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Inhibition of Human DNA Polymerases Eta and Kappa by Indole-Derived Molecules Occurs through Distinct Mechanisms

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Abstract

Overexpression of human DNA polymerase kappa (hpol κ) in glioblastoma is associated with shorter survival time and resistance to the alkylating agent temozolomide (TMZ), making it an attractive target for the development of small-molecule inhibitors. We previously reported on the development and characterization of indole barbituric acid-derived (IBA) inhibitors of translesion DNA synthesis polymerases (TLS pols). We have now identified a potent and selective inhibitor of hpol κ based on the indole-aminoguanidine (IAG) chemical scaffold. The most promising IAG analogue, IAG-10, exhibited greater inhibitory action against hpol κ than any other human Y-family member, as well as pols from the A-, B-, and X-families. Inhibition of hpol κ by IAG analogues appears to proceed through a mechanism that is distinct from inhibition of hpol η based on changes in DNA binding affinity and nucleotide insertion kinetics. By way of comparison, both IAG and IBA analogues inhibited binary complex formation by hpol κ and ternary complex formation by hpol η . Decreasing the concentration of enzyme and DNA in the reaction mixture lowered the IC₅₀ value of IAG-10 to submicromolar values, consistent with inhibition of binary complex formation for hpol κ . Chemical footprinting experiments revealed that IAG-10 binds to a cleft between the finger, little finger, and N-clasp domains on hpol κ and that this likely disrupts the interaction between the N-clasp and the TLS pol core. In cell culture, IAG-10 potentiated the antiproliferative activity and DNA damaging effects of TMZ in hpol κ -proficient cells but not in hpol κ -deficient cells, indicative of a target-dependent effect. Mutagenic replication across alkylation damage increased in hpol κ -proficient cells treated with IAG-10, while no change in mutation frequency was observed for hpol κ -deficient cells. In summary, we developed a potent and selective small-molecule inhibitor of hpol κ that takes advantage of structural features unique to this TLS enzyme to potentiate TMZ, a standard-of-care drug used in the treatment of malignant brain tumors. Furthermore, the IAG scaffold represents a new chemical space for the exploration

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Supporting Information

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Supplementary Figure S1 showing results from the clonogenic assay for hpol κ -proficient and hpol κ -deficient HAP-1 cells treated with IAG-10 as well as Tables S1 and S2 detailing the chemical structures and activity against hpol κ for all compounds tested in our study (PDF)

The authors declare no competing financial interest.

of TLS pol inhibitors, which could prove useful as a strategy for improving patient response to genotoxic drugs.

Graphical Abstract

The ability of tumor cells to bypass DNA damage inflicted by cancer therapeutics is one mechanism that promotes resistance to these drugs.¹ Central to this process are the so-called translesion DNA polymerases (TLS pols), and included among these nonessential enzymes is human pol kappa (hpol κ).^{2,3} In evolutionary terms, hpol κ is considered to be a homologue of bacterial pol IV encoded by the *dinB* gene in *Escherichia coli*.³ A number of studies have highlighted a role for hpol κ in tolerating bulky DNA adducts such as those induced following bioactivation of polycyclic aromatic hydrocarbons (PAHs).^{4–8} Animals and cells lacking pol κ exhibit sensitivity to other sources of DNA damage such as UV light, mitomycin C (MMC), and conditions that promote oxidative stress.^{9–11} There also appears to be a role for pol κ in the maintenance of endogenous barriers to replication, including AT-rich microsatellites, common fragile sites, and G-rich quadruplex forming motifs.^{12–15} Emerging evidence supports the idea that hpol κ may also serve as a means of resolving replication intermediates by protecting regressed forks¹⁶ and by activating the replication stress response (RSR) by synthesizing short DNA primers near stalled forks, which then facilitate recruitment of proteins and enzymes involved in signaling through the ATR kinase.^{17,18} Other studies have implicated misregulation of hpol κ in the etiology of cancer.

In 2010, overexpression of hpol κ in glioblastoma patients was reported to be an independent prognostic indicator of shorter survival.¹⁹ A follow-up to that study revealed that hpol κ expression promotes resistance to temozolomide (TMZ), an alkylating agent often used to treat aggressive brain tumors.²⁰ The mechanism of hpol κ -mediated resistance to TMZ involved activation of ATR/Chk1 signaling to stimulate resolution of replication stress and homologous recombination mediated repair of alkylation-induced DNA damage. Yet, the mechanisms driving overexpression of hpol κ in glioblastoma remained unknown.

Building off these reports, we found that activation of the kynurenine pathway (KP) increased hpol κ expression in glioblastoma-derived cells through the action of the aryl hydrocarbon receptor (AhR).²¹ Aberrant activation of the KP occurs in glioblastoma and exerts a multifaceted effect on cancer phenotypes that includes suppression of antitumor immune response and the promotion of malignancy, at least in part, through activation of the AhR.^{22,23} Pioneering work from the laboratory of Dr. Haruo Ohmori established a

regulatory link between AhR activation and upregulation of pol κ expression in a murine model.²⁴ Experiments in rats also support a role for the AhR in regulation of pol κ .²⁵ Subsequent work helped elucidate a crucial role for hpol κ in the tolerance of DNA damage induced by AhR ligands such as benzo[*a*]-pyrene (B[a]P).^{4,7} Thus, a picture begins to emerge, painting hpol κ as having evolved to promote tolerance of bioactivated AhR ligands with some aspect of this pathway contributing to glioblastoma biology. Additional studies have illustrated that upregulation of hpol κ can have a detrimental effect on genome stability, including disruption of fork dynamics, aberrant stimulation of homologous recombination, increased micronuclei formation, and aneuploidy, supporting the notion that overexpression of hpol κ can have a detrimental impact on genome stability.^{26,27} However, it is clear that the relationships among pol κ , tolerance of replication stress, and genome stability involve multiple variables and are not simple affairs, as demonstrated by the fact that pol κ protects stalled forks from degradation¹⁶ and helps resolve DNA replication intermediates in cells that have CDK2 activity artificially induced.²⁸ Despite the double-edged nature of pol κ action, it is generally accepted that pol κ inhibition might help improve the anticancer activity of genotoxic drugs such as TMZ or MMC.

As efforts to develop targeted cancer therapeutics progress, several groups have sought to identify inhibitors of DNA damage tolerance as a way of improving existing treatments. A number of studies have identified small-molecule TLS pol inhibitors,^{29–35} and new strategies include targeting mechanisms that promote TLS pol recruitment to sites of replication stress/DNA damage.^{36–40} Working with our collaborators, we identified candesartan cilexetil and MK-886 as inhibitors of the Y-family members.^{35,41} We also reported on the inhibitory action of indole barbituric/thiobarbituric acid (IBA) derivatives against hpol η .²⁹ Although MK-886 seemed to exhibit some specificity for hpol ι and the IBA derivatives inhibited hpol ι with modest specificity, we struggled to identify a compound that selectively inhibits hpol κ over the other Y-family members. Most recently, we synthesized a compound that inhibits hpol η with a low micromolar IC₅₀ value and acts synergistically to potentiate the cytotoxic effects of cisplatin in a target-dependent manner.⁴² In the current study, we overcame the barrier to specificity for hpol κ . Remarkably, the novel indole aminoguanidine (IAG) scaffold reported here acts through a mechanism that is distinct from IBA derivatives and appears to disrupt the interaction between the N-clasp, which is unique to hpol κ , and the core Y-family pol structure to inhibit TLS action. Finally, we observe target-dependent potentiation of TMZ, a standard-of-care treatment for glioblastoma.

