Published in final edited form as:

Chem Res Toxicol. 2017 February 20; 30(2): 715–725. doi:10.1021/acs.chemrestox.6b00451.

Honokiol Inhibits DNA Polymerases β and λ and Increases Bleomycin Sensitivity of Human Cancer Cells

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Abstract

A major concept to sensitize cancer cells to DNA damaging agents is by inhibiting proteins in the DNA repair pathways. X-family DNA polymerases play critical roles in both base excision repair (BER) and nonhomologous end joining (NHEJ). In this study, we examined the effectiveness of honokiol to inhibit human DNA polymerase β (pol β), which is involved in BER, and DNA polymerase λ (pol λ), which is involved in NHEJ. Kinetic analysis with purified polymerases showed that honokiol inhibited DNA polymerase activity. The inhibition mode for the polymerases was a mixed-function noncompetitive inhibition with respect to the substrate, dCTP. The X-family polymerases, pol β and pol λ , were slightly more sensitive to inhibition by honokiol based on the K_i value of 4.0 μ M for pol β , and 8.3 μ M for pol λ , while the K_i values for pol η and Kf were 20 and 26 μ M, respectively. Next we extended our studies to determine the effect of honokiol on the cytotoxicity of bleomycin and temozolomide in human cancer cell lines A549, MCF7, PANC-1, UACC903, and normal blood lymphocytes (GM12878). Bleomycin causes both single strand DNA damage that is repaired by BER and double strand breaks that are repaired by NHEJ, while temozolomide causes methylation damage repaired by BER and *O*⁶-alkylguanine-DNA alkyltransferase. The greatest effects were found with the honokiol and bleomycin combination in MCF7, PANC-1, and UACC903 cells, in which the EC₅₀ values were decreased 10-fold. The temozolomide and honokiol combination was less effective; the EC₅₀ values decreased three-fold due to the combination. It is hypothesized that the greater effect of honokiol on bleomycin is due to inhibition of the repair of the single strand and double strand damage. The synergistic activity shown by the combination of bleomycin and honokiol suggests that they can be used as combination therapy for treatment of cancer, which will decrease the therapeutic dosage and side effects of bleomycin.

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00451.

Figures of steady-state kinetics for inhibition of DNA polymerases by HNL and EC₅₀ plots for all cell lines (PDF)

Graphical Abstract

INTRODUCTION

Recent studies show that novel plant-derived compounds act as antitumor agents through modulation of biological pathways.¹ Honokiol (2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enyl-phenol (Figure 1), a biologically active biphenolic compound isolated from the *Magnolia officinalis/grandiflora*, has received significant attention due to its potent antineoplastic and antiangiogenic properties.^{2,3} It has yielded promising results against skin, colon, lung, and breast cancers.^{2,4–7} Furthermore, honokiol (HNL) is less toxic to normal cells than tumor cells, it has preferential activity against patient-derived chronic lymphocytic leukemia cells versus normal lymphocytes,⁸ and HNL killed myeloma cells but not peripheral blood mononuclear cells from relapsed patients.⁹ HNL and analogs are attractive therapeutic agents because HNL is nontoxic with no side effects in rats treated with a dose of 100 mg/kg.¹⁰ In addition, HNL crosses the blood–brain and blood–cerebrospinal fluid barriers³ and is effective in prolonging life in mice xenografted with HL60 promyelocytic leukemia cells.^{10,11} HNL has been shown to produce its anticancer effects via multiple mechanisms including NF- κ B, STAT3, EGFR, mTOR HDAC, and caspase-mediated common pathways.^{12–14} The typical concentrations used to observe these effects have been in the micromolar range. However, it is still unclear if there is a single therapeutic target or if the effective in vivo target has been found. Since HNL is relatively nontoxic, a potentially useful application of HNL may be its ability to potentiate the actions of chemotherapeutic agents. In this regard, we evaluated the potentiation of two chemotherapeutic DNA damaging agents, bleomycin and temozolomide, and examined two novel targets of HNL, the X-family polymerases β and λ .

