibitory effects of MK886 were measured using two recombinant versions of hpol ι (Figure 1B, bottom two gels). The two versions of hpol ι was used because the original annotation of the hpol ι-coding region erroneously started downstream of the actual translational start site. The first version of hpol ι was predicted to be 715 amino acids in length, and the newly annotated protein has an additional 25 amino acids at the N-terminus (740 a.a.). Thus, the polymerase core that has been utilized for crystallization purposes on many occasions by our group and others was originally denoted as being residues 1–420. The initial hpol ι construct contained residues 26–446 (hpol ι26–446), and the construct that resulted from the updated annotation, with the extended N-terminus, comprised residues 1–446 (hpol ι1–446).

DNA synthesis by the two recombinant versions of hpol ι was tested in the presence of MK886 (Figure 1). The measured IC50 values were 8.2 ± 0.6 µM and 15.1 ± 2.1 µM for hpol ι26–446 and hpol ι1–446, respectively. Analyses of these data revealed that the IC50 is lower for both enzymes relative to what was observed with hpol η and hpol κ and that there was a greater inhibition against the shorter version of hpol ι26–446 which lacks the N-terminal 25 a.a. extension (IC50 = 8.2 ± 0.6 µM). These assays were repeated using different time points to verify these results (Supporting Information, Figure S1). The more potent inhibition of the hpol ι construct that lacks the N-terminal extension was suggestive of the potential site of interaction between the small-molecule inhibitor MK886 and hpol ι26–446. These results are interesting when one considers the relative importance of N-terminal extensions in the determination of Y-family polymerase mechanisms of action. For example, hpol κ lacking the N-terminal 70 amino acids is inactive, and Rev1 requires an arginine residue in the N-digit for the protein–template-directed mechanism of nucleotide selection. The stronger inhibition of hpol ι26–446 by MK886 is discussed later with regard to structural features that are only observed for this Y-family member. Perhaps most importantly, the IC50 experiments show that of the Y-family members tested here, hpol ι is most strongly inhibited by MK886 with the shorter version, hpol ι28–446, exhibiting the lowest IC50 value.

**MK886 Inhibits a Model B-Family DNA Polymerase.**

The small molecule MK886 was clearly able to inhibit all of the Y-family DNA polymerases tested, exhibiting some specificity against hpol ι. To extend these analyses, inhibition of a model B-family DNA polymerase, Dpo1 from *Sulfolobus solfataricus*, was tested (Figure 2). B-family DNA polymerases are typically high-fidelity enzymes involved in processive replication events, though there are well-studied examples of B-family polymerases, such as pol ζ, that participate in DNA repair/translesion synthesis. Dpo1 is a high-fidelity enzyme that appears to function as the primary replicative enzyme in the crenarchaeote *S. solfataricus.* It is a robust enzyme that consists of a single subunit and serves as an excellent model system for understanding the more complex eukaryotic B-family members. An exonuclease-defective mutant form of Dpo1 was utilized for our inhibition assays. Dpo1 was inhibited by MK886 with an IC50 value (59.8 ± 18.5 µM) that is similar to that observed for the Y-family members hpol η and hpol κ (19–526) (Figure 2). These results show that MK886 can inhibit DNA synthesis by enzymes from different families but that it shows the most potency against hpol ι. On the basis of these results, experimental strategies were designed to understand the mechanism of polymerase inhibition by MK886.

**Effect of MK886 upon DNA Binding by hpol ι and hpol κ.** The molecular rationale for increased potency against hpol ι was investigated further in order to determine the mechanism of action. The ability of MK886 to interfere with polymerase binding to primer–template DNA was determined by measuring the equilibrium dissociation constant (Kd,DNA) for hpol ι26–446 in the presence of increasing concentrations of MK886 (Figure 3A and B). For hpol ι26–446, we obtained Kd,DNA values of 5.3 ± 0.7 nM, 5.5 ± 0.7 nM, 6.3 ± 0.9 nM, 8.8 ± 1.3 nM, 9.1 ± 1.6 nM, 18.8 ± 2.7 nM, 16.4 ± 2.1 nM, and 38.8 ± 17.1 nM at MK886 concentrations of 0, 1, 10, 25, 50, 100, 150, and 250 µM, respectively. There was no observed change in the affinity of hpol ι26–446 for DNA at 10 µM MK886 relative to the DMSO control, which is near the IC50 value for inhibition of hpol ι26–446. There appears to be a linear relationship between the amount of MK886 present and the observed binding...
constant for hpol $k_{26-446}$, with the $K_{d,DNA}$ increasing $\sim0.12$ nM for every 1 $\mu$M MK886 in solution. Only MK886 concentrations at or above 100 $\mu$M was there a substantial decrease in affinity for DNA binding (i.e., >2-fold increase in the $K_{d,DNA}$), suggesting that while high-concentrations of MK886 may inhibit hpol $k_{26-446}$ activity by interfering with binding to primer-template DNA, this is not the primary mode of polymerase inhibition observed at lower concentrations of the inhibitor.

