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N-Aroyl Indole Thiobarbituric Acids as Inhibitors of DNA Repair and Replication Stress Response Polymerases

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Abstract

Using a robust and quantitative assay, we have identified a novel class of DNA polymerase inhibitors that exhibits some specificity against an enzyme involved in resistance to anti-cancer drugs, namely human DNA polymerase eta (hpol η). In our initial screen, we identified the indole thiobarbituric acid (ITBA) derivative 5-((1-(2-bromobenzoyl)-5-chloro-1*H*-indol-3-yl)methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione (ITBA-12) as an inhibitor of the Y-family DNA member hpol η , an enzyme that has been associated with increased resistance to cisplatin and doxorubicin treatments. An additional seven DNA polymerases from different sub-families were tested for inhibition by ITBA-12. Hpol η was the most potently inhibited enzyme ($30 \pm 3 \mu\text{M}$), with hpol β , hpol γ and hpol κ exhibiting comparable but higher IC_{50} values of $41 \pm 24 \mu\text{M}$, $49 \pm 6 \mu\text{M}$ and $59 \pm 11 \mu\text{M}$, respectively. The other polymerases tested had IC_{50} values closer to $80 \mu\text{M}$. Steady-state kinetic analysis was used to investigate the mechanism of polymerase inhibition by ITBA-12. Based on changes in the Michaelis constant, it was determined that ITBA-12 acts as an allosteric (or partial) competitive inhibitor of dNTP binding. The parent ITBA scaffold was modified to produce 20 derivatives and establish structure-activity relationships by testing for inhibition of hpol η . Two compounds with *N*-naphthoyl Ar-substituents, ITBA-16 and ITBA-19, were both found to have improved potency against hpol η with IC_{50} values of $16 \pm 3 \mu\text{M}$ and $17 \pm 3 \mu\text{M}$, respectively. Moreover, the specificity of ITBA-16 was improved relative to ITBA-12. The presence of a chloro substituent at position 5 on the indole ring appears to be crucial for effective inhibition of hpol η , with the indole *N*-1-naphthoyl and *N*-2-naphthoyl analogs being the most potent inhibitors of hpol η . These results provide a framework from which second-generation ITBA derivatives may be developed against specialized polymerases that are involved in mechanisms of radio- and chemo-resistance.

Keywords

DNA replication; DNA polymerase; small-molecule inhibitors; indole thiobarbituric acids

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Supporting Information. Tables S1-S2 summarizing the IC_{50} determination and figures S1-S3 detailing the results of the screen for inhibitors, validation of ITBA-12, and molecular docking results may be found in the Supporting Information section. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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INTRODUCTION

Efficient DNA replication is a barrier to genomic instability.¹⁻⁴ The process of replicating DNA in a timely manner can be perturbed by both exogenous and endogenous processes.⁵ DNA adducts and/or natural replication fork barriers, such as G-quadruplex forming sequences, can impede progress by inhibiting the replication machinery.^{3, 6, 7} In these instances of perturbed replication, there are cellular mechanisms in place that recruit specialized polymerases to sites of replication stress.⁸ These so-called replication stress response (RSR) polymerases assist replication fork progression during S-phase and participate in post-replication repair events that occur during the G2/M transition at sites where the replisome has collapsed and ssDNA gaps persist.^{9, 10} Replication stress is a hallmark of cancer and many existing chemo- and radiotherapies act to limit tumor growth primarily through the induction of DNA damage, which blocks replication.^{11, 12} Moreover, recent studies have shown that in some tumors, such as highly malignant brain tumors, markers of replication stress are constitutively activated prior to treatment with genotoxic agents.¹³ Up-regulation of specialized RSR polymerases in these tumors may contribute to the progression of the disease by promoting increased genomic instability, as has been demonstrated by examination of clinical specimens and through *in vitro* experiments with the human Y-family DNA polymerase kappa (hpol κ).¹⁴⁻¹⁶

As indicated above, anti-cancer treatments that use DNA damage as a means of eliminating tumor cells are often rendered ineffective through the stimulation of DNA repair mechanisms or through other acquired mutations, which result in resistance to the damaging effects of the compound.¹⁷⁻¹⁹ Another way of acquiring resistance to genotoxic agents is through pathways that allow the cell to tolerate the DNA damage by performing translesion DNA synthesis (TLS) past the offending lesion instead of performing repair.²⁰⁻²² DNA damage tolerance pathways are utilized when DNA adducts are not repaired prior to S-phase or when the repair mechanism requires a specialized polymerase to complete the repair process (e.g. nucleotide excision repair of cross-linked DNA).⁷ TLS is an important part of the replication stress response mediated by the RSR-associated ATR/Chk1 kinase signaling pathway.^{3, 4, 23-27} The nature of TLS involves bypassing lesions that are often incapable of forming normal Watson-Crick base pairs and as such, is generally thought to be somewhat error-prone.²⁸⁻³⁰ Thus, activation of TLS pathways in response to anti-cancer treatments can directly contribute to cell survival by bypassing DNA adducts, while simultaneously producing mutations associated with the development of resistance and tumor heterogeneity. The ability to specifically target these processes in tumor cells could be of great potential benefit.