MATERIALS AND METHODS

Reagents.

All chemicals were of molecular biology grade or higher. Only ultrapure H₂O was used. The novel IAG compounds tested for inhibitory properties against the DNA polymerases were synthesized utilizing previously published procedures.⁴³ TMZ was purchased from Millipore Sigma (Burlington, MA). TMZ is an alkylating agent used in the treatment of cancer. It is an irritant, carcinogen, teratogen, and mutagen. Appropriate safety precautions should be taken in handling and disposal.

Protein Expression and Purification.

Bacterial overexpression and purification of the core polymerase domain of Y-family members hpol κ (residues 19–526), hpol η (residues 1–437), hpol η (residues 1–446), and hRev1 (residues 330–833) have been described previously.^{29,41} Similarly, the expression and purification for representative members of the B-family (pol Dpo1 from *Sulfolobus solfataricus*) and A-family (DNA pol I from *Mycobacterium tuberculosis*) have been previously described.⁴² Purified human DNA pol beta (hpol β) was a kind gift from Dr. Samuel Wilson (NIEHS). The purified catalytic core of human DNA pol lambda (hpol λ , residues 1–325) was a kind gift from Dr. Miguel Garcia-Diaz (Stony Brook University). Both hpol β and hpol λ served as representative members from the X-family of polymerases.

Fluorescence-Based Assay to Screen for Inhibition of DNA Polymerase Activity.

A total of 44 compounds based on IBA and IAG chemical scaffolds were evaluated for inhibition of polymerase activity of hpol κ and the other polymerases described above using an assay monitoring fluorescence of a 5-carboxytetramethylrhodamine (TAMRA) labeled oligonucleotide. The DNA substrate was prepared as described previously.²⁹ Briefly, 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 AAAAAA GAG TCG TAC GAG GGT GA-3') in 10 mM Tris (pH 8.0) buffer containing 50 mM NaCl, 2 mM MgCl₂, and H₂O. 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 3 min at 95 °C, the DNA was allowed to slowly cool to RT overnight. The assay was initially used to screen 44 compounds for inhibition of hpol κ activity (Table S1). The assay conditions included 1 nM hpol κ , 50 nM DNA, 40 μ M test compound (dissolved in DMSO), 100 μ M dTTP, and 1 mM MgCl₂. The reactions were performed at 25 °C in 50 mM Tris (pH 8.0) buffer containing 40 mM NaCl, 2 mM dithiothreitol, and 0.01% (v/v) Tween-20. In control reactions, DMSO was added instead of the compound to a similar final concentration of 10% (v/v). The enzyme, test compound (or DMSO), and dTTP were combined with the reaction buffer in individual wells and incubated for 5–10 min to allow the compound to bind to the enzyme. Subsequently, the reaction was initiated by addition of DNA substrate, 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 typically 200 μ L. Fluorescence was monitored for 60 to 90 min for most reactions. The initial linear portion of the velocity curve was analyzed to calculate an observed rate of product formation. For each data set, we averaged 8 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. The experiments were performed in triplicate, and the mean ($\pm$ standard deviation) of the IC₅₀ values calculated for each data set is reported.

The TAMRA-based strand-displacement assay was also used to examine selectivity against hpol κ and elucidate the mechanism of inhibition of hpol κ by IAG-10. For the selectivity experiments, members representing the other DNA pol families (25 nM hpol κ , 1 nM hpol β ,

10 nM hpol λ X-family; 10 nM TB pol I A-family; 25 nM Dpo1 B-family) were tested at a single dose of 10 μ M IAG-10 using the reaction conditions described above. The steady-state kinetic parameters for hpol κ activity were determined in the presence of multiple concentrations of dTTP (0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 20, 30, 40, and 50 μ M). For steady-state experiments, hpol κ concentration was 0.5 nM, while the TAMRA-labeled DNA substrate (described above) was used at 25 nM. The activity of hpol κ was determined for each dTTP concentration by monitoring the change in fluorescence as a function of time and using the linear portion of each curve to calculate the reaction velocity. The rate of product formation was then plotted as a function of dTTP concentration and fit to a hyperbola to estimate the Michaelis constant ($K_{M,dTTP}$). The turnover number (k_{cat}) was calculated after correcting for the enzyme concentration. These experiments were then repeated in the presence of IAG-10 (0–10 μ M) to determine the effect of this small molecule upon Michaelis–Menten kinetics and help elucidate the mechanism of inhibition of hpol κ .

Determination of IC₅₀ Values against hpol η and κ .

DNA polymerase-catalyzed TAMRA-strand displacement experiments were performed with varying concentrations of IAG to determine the IC₅₀ value against recombinant enzyme. The assay conditions for the IC₅₀ values reported in Table 1 included 1 nM hpol κ , 50 nM DNA, varying concentrations of IAG compound (dissolved in DMSO), 100 μ M dTTP, and 1 mM MgCl₂. The reactions were performed at 25 °C in 50 mM Tris (pH 8.0) buffer containing 40 mM NaCl, 2 mM dithiothreitol, and 0.01% (v/v) Tween-20. In control reactions, DMSO was added instead of the compound to a similar final concentration of 10% (v/v). For hpol κ , the IC₅₀ for IAG-10-mediated inhibition was determined at six enzyme:DNA concentrations: 1 nM hpol κ :50 nM DNA, 1 nM hpol κ :25 nM DNA, 0.5 nM hpol κ :10 nM DNA, 0.2 nM hpol κ :2.5 nM DNA, 0.2 nM hpol κ :1 nM DNA, and 0.1 nM hpol κ :0.5 nM DNA. For hpol η , we measured the IC₅₀ at two different enzyme:DNA concentrations: 1 nM hpol η :50 nM DNA and 0.2 nM hpol η :2 nM DNA. The IC₅₀ value was estimated using a four-parameter logistic model defined by eq 1:

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

The experiments were performed in triplicate, and the mean ($\pm$ standard deviation) of the IC₅₀ values calculated for each data set is reported.

Gel-Based Primer Extension Assay Measuring DNA Polymerase Activity.

The Y-family member hRev1 was not easily amenable to the fluorescence-based assay used for the other polymerases. Therefore, we used a gel-based primer extension assay to determine the polymerase activity of all four human Y-family polymerases (hpol κ , hpol η , hpol ι , and hRev1) in the absence or presence of IAG-10 to determine the degree of specificity of inhibition of the compound toward hpol κ . For this assay, extension of a 13/18-mer primer-template DNA substrate by each DNA polymerase was studied. The DNA substrate was prepared by annealing the FAM-labeled primer to a complementary template oligonucleotide (1:2, primer:template molar ratio) by incubating at 95 °C for 5 min followed

by slow cooling to RT. The polymerase extension assay was performed in 50 mM HEPES (pH 7.5) buffer containing 50 mM NaCl, 1 mM DTT, 0.1 mg mL⁻¹ BSA, and 10% (v/v) glycerol. The enzymes were used at the following concentrations: 1 nM hpol κ , 2 nM hpol η , 10 nM hpol ι , and 10 nM hRev1. Each enzyme was incubated with 10% DMSO (control) or 10 μ M of IAG-10 for 5 min before initiating the reaction by the addition of DNA-dNTP-Mg²⁺ (200 nM DNA, 100 μ M of each dNTP, and 5 mM MgCl₂) at 37 °C. The reactions were terminated by adding quench solution (20 mM EDTA and 95% v/v formamide) to each reaction followed by heating at 95 °C. The samples were separated using a 15% (w/v) polyacrylamide–7 M urea gel, and the product formation was analyzed as described previously.⁴¹ The experiments were performed in triplicate, and for each enzyme, percent (%) activity was calculated by normalizing to the specific activity in the DMSO-control condition (taken as 100%).