The ability of MK886 to inhibit DNA binding by hpol $\kappa$ was also tested (Figure 3C and D). Like hpol $k_{26-446}$, a moderate increase in the equilibrium dissociation constant was observed as the concentration of MK886 was increased. For hpol $k_{19-526}$, $K_{d,DNA}$ values of 11.1 $\pm$ 5.1 nM, 13.7 $\pm$ 5.1 nM, 19.0 $\pm$ 5.6 nM, 26.0 $\pm$ 9.1 nM, 24.1 $\pm$ 9.4 nM, 31.3 $\pm$ 12.1 nM, 70.4 $\pm$ 15.7 nM, and 67.9 $\pm$ 17.1 nM were obtained at MK886 concentrations of 0, 1, 10, 25, 50, 100, 150, and 250 $\mu$M, respectively. Again, the trend appeared to be fairly linear in nature, and linear regression analysis indicated that the $K_{d,DNA}$ for hpol $k_{19-526}$ binding to primer-template DNA increased $\sim0.24$ nM for every 1 $\mu$M increase in MK886 concentration. The small molecule inhibitor produces a change in binding affinity for hpol $\kappa$ that was $\sim2$-fold greater than that observed for hpol $k_{26-446}$. The $K_{d,DNA}$ for hpol $\kappa$ binding to DNA was increased $\sim1.8$-fold at concentrations near the measured $IC_{50}$ value for inhibition of polymerase activity, which would indicate that this is an important feature in the mechanism of MK886 inhibition of hpol $k_{19-526}$. These data are interpreted as evidence of increased specificity for interactions between MK886 and hpol $k_{26-446}$ that occur at sites on the protein that are not involved in DNA binding.

Kinetic Analysis of the Mechanism of Polymerase Inhibition by MK886. In order to further assess the mechanism of polymerase inhibition by MK886, single-nucleotide insertion kinetic analyses of hpol $k_{26-446}$ activity were performed in the presence of increasing concentrations of the inhibitor. The concentration of nucleotide triphosphate was varied (1 $\mu$M to 1.5 mM), and the initial velocity of dNTP insertion was measured. These experiments were repeated in the presence of 20 nM, 50 nM, 1 $\mu$M, and 10 $\mu$M MK886. Steady-state kinetic analyses showed that both the turnover number ($k_{cat}$) and the Michaelis constant ($K_{m,dNTP}$) were altered by the inclusion of MK886 (Table 1), a result that was consistent with a mixed-model of inhibition of DNA polymerase activity by MK886. A fluorescence-based assay was also used to determine the effect of MK886 upon the steady-state kinetic parameters that define hpol $k_{26-446}$-catalyzed insertion of dNTPs in the presence of increasing amounts of inhibitor (Supporting Information, Figure S2). Again, a diminished $k_{cat}$ and increased $K_{m,dNTP}$ were observed as the concentration of MK886 was increased.
MK886 was in the reaction mixture (Table 2), a result that is indicative of mixed-type inhibition by MK886.

Table 2. Steady-State Kinetic Parameters for hpol Y–446. Catalyzed Primer Extension in the Presence of MK886

<table>
<thead>
<tr>
<th>[MK886] (μM)</th>
<th>[MK886] (μM)</th>
<th>K_{M,dNTP} (μM)</th>
<th>k_{cat}/K_{M,dNTP} (min^{-1} μM^{-1})</th>
<th>Fold decrease in k_{cat}/K_{M,dNTP}</th>
<th>k_{cat} (min^{-1})</th>
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<td>0</td>
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<td>28.4 ± 7.6</td>
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<tr>
<td>0.02</td>
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<td>27.1 ± 12.3</td>
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<td>33.1 ± 11.8</td>
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<tr>
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<td>0.036</td>
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</tr>
<tr>
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<td>56.0 ± 19.4</td>
<td>0.027</td>
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</tbody>
</table>

Results of Molecular Docking of MK886 to the Y-Family DNA Polymerases. We attempted to crystallize hpol Y–446 in the presence of MK886 in order to assess the structural rationale for polymerase inhibition. Diffraction crystals were obtained of both binary and ternary complexes of hpol Y–446. MK886 was either co-crystallized or soaked in after the crystals were formed. However, none of the diffraction patterns revealed clear density for the small molecule, even after multiple rounds of refinement (data not shown). After several unsuccessful attempts to obtain data sets with density for MK886, we turned to in silico docking with the web server-based SwissDock in an effort to better understand the molecular features associated with polymerase inhibition.