The enzymes primarily responsible for DNA adduct bypass include the Y-family DNA polymerases (pols).^{31, 32} These specialized polymerases exhibit unique structural and functional properties that allow for the successful copying of DNA adducts, but these features also make them targets for small-molecule inhibitors.^{33, 34} The mis-regulation and mutation of Y-family pols has been observed in many tumor types.^{16, 35-39} Importantly, recent studies have shown that Y-family polymerases, particularly human DNA polymerase eta (hpol η), participate in mechanisms that promote resistance to anti-cancer treatments, such as cisplatin and doxorubicin.²⁰⁻²² We have attempted to identify novel inhibitors of DNA polymerase activity by utilizing a previously reported fluorescence-based assay that measures polymerase-catalyzed strand displacement, which is dependent upon nucleotidyl transfer by the enzyme.⁴⁰ We screened a targeted collection of over 300 compounds that were designed to target nucleic acid-interacting proteins and enzymes. Of these 320 compounds, one of the more potent inhibitors of DNA polymerase activity was found to contain an indole thiobarbituric acid (ITBA) chemical scaffold. A number of ITBA

derivatives were then prepared to assess structure-activity relationships and steady-state kinetic analysis of the compounds identified in the screen was performed to determine the mechanism of polymerase inhibition. Our results report on the identification and characterization of first generation DNA polymerase η inhibitors derived from a novel chemical scaffold.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were molecular biology grade or better. All dNTPs were purchased from GE Healthcare Life Sciences (Piscataway, NJ). All oligonucleotides used in this work were synthesized by either Integrated DNA technologies (Coralville, IA) or Biosearch Technologies (San Diego, 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 gel-based extension assays and inhibition assays was 5'-(FAM-TTT)-GGG GGA AGG ATT C-3'. The template DNA sequence used in the gel-based extension assays and inhibition assays was 5'-TCA CGG AAT CCT TCC CCC-3'.

Expression and purification of recombinant proteins

The pBG101 plasmid was used to prepare constructs encoding human DNA polymerases η (amino acids 1-437), ι (amino acids 26-446) and κ (amino acids 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 *Escherichia coli* (strain BL21 DE3) and purified in an identical manner. Briefly, pBG101 vector encoding the polymerases 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 (OD600 = 0.5-0.6), followed by induction for three hours (37 °C and 250 rpm) by addition of isopropyl β -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 β -mercaptoethanol (β -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 (35,000 g, 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 β -ME, 10% glycerol and 20 mM imidazole to remove non-specifically 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 β -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 the HEPES buffer (pH 7.5) containing 0.1 M NaCl, 5 mM β -ME, and 30% glycerol. The model B-family DNA polymerases, Dpo1 and Dpo4, from *S. solfataricus* were expressed and purified as described previously.^{41, 42} Human DNA polymerase beta (hpol β) was purchased from Enzymax (Lexington, KY). HIV-1 RT was kindly provided by Prof. F. Peter Guengerich (Vanderbilt University School of Medicine). Purified hetero-

dimeric hpol γ was generously provided by Dr. William Copeland (Mitochondrial DNA replication group, NIEHS).

Fluorescence-based assay to screen for inhibition of DNA polymerase activity

A library of 320 compounds targeted against nucleic acid interacting proteins was screened for inhibition of polymerase activity using an assay that monitors fluorescence from a 5-carboxytetramethylrhodamine (TAMRA) labeled oligonucleotide. In order to prepare the DNA for the experiment, a TAMRA-labeled reporter (or displaced) strand (5'-TTT TTT TTG C-TAMRA-3') and unlabeled primer strand (5'-TCA CCC TCG TAC GAC TCT T-3') were annealed to a Black Hole Quencher (BHQ)-labeled template strand (5'-BHQ2-GCA AAA AAA AAA GAG TCG TAC GAG GGT GA-3') in a solution containing 10 mM Tris (pH 8.0), 50 mM NaCl, 2 mM MgCl₂, and dH₂O. The template (T), primer (P) and displaced strand (D) oligonucleotides were mixed in a 1:1.5:1.5 (T:P:D) molar ratio for annealing. After an incubation period of three minutes at 95 °C, the DNA was allowed to slowly cool to room temperature overnight.

The fluorescence-based assay used to screen for polymerase inhibitors measures polymerase-catalyzed displacement of a TAMRA-labeled oligonucleotide.⁴⁰ For the initial screen, the experimental setup included 50 nM hpol η , 50 nM DNA, 6 μ M compound, 100 μ M dTTP and 1 mM MgCl₂ (8). The reactions were performed in 50 mM Tris (pH 8.0) buffer containing 40 mM NaCl, 2 mM dithiothreitol (DTT), and 0.01% (v/v) Tween-20. The concentration of dimethyl sulfoxide (DMSO) was 3.5% (v/v) for the initial screen. The enzyme, the compounds (including a DMSO control) and dTTP were combined with the reaction buffer in individual wells of each half-plate and allowed to incubate for 5-10 minutes. The DNA substrate was subsequently added to initiate the reaction and the plate was read immediately using a BioTek SynergyH4 plate reader (λ_{ex} = 525 nm, λ_{em} = 598 nm). The final reaction volume was 200 μ L. Fluorescence was monitored for 90 minutes for most reactions. The initial portion of the velocity curve was analyzed by linear regression to calculate an observed rate of product formation. For each data set, we averaged eight DMSO control experiments to obtain our measure of 100% activity. Rates of product formation in the presence of each compound were then divided by the rate of the DMSO control to produce a relative measure of polymerase activity.

Gel-based assay measuring DNA polymerase activity

In order to provide a second measure of enzyme inhibition, polymerase extension assays were performed. Briefly, hpol η (2 nM) was pre-incubated with FAM-16/18-mer primer-template DNA (100 nM) and either DMSO (final concentration = 10%) or compound (6 μ M, 13 μ M and 60 μ M; maintaining 10% DMSO for all experiments). Polymerase catalysis was initiated by the addition of dNTP (1 mM) and MgCl₂ (5 mM). The reaction was allowed to proceed at 37°C for varying time and then terminated by the addition of 5 μ L aliquots of the reaction mix to 25 μ L of the quench solution (20 mM EDTA and 95% formamide). The samples were separated using a 16% polyacrylamide/7M urea gel and the products analyzed using a Typhoon imager and ImageQuantTM software (GE Healthcare Life Sciences).