Bleomycins are a family of glycopeptide antibiotics that bind to Fe(II) and DNA. Upon binding oxygen and undergoing a one electron reduction, the activated bleomycin initially produces 4'-oxidized abasic sites, or single-strand breaks containing 3'-phosphoglycolate/5'-phosphate ends.¹⁵ Subsequent reactivation of the DNA-bound bleomycin and a second reaction can lead to double strand breaks (DSBs). While the ssDNA breaks can be repaired by the short-patch base-excision repair pathway that utilizes DNA polymerase β (pol β),^{16,17} the oxidized abasic sites react with the lyase domain of pol β to form a DNA–protein adduct during short-patch BER.¹⁸ Even though the protein–DNA cross-links can be repaired by a pathway involving the proteasome, the protein adducts are toxic.¹⁹ However, the oxidized abasic sites can be effectively repaired by long-patch BER,²⁰ a process that involves pol β ^{21–23} and perhaps δ or ϵ .²⁴ Bleomycin-induced DSBs can be

repaired by nonhomologous end joining (NHEJ), a process that utilizes the X-family polymerases λ and μ .^{25,26} Thus, the X-family polymerases β and λ are integral in attenuating the toxicity of BLM. In fact, inhibition of pol β sensitizes the cells to bleomycin damage,^{27–29} while increased expression of pol β attenuates BLM toxicity.³⁰

Temozolomide (TMZ) is a chemotherapeutic agent that produces methyl diazonium ions that react with DNA to form adducts such as 7-methylguanine (7mG), 3-methyladenine (3mA), and O^6 -methylguanine (O^6 mG). Each of these adducts contributes to the toxicity of TMZ. While O^6 mG is repaired by O^6 -alkylguanine-DNA alkyltransferase, 7mG and 3mA, which comprise over 80% of the TMZ DNA adducts, are repaired by BER. BER inhibition, via PARP1 or pol β inhibition, increases the toxicity of TMZ, while pol β activity decreases the cytotoxicity of TMZ.^{31–33}

While BLM and TMZ produce different types of DNA damage, the X-family polymerases, pol β and λ , are involved in the repair their damage. Pol β is the primary polymerase involved in BER, while pol λ functions as a backup.³⁴ Pol λ is also involved in NHEJ. In this manuscript, we evaluated the inhibitory activity of HNL toward pol β and pol λ , and two other polymerases, pol η and *E. coli* DNA polymerase I (Klenow fragment) with the proofreading activity inactivated (KF(exo-)), to evaluate the selectivity of the inhibition. In addition, we studied the effect that HNL has on the cytotoxicity of BLM and TMZ. Our results show that HNL inhibits eukaryotic pol β , pol λ , and pol η activities but has lesser inhibition of prokaryotic Kf(exo-). In addition, we found that HNL chemosensitizes the cancer cell lines to cytotoxic effects of BLM to a greater extent than does TMZ.

MATERIALS AND METHODS

Caution: *These chemicals are dangerous*

The following chemicals are hazardous and should be handled carefully: bleomycin, temozolomide, and honokiol.

Chemicals and Reagents

T4 polynucleotide kinase was purchased from Epicenter (Lexington, KY) and [γ -³²P]ATP (6000 Ci/mmol) from PerkinElmer (Waltham, MA). DNA oligomers were purchased from IDT (Coralville, IA). The concentrations of the dNTPs (Promega, Madison WI) were each determined by UV absorbance.³⁵ HNK, BLM, and TMZ, purchased from Sigma-Aldrich (St. Louis, MO), were freshly prepared in DMSO, stored at -80°C , and diluted in buffer or complete medium just before use. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA), and phenazine metho-sulfate (PMS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). MTS/PMS solution was prepared at a concentration of 2 mg/mL of MTS and 0.92 mg/mL of PMS in phosphate-buffered saline (PBS) and stored in dark bottle at 4°C . Fetal bovine serum was purchased from Atlanta Biologicals Inc. The human cancer cell lines, MCF7, A549, PANC-1, UACC903, were purchased from American Type Culture Collection (ATCC), and GM12878 cells were purchased from Coriell Institute for Medical Research.

DNA Substrates

The single gapped DNA substrate for pol β and pol λ contained a template, a primer, and a downstream blocking oligodeoxynucleotide as illustrated in Table 1. The primer strand was 5'-end-labeled with T4 polynucleotide kinase and [γ - ^{32}P]ATP (6000 Ci/mmol) as previously described.³⁶ The blocking strands were phosphorylated on the 5'-end with nonradioactive ATP. The unreacted [γ - ^{32}P]ATP and ATP were removed using a Sephadex G-25 spin column and annealed at a primer/blocker/template ratio of 1:3:1. The DNA substrates for pol η and Kf(exo-) were prepared by mixing a ^{32}P labeled 15-mer primer with the 24-mer template at a molar ratio of 1:1.2.