Prior to performing docking analyses with MK886 and the Y-family polymerases, a series of control docking experiments were performed to establish that the algorithms would provide reasonable models for potential small-molecule binding sites. For a positive control, the previously solved crystal structure for HIV-1 protease in complex with the small-molecule inhibitor VX-478 was used. For negative controls, either VX-478 or MK886 were docked onto bovine serum albumin (BSA). Importantly, SwissDock correctly predicted the binding mode observed for VX-478 in the HIV-1 protease crystal structure (Supporting Information, Figure S3). Both VX-478 and MK886 molecules were distributed across multiple sites on BSA (Supporting Information, Figures S3 and S4). Notably, docking runs with BSA produced variable results, with no consistently identified binding pockets (Supporting Information, Figure S4). Thus, it was confirmed that SwissDock could accurately identify a small-molecule binding site for a previously solved structure and that the program did not identify binding pockets for either VX-478 or MK886 on BSA.

Docking submissions were next performed with the apo-forms of three Y-family DNA polymerases: hpol Y–446 (4EBC), hpol Y–437 (3MR2), and hpol Y–526 (2OH2). An average of 34 clusters was obtained for hpol Y, 32 clusters for hpol Y, and 32 clusters for hpol K from five docking runs of MK886 with each of the three Y-family polymerases (Supporting Information, Table S1). The term cluster refers to a group of MK886 molecules with binding modes occurring at one site on the target protein. The binding modes within a cluster differ only by small rotational changes or rotations around bonds and are ranked according to the predicted binding energy.

In docking analyses, three MK886 binding pockets for hpol Y–446 were consistently identified (Figure 4A) and only two MK886 binding pockets for hpol Y–437 (Figure 4B) and hpol Y–526 (Figure 4C). For all three polymerases, the highest number of clusters was found to localize at the interface between the DNA-binding cleft and the active site of each Y-family member, which we refer to as pocket A (Figure 4). In addition to this pocket, two more distinct binding pockets were observed for hpol Y. The first of these, which we call pocket B, lies at the junction between the finger and palm subdomains of hpol Y (Figure 4A). Pocket B was also identified in docking analyses with hpol Y–437 and hpol Y–526. The final binding pocket for MK886 on hpol Y–446, which we refer to as pocket C, lies at the junction between the palm and thumb subdomains (Figure 4A). Consistently, it was observed that at least one cluster from the top 5 (top 10 in the case of pocket C) localized to these three pockets on hpol Y–446. It is interesting to note that nearly all of the binding modes identified in our docking analyses for the polymerases localized to one of these three pockets, with only an occasional outlier cluster, which localized to a completely different region of the polymerase. The consistent identification of MK886 binding pockets on the DNA polymerases is in stark contrast to what was observed with BSA where the top 10 binding modes changed positions dramatically between docking experiments (Supporting Information, Figure S4).

Docking runs were performed with two versions of the target polymerase PDB files, one in which the coordinates for the DNA atoms were left in place (binary form) and the other in which these were removed to give an “apo” form. The only difference observed in the results obtained with these two versions was in the number of clusters observed in pocket A. As expected, fewer clusters were found localized to pocket A when the binary form of the target PDB was used, as compared to when we used the apo form PDB coordinates since the DNA occludes much of pocket A for each enzyme. However, the number and positions of clusters obtained in the pockets B and C using these two versions did not vary significantly, and hence, in subsequent discussions, only the results with the apo form are considered since this gave the maximum number of hits.

DISCUSSION

The number of polymerases encoded by the human genome includes at least 15 enzymes. It has been recognized that some of the more recently identified polymerases appear to play minimal roles in genomic replication (i.e., processive/high-fidelity events), but rather participate in specialized replication events, such as DNA repair, somatic hypermutation, and replication stress response. The Y-family DNA polymerases represent a class of enzymes that can bypass DNA adducts because of a relatively spacious active site and lack of proofreading activity. Prokaryotic Y-family enzymes constitute the central polymerase activity in the so-called “S.O.S” response. To a large extent, eukaryotes also rely upon the Y-family polymerases to bypass unrepaired DNA adducts. The relevance of Y-family polymerases to human disease is best illustrated by studies with the skin-cancer-prone disease...
The first conclusion that can be drawn from our results is that MK886 inhibits hpol \(_{26-446}\) more potently than the other polymerases tested here. The IC\(_{50}\) values indicate that MK886 inhibits the truncated version of hpol \(\iota\) 6- to 8-fold more effectively than either hpol \(\eta_{1-437}\) or hpol \(\kappa_{19-526}\) (Figure 1 and Figure S1 (Supporting Information)). The increased potency observed for hpol \(\iota\) could be due to a number of factors. Interestingly, hpol \(\iota\) is the only DNA polymerase that preferentially utilizes Hoogsteen base pairing modes during nucleotide selection opposite undamaged templates. This unusual mechanism of nucleotide selection is due in large part to a narrowed active site that constrains the distance between C1’-C1’ atoms to around 9 Å and induces purines to adopt a syn-orientation about the glycosyl bond in order to accommodate the purine/pyrimidine base pair.