DNA Binding Analysis.

Fluorescence anisotropy DNA binding assays were performed as described previously⁴¹ to determine the effect of the compounds IAG-10 and IBA-32 on the DNA-binding affinities of the enzymes hpol κ and hpol η . Briefly, an 11-mer oligo 5'-ATCCTCCCCTA-3' was mixed with a fluorescein-labeled 28-mer oligo 5'-FAM/TATGTGACTATGCTGGGTAGGGGAGGAT-3' in a 1:1.2 molar ratio, incubated at 95 °C for 5 min, followed by slow-cooling to RT. The annealed FAM-labeled duplex DNA substrate (2 nM) was incubated with increasing concentrations of hpol κ or hpol η , and fluorescence polarization was measured in a Biotek SynergyH4 plate reader using the appropriate filter sets ($\lambda_{\text{ex}} = 485 \pm 20$ nm and $\lambda_{\text{em}} = 525 \pm 20$ nm). All titrations were performed at 25 °C in 50 mM HEPES buffer (pH 7.5) containing 10 mM KOAc, 10 mM MgCl₂, 10 mM KCl, 2 mM β -mercaptoethanol (β -ME), 10% (v/v) dimethyl sulfoxide (DMSO), and 0.1 mg mL⁻¹ BSA. The experiments were performed in the presence of increasing concentrations of IAG-10 and IBA-32. The range of concentrations was chosen such that it bracketed the IC₅₀ of each compound for the respective enzyme. Thus, IAG-10 was used in the range 0–10 μ M for hpol κ and 0–20 μ M for hpol η , while IBA-32 was used at 0–24 μ M for hpol κ and 0–12 μ M for hpol η . The resulting polarization data were fit to a quadratic equation to estimate the equilibrium dissociation constant ($K_{\text{D,DNA}}$) for hpol η binding to the duplex DNA.

p-Hydroxyphenylglyoxal (HPG) Labeling of hpol κ to Identify Binding Sites for IAG-10.

HPG modifies the L-arginine side chains of proteins selectively. The HPG labeling experiments were performed with hpol κ (10 μ M) in the presence or absence of IAG-10. Briefly, hpol κ was preincubated with either 20 μ M (1:2 molar ratio) or 100 μ M (1:10 molar ratio) IAG-10 for 10 min at 37 °C in 50 mM HEPES buffer (pH 7.8) containing 200 mM NaCl, 10 mM MgCl₂, and 10% (v/v) DMSO. The HPG labeling was carried out at 37 °C in the dark for 1 h by adding 500 μ M of HPG to the preincubated reaction mix, followed by addition of 200 mM of free L-arginine to quench the reaction after 1 h. The samples were then resolved on an SDS-PAGE gel and submitted to the UAMS Proteomics Core Facility for analysis by mass spectrometry. In-gel trypsin digestion was employed to obtain peptides, which were then subjected to tandem liquid chromatography–mass spectrometric (LC-MS) analysis.

Quantification of HPG-Labeled Peptides.

Unmodified and HPG-modified peptides were both identified as described previously,⁴² using Scaffold Viewer (Proteome Software Inc.). The modified peptides have a molecular weight of the peptide fragment plus the 132-Da HPG adduct (Table S2). The peak intensity was then quantified manually using the corresponding m/z value and the relative abundance for each modified peptide was calculated as a function of area under the peak in Xcalibur software (Thermo Fisher Scientific). The percentage of modified arginine measured by mass spectrometry was calculated as a fraction of total arginine (i.e., unmodified + HPG modified) quantified for a specific residue. The data for each peptide was normalized to the hpol κ -HPG alone sample to calculate the change in HPG modification after treatment with IAG-10.

Human Cell Culture and Clonogenic Survival Assay.

HAP-1 and hpol κ^{KO} cells were purchased from Horizons Discovery Group LLC (Cambridge, UK). The company generated the HAP-1 cells using CRISPR-Cas9 technology, and genome editing was confirmed by sequencing. We further confirmed the elimination of hpol κ protein expression by Western blotting (data not shown). HAP-1 cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, CA, United States) supplemented with 10% (v/v) fetal bovine serum (FBS; Atlas Biologicals, CO, United States) and 1% (v/v) antibiotic/antimycotic solution (Sigma-Aldrich, MO, United States).

For the clonogenic survival assay, both HAP-1 and hpol κ^{KO} cells were plated (500 cells per well) in 6-well dishes. The next day, cells were treated with TMZ (0, 1, 5, 50, 125, and 250 μM) alone or in combination with IAG-10 (0.1 and 0.5 μM) for 1 h. After treatment, cells were cultured in fresh IMDM supplemented with 10% (v/v) FBS for 10 days. The colonies were gently washed with 1 $\times$ PBS, fixed with 3.7% (v/v) formaldehyde for 20 min at RT, and stained with crystal violet solution (Sigma-Aldrich) for 30 min at RT.

The fixed crystal violet stained colonies of HAP-1 cells (hpol κ -proficient and hpol κ -deficient) in each 6-well plate were examined under an EVOS XL Core imaging system (Thermo Fisher Scientific) under bright-field, and the colonies were inspected manually. By considering a group of ~50 cells as one colony for hpol κ -proficient cells (hpol κ -deficient cells showed consistently smaller colonies, hence for these, ~25 cells together were considered one colony), an average minimum radius was determined for one colony. Color images of each 6-well plate were captured and opened for processing using the Fiji version of the freely available software ImageJ. Setting the appropriate threshold for counting colonies for each cell type, corresponding to the respective radii calculated previously, the colonies were counted using the Colony Area plugin of ImageJ.⁴⁴ Manual colony counting was compared with the colony number obtained using ImageJ to ensure that they matched reasonably well. All experiments were performed in triplicate, and the surviving fraction for each condition was expressed as the mean ($\pm$ standard deviation). The results were plotted using Graphpad Prism software.

Alkaline Comet Assay.

To evaluate DNA damage upon treatment with TMZ and the compound IAG-10, the alkaline comet assay was performed using protocol as described by the manufacturer (Trevigen, Gaithersburg, MD). Briefly, 100 000 HAP-1 cells per well for both the hpol κ -proficient and hpol κ -deficient cell types were plated in a 12-well dish. Both types of cells were treated with 0.5 μ M of IAG-10 alone or in combination with TMZ (100 μ M) for 1 h. Cells treated with DMSO served as the control. Following treatment, cells were harvested, washed in ice-cold 1 $\times$ PBS, and counted. The cells were then suspended in low-melting agarose to a final dilution of 200–300 cells per well on the comet slide. The comet slides with wells coated with cell/agarose mixture were incubated at 4 $^{\circ}$ C in the dark to allow the agarose to solidify before immersing the slides in proprietary lysis solution for 1 h. Each comet slide was then immersed in an alkaline unwinding solution (200 mM NaOH, 1 mM EDTA) for 20 min at RT before placing them in the electrophoresis unit in ~850 mL of the same solution. Samples were electrophoresed at 21 V for 30 min at 4 $^{\circ}$ C. The comet slides were washed and dried completely at 37 $^{\circ}$ C before staining with SYBR Gold diluted in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The comets were visualized using an EVOS FL Auto microscope (Life Technologies, Carlsbad, CA) set at 10 $\times$ magnification and were scored using CometScore software. All experiments were repeated in 3 biological replicates with at least 60 comets scored for each condition. Results were plotted using Graphpad Prism, as the percent of DNA in the tail of each comet, and statistical significance between treatment conditions was calculated using one-way ANOVA with the Bonferroni posthoc correction.

supF Mutagenesis Assay.