Cell Lines and Culture Conditions

The human cancer cell lines MCF7, A549, PANC-1, and UACC903 were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (v/v). The normal blood lymphoblasts (GM12878) were cultured in RPMI 1640 medium containing 15% fetal bovine serum. All cell lines were cultured at 37 °C in a humidified incubator with 5% CO₂ (v/v).

DNA Polymerases

DNA polymerase I from *E. coli* (Klenow fragment) with the proofreading activity inactivated (Kf(exo-)) was purchased from USB scientific. His-tagged human DNA polymerases β ,³⁷ η ,³⁸ and λ ³⁹ were expressed and purified as previously described. The polymerase concentrations were determined by analyzing the burst intensity as described previously.³⁸

Steady-State Primer Extension Assay

The DNA polymerase reaction was initiated by adding equal volumes of DNA polymerase, DNA substrate, and the appropriate amount of honokiol in buffer with dNTP and MgCl₂ at 37 °C. The final buffer concentrations depended on the polymerase: Kf, 50 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 0.1 mM EDTA, 3 mM DTT, and 100 $\mu\text{g}/\text{mL}$ of BSA; pol η , 40 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.1 mM EDTA, 3 mM DTT, and 100 $\mu\text{g}/\text{mL}$ of BSA with 2.5% glycerol (v/v); pol β and λ , 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, 100 $\mu\text{g}/\text{mL}$ of BSA, 1 mM DTT. The concentrations of the polymerases varied from 0.01–0.2 nM, and the DNA concentration was 10 nM during the reactions. The gapped DNA substrate was used for pol β and λ , while the simple primer template was used for Kf and pol η (Table 1). The reaction was quenched by addition of an equal volume of STOP solution (95% formamide, 20 mM Na₂EDTA, 0.025% bromphenol blue (w/v), and 0.025% xylene cyanol (w/v)).

Polymerase in Excess Primer Extension Assay

The reaction was initiated by adding equal volumes of DNA polymerase, DNA substrate, and the appropriate amount of honokiol in buffer (as above) with dNTP and MgCl₂ at 37 °C with a rapid quench instrument (RQF-3, KinTek Corp). The concentrations of the polymerase and DNA during the reaction were 100 nM and 15 nM, respectively. The reaction was quenched by addition of 0.3 M EDTA (pH 8.0).

Analysis of Reactions

The reaction products were separated by electrophoresis on a 15% (w/v) polyacrylamide (19:1 (w/w), acrylamide/bis-acrylamide) gel containing 8 M urea in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA, pH 8.3). The amounts of radioactivity in the reactant and product bands were quantified using a Typhoon 9200 and ImageQuant software (GE Healthcare).

Polymerase–DNA Interaction

The binding affinities of DNA and the polymerases were examined with an electrophoretic mobility shift assay.⁴⁰ The DNA, polymerase, and honokiol were incubated for 20 min in the correct DNA polymerase buffer at 37 °C. Samples were loaded onto a 6% native polyacrylamide gel (0.5 × TBE) and run at 100 V for 2 h. Bound protein was quantified using ImageQuant software after the gel was scanned using a Typhoon 9200 and ImageQuant software (GE Healthcare). Protein bound to DNA resulted in a shift of the DNA on the gel when compared to DNA without bound protein.

MTS Assay of Cell Proliferation

A colorimetric cell proliferation assay^{7,10} was used to assess the effect on cell proliferation of potentiation of the cytotoxicity of BLM with HNL in cancer cell lines A549, MCF7, PANC-1, UACC903, and the immortalized normal cell line GM12878. For these studies, 5×10^3 cells were plated and grown for 24 h in 100 μ L of growth medium in 96-well microtiter plates at 37 °C in a humidified 5% CO₂ atmosphere. For treatments, stock solution of HNL (10 mM), BLM (10 mM), or TMZ (100 mM) in DMSO were diluted with fresh complete medium immediately before use. Cells were treated for 24 and 72 h in 200 μ L of fresh media containing various concentrations of HNL, BLM, or TMZ alone or in combination. Control cells were treated with equivalent concentrations of DMSO. In all cases, final concentration of DMSO was 0.2%, well below the concentrations that interfere with proliferation in the above cell lines. After a 24 or 72 h incubation period, the number of viable cells was determined by measuring the bioreduction by intracellular dehydrogenases of the tetrazolium compound MTS in the presence of the electron coupling reagent PMS. To perform the assay, 20 μ L of combined MTS/PMS solution containing 2 mg/mL MTS and 0.92 mg of PMS in PBS, pH 7.2, was added to each well, and the mixture was incubated for 4 h at 37 °C in a humidified 5% CO₂ atmosphere. Absorbance at 492 nm was measured using an ELISA microplate reader (Flexstation 3 Molecular Devices, Softmax Pro 5). Background absorbance of the medium was measured in wells that contained medium and the MTS/PMS solution without added cells.