Determination of IC₅₀ values for individual DNA polymerases

In order to determine the IC₅₀ value for each enzyme, we repeated the fluorescence-based polymerase assay with increasing concentrations of inhibitor. The conditions varied slightly for each enzyme. With the exception of hpol ι , all IC₅₀ experiments were performed in 50 mM Tris HCl (pH 8.0) buffer containing 1 mM MgCl₂, 0.1 mM dTTP, 40 mM NaCl, 2 mM DTT, 0.01% (v/v) Tween 20 and 10% (v/v) DMSO. For experiments with hpol ι , KCl was substituted for NaCl and 0.25 mM MnCl₂ was substituted for MgCl₂. The concentration of inhibitor was 0, 1, 5, 10, 20, 30, 50, 75 and 100 μ M. The enzyme and DNA concentrations

were as follows: 10 nM hpol η , 50 nM DNA; 3 nM hpol κ , 60 nM DNA; 50 nM hpol ι , 50 nM DNA; 10 nM Dpo4, 50 nM DNA; 100 nM Dpo1, 50 nM DNA; 50 nM hpol β , 50 nM DNA; 10 nM hpol γ , 50 nM DNA; 50 nM HIV-1 RT, 50 nM DNA. The percent activity was plotted as a function of the log of inhibitor concentration and fit to a four-parameter logistic model (equation 1) using Prism software (GraphPad, San Diego, CA):

$$y = \text{bottom} + \frac{(\text{top} - \text{bottom})}{1 + (x/IC_{50})^{\text{slope}}} \quad \text{Eq.1}$$

The experiments were performed in triplicate and the mean ($\pm$ standard deviation) of the IC_{50} values calculated for each data set is reported.

Steady-state kinetic analysis of DNA polymerase activity

The steady-state kinetic parameters defining polymerase activity in the presence of inhibitor were determined using the fluorescence-based reporter assay. Hpol η activity was monitored in the presence of increasing concentrations of dTTP (1, 5, 10, 20, 30, 50, 75 and 100 μ M). The measured relative fluorescence units (RFUs) were converted to a nanomolar quantity by calculating the total change in fluorescence observed between the start of the reaction and the time point at which the fluorescence change was maximal and considering that change to be 100% of substrate converted to product. The percentage of substrate converted to product was multiplied by the concentration of dsDNA in the reaction mixture. Product formation was then plotted as a function of time, and by considering only the linear portion of each curve, velocities were calculated for each dTTP concentration. These were then plotted as a function of dTTP concentration, and fit to a hyperbola. After correcting for enzyme concentration, the steady-state kinetic parameters were obtained as described previously. The experiments were then repeated in the presence of inhibitor (1, 10, 20 40 and 50 μ M) to determine the effect of the small-molecule upon Michaelis-Menten kinetics.

RESULTS & DISCUSSION

Identification of small-molecule inhibitors of hpol η

We initially screened a small library of some 320 compounds using a robust and quantitative assay that measures polymerase activity over time, which has been validated previously as a means of identifying small-molecule inhibitors of Y-family members and other DNA polymerase families (Figure 1A).^{33, 40} The assay relies upon polymerase-catalyzed displacement of a fluorescently-labeled oligonucleotide and shows excellent reproducibility (Figure 1B). Our initial screen to identify inhibitors of hpol η was performed with a final concentration of 6 μ M for each compound. The experiments were performed in triplicate. The means and standard deviation for polymerase activity (i.e. the rate of change in fluorescence) from all samples were calculated and compounds exhibiting a decrease in activity of greater than one standard deviation from that observed for the control experiment were considered as possible inhibitors (Figure S1).

From this set of experiments, we identified 28 potential polymerase inhibitors. The success rate (~9% of the compounds tested were found to inhibit polymerization) can be attributed in part to the targeted nature of the compound library. One of the compounds identified in our screen was 5-((1-(2-bromobenzoyl)-5-chloro-1*H*-indol-3-yl)methylene)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione, which is an indole thiobarbituric acid (ITBA) derivative (Figure 2A). Since this molecule was the twelfth ITBA derivative prepared for our screen, we refer to it as ITBA-12. We re tested ITBA-12 for polymerase inhibition by monitoring polymerase activity with the fluorescence assay at increasing concentrations of inhibitor (6 μ M, 13 μ M and 20 μ M). We observed a dose-dependent decrease in polymerase

activity using both fluorescence and gel-based analyses (Figure S2) that led us to perform a more rigorous determination of the IC_{50} value for ITBA-12 mediated inhibition of hpol η (Figure 2B). The measured IC_{50} value for ITBA-12 was found to be $29.8 \pm 2.7 \mu\text{M}$ (Figure 2C). From these results, we determined ITBA-12 to be a reasonable starting point for the development of novel polymerase inhibitors. However, before proceeding further, we attempted to ascertain the specificity of ITBA-12 against hpol η .

Determination of the *in vitro* specificity of ITBA-12 against different DNA polymerases

In order to determine the specificity of ITBA-12 against the Y-family member hpol η , we measured the IC_{50} values for inhibition of seven other polymerases (Figure 3 and Table S1). We found that ITBA-12 exhibited the most potent inhibition of hpol η when compared with the other polymerases tested. Of the other Y-family polymerases tested, only hpol κ showed an IC_{50} value that was noticeably reduced relative to hpol ι and Dpo4 from *Sulfolobus solfataricus* (Sso). However, the IC_{50} value for ITBA-12 inhibition of hpol κ is twice as high as that measured for hpol η , suggesting some discrimination between the Y-family enzymes tested here. We next tested the model B family polymerase Dpo1 from *S. solfataricus* for inhibition by ITBA-12 and found the IC_{50} value to be near $80 \mu\text{M}$. A similar value was observed for HIV-1 RT. Besides hpol η , the closest IC_{50} values were those obtained for the X-family member hpol β and the A-family member hpol γ , which showed IC_{50} values of $41 \pm 24 \mu\text{M}$ and $49 \pm 6 \mu\text{M}$, respectively. Based on these results we concluded that ITBA-12 exhibits modest selectivity against hpol η but we hesitate to call it a specific inhibitor of this enzyme.