HAP-1 hpol κ -proficient and hpol κ -deficient cells were cultured in 10 cm dishes to ~80% confluency in Iscove's modified Dulbecco's medium (Invitrogen) containing 10% (v/v) FBS supplemented with 1 $\times$ antibiotic antimycotic solution (Sigma). They were transfected with unmodified (control) and methylmethanesulfonate (MMS)-modified pSP189 plasmid harboring the *supF* gene either in the absence or presence of 1 μ M IAG-10. For plasmid modification with MMS, the pSP189 plasmid was first incubated with 100 mM MMS in 10 mM HEPES buffer (pH 7.5) containing 50 mM KCl, 5 mM MgCl₂, and 1 mM dithiothreitol at RT for 1 h. The MMS-modified plasmid was then purified by ethanol precipitation and used in subsequent transfections, similar to the unmodified plasmid.

The HAP-1 hpol κ -proficient and hpol κ -deficient cells were transfected for 24 h with the unmodified or MMS-modified pSP189 plasmids (10 μ g plasmid per 10 cm dish) using Lipofectamine 3000 as per manufacturer's instructions, either in the presence or absence of 1 μ M IAG-10, followed by harvesting of the cells. Plasmids were extracted using plasmid miniprep kit (Qiagen) from the cell pellets of all samples and digested with the Dpn I endonuclease to eliminate unreplicated plasmids. The recovered plasmids (75–100 ng) were then transformed into *E. coli* MBM7070 strain by electroporation, and the transformed bacterial cells were plated on LB-agar plates containing 100 μ g/mL ampicillin, 1 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) and 100 μ g/mL X-gal. The resulting blue (nonmutated) and white (mutated) colonies were counted. The mutation frequency was calculated as the ratio of mutant (white) colonies to total (blue + white) colonies. At least 10

000 colonies were counted for each condition, and the results reported represent the mean ($\pm$ SD) of experiments performed in biological triplicate.

RESULTS AND DISCUSSION

Expansion of Chemical Library and Identification of Indole-Aminoguanidine Analogues As Inhibitors of hpol κ .

As with previous work, we used a fluorescence-based assay developed at NIH to screen for pol inhibitors. Briefly, the assay involves pol-catalyzed extension of an unlabeled DNA primer, as well as a 5-carboxytetramethylrhodamine (TAMRA)-labeled (displaced) strand, both annealed to a BHQ-labeled template.³⁰ Primer extension leads to displacement of the TAMRA-labeled strand and an increase in TAMRA fluorescence due to separation from the BHQ-labeled template. The rate of increase in fluorescence is reduced or prevented if the pol is inhibited. This assay had led to our successful identification of IBA analogues as inhibitors of hpol η activity.

In the present study, we focused on the identification of hpol κ inhibitors and expanded the IBA scaffold library to include compounds in which the barbituric acid moiety had been replaced with an aminoguanidine moiety (Figure 1A and B). We tested a total of 44 IBA and IAG derivatives at a final concentration of 40 μ M for inhibition of hpol κ activity. Several compounds inhibited enzyme activity by more than 50% in the initial screen (Figure 1C and 1D). The chemical structures and a summary of the results of the screen against hpol κ may be found in Table S1. Of the compounds tested, IAG-10 exerted the greatest inhibitory action against hpol κ (Figure 1D). The chemical structure of IAG-10 is reminiscent of the most potent inhibitors of hpol η identified in our previous work in that the indole substituent is a *N*-naphthoyl moiety (Figure 2A). Replacement of the barbituric acid moiety with an aminoguanidine moiety is of interest because of the improved water-solubility afforded by the latter group. We next sought to determine the structure–activity relationships important for inhibition of hpol κ by the IAG compounds.

Structure–Activity Studies with the IAG Derivatives.

Analogue (*E*)-2-((1-(1-naphthoyl)-5-chloro-1*H*-indol-3-yl)-methylene)hydrazine-1-carboximidamide hydrochloride (IAG-10) exhibited the most potent inhibitory action against hpol κ (IC_{50} = 7.2 μ M, Table 1). We then measured the IC_{50} value for 13 IAG derivatives (Table 1) related to IAG-10. While none of the other chemical changes improved activity over that of IAG-10, some interesting trends emerged. The presence of a halogen moiety at the fifth position of the indole ring combined with an indolic *N*-naphthoyl (IAG-10 and IAG-9) or *N*-naphthyl group (IAG-8, IAG-13, and IAG-7) generated the top five most potent inhibitors with IC_{50} values ranging from 7.2 to 24.2 μ M. Replacement of the indole *N*-1-(1-naphthoyl) moiety in IAG-10 with an isomeric *N*-1-(2 naphthoyl) moiety (IAG-9) reduced inhibitory potency against hpol κ by about half (IC_{50} = 18.8 μ M) (Table 1). Replacement of the 5-chloro substituent on the indole ring with a methoxy group (IAG-2) in IAG-10 decreased potency against the target (IC_{50} = 68.6 μ M, Table 1). Introducing a methyl group at the 2-position of the indole ring also reduced potency (IAG-1; IC_{50} = 84.6 μ M) against hpol κ , and removal of the *N*-1-(1-naphthoyl) moiety from the indole ring (IAG-11)

completely eliminated inhibitory activity ($IC_{50} > 200 \mu M$; Table 1). Replacement of the indole *N*-1-(1-naphthoyl) substituent in IAG-10 with either an *N*-1-(1-naphthylidene) or *N*-1-(2-naphthylidene) moiety (IAG-7 and IAG-13, respectively) led to reduced inhibitory potency against hpol κ (24.2 and 22.7 μM , respectively), and replacement of the indole 5-chloro substituent in IAG-7 with bromo, methoxy, or hydrogen afforded analogues with IC_{50} values in the range 22.3–52.0 μM . Similarly, replacement of the indole 5-chloro substituent in IAG-13 with methoxy or hydrogen resulted in analogues with reduced potency (IAG-5 and IAG-4 IC_{50} values of 43.7 and 48.3 μM , respectively). Thus, the presence of 5-chloro and *N*-naphthoyl/naphthyl indole substituents combined to inhibit hpol κ with the greatest potency. This is similar to our previous observations for IBA-mediated inhibition of hpol η .⁴² Next, we investigated the selectivity of IAG-10 for hpol κ by testing for inhibition of other DNA pols.

Selectivity of hpol κ Inhibition by IAG-10.