Data Analysis

IC₅₀ values for the inhibition of the polymerases were obtained by fitting the data to eq 1, in which Y was the amount of product, A was the amount of product with no honokiol, and X was the concentration of honokiol and IC₅₀, the concentration of honokiol that reduces the amount of product to 50%. IC₅₀ values for the inhibition of cell viability were also obtained by fitting the data of eq 1, in which Y was the normalized absorbance value, A was 100, and

X was the concentration of the test compound and IC_{50} , the concentration that reduces the normalized absorbance to 50%.

$$Y = \frac{A}{1 + 10^{(\log(IC_{50}) - \log(X))}} \quad (1)$$

$V_{\max}^{\text{app}}$ and K_m^{app} values were obtained by fitting the initial rates (v_0) versus $[dNTP]$ to eq 2, where $[E]_0$ and $[S]_0$ are the initial concentrations of the polymerase and dNTP. Eqs 1 and 2 were fitted using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA):

$$v_0 = \frac{V_{\max}^{\text{app}} [S]_0}{K_m^{\text{app}} + [S]_0} \quad (2)$$

The k_{cat} , K_m , and K_i were determined by fitting v_0 directly to the hyperbolic multitype inhibition eq 3 using Matlab 2015b:

$$v_0 = \frac{V_{\max} \left(\frac{[S]}{K_m} + \frac{\beta [S][I]}{\alpha K_m K_i} \right)}{1 + \frac{[S]}{K_m} + \frac{[I]}{K_i} + \frac{[S][I]}{\alpha K_m K_i}} \quad (3)$$

The time course experiments were fit to the burst eq 3 where P is the total product, A the burst amplitude, k_b the burst, and k_{ss} the steady-state rate constants:

$$P = A(1 - e^{-k_b t}) + k_{ss} t \quad (4)$$

The K_d for honokiol was evaluated from the burst amplitudes by fitting the amplitudes to eq 5 in which A represents the burst amplitude, $A_{\min}$ and $A_{\max}$ the minimum and maximum burst amplitudes, and $[I]$, the HNL concentration:

$$A = A_{\max} - \frac{(A_{\max} - A_{\min})[I]}{K_d + [I]} \quad (5)$$

RESULTS

Effects of HNL on the Activities of Select Eukaryotic and Prokaryotic DNA Polymerases

We initially examined the inhibitory activity of honokiol on selected polymerases using the primer extension assay with 1 nM polymerase, 10 nM DNA, and 50 μM dCTP and various concentrations of HNL. As illustrated in Figure 2, HNL inhibited the X-family polymerases (β and λ) to a greater degree than all of the polymerases studied with IC_{50} values of 0.6 μM for pol β , 0.8 μM for pol λ , 10 μM for pol η , and 80 μM for Kf(exo-). The low IC_{50} values

for pol β and λ lead us to evaluate the mechanism underlying the inhibition. The inhibition could be at several stages including inhibition of DNA or dNTP binding or by allosteric inhibition of the catalytic activity.

Honokiol Does Not Inhibit the Binding of Polymerases to DNA

Since the honokiol effects were greatest with pols β and λ , we examined whether honokiol inhibited the binding of the DNA substrates to these enzymes. As shown in Figure 3, we used the EMSA to determine a K_d^{DNA} of 10.0 ± 1.5 nM for pol β and 11.4 ± 3.0 nM for pol λ , values consistent with previous results for pol β .^{41–43} We then incubated honokiol with the polymerases and the appropriate DNA substrate and evaluated DNA binding by EMSA. Figure 3 shows experiments with polymerase in excess of DNA so that the amount of unbound radiolabeled DNA is minimal and consequently we would be able to detect unbound DNA. Up to its solubility limit of 200 μM , honokiol did not inhibit the binding of the DNA to the polymerase. On the basis of equilibria equations, a conservative estimate for the K_i^{HNL} would be greater than 500 μM . Since we observed polymerase inhibition at lower concentrations of honokiol, we concluded that competitive inhibition DNA binding is not the mechanism by which honokiol inhibits these DNA polymerases.