Mechanism of hpol η inhibition by ITBA-12

Next, we sought to determine the mechanism of polymerase inhibition by ITBA-12. We measured the Michaelis-Menten kinetic parameters describing hpol η activity in the presence of increasing concentrations of inhibitor. By varying the concentration of dTTP in the reaction mixture we determined the turnover number (k_{cat}) and Michaelis constant ($K_{\text{m,dTTP}}$) for hpol η in the absence of inhibitor and at five concentrations of ITBA-12 (Table 1). Increasing the amount of inhibitor in the reaction mixture results in an increase in the Michaelis constant but does not appear to affect the turnover number. These results are indicative of a competitive mode of inhibition by ITBA-12. However, there is a non-linear relationship between the concentration of inhibitor and the Michaelis constant, which is suggestive of allosteric or partial inhibition of pol activity by ITBA-12. In such a model, the enzyme•substrate•inhibitor complex would still be able to adopt a catalytically competent state. Docking analyses position ITBA derivatives in the cleft between the finger and little finger domains of hpol η (Figure S3). Single-stranded template DNA normally occupies this pocket, and binding of ITBA-12 could either distort the templating base or induce a conformational change in hpol η that impairs nucleotidyl transfer.

Structure-activity relationships for inhibition of hpol η by ITBA derivatives

In order to improve the potency of ITBA-12 and establish structure-activity relationships, a series of ITBA derivatives was prepared as described elsewhere.⁴³ In total, 20 compounds derived from the ITBA scaffold were tested for their ability to inhibit hpol η (Figure 4). The compounds were tested at a concentration of $50 \mu\text{M}$ and the percent activity relative to the control assay was plotted (Figure 4C). The parent compound (ITBA-1; R=H, Ar=C₆H₅) shows almost no inhibitory action at $50 \mu\text{M}$. The addition of a chloro substituent at the 5 position of the indole ring results in a modest drop in polymerase activity (Figure 4C, ITBA-2), but substitution at this same position with a bromo group fails to inhibit the polymerase at the concentration tested (Figure 4C, ITBA-3). We then tested indole *N*-(4-substituted benzoyl) derivatives of ITBA (ITBA-4 through ITBA-10). With the exception of ITBA-7, the *N*-4-substituted benzoyl analogs do not inhibit hpol η . Notably, of these 4-

substituted derivatives only ITBA-7 has a 5-chloro substituent on the indole ring. The addition of a bromo substituent at the 2 position of the *N*-benzoyl moiety (ITBA-11) somewhat improves the inhibition of hpol η relative to the parent compound, but it is the addition of a chloro substituent at position 5 on the indole ring *combined* with a 2-bromo substituent on the *N*-benzoyl moiety (ITBA-12) that causes a dramatic improvement in activity against the polymerase (Figure 4C, ITBA-12). When R is either a bromo or a methoxy group, the activity is attenuated (Figure 4C, ITBA-13 and 14).

The most potent ITBA derivatives prepared in our study possessed a naphthoyl moiety as the Ar substituent (ITBA-15 through ITBA-20). Both indole *N*-1-naphthoyl- and indole *N*-2-naphthoyl-substituted ITBA derivatives were compared. The indole *N*-1-naphthoyl substituted molecules (ITBA-15, 16 and 17) display large differences in the observed level of polymerase inhibition that appears to be dependent upon the nature of the R substituent. ITBA-16 was found to be the most potent inhibitor of hpol η activity identified in our study. This molecule retained a 5-chloro substituent on the indole ring but incorporated a 2-naphthoyl moiety as the indole *N*-substituent. The measured IC_{50} value for ITBA-16 inhibition of hpol η was found to be $15.8 \pm 3.3 \mu M$ (Figure 5), which is about half the value measured for ITBA-12. Three ITBA derivatives bearing an indole *N*-2-naphthoyl substituted moiety each show roughly equal activity against hpol η when tested at a final inhibitor concentration of $50 \mu M$ (Figure 4 compare results for ITBA-18, 19 and 20). We measured the IC_{50} values for ITBA-18, 19 and 20 and found that substitution of a chloro group at the 5 position of the indole ring (ITBA-19, $IC_{50} = 16.6 \pm 3.3 \mu M$) does indeed show improved inhibitory action, relative to the ITBA-18 (R=H; $IC_{50} = 67.8 \pm 3.3 \mu M$) or ITBA-20 (R=O-methoxy; $IC_{50} = 72.4 \pm 3.3 \mu M$). Thus, the presence of a 5-chloro substituent appears to be of some importance for effective inhibition of hpol η .

Specificity of ITBA-16 for inhibition of hpol η

The improved IC_{50} for ITBA-16 against hpol η led us to examine the specificity of this *N*-1-naphthoyl derivative. As with ITBA-12, the IC_{50} values of ITBA-16 for inhibition of seven additional DNA pols were measured (Figure 6 and Table S2). Once again, hpol η was most strongly inhibited by ITBA-16 ($IC_{50} 15.8 \pm 3.3 \mu M$). The IC_{50} values closest to that of hpol η were $32.9 \pm 3.2 \mu M$ for hpol κ and $35.3 \pm 2.9 \mu M$ for hpol γ . These results suggest that the specificity of ITBA-16 against hpol η , though not great, is increased slightly relative to ITBA-12 since the IC_{50} of ITBA-16 for hpol η is a little over 2-fold lower than any of the other pols tested. ITBA-12 showed only ~1.4-fold difference between hpol η and hpol β (Figure 3). Thus, the addition of *N*-1-naphthoyl to the Ar position on the parent scaffold results in more specific inhibition of hpol η .