We showed previously that several indole-derived compounds inhibit the DNA polymerase activity of hpol κ , but in every instance, those molecules inhibited hpol η (or another TLS pol) more potently than hpol κ .^{29,41} We tested IAG-10 (10 μM) for activity against the other three human Y-family members, but only hpol κ was inhibited under these conditions (Figure 2A). Then, we measured the activity of enzymes from different pol families (A, B, and X) in the presence of IAG-10 and found that, once again, only hpol κ activity was inhibited (Figure 2B). To our knowledge, these results make IAG-10 one of the most selective inhibitors of hpol κ identified to date.

IAG-10 Disrupts hpol κ Binding to Primer-Template DNA.

We next investigated the ability of IAG derivative IAG-10 and IBA derivative IBA-32 (Figure 3A) to disrupt hpol κ binding to primer-template DNA (p/t-DNA). The equilibrium dissociation constant ($K_{d,DNA}$) was determined by titrating protein (0–400 nM) into a solution containing FAM-labeled p/t-DNA (2 nM) at various concentrations of inhibitor and measuring changes in fluorescence anisotropy. Fitting the results to a quadratic equation produced an estimate of DNA binding affinity for hpol κ in the presence of the inhibitors. Both compounds IAG-10 and IBA-32 inhibited hpol κ binding to p/t-DNA (Figure 3B). The equilibrium dissociation constant ($K_{d,DNA}$) increased approximately 2- to 3-fold (100–200%) at concentrations of inhibitor near the IC_{50} measured for inhibition of pol activity. The increase in the $K_{d,DNA}$ that occurred as a function of inhibitor concentration clearly followed a nonlinear trend upward, indicative of an allosteric effect of the inhibitors on pol binding to DNA. We also measured the effect of IAG-10 and IBA-32 on DNA binding by hpol η . In contrast to what was observed with hpol κ , there is only about a 20–30% increase in the $K_{d,DNA}$ at concentrations of inhibitor near the IC_{50} for inhibition of pol activity (Figure 3C). Also, the small effect of the inhibitors on the affinity of hpol η for p/t-DNA is linear in nature. We conclude from these results that the effect of IAG-10 and IBA-32 on the DNA binding affinity of hpol κ is quite distinct from the modest effects on DNA binding observed for hpol η , indicative of a different mechanism of inhibition for the two enzymes.

Effect of Enzyme and DNA Concentration on the Measured IC_{50} against hpol η and κ .

The impact of IAG-10 on the affinity of hpol κ for DNA was an important insight and one that we had not observed previously with MK-886.⁴¹ We reasoned that by decreasing the concentration of either enzyme or DNA, we might affect the IC_{50} value by shifting the equilibrium away from the formation of binary hpol κ :DNA complex and toward the enzyme-inhibitor complex. To investigate this idea, we varied the concentration of hpol κ and p/t-DNA in the reaction mixture and measured the IC_{50} value for IAG-10. Decreasing the enzyme and DNA in solution led to a dramatic decrease in the IC_{50} for IAG-10 against hpol κ (Figure 4A). At the lowest concentration of hpol κ and DNA tested (0.1 and 0.5 nM, respectively), the IC_{50} was 0.72 μ M (Figure 4A, open circles), an order of magnitude lower than that measured at p/t-DNA concentrations >10 nM. We then performed similar experiments with hpol η by varying the concentration of enzyme and DNA in the reaction mixture and determining the IC_{50} value for IAG-10-mediated inhibition. Notably, the IC_{50} value for inhibition of hpol η by IAG-10 increased slightly when the concentration of DNA and enzyme was decreased (Figure 4B). These results were again supportive of IAG-10 inhibiting hpol η and κ through different modes of action with hpol κ being much more sensitive to destabilization of binary complex formation.

IAG-10 Inhibits hpol κ and hpol η through Distinct Mechanisms of Action.

Next, the impact of IAG-10 and IBA-32 on nucleotide selection by hpol κ was examined. Steady-state kinetic analysis of nucleotide insertion in the presence of either IAG-10 or IBA-32 revealed that both compounds had a similar effect on hpol κ activity. The turnover number (k_{cat}) exhibited a sharp decrease when the concentration of IAG-10 exceeded the IC_{50} value, whereas the Michaelis constant ($K_{M,dNTP}$) exhibited very little change at inhibitor concentrations in the range of 2–10 μ M (Figure 5A and B). The effect was somewhat less acute with IBA-32, but again, it was the k_{cat} and not the $K_{M,dNTP}$ that was altered by the presence of the inhibitor (Figure 5C and D). These results are consistent with a model for inhibition where IAG-10 impedes a step in the hpol κ catalytic cycle that occurs prior to nucleotide binding and are in direct contrast to those reported previously for IBA derivatives tested against hpol η . Previously, we observed a nonlinear increase in the $K_{M,dNTP}$ when hpol η activity was measured in the presence of IBA-32. This led us to conclude that IBA-32 inhibited hpol η through a partial competitive mechanism of action. We performed similar steady-state kinetic analysis with hpol η and IAG-10. Intriguingly, there was no measurable change in the k_{cat} , but a linear increase in the Michaelis constant accompanied the increase in IAG-10 concentration (Figure 5E and F). These results are consistent with a competitive mechanism of inhibition distinct from what we reported for IBA-32 but also quite different from that observed for hpol κ and IAG-10.

Chemical Footprinting Helps Identify Changes in hpol κ Structure Induced by IAG-10.

Chemical foot-printing was used in an effort to ascertain the binding site of IAG-10 on hpol κ . Recombinant hpol κ was incubated with the arginine-reactive probe HPG alone or in the presence of IAG-10 (20 and 100 μ M). After quenching the reaction and gel electrophoresis, the modified protein was trypsin-digested, and the resulting peptides were analyzed by LC-MS. By quantifying the peak intensity of HPG-modified peptides as a fraction of the total

intensity measured for peptides containing a given arginine residue, we determined whether the addition of IAG-10 alters the reactivity of that site (Figure 6A–C). A decrease in the fraction of HPG-modified peptides was interpreted as evidence for protection from HPG-reactivity by IAG-10. We identified 15 arginine residues (out of 30 arginine residues occurring between amino acids 19–526) that were modified by HPG (Figure 6C). Of those 15 residues, three of them (Arg145, Arg149, and Arg505) exhibited a >50% decrease in the fraction of HPG-modified peptides when IAG-10 was added to the reaction mixture (Figure 6C). These three residues map to the finger and little finger domains of hpol κ (Figure 6D) in a cleft similar to the one identified in our study with hpol η .⁴² Perhaps most striking of all was the large increase in the fraction of HPG-modified peptides containing Arg48 upon addition of IAG-10 (Figure 6C).

The increase in HPG reactivity for Arg⁴⁸ is indicative of increased exposure in the presence of IAG-10. Because Arg48 resides in the N-clasp, it is reasonable to assume that binding of IAG-10 disrupts contacts occurring between the N-clasp, finger, and little finger domains of hpol κ and results in a conformational change that exposes the N-clasp to solvent. Indeed, the N-clasp allows hpol κ to encircle the DNA substrate and is required for optimal polymerase activity. An important function of the N-clasp is to stabilize the position of the little finger domain, which makes the largest number of contacts with the DNA substrate. Loss of this stabilization would presumably impact DNA binding affinity.

IAG-10 Potentiates the Antiproliferative Effects of TMZ in a Target-Dependent Manner.