Honokiol Inhibits Polymerases by Mixed-type Inhibition

To determine the kinetic mechanism by which honokiol inhibits polymerase activity, the initial rates were determined over a range of dCTP and honokiol concentrations. The data were fitted to the Michaelis–Menten eq 2 by nonlinear least-squares analysis. The plots for pol β and λ are shown in Figure 4. These plots clearly show that the $V_{\text{max}}^{\text{app}}$ decreases as honokiol concentrations increase. As shown in Figures S1 and S2, both $V_{\text{max}}^{\text{app}}$ and K_m^{app} decrease for all four polymerases with increasing HNL concentrations. The data were visualized with Lineweaver–Burk plots as shown in Figure 4C and D (also Figure S4). The observation that the lines intersect to the left of the y -axis and below the x -axis is consistent with hyperbolic mixed type inhibition, illustrated in Scheme 1 with kinetic eq 3 in which $\alpha < 1$ and $\beta < 1$.⁴⁴ The experiment was conducted in triplicate, the data were fit directly to the hyperbolic mixed type inhibition eq 3, and the results are presented in Table 2. The β values for pol β , λ , and Kf(exo-) are small, but greater than 0, indicating that a small amount of catalysis occurs with HNL bound to the polymerase complex. The β value for pol η has 95% confidence limits that includes 0. With $\beta = 0$, the mechanism would be described as linear mixed-type inhibition. The α values are approximately 0.5, which indicate that binding of dCTP or HNL increases the affinity of the other ligand.

Presteady State Burst Kinetics of Pol β and Pol λ in the Presence of HNL

Presteady-state kinetic analysis of enzyme inhibition is a powerful approach for determining which step or steps are affected by a given inhibitor. This technique has been applied to study a number of systems including D-3-phosphoglycerate dehydrogenase,⁴⁵ beta-site amyloid precursor protein-cleaving enzyme,⁴⁶ ATP-phosphoribosyl-transferase,⁴⁷ DNA polymerase γ ,⁴⁸ and HIV-RT.^{49,50}

Presteady-state analysis was carried out using a rapid quench apparatus to further characterize the mechanism of inhibition of pol β and pol λ . We initially examined the

reaction with no inhibitor and found $k_{\text{pol}} = 18 \pm 1 \text{ s}^{-1}$ and $K_d^{\text{dCTP}} = 20 \pm 3 \text{ }\mu\text{M}$ for pol β (data not shown), values similar literature values.^{41,42} Preincubated gapped DNA (15 nM), pol β (100 nM), and honokiol were added to dCTP (100 μM) with Mg^{2+} . The reactions were quenched at various times and the data presented in Figure 5A. The data were fit to the burst eq 4. With no inhibitor, the burst amplitude is close to the polymerase concentration. The addition of HNL decreased the burst amplitude but neither the burst (k_b) nor the steady-state (k_{ss}) rate constants. The burst amplitude was fitted to the hyperbolic eq 5 to obtain $K_d^{\text{HNL}} = 20 \pm 4 \text{ }\mu\text{M}$. The minimum burst amplitude is close to zero. A decrease in burst amplitude indicates that honokiol inhibits pol β by forming a catalytically inactive quaternary species that reverses slowly. The off rate for HNL would have to be slower than the k_{cat} . This type of inhibition was observed in the non-nucleoside inhibitors of HIV-RT.^{49,50} We found similar results with pol λ (Figure S4).

Honokiol Chemosensitizes the Cancer Cells for BLM Toxicity

The inhibition of pol β and λ may also reduce the activity of the BER pathway in cells. If this occurs, then cytotoxic DNA damaging agents that are repaired by BER may become more toxic with coadministration of HNL. Thus, we examined the ability of HNL to potentiate the cytotoxicity of BLM and TMZ in human cancer cell lines A549, MCF7, PANC1, UACC903, and GM12878. The cells were treated with various concentrations of honokiol, BLM, and TMZ to determine EC_{50} values. Figure 6 shows the effect on the EC_{50} values with PANC1 cells. The open markers represent treatment with a single compound, while the filled symbols represent dual treatment in which 10 μM BLM or 250 μM TMZ was added to HNL, or 10 μM HNL was added to BLM or TMZ. Panels A and B show the effect of HNL on TMZ. The small leftward shift of the curves indicates that HNL and TMZ weakly enhance each other's cytotoxicity. The EC_{50} values decreased up to three-fold. Panels C and D illustrate that HNL and BLM more strongly enhance each other's cytotoxicity. In these graphs, larger leftward shifts in the curves are more apparent. The EC_{50} values decreased 10-fold. The $\log\text{EC}_{50}$ values for all cell lines are presented in Table 3, and the plots for the other cell lines are presented in Figure S5–S9. The effect on the $\log\text{EC}_{50}$ of the dual administration of compounds is graphically illustrated in Figure 6. The data indicate that the BLM and HNL combination increased cellular toxicity to a greater extent than TMZ and HNL in the MCF7, PANC1, and UCAA cell lines.