CONCLUSIONS

We have screened a library of novel compounds in order to identify potential small molecule inhibitors of translesion DNA polymerases, such as hpol η . Of the 28 leads identified in the screen, ITBA-12 was determined early on to be a true polymerase inhibitor, as assessed by complementary assays (Figure S2). In terms of polymerase specificity, ITBA-12 and ITBA-16 both exhibit the most potency against hpol η , with comparable IC_{50} values for inhibition against the X-family member hpol β , the A-family member hpol γ , and another Y-family member, hpol κ . The mechanism of inhibition by ITBA-12 was probed by both steady-state kinetic analysis and by chemical modification of the ITBA scaffold. The allosteric or partial competitive mode of inhibition suggests that ITBA may interfere with some aspect of dNTP binding. We performed molecular docking analyses with SwissDOCK and the top binding modes identified localize ITBA-12 (data not shown) and ITBA-16 (Figure S3) to a pocket between the finger and little finger domains. This pocket was also identified when we performed docking with another small-molecule inhibitor of Y-family

members, candesartan cilexetil (Figure S3). Notably, the finger domain possesses residues that are crucial for stabilization of the incoming dNTP within the active site of all DNA polymerases, although the secondary structures defining the “finger” domain vary considerably between polymerase families. Alternatively, recent crystal structures with hpol η bound to cisplatin-modified DNA reported the identification of a second nucleotide binding site near Trp²⁹⁷, when crystals were soaked with high concentrations of dATP (>0.5 mM).⁴⁴ Binding of the extra nucleotide at this hydrophobic site appears to inhibit hpol η activity based on mutational analysis. The hydrophobic pocket identified in the crystal structure is located near the thumb domain of the protein and could interfere with conformational changes identified in this region for other Y-family members. Structural investigations into ITBA-mediated inhibition of hpol η are underway.

Following identification of ITBA-12 as an inhibitor of hpol η , we were able to use structure-activity relationships to improve the potency of our initial finding. The presence of a chloro substituent at position 5 of the indole ring of ITBA appears to be necessary to impart the maximum inhibitory effect observed in our assays. The comparative improvement on polymerase inhibition by incorporating a naphthoyl group as the Ar substituent is interesting. Examination of both the indole *N*-1- and *N*-2-naphthoyl analogs shows that the activity of the indole *N*-1-naphthoyl analogs appears to be dependent upon the presence of a 5-chloro substituent on the indole ring (Figure 4). Optimization strategies are being devised to expand upon our first generation of ITBA polymerase inhibitors in an effort to improve potency and specificity against specialized polymerases.

Several groups have reported the identification of small-molecule inhibitors of non essential DNA polymerases. A study in 2009 identified aurintricarboxylic acid and ellagic acid as inhibitors of hpol η , hpol β and hpol ι .⁴⁰ These compounds exhibit very promising sub-micromolar IC₅₀ values. However, structure-activity relationship studies are lacking for aurintricarboxylic acid and ellagic acid. Also, it remains to be seen whether these compounds show potent anti-proliferative or anti cancer activities. The potency of either ITBA-16 or ITBA-19 against hpol η must be significantly improved in order to prove comparable to either aurintricarboxylic acid or ellagic acid. Nevertheless, the specificity of the ITBA compounds tested here against hpol η is novel, as both aurintricarboxylic acid and ellagic acid exhibited greater than 2-fold specificity against hpol β .⁴⁰

With regard to specificity against hpol η , recent work from our group has shown that the leukotriene biosynthesis inhibitor MK886 inhibits hpol ι (IC₅₀ = 9 μ M) about five- to eight-times more effectively than other Y-family pols.⁴⁵ In that study, hpol η was inhibited by MK886 with an IC₅₀ of ~46 μ M. Potency and specificity comparable to our results with MK886 was observed for inhibitors of the X-family member hpol λ with compounds built off of a rhodanine scaffold.⁴⁶ Notably, the specificity of the rhodanine compounds towards hpol λ was quite good, as they were ~10-fold less active against hpol β , similar to our results with MK886 and hpol ι . Thus, we hope to substantially improve the specificity against hpol η with second-generation ITBA derivatives.

The small-molecule inhibitors identified in our study are structurally related to *N*-benzoyl indolylbarbituric acids that possess potent anti-cancer activity. Additionally, *N*-benzylindole analogs have been shown exhibit potent cytotoxic and radiosensitizing activities against colorectal adenocarcinoma cells (HT-29).⁴⁷ Moreover, the combination of anti-cancer and anti-inflammatory properties in a single agent presents an attractive approach to treating cancer. At least two of the ITBA derivatives tested here (ITBA-9 and 17) appear to possess properties required for the anti-cancer/anti-inflammatory type of approach.⁴³ Based on the results reported here, it may be possible to combine the anti-inflammatory and cytotoxic properties with the suppression of DNA damage tolerance.