Earlier work has shown that hpol κ activity is important for cellular responses to alkylation damage, including abasic sites and *O*⁶-methylguanine adducts produced by TMZ, and further, that the enzyme promotes TMZ-resistance in both *in vitro* and *in vivo* models of glioblastoma multiforme.^{20,45} We wanted to test the ability of IAG-10 to inhibit hpol κ in the cellular context and also explore the potential of using IAG-10 or other potent hpol κ -inhibitors as a potential way of enhancing the antiproliferative properties of TMZ. Toward this goal, we measured clonogenic survival for hpol κ -proficient (wt HAP-1) and hpol κ -deficient (hpol κ knockout) cell lines treated with the chemotherapeutic drug TMZ, either alone or in combination with IAG-10.

We first measured the number of colonies formed by hpol κ -proficient HAP-1 cells exposed to increasing concentrations of TMZ. As expected, there was a dose-dependent decrease in the number of colonies formed in the presence of TMZ (Figure 7A). For cotreatment experiments, we used 0.5 μ M IAG-10: a concentration that does not impair colony formation by either cell line (Figure S1). We found that cotreatment with TMZ and IAG-10 reduced the number of colonies formed by hpol κ -proficient cells at every TMZ concentration tested compared to those treated with TMZ alone (Figure 7A). At the highest concentration (250, μ M) of TMZ, the IAG-10 cotreated cells formed only ~40% as many colonies as untreated cells, while the cells treated with 250 μ M TMZ alone were able to form ~75% of control (Figure 7B). In stark contrast, the hpol κ -deficient cells did not show any significant reduction in colony forming ability when cotreated with TMZ and IAG-10 over TMZ alone (Figure 7B). The number of colonies formed by the hpol κ -deficient cells exposed to 250 μ M TMZ was reduced to less than 50% of the untreated cells (Figure 7A and B). Thus,

compared to the hpol κ -proficient cells, the hpol κ -deficient HAP-1 cells were twice as sensitive to TMZ treatment, consistent with similar observations reported in earlier studies with different cell lines.²⁰

The results of our clonogenic survival experiments strongly suggest that the synergistic effect on TMZ toxicity exhibited by IAG-10 is dependent upon the presence of hpol κ , although secondary targets for IAG-10 action cannot be ruled out. Yet, the clear difference observed between the hpol κ -proficient and the hpol κ -deficient HAP-1 cells in their response to cotreatment with IAG-10 and TMZ indicates that the secondary, nonhpol κ mediated effects, if present, are minimal and may not be relevant in the context of TMZ toxicity. Thus, our results with the clonogenic assay demonstrate the possibility of using compounds like IAG-10 as an effective adjuvant treatment for existing anticancer chemotherapeutics like TMZ.

IAG-10 Increases TMZ-Induced DNA Damage in hpol κ -Proficient Cells.

Alkaline comet assays were performed with the hpol κ -proficient and hpol κ -deficient cells to determine whether inhibition of hpol κ by IAG-10 potentiated the genotoxic effects of TMZ in a target-dependent manner. Both the hpol κ -proficient and hpol κ -deficient HAP-1 cells were treated with 100 μ M TMZ alone or in combination with 0.5 μ M IAG-10, and the resulting DNA damage compared to control (DMSO-treated) was estimated by measuring the percent DNA in the tail in a comet assay (Figure 7C and D). For every experiment, an estimation of DNA damage was performed in at least 20 cells per biological replicate. Thus, there was a minimum of 60 cells used for calculating DNA damage per experimental condition. Treatment with IAG-10 alone increased the amount of DNA damage in the hpol κ -proficient HAP-1 cells approximately twofold compared to control (Figure 7C). Treatment with TMZ alone increased DNA damage approximately sevenfold over that of untreated cells (Figure 7C). The extent of damage increased even further when cells were treated with both TMZ and IAG-10 simultaneously (Figure 7C). In contrast, treatment of hpol κ -deficient cells with IAG-10 alone did not increase DNA damage over control (Figure 7D), again indicating that IAG-10-induced effects involve hpol κ . TMZ treatment did produce a significantly greater amount of DNA damage over control in the hpol κ -deficient cells, but unlike in the hpol κ -proficient cells, the extent of damage did not increase further upon cotreatment with TMZ and IAG-10 (Figure 7D; compare the red and brown scatter plots). The comet assay results lend further support to the notion that IAG-10 targets hpol κ in cells, which impacts DNA damage formation.

Inhibition of hpol κ by IAG-10 Increases the Mutation Frequency on MMS-Damaged DNA.

Previous studies have implicated hpol κ in the bypass of multiple DNA adducts, including MMS-induced DNA damage. We hypothesized that inhibition of hpol κ activity by IAG-10 might potentiate the mutagenic potential of MMS-damaged DNA. To test this idea, we employed the *supF* forward mutagenesis assay.

Both the hpol κ -proficient and hpol κ -deficient HAP-1 cells were transfected with either the undamaged pSP189 plasmid harboring the *supF* gene (control) or the MMS-treated pSP189 plasmid. The transient transfections were performed either alone or coupled with treatment

of the cells with 1 μ M IAG-10 for 24 h, followed by harvesting the cells and extraction of plasmid DNA. The recovered plasmids were then transformed into the MBM7070 strain of *E. coli* competent cells and screened for blue (wild-type) and white (mutant) colonies to estimate the mutation frequency.

In hpol κ -proficient HAP-1 cells, we did not observe a significant difference in mutation frequency between the control and MMS-treated plasmid DNA (Figure 8A). Likewise, the addition of IAG-10 did not change the mutation frequency for the undamaged plasmid. Replication of the MMS-damaged plasmid was more mutagenic in cells treated with IAG-10 than it was in untreated cells (Figure 8A). Also, treatment with IAG-10 increased the mutation frequency for MMS-damaged DNA approximately twofold over that of the undamaged control plasmid (with or without IAG-10). As expected, the hpol κ -deficient cells replicated the MMS-damaged in a more errorprone manner than the control plasmid (Figure 8B). However, treatment with IAG-10 did not alter the mutation frequency for either control or MMS-damaged DNA in hpol κ -deficient HAP-1 cells. From these results, we conclude that treating hpol κ -proficient cells with IAG-10 facilitated an increase in the mutation frequency for MMS-damaged DNA. This increase did not manifest in cells lacking hpol κ , once again indicating that the effects of IAG-10 are hpol κ -dependent.

CONCLUSIONS

The development of strategies to improve therapies for aggressive cancers remains an important area of research. Cellular responses to genotoxins are a potential target for these advancements in clinical care. DNA repair and damage response mechanisms have received considerable attention in this regard, and more recent work has identified damage tolerance mechanisms as playing important roles in resistance to anticancer drugs.^{46–51} Many groups have pursued studies targeting DNA repair and TLS as a means of potentiating the effects of genotoxic agents, specifically targeting the DNA pols that promote direct bypass of DNA damage.^{30–32,35,46} From our work, several indole-derived compounds have emerged as potential TLS pol inhibitors.^{29,41,42} Until now, our focus has been on the identification of hpol η inhibitors, as the *in vivo* and clinical evidence supporting a role for this enzyme in chemoresistance is fairly robust.^{49,52–56} While the rationale for targeting hpol κ is also sound, the identification of potent inhibitors has proven challenging. We built upon our initial findings and have now elucidated key differences between the mechanism of inhibition by indole-derived compounds for hpols η and κ .