On the basis of our analyses, it would appear that MK886 only interferes with hpol \(_{26-446}\) DNA binding at concentrations above the measured IC\(_{50}\) values (Figure 3A and B). This indicates that the small-molecule acts through a mechanism that is not dependent upon direct interference with the polymerase-primer–template DNA interactions. MK886 has a more pronounced effect upon hpol \(\kappa_{19-526}\) DNA binding affinity, increasing the \(K_{d,\text{DNA}}\) about 2-fold at concentrations of MK886 near the IC\(_{50}\) value (Figure 3C and D). Steady-state results with hpol \(_{26-446}\) in which the dNTP concentration was varied point to a mixed-mode of inhibition by MK886 (i.e., an increased \(K_{m,\text{dNTP}}\) was accompanied by a diminished turnover number; see Tables 1 and 2). Thus, at least for hpol \(_{26-446}\), MK886 presumably acts through a mechanism that interferes with the ternary complex in some fashion.

We attempted to reconcile our functional results with structural determinants associated with polymerase inhibition by MK886. Our attempts to crystallize hpol \(_{26-446}\) were successful in that they produced X-ray diffraction data sets, but we did not observe convincing electron density for MK886 in the resulting maps (data not shown). As an alternative strategy, we chose to utilize a freely available docking program (SwissDock) to analyze potential binding pockets for MK886, using coordinates from previously reported crystal structures of three Y-family DNA polymerases tested in our functional assays. The web server (http://www.swissdock.ch) allows for automated docking to be performed with existing coordinate files. For our docking submissions, we focused on the Y-family DNA polymerases. Multiple crystal structures have been reported for hpol \(\eta_{1-437}\), hpol \(\kappa_{19-526}\), and hpol xeroderma pigmentosum variant (XPV), a severe cancer-prone disease that results from inactivating mutations in the gene that codes for hpol \(\eta\) and leads to inaccurate replication of UV-induced cyclobutane pyrimidine dimers. Perhaps not surprisingly, altered activity/expression of Y-family members has been observed in multiple cancer types. The importance of these enzymes to mechanisms of mutagenesis and a potential role for them in cancer initiation/progression led us to identify small molecule inhibitors against one of the human enzymes, hpol \(\kappa\), which is thought to play important roles in the relatively accurate bypass of bulky \(N^2\)-guanyl DNA adducts and in the mutagenic bypass of the common oxidative lesion 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG or o xoG). These investigations identified the small molecule MK886 that was originally pursued as an inhibitor of leukotriene biosynthesis. The current study was an attempt to determine the in vitro specificity and mode of polymerase inhibition by MK886.

The docking results for MK886 reveal potential binding sites, including a pocket specific to hpol \(_{26-446}\). In silico docking runs were performed as described in Experimental Procedures. (A) The crystal structure of hpol \(_{26-446}\) (PDB ID 4EBC) is shown as a cartoon representation, with the subdomains colored as follows: finger, blue; thumb, green; palm, red; little finger (or palm-associated domain), purple. The MK886 binding modes with the highest FF scores from each of the five docking runs are shown as sticks, localized to each of the three binding pockets (A, B, and C, indicated by dashed yellow, green, and magenta circles, respectively). MK886 clusters binding to the pocket A are shown in yellow, pocket B clusters (junction of fingers and palm subdomains) are in green, while those at pocket C are shown in magenta. Panels B and C show crystal structures of hpol \(\eta_{1-437}\) (PDB ID 3MR2) and hpol \(\kappa_{19-526}\) (PDB ID 2OH2), respectively, in the same relative orientation with respect to hpol \(_{26-446}\) in panel A and with the domains colored identically. The N-clasp subdomain in hpol \(\kappa_{19-526}\) is shown in orange in panel C. Note the absence of any MK886 clusters in hpol \(\eta_{1-437}\) and hpol \(\kappa_{19-526}\), in the region corresponding to pocket C of hpol \(_{26-446}\). All crystal structure representations shown were generated using the software PyMol (DeLano Scientific, San Carlos, CA).