Our ultimate goal is to find small-molecule inhibitors of translesion DNA polymerases, such as hpol η , in order to augment existing anti-cancer treatments. Experiments reported elsewhere have shown that ITBA-9, 14, 15 and 17 exhibit potent anti-proliferative and cytotoxic properties against a number of human tumor cell lines.⁴³ Of these molecules, only ITBA-9 shows a modest inhibition of hpol η activity, although we cannot rule out the possibility that these compounds might inhibit other polymerases. Notably, the isomeric *N*-2-naphthoyl ITBA analogs do not exhibit the same growth inhibitory and cell killing properties observed for the *N*-1-naphthoyl ITBA-15 and 17 analogs. It is possible that the *N*-2-naphthoyl ITBA derivatives, which do show relatively potent inhibition of hpol η , could exhibit improved anti-proliferative properties if they were used to treat cells that had also been exposed to DNA damaging agents. Experiments to determine whether these compounds can modulate cell growth in the face of DNA damage are ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FAM	fluorescein
HIV-1 RT	human immunodeficiency virus type-1 reverse transcriptase
Pol	polymerase

REFERENCES

- (1). Admire A, Shanks L, Danzl N, Wang M, Weier U, Stevens W, Hunt E, Weinert T. Cycles of chromosome instability are associated with a fragile site and are increased by defects in DNA replication and checkpoint controls in yeast. *Genes Dev.* 2006; 20:159–173. [PubMed: 16384935]
- (2). Bester AC, Roniger M, Oren YS, Im MM, Sarni D, Chaoat M, Bensimon A, Zamir G, Shewach DS, Kerem B. Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell.* 2011; 145:435–446. [PubMed: 21529715]
- (3). Cha RS, Kleckner N. ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. *Science.* 2002; 297:602–606. [PubMed: 12142538]
- (4). Cimprich KA, Cortez D. ATR: an essential regulator of genome integrity. *Nat. Rev. Mol. Cell. Biol.* 2008; 9:616–627. [PubMed: 18594563]
- (5). Friedberg, EC.; Walker, GC.; Siede, W.; Wood, RD.; Shultz, RA.; Ellenberger, T. *DNA Repair and Mutagenesis*. 2nd ed. ASM Press; Washington, D.C.: 2006.
- (6). Azvolinsky A, Giresi PG, Lieb JD, Zakian VA. Highly transcribed RNA polymerase II genes are impediments to replication fork progression in *Saccharomyces cerevisiae*. *Molecular cell.* 2009; 34:722–734. [PubMed: 19560424]

- (7). Chang DJ, Cimprich KA. DNA damage tolerance: when it's OK to make mistakes. *Nat. Chem. Biol.* 2009; 5:82–90. [PubMed: 19148176]
- (8). Friedberg EC, Wagner R, Radman M. Specialized DNA polymerases, cellular survival, and the genesis of mutations. *Science*. 2002; 296:1627–1630. [PubMed: 12040171]
- (9). Edmunds CE, Simpson LJ, Sale JE. PCNA ubiquitination and REV1 define temporally distinct mechanisms for controlling translesion synthesis in the avian cell line DT40. *Molecular cell*. 2008; 30:519–529. [PubMed: 18498753]
- (10). Phillips LG, Sale JE. The Werner's Syndrome protein collaborates with REV1 to promote replication fork progression on damaged DNA. *DNA Repair (Amst)*. 2011; 9:1064–1072. [PubMed: 20691646]
- (11). Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646–674. [PubMed: 21376230]
- (12). Lord CJ, Ashworth A. The DNA damage response and cancer therapy. *Nature*. 2012; 481:287–294. [PubMed: 22258607]
- (13). Bartkova J, Hamerlik P, Stockhausen MT, Ehrmann J, Hlobilkova A, Laursen H, Kalita O, Kolar Z, Poulsen HS, Broholm H, Lukas J, Bartek J. Replication stress and oxidative damage contribute to aberrant constitutive activation of DNA damage signalling in human gliomas. *Oncogene*. 2010; 29:5095–5102. [PubMed: 20581868]
- (14). Bavoux C, Leopoldino AM, Bergoglio V, J OW, Ogi T, Bieth A, Judde JG, Pena SD, Poupon MF, Helleday T, Tagawa M, Machado C, Hoffmann JS, Cazaux C. Up-regulation of the error-prone DNA polymerase κ promotes pleiotropic genetic alterations and tumorigenesis. *Cancer Res*. 2005; 65:325–330. [PubMed: 15665310]
- (15). Jones MJ, Colnaghi L, Huang TT. Dysregulation of DNA polymerase kappa recruitment to replication forks results in genomic instability. *EMBO J*. 2012; 31:908–918. [PubMed: 22157819]
- (16). Wang H, Wu W, Wang HW, Wang S, Chen Y, Zhang X, Yang J, Zhao S, Ding HF, Lu D. Analysis of specialized DNA polymerases expression in human gliomas: association with prognostic significance. *Neuro. Oncol*. 2010; 12:679–686. [PubMed: 20164241]
- (17). Fukushima T, Takeshima H, Kataoka H. Anti-glioma therapy with temozolomide and status of the DNA-repair gene MGMT. *Anticancer Res*. 2009; 29:4845–4854. [PubMed: 20032445]
- (18). Kitange GJ, Carlson BL, Schroeder MA, Grogan PT, Lamont JD, Decker PA, Wu W, James CD, Sarkaria JN. Induction of MGMT expression is associated with temozolomide resistance in glioblastoma xenografts. *Neuro. Oncol*. 2009; 11:281–291. [PubMed: 18952979]
- (19). Wang L, Mosel AJ, Oakley GG, Peng A. Deficient DNA damage signaling leads to chemoresistance to cisplatin in oral cancer. *Mol. Cancer Ther*. 2012
- (20). Albertella MR, Green CM, Lehmann AR, O'Connor MJ. A role for polymerase eta in the cellular-tolerance to cisplatin induced damage. *Cancer Res*. 2005; 65:9799–9806. [PubMed: 16267001]
- (21). Ceppi P, Novello S, Cambieri A, Longo M, Monica V, Lo Iacono M, Giaj-Levra M, Saviozzi S, Volante M, Papotti M, Scagliotti G. Polymerase eta mRNA expression predicts survival of non-small cell lung cancer patients treated with platinum-based chemotherapy. *Clin. Cancer Res*. 2009; 15:1039–1045. [PubMed: 19188177]
- (22). Moraes MC, de Andrade AQ, Carvalho H, Guecheva T, Agnoletto MH, Henriques JA, Sarasin A, Sary A, Saffi J, Menck CF. Both XPA and DNA polymerase eta are necessary for the repair of doxorubicin-induced DNA lesions. *Cancer Lett*. 2012; 314:108–118. [PubMed: 21999933]
- (23). Ammazalorso F, Pirzio LM, Bignami M, Franchitto A, Pichierri P. ATR and ATM differently regulate WRN to prevent DSBs at stalled replication forks and promote replication fork recovery. *EMBO J*. 2010; 29:3156–3169. [PubMed: 20802463]
- (24). Bi X, Barkley LR, Slater DM, Tateishi S, Yamaizumi M, Ohmori H, Vaziri C. Rad18 regulates DNA polymerase kappa and is required for recovery from S-phase checkpoint-mediated arrest. *Mol. Cell. Biol*. 2006; 26:3527–3540. [PubMed: 16611994]
- (25). Bienko M, Green CM, Crosetto N, Rudolf F, Zapart G, Coull B, Kannouche P, Wider G, Peter M, Lehmann AR, Hofmann K, Dikic I. Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. *Science*. 2005; 310:1821–1824. [PubMed: 16357261]