DISCUSSION

Chemotherapeutic agents have a concentration window where they kill tumor cells but not normal cells. The idea of chemotherapeutic cocktails is to use low nontoxic doses of two or more compounds that take advantage of the altered protein expression levels in tumor cells to selectively kill these cells. Many tumor cells have upregulated DNA polymerase β levels,^{51,52} perhaps to compensate for a higher oxidative stress levels in the tumors. Our strategy is to stress these tumor cells by simultaneously inhibiting DNA polymerase β while stressing the cells with a DNA damaging agent that would need the BER pathway. In this, we hope to enlarge the therapeutic window between toxicity for tumor cells and normal cells. This strategy has been successful with many pol β inhibitors that have been identified in the past two decades. For example, a modified benzenesulfonamide inhibits the binding of

the APC-protein to pol β and thus inhibits long-patch BER activity.³² This action sensitizes colon cancer cells to TMZ. Similarly, triterpenoids and fatty acid-like compounds have increased the cytotoxic activity of BLM^{27–29} and methylmethanesulfonate.³²

Several triterpenes, such as koetjapic acid, inhibit pol β via a linear mixed-mode inhibition mechanism with K_i values in the low micromolar range.⁵³ Here we show that HNL also inhibits both pol β and λ with K_i values as low as these natural products. Steady-state kinetic analysis indicated that HNL inhibition versus dCTP is mixed-type noncompetitive for pol β , pol λ , and KF(exo-), and linear mixed-type noncompetitive for pol η . While the formal nomenclatures of the inhibition mechanisms are different, essentially the inhibition is of a noncompetitive nature with a kinetic mechanism illustrated in Scheme 1.

NMR studies show that koetjapic acid and related triterpenoids bind in a cleft on the *N*-terminal 8 kDa domain of pol β , which is responsible for the deoxyribose phosphate lyase activity of the enzyme.^{54,55} Binding to the *N*-terminal 8 kDa domain of pol β occurs with other large fatty acid-like compounds.^{55,56} HNL can potentially bind to the same region. These compounds are similar to HNL in that they contain large hydrophobic hydrocarbon regions punctuated with oxygen to provide some hydrophobicity. However, HNL is smaller than these compounds and does not contain a carboxylic acid moiety. In addition, while this mechanism may also occur with the X-family pol λ , pol η and KF(exo-) do not have an analog to the *N*-terminal 8 kDa lyase domain, so inhibition of these polymerases must occur via a different mechanism. Thus, the molecular mechanism by which honokiol inhibits DNA polymerases is unknown.

Triterpenes require preincubation with the polymerase for successful inhibition; an indication that the binding of the inhibitor to the polymerase is slow or that inhibition requires a slow conformational change to form an inactive complex. Using presteady-state kinetics, we have found that HNL inhibits the reaction by decreasing the burst amplitude of the reaction. This type of kinetic plot would be observed only if the inhibitor complexes (EDI and EDNI) are slow to dissociate. If the binding and dissociation of HNL were rapid, then addition of HNL would decrease the rate constant, not the amplitude. Modeling the reaction with a noncompetitive inhibition scheme results in a honokiol off rate constant of 0.16 s^{-1} (Figure S10). If HNL and triterpenes inhibit pol β by similar mechanisms, then the need for preincubation and the slow off rates are consistent. This type of inhibition was observed previously with non-nucleoside inhibitions and HIV-RT.⁴⁹

In summary, HNL is an inhibitor of pol β with a $K_i < 10 \mu\text{M}$. This K_i value, while not very low, is similar to IC_{50} and K_i values of other compounds.^{27,29,55–66} The kinetic mechanism involves HNL sequestering the polymerase–DNA–dNTP complex in an unreactive configuration that decomposes slowly. To evaluate if HNL can be utilized to chemosensitize damage DNA, which is repaired by BER, we examined two reagents TMZ and BLM.