An initial screen identified candesartan cilexetil and MK-886 as inhibitors of hpol κ .³⁵ Both compounds possess bicyclic ring systems. Candesartan cilexetil contains a benzimidazole ring system and is an angiotensin II receptor antagonist, while MK-886, an inhibitor of 5-lipoxygenase activating protein inhibitor, contains a central indole moiety. Both candesartan and MK-886 were found to inhibit recombinant hpol κ with IC₅₀ values in the tens of micromolar, but neither was specific to the target, as the compounds inhibited other TLS pols with comparable or greater potency.^{35,41} In the case of MK-886, there was some effect on the affinity of hpol κ but mostly at concentrations of inhibitor well above the IC₅₀ value. By way of comparison, the IAG and IBA compounds reported here (IAG-10 and IBA-32) increased the $K_{d,DNA}$ approximately threefold when incubated at concentrations

near the IC₅₀ for inhibition of pol activity (Figure 3). The realization that IAG-10 likely destabilizes binary complex formation led us to perform experiments in which the concentration of enzyme and DNA in the reaction mixture was varied. In this way, we not only improved the apparent potency of IAG-10 against hpol κ but we were also able to increase the apparent selectivity of the compound for hpol κ , as the IC₅₀ for inhibition of hpol η increased slightly at lower concentrations of enzyme and DNA (Figure 4). Thus, altering the reaction conditions changed the specificity of IAG-10 for hpol κ over η from ~2-fold to ~30-fold.

Our results with IAG-10 led us to propose two distinct models for inhibition of hpol η and κ (Figure 9). First, we observe either a partial competitive or purely competitive mechanism of hpol η inhibition for a variety of indole-derived compounds. Based on HPG-footprinting and molecular docking studies, we propose that these inhibitors bind somewhere between the finger and little finger domains of hpol η and that this disrupts the template strand near the nascent base pair (Figure 9A). Binding to a similar site on hpol κ produces a stronger effect on binary complex formation because the normal position of the N-clasp is disrupted (Figure 9B). Without the N-clasp to stabilize the position of the little finger, hpol κ cannot effectively bind DNA, which is consistent with the structure and activity of hpol κ constructs lacking the N-clasp.⁵⁷

Structural studies have noted differences in the architecture of the pol κ and η active sites that undoubtedly emerged as selective pressure to bypass distinct DNA lesions shaped the evolution of these enzymes.^{58,59} For example, pol η is an important means of tolerating fused pyrimidine lesions that occur following exposure to UV irradiation. As such, the crystal structures of pol η reveal an active site that can readily accommodate two template bases.⁶⁰ This structural feature also appears to be important for the ability of pol η to act as a “double-agent” when it bypasses damage induced by platinum-based chemotherapeutics.⁶¹

In contrast to pol η , pol κ relies upon residues in the N-clasp, finger, and little finger domains to guide and hold template residues in the pol active site.⁶² Residues such as Phe-49 in the N-clasp, Ser134 and Phe155 in the finger domain, and Lys461 and Arg507 in the little finger domain help to guide the template into an active site that is smaller than that observed for pol η or the archaeal homologue Dpo4 from *Sulfolobus solfataricus*. Amino acid side chains, including Met135 and Ala151, present a loop in the finger domain that limits the orientation of the nascent base pair in the hpol κ active site. Moreover, the unique orientation of the little finger domain of hpol κ exposes the minor groove side of the DNA substrate to solvent, which facilitates the accommodation of bulky, minor groove adducts like those formed from bioactivated benzo[*a*]-pyrene.⁶³ Compared to Dpo4, the little finger domain of hpol κ is rotated ~19° away from the pol core. This precarious positioning of the little finger places an increased reliance upon the N-clasp to stabilize hpol κ binary and ultimately ternary complex formation. In the case of IAG-10, these structural features likely represent the Achilles’ heel of hpol κ that allows effective inhibition of the enzyme.

The mechanism by which IAG-10 inhibits hpol κ presents some interesting possibilities for what might occur in cells or *in vivo*. Misregulated expression or recruitment of hpol κ to replication forks in the absence of DNA damaging agents increases markers of replication

stress and genomic instability.^{26,27} In tumors that overexpress hpol κ , such as glioblastomas, inhibiting the aberrant recruitment of hpol κ could alleviate some of the stress imparted by misregulated TLS. Of course, there is a strong possibility that hpol κ inhibition could alleviate problems associated with chemo-resistance, and proof-of-principle experiments in a mouse model have illustrated that resistance to TMZ is facilitated in part by expression of hpol κ .²⁰ The key role for hpol κ activity in promoting recruitment of the 9-1-1 complex, activation of the ATR-Chk1 checkpoint, and subsequent resolution of fork stress through TLS or repair pathways such as homology directed repair make it an attractive target for drug design. The mechanism of inhibition exhibited by IAG-10, which depends on a domain that is unique to hpol κ , makes it an interesting molecule for further study. Moving forward, it will be important to study the effect of hpol κ inhibitors in the context of glioblastoma and explore the role of potential secondary targets of IAG-10 such as human replicative pols and the TLS enzyme pol ζ in potentiating the effects of genotoxic agents. Efforts to combine TLS pol inhibitors with traditional DNA damaging therapeutics are ongoing and represent an important strategy for studying mechanisms of resistance and perhaps even attenuating these processes in cancer. Our findings pave the way for development of combination treatments that target hpol κ in cancer where it likely facilitates multiple aspects of tumor etiology through its ability to bypass DNA damage and its role in the resolution of replication stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Screen of indole-derived molecules for inhibitory properties against hpol κ . Chemical structures of the (A) IBA and (B) IAG scaffolds. hPol κ activity, relative to a DMSO control experiment, is shown for all the compounds containing the IBA scaffold (C) and the IAG scaffold (D). Each compound was used at a concentration of 40 μ M in the screen. The compound identification is noted below each histogram. The chemical structure and detailed activity information for each compound are listed in Table S1a and Table S1b. The results represent the mean ($\pm$ SD) for experiments performed in triplicate.

Figure 2.

IAG-10 is a selective inhibitor of hpol κ . (A) The chemical structure of IAG-10 is shown. (B) The activity of the four human Y-family pols was measured in the presence of IAG-10 (10 μ M). The concentrations of the enzymes were: 1 nM hpol κ , 2 nM hpol η , 10 nM hpol ι , and 10 nM Rev1. The percent activity relative to a DMSO control experiment is shown. (C) The activity of hpol κ (25 nM), Tb pol I (10 nM), *S. solfataricus* Dpo1 (25 nM), hpol β (1 nM), and hpol λ (10 nM) was measured in the presence of IAG-10 (10 μ M). The percent activity relative to a DMSO control experiment is shown. The results shown represent the mean ($\pm$ SD) for three independent replicates. A two-tailed unpaired Student's *t* test was used to compare the results for hpol κ with those of the other DNA pols (** = $P < 0.005$ and *** = $P < 0.0001$).

Figure 3.

Indole-derived compounds decrease the affinity of hpol κ for primer-template DNA. (A) The chemical structure of IBA-32, an IBA derivative. The equilibrium dissociation constants for (B) hpol κ and (C) η binding to DNA ($K_{D,DNA}$). The $K_{D,DNA}$ values measured for hpol κ were replotted as a function of inhibitor concentration and fit to a second order polynomial equation ($Y = b + m_1x + m_2x^2$). The $K_{D,DNA}$ values measured for hpol η were replotted as a function of inhibitor concentration, and linear regression was used to calculate the slope and y -intercept.

Figure 4.