- (26). Chen YW, Cleaver JE, Hatahet Z, Honkanen RE, Chang JY, Yen Y, Chou KM. Human DNA polymerase η activity and translocation is regulated by phosphorylation. *Proc. Natl. Acad. Sci. (U. S. A.)*. 2008; 105:16578–16583. [PubMed: 18946034]
- (27). Göhler T, Sabbioneda S, Green CM, Lehmann AR. ATR-mediated phosphorylation of DNA polymerase η is needed for efficient recovery from UV damage. *J. Cell Biol.* 2011; 192:219–227. [PubMed: 21242293]
- (28). Goodman MF. Error-prone repair DNA polymerases in prokaryotes and eukaryotes. *Annu. Rev. Biochem.* 2002; 71:17–50. [PubMed: 12045089]
- (29). Kunkel TA. DNA replication fidelity. *J. Biol. Chem.* 2004; 279:16895–16898. [PubMed: 14988392]
- (30). Kunkel TA. Evolving views of DNA replication (in)fidelity. *Cold Spring Harb Symp Quant Biol.* 2009; 74:91–101. [PubMed: 19903750]
- (31). Prakash S, Johnson RE, Prakash L. Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu. Rev. Biochem.* 2005; 74:317–353. [PubMed: 15952890]
- (32). Yang W, Woodgate R. What a difference a decade makes: insights into translesion DNA synthesis. *Proc. Natl. Acad. Sci. (U. S. A.)*. 2007; 104:15591–15598. [PubMed: 17898175]
- (33). Yamanaka K, Dorjsuren D, Eoff RL, Egli M, Maloney DJ, Jadhav A, Simeonov A, Lloyd RS. A comprehensive strategy to discover inhibitors of the translesion synthesis DNA polymerase κ . *PLoS One*. 2012; 7:e45032. [PubMed: 23056190]
- (34). Yamanaka, K.; Lloyd, RS. Functions of Translesion DNA polymerases: Implications for cancer risk and opportunities as therapeutic targets. In: Madhusudan, S.; Wilson, DM., 3rd, editors. *DNA Repair and Cancer: Bench to Clinic*. Science Publishers; Enfield, NH: 2012.
- (35). Albertella MR, Lau A, O'Connor MJ. The overexpression of specialized DNA polymerases in cancer. *DNA Repair (Amst)*. 2005; 4:583–593. [PubMed: 15811630]
- (36). Lemée F, Bavoux C, Pillaire MJ, Bieth A, Machado CR, Pena SD, Guimbaud R, Selves J, Hoffmann JS, Cazaux C. Characterization of promoter regulatory elements involved in downexpression of the DNA polymerase κ in colorectal cancer. *Oncogene*. 2007; 26:3387–3394. [PubMed: 17099721]
- (37). Makridakis NM, Reichardt JK. Translesion DNA polymerases and cancer. *Front. Genet.* 2012; 3:174. [PubMed: 22973298]
- (38). Wang Y, Seimiya M, Kawamura K, Yu L, Ogi T, Takenaga K, Shishikura T, Nakagawara A, Sakiyama S, Tagawa M, J OW. Elevated expression of DNA polymerase κ in human lung cancer is associated with p53 inactivation: Negative regulation of POLK promoter activity by p53. *Int. J. Oncol.* 2004; 25:161–165. [PubMed: 15202001]
- (39). Yang J, Chen Z, Liu Y, Hickey RJ, Malkas LH. Altered DNA polymerase ι expression in breast cancer cells leads to a reduction in DNA replication fidelity and a higher rate of mutagenesis. *Cancer Res.* 2004; 64:5597–5607. [PubMed: 15313897]
- (40). Dorjsuren D, Wilson DM 3rd, Beard WA, McDonald JP, Austin CP, Woodgate R, Wilson SH, Simeonov A. A real-time fluorescence method for enzymatic characterization of specialized human DNA polymerases. *Nucleic Acids Res.* 2009; 37:e128. [PubMed: 19684079]
- (41). Choi JY, Eoff RL, Pence MG, Wang J, Martin MV, Kim EJ, Folkman LM, Guengerich FP. Roles of the four DNA polymerases of the crenarchaeon *Sulfolobus solfataricus* and accessory proteins in DNA replication. *J. Biol. Chem.* 2011; 286:31180–31193. [PubMed: 21784862]
- (42). Zang H, Goodenough AK, Choi JY, Irimia A, Loukachevitch LV, Kozekov ID, Angel KC, Rizzo CJ, Egli M, Guengerich FP. DNA adduct bypass polymerization by *Sulfolobus solfataricus* DNA polymerase Dpo4: analysis and crystal structures of multiple base pair substitution and frameshift products with the adduct 1, N^2 -ethenoguanine. *J. Biol. Chem.* 2005; 280:29750–29764. [PubMed: 15965231]
- (43). Penthala NR, Ponugoti PR, Kasam V, Crooks PA. 5-((1-Aroyl-1H-indol-3-yl)methylene)-2-thioxodihydropyrimidine-4,6(1H,5H)-diones as potential anticancer agents with anti-inflammatory properties. *Bioorg. Med. Chem. Lett.* 2013; 23:1442–1446. [PubMed: 23339966]