TMZ is a methylating agent approved for use with anaplastic astrocytoma and glioblastoma,⁶⁷ has clinical activity in metastatic melanoma, and is under clinical evaluation for use in other cancers including leukemia, lymphoma, aerodigestive tract, pancreatic, and neuroendocrine tumors. TMZ causes cancer cell cytotoxicity by methylating genomic DNA,

producing cytotoxic or mutagenic abnormal DNA bases.⁶⁸ The major DNA adducts studied include 7mG, 3mA, and *O*⁶mG. 7mG and 3mA are cytotoxic because they depurinate and form abasic sites. 3mA is also block to DNA polymerases.⁶⁹ These adducts are repaired by BER which utilizes pol β as the primary polymerase. The inhibition of this repair system with PARP or pol β inhibitors leads to increased efficacy of TMZ.^{31–33,70,71} *O*⁶mG is mutagenic, and its cytotoxicity is derived from the futile cycling of the mismatch repair system.⁷² It is not repaired by BER, but by *O*⁶-alkylguanine DNA alkyltransferase. Low activity of AGT also increases the efficacy of TMZ.^{73–75} The net toxicity of TMZ is determined through the interaction of at least two DNA repair pathways.

Bleomycin produces 4'-oxidized abasic sites, single-strand breaks containing 3'-phosphoglycolate/5'-phosphate ends as well as double stranded breaks.¹⁵ Pol β is involved in the repair of the single-strand breaks via the BER pathway.^{16,17} While short-patch BER of oxidized abasic sites produces a DNA-pol β cross-link, the adduct can be repaired via long patch BER. Inhibition of pol β sensitizes the cells to bleomycin damage.^{27–29} Links between DNA pol β expression and sensitivity to BLM also support the ability of pol β to attenuate BLM toxicity.³⁰ BLM also caused double-strand breaks that may be the primary source of toxicity. Double-strand breaks are repaired by homologous recombination (HR), nonhomologous end joining (NHEJ), and alternative NHEJ. NHEJ operates during all phases of the cell cycle and is predominant during G0/G1.⁷⁶ HR is absent in G1 and most active in S and G2.²⁶ Alternative NHEJ appears to operate as a backup repair system for HR and NHEJ.⁷⁷ Each of these pathways involves many proteins, but relevant to our study, NHEJ utilizes the X-family polymerases λ and μ .⁷⁶

HNL is relatively nontoxic to normal cells and may be an alternative to stronger inhibitors that have side effects. The EC₅₀ values for a 24 h incubation in our GM12878 cells were >1 mM in one study and >100 μ M in another. We evaluated the toxicity of HNL, BLM, and TMZ in five cell lines, GM12878, a normal cell line immortalized with the Epstein Bar virus, with four tumor derived lines A549, MCF7, PANC1, and UACC903. As shown in Figure 7, the EC₅₀ values for TMZ and BLM are decreased in the presence of HNL (Figure 7A,C), and the EC₅₀ for HNL is decreased in the presence of both TMZ and BLM (Figure 7B,D). Importantly, in the nontumor derived GM12878 cells, HNL does not increase the toxicity of TMZ and has a very small effect with BLM. This property would be useful in therapy.

HNL increases the toxicity of both BLM and TMZ, but the effect is greater for BLM. The EC₅₀ values decreased up to 10-fold in MCF7, PANC1, and UACC903 cells due to the HNL/BLM combination (Figure 7C). The effect with TMZ is less, with the EC₅₀ values being decreased up to three-fold with HNL (Figure 7A). We speculate that the increased activity with BLM is because HNL inhibits repair of both single-strand damage and DSBs caused by BLM. As discussed above, both BLM and TMZ produce various types of DNA damage. TMZ produces 3mA and 7mG, adducts that are repaired by BER, and *O*⁶mG, which is repaired by *O*⁶-alkylguanine-DNA alkyltransferase. BLM produces both single-strand damage (single-strand breaks and oxidized abasic sites) and DSBs. The single-strand breaks are repaired by short patch BER, the oxidized abasic sites can be repaired by long patch BER, and the DSBs are repaired by NHEJ. In this work, we found that HNL inhibits

both pol β , which is involved in BER, and pol λ , which is involved in both BER and NHEJ. Thus, HNL can inhibit the repair of the major DNA damage produced by BLM. In contrast, HNL inhibits the BER repair of 7mG and 3mA but not the repair of 8mG.

The effects of HNL on modulating the cytotoxicity of TMZ and BLM are similar for the four cancer cell lines. In Figure 7A, the effect of HNL on TMZ toxicity was much less than the other cell lines at the 72 h time point. However, this effect was not observed in the 24 h time point nor in Figure 7B, so we do not want to draw any conclusion based upon this result. In the BLM studies, the A549 cell line is more resistant to the effects of HNL than the other cell lines tested (Figure 7C,D). HNL has multiple possible targets for its anticancer effects such as the NF- κ B, STAT3, EGFR, mTOR HDAC, and caspases.^{12–14} We have identified pol β and λ as possible HNL targets. The effect that HNL has in different cell lines will be affected by the interplay between these pathways. More work needs to be done to understand how the different cell lines respond to HNL treatment.