Figure 4. Docking results for MK886 reveal potential binding sites, including a pocket specific to hpol \(_{26-446}\). In silico docking runs were performed as described in Experimental Procedures. (A) The crystal structure of hpol \(_{26-446}\) (PDB ID 4EBC) is shown as a cartoon representation, with the subdomains colored as follows: finger, blue; thumb, green; palm, red; little finger (or palm-associated domain), purple. The MK886 binding modes with the highest FF scores from each of the five docking runs are shown as sticks, localized to each of the three binding pockets (A, B, and C, indicated by dashed yellow, green, and magenta circles, respectively). MK886 clusters binding to the pocket A are shown in yellow, pocket B clusters (junction of fingers and palm subdomains) are in green, while those at pocket C are shown in magenta. Panels B and C show crystal structures of hpol \(\eta_{1-437}\) (PDB ID 3MR2) and hpol \(\kappa_{19-526}\) (PDB ID 2OH2), respectively, in the same relative orientation with respect to hpol \(_{26-446}\) in panel A and with the domains colored identically. The N-clasp subdomain in hpol \(\kappa_{19-526}\) is shown in orange in panel C. Note the absence of any MK886 clusters in hpol \(\eta_{1-437}\) and hpol \(\kappa_{19-526}\), in the region corresponding to pocket C of hpol \(_{26-446}\). All crystal structure representations shown were generated using the software PyMol (DeLano Scientific, San Carlos, CA).
MK886 is an indole-derived compound that has been shown to perturb leukotriene biosynthesis. MK886 inhibits DNA synthesis in cell culture at concentrations as low as 1 μM and can induce DNA fragmentation at 0.1 μM. At higher concentrations, MK886 has antiproliferative properties, inducing apoptosis through inhibition of multiple pathways. We recently discovered that MK886 is also an inhibitor of hpol η. The initial discovery that MK886 can inhibit a translesion polymerase led us to study the in vitro specificity of the molecule as an inhibitor of DNA replication and to investigate the mode of action against these enzymes. Our results clearly show that MK886 can inhibit both the B-family polymerase Sso Dpo1 and the Y-family DNA polymerases hpol η and κ with similar efficiency. The relatively high IC_{50} values for MK886 inhibition observed for these enzymes make it unlikely that it could be used to target translesion DNA synthesis properties in vivo. However, future studies with MK886 derivatives may identify more potent and/ or specific inhibitors of translesion DNA polymerase activity.

MK886 exhibits the most potent inhibitory properties against the highly error-prone Y-family member, hpol η. The mechanism of inhibition appears to be mixed in nature. The mixed-mode of inhibition is supported by steady-state kinetic results and is likely the result of MK886 binding to multiple pockets identified by our docking experiments. The docking experiments with MK886 reveal a third binding pocket (pocket C) for MK886 near the N-terminus that does not exist for either hpol η or hpol κ. The existence of multiple binding pockets is supported by our results showing increased potency of MK886 inhibition against a truncated version of hpol η.

CONCLUSIONS

MK886 binds to both the B-family polymerase Sso Dpo1 and the Y-family DNA polymerases hpol η and κ with similar efficiency. The relatively high IC_{50} values for MK886 inhibition observed for these enzymes make it unlikely that it could be used to target translesion DNA synthesis properties in vivo. However, future studies with MK886 derivatives may identify more potent and/ or specific inhibitors of translesion DNA polymerase activity.

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In summary, our results show that MK886 exerts a previously unrecognized biochemical effect by acting as a polymerase inhibitor. It is difficult to make direct comparisons with the IC_{50} values determined in our in vitro polymerase assays and those values reported for MK886 inhibition of 5-lipoxygenase activating protein (FLAP) since most of the previous studies
utilized cell culture assays or simple binding assays to measure the inhibition of FLAP.25,62 Still, it is worth noting that the IC_{50} value for MK886 inhibition of hpol ι is comparable to the IC_{50} value for COX1 inhibition (8 μM) by MK886 that was determined using in vitro activity assays.27 However, it remains unclear whether MK886 can provide targeted modulation of hpol ι translesion polymerase activity in vivo.

■ ASSOCIATED CONTENT

① Supporting Information
Summary of the docking results and results from our kinetic analysis and docking studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS

dNTP, deoxynucleoside triphosphate; FAM, fluorescein; HIV-1, human immunodeficiency virus type 1; pol, polymerase; Sso, Sulfolobus solfataricus; TLS, translesion DNA synthesis

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