- (44). Zhao Y, Biertumpfel C, Gregory MT, Hua YJ, Hanaoka F, Yang W. Structural basis of human DNA polymerase eta-mediated chemoresistance to cisplatin. *Proc. Natl. Acad. Sci. (U. S. A.)*. 2012; 109:7269–7274. [PubMed: 22529383]
- (45). Ketkar A, Zafar MK, Maddukuri L, Yamanaka K, Banerjee S, Egli M, Choi JY, Lloyd RS, Eoff RL. Leukotriene biosynthesis inhibitor MK886 impedes DNA polymerase activity. *Chem. Res. Toxicol.* 2013; 26:221–232. [PubMed: 23305233]
- (46). Strittmatter T, Bareth B, Immel TA, Huhn T, Mayer TU, Marx A. Small Molecule Inhibitors of Human DNA Polymerase lambda. *ACS Chem. Biol.* 2011; 6:314–319. [PubMed: 21194240]
- (47). Reddy YT, Sekhar KR, Sasi N, Reddy PN, Freeman ML, Crooks PA. Novel substituted (Z)-5-((N-benzyl-1H-indol-3-yl)methylene)imidazolidine-2,4-diones and 5-((N-benzyl-1H-indol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-triones as potent radio-sensitizing agents. *Bioorg. Med. Chem. Lett.* 2010; 20:600–602. [PubMed: 20005706]

FIGURE 1.

Assay to screen for polymerase inhibitors. (A) Polymerase activity separates a short TAMRA-labeled oligonucleotide from its BHQ2-labeled complement. The TAMRA-labeled oligonucleotide was exposed to an excitation wavelength of $\lambda_{ex} = 525$ nm and fluorescence emission at $\lambda_{em} = 598$ nm was monitored over time. (B) Hpol η^{1-437} activity was monitored and the mean ($\pm$ standard deviation) from the resulting data sets was plotted as a function of time and the slope of the initial portion of the velocity curve (inset) was used to estimate the rate of polymerase-catalyzed strand-displacement: $v_0 = 10.2 \pm 0.4$ nM min⁻¹.

FIGURE 2.

Determination of IC₅₀ for ITBA-12 mediated inhibition of hpol η activity. (A) The chemical structure of ITBA-12, the first inhibitor identified in the screen, is shown. (B) Hpol η (10 nM) activity was monitored using the fluorescence-based assay in the presence of increasing amounts of ITBA-12: DMSO control (black), 1 μM (blue), 5 μM (cyan), 10 μM (green), 25 μM (orange), 50 μM (red), 100 μM (magenta) and 250 μM (purple). (C) Hpol η activity was plotted as a function of the log of inhibitor concentration and fit to equation 1 to determine the IC₅₀ value. The mean (± standard deviation) of three data sets is shown.

FIGURE 3.

Determination of ITBA-12 specificity for inhibition of hpol η . The IC₅₀ values for ITBA-12 inhibition of different polymerases are shown. The mean ($\pm$ standard deviation) of IC₅₀ values obtained for three data sets is shown.

FIGURE 4.

Structure-activity relationships for ITBA derivatives and inhibition of hpol η . (A) The chemical structure of the ITBA scaffold with the position of the R and Ar substituents indicated. (B) The identity of the R and Ar substituents is listed for each of the twenty ITBA derivatives tested for activity against hpol η . (C) Hpol η activity was measured in the presence of either DMSO or 50 μ M of the indicated ITBA derivative. The numbering scheme on the X-axis matches the numbering in panel B.

FIGURE 5.

Determination of the IC_{50} value for ITBA-16 mediated inhibition of hpol η . (A) The chemical structure of ITBA-16 is shown. (B) Hpol η activity was measured in the presence of increasing amounts of ITBA-16. The IC_{50} was found to be roughly half that of ITBA-12.

FIGURE 6.

Determination of ITBA-16 specificity for inhibition of hpol η . The IC₅₀ values for ITBA-16 inhibition of different polymerases are shown. The mean ($\pm$ standard deviation) of IC₅₀ values obtained for three data sets is shown.

Table 1

Steady-state kinetic parameters for hpol η activity in the presence of ITBA-12 and varying concentrations of dTTP

[ITBA-12] (μM)	k_{cat} (min^{-1})	$K_{\text{m,dTTP}}$ (μM)
-	3.9 ± 0.1	6.0 ± 0.8
1	3.9 ± 0.1	6.7 ± 0.9
10	4.3 ± 0.1	7.6 ± 1.0
20	4.0 ± 0.2	8.5 ± 1.4
40	4.2 ± 0.2	15.0 ± 2.0
50	3.5 ± 0.3	26.7 ± 5.6