IC_{50} value for inhibition of hpol κ by IAG-10 is sensitive to the concentration of enzyme and DNA. (A) The concentration of enzyme and DNA in the reaction mixture was varied, and the IC_{50} for inhibition of hpol κ by IAG-10 was measured. Fitting the results to eq 1 resulted in the following IC_{50} values: 50 nM DNA (black circles), 7.2 (6.7 to 7.8) μ M; 25 nM DNA (orange squares), 8.9 (8.7 to 9.2) μ M; 10 nM DNA (blue triangles), 8.9 (8.6 to 9.2) μ M; 2.5 nM DNA (red triangles), 1.4 (1.3 to 1.6) μ M; 1 nM DNA (purple diamonds), 1.1 (1.0 to 1.1) μ M; 0.5 nM DNA (open circles), 0.72 (0.69 to 0.76) μ M. (B) The concentration of enzyme and DNA in the reaction mixture was varied, and the IC_{50} for inhibition of hpol η by IAG-10 was measured. Fitting the results to eq 1 resulted in the following IC_{50} values: 50 nM DNA (black circles), 14.3 (12.8 to 15.9) μ M; 2 nM DNA (open squares), 22.5 (21.7 to 23.3) μ M. The results shown in both panels represent the mean ($\pm$ SD) for three independent replicates. Values in parentheses represent the 95% confidence interval derived from the fit of the data.

Figure 5.

Steady-state kinetic analysis of the mechanism of pol inhibition by indole-derived compounds. The (A) turnover number (k_{cat}) and (B) Michaelis constant ($K_{M,dTTP}$) were measured for hpol κ -catalyzed insertion of dTTP in the presence of varying concentrations of IAG-10. Similar experiments were performed for hpol κ catalysis in the presence of IBA-32 (panels C and D) and hpol η catalysis in the presence of IAG-10 (panels E and F). The results shown in all panels represent the mean ($\pm$ SD) for three independent replicates.

Figure 6.

LC-MS analysis of HPG labeling identified a possible binding interface for IAG-10 on hpol κ . Total ion chromatograms are shown for peptides containing HPG-modified (A) Arg48 and (B) Arg505 following incubation with HPG alone (black), HPG + IAG-10 (20 μ M; magenta), or HPG + IAG-10 (100 μ M; cyan). (C) The fraction of HPG-modified peptide was calculated for each of the 15 peptides. (D) The structure of hpol κ is shown with HPG modified peptides highlighted in cyan. HPG-modified peptides that exhibited a decrease of >1.5-fold upon addition of IAG-10 (i.e., protected) are shown in red, and those that exhibited

an increase of >1.5-fold (i.e., exposed) are shown in blue. Arginine residues showing the most change in HPG modification upon addition of IAG-10 are represented in ball-and-stick and are labeled.

Figure 7.

IAG-10 potentiates the antiproliferative and DNA damaging effects of TMZ. Clonogenic assays were performed to assess the effect of IAG-10 on the survival of HAP-1 cells (both hpol κ -proficient and hpol κ -deficient) upon treatment with increasing concentration of TMZ. (A) Representative images for hpol κ -proficient HAP-1 cells are shown. The top panel represents colonies formed by untreated cells (CTL), the middle panel shows an image of HAP-1 cells treated with 250 μ M TMZ (TMZ), while the bottom panel shows cells treated with 250 μ M TMZ and 0.5 μ M IAG-10 in combination (TMZ + IAG-10). Results at all concentrations of TMZ (as indicated) are shown as curves on the right. The number of colonies formed as a function of TMZ concentration is represented as a dose-response plot for the hpol κ -proficient cells treated with TMZ (black circles) or TMZ + 0.5 μ M IAG-10 (red circles). (B) Similar results as in panel A for the clonogenic assay performed with hpol κ -deficient HAP-1 cells are shown. Results shown in A and B represent mean ($\pm$ SD) for three biological replicates. Results from the alkaline comet assay performed with the hpol κ -proficient and hpol κ -deficient HAP-1 cells are shown in C and D, respectively. The four vertical panels on the left of both C and D are the representative images of single cells showing the SYBR-Gold stained DNA. The four panels show images from a control cell, a cell treated with 0.5 μ M IAG-10 alone or 100 μ M TMZ alone, or a combination of both IAG-10 and TMZ, respectively. To the right, the graph depicts results from the comet assay measurements for at least 60 cells per condition from three biological replicates (minimum of 20 cells per replicate). The mean ($\pm$ SD) is shown for each experimental condition. Black open circles represent control cells; blue represent the IAG-10 treated cells, and red

represent the TMZ treated cells. Brown circles represent cells treated with a combination of the two. The % DNA in the tail calculated for each cell from the comet assay is shown on the *Y*-axis. Significance among treatment conditions was calculated using one-way ANOVA with the Bonferroni posthoc correction.

Figure 8.

IAG-10 alters the mutation frequency of MMS-damaged DNA in hpol κ -proficient cells.

The hpol κ -proficient and hpol κ -deficient HAP-1 cells were both transfected with *supF* carrying pSP189 plasmids that were either undamaged (CTL) or MMS-damaged. The HAP-1 cells were grown for 24 h either in the presence of vehicle (DMSO) or 1 μ M IAG-10. The plasmids were extracted and transformed into the *E. coli* MBM7070 competent cells for blue-white screening. Results for the calculated mutational frequency for each experimental sample are shown in A (hpol κ -proficient) and B (hpol κ -deficient). For each condition, at least 10 000 colonies were counted. Black circles represent the untreated cells, while magenta squares show the IAG-10 treated cells. The results are shown as mean ($\pm$ SD) for each condition. *P*-values were calculated using a two-way ANOVA with Sidak's multiple comparison test.

Figure 9. Models for inhibition of hpol η and κ by indole-derived compounds. Cartoon illustrations of (A) hpol η and (B) hpol κ inhibition by indole-derived compounds are shown.

Table 1.IC₅₀ Values for the Inhibition of hpol α by Different IAG Compounds

	compound ID	R1	R2	R3	IC ₅₀ (μ M)	95% confidence interval
1	IAG-2	CO-1-naphthyl	H	OCH ₃	68.6	66.1–71.3
2	IAG-10	CO-1-naphthyl	H	Cl	7.2	6.7–8.3
3	IAG-1	CO-1-naphthyl	CH ₃	H	84.6	79.1–90.5
4	IAG-9	CO-2-naphthyl	H	Cl	18.8	18.3–19.3
5	IAG-8	CH ₂ -1-naphthyl	H	Br	22.3	21.1–23.6
6	IAG-3	CH ₂ -1-naphthyl	H	OCH ₃	52.0	48.7–55.5
7	IAG-7	CH ₂ -1-naphthyl	H	Cl	24.2	21.9–26.7
8	IAG-6	CH ₂ -1-naphthyl	H	OCH ₃	37.1	35.4–38.9
9	IAG-12	CH ₂ -1-naphthyl	H	H	38.9	34.3–41.4
10	IAG-5	CH ₂ -2-naphthyl	H	OCH ₃	43.7	41.7–45.9
11	IAG-4	CH ₂ -2-naphthyl	H	H	48.3	46.6–50.0
12	IAG-13	CH ₂ -2-naphthyl	H	Cl	22.7	19.8–23.3
13	IAG-11	H	CH ₃	Cl	>200	n.d.

^a All IC₅₀ values represent the mean ($n = 3$), and the 95% confidence interval was derived from the fit of the data to a four parameter logistic model.