In summary, HNL is an inhibitor for X-family polymerases β and λ . The mechanism is noncompetitive inhibition in which the complex between HNL/polymerase/DNA/dNTP, while not extremely stable, is slow to dissociate. HNL modulates the cytotoxicity of TMZ and BLM, possibly through the inhibition of pol β and λ . More work needs to be done to test this mechanism. The approach of combining HNL with BLM shows promise as HNL can inhibit polymerases involved in the repair of single strand damage and double strand breaks. Thus, by developing a target defined strategy of chemotherapy and with the appropriate understanding of the mechanism of action, the strategy of combination of HNL with BLM may provide a foundation for both clinical and scientific development of cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

The work was supported by the National Institutes of Health Grant No. ES021762 to T.E.S.

ABBREVIATIONS

BML	bleomycin
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
HNL	honokiol
Kf(exo-)	Klenow fragment of <i>E. coli</i> DNA polymerase I with the proofreading exonuclease activity inactivated

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PMS	phenazine metho-sulfate
PBMCs	peripheral blood mononuclear cells
pol β	DNA polymerase β
pol η	DNA polymerase η
pol λ	DNA polymerase λ
TBE	Tris-HCl, boric acid, and EDTA buffer

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honokiol (HNK)

temozolomide (TMZ)

bleomycin (BLM)

Figure 1.
Structures of molecules used in this study.

Figure 2.

Dose–response curves of honokiol with DNA polymerases. Each polymerase (1 nM) enzyme was preincubated with honokiol and 10 nM DNA substrate for 10 min prior to initiation of the reaction by addition of dCTP and MgCl_2 . After a 15 min incubation at 37 °C for 15 min. The DNA polymerase activity in the absence of HNL was taken as 100% activity.

Figure 3. Honokiol does not inhibit DNA binding to polymerases. EMSA showing native PAGE of of (A) pol β , (B) pol λ , (C) pol η , and (D) KF(exo-).

Figure 4.

Inhibition of the pol β and pol λ catalyzed incorporation of dCTP into DNA by honokiol. Initial rate versus dNTP plot for (A) pol β and (B) pol λ . The lines are the best fit to eq 2. Lineweaver–Burk plots for (C) pol β and (D) pol λ . The lines are the best fit to a linear equation. The HNL concentrations are 0 (open circle), 25 (diamond), 50 (down triangle), 100 (up triangle), 250 (square), and 500 (filled circle) μ M. Each point represents the mean $\pm$ SEM of three experiments.

Figure 5.

Reduced pol β burst amplitudes in the presence of honokiol. (A) Time course for the insertion of 200 μM dCTP opposite dG in the presence of 0 (square), 10 (down triangle), 50 (circle), and 100 (up triangle) μM honokiol. The lines are the best fit to the burst equation. The k_b and k_{ss} were unchanged with increasing honokiol concentrations at $11.7 \pm 1.0 \text{ s}^{-1}$ and $0.2 \pm 0.6 \text{ nM}^{-1} \text{ s}^{-1}$, respectively. (B) Plot of burst amplitude versus honokiol concentration. The line is the best fit to the hyperbolic equation with $A_{\text{max}} = 12.8 \pm 0.3 \text{ nM}$, $A_{\text{min}} = 1.0 \pm 0.5$, $K = 21 \pm 4 \mu\text{M}$. The data points are the mean $\pm$ SEM of three experiments.

Figure 6.

Changes in EC_{50} due to combination of HNL with BLM and TMZ in PANC1 cells. The cell viability was determined at different concentrations of HNL, TMZ, or BLM alone (represented by the open symbols) and in combination (represented by solid symbols). The data are the mean $\pm$ SE of three experiments.

Figure 7.

Changes in log EC₅₀ due to dual administration of HNL and either BLM or TMZ. Change in log EC₅₀ of (A) TMZ caused by 10 μ M HNL; (B) HNL caused by 250 μ M HNL; (C) BLM cause by 10 μ M HNL; and (D) HNL caused by 10 μ M BLM.

Scheme 1.

Hyperbolic Mixed-type Inhibition of DNA Polymerases by HNL

Table 1

DNA Substrates for Polymerases

5'- 3'-	pol β and λ																-3'- -5'
	G	A	T	G	A	G	T	A	C	T	G	A	T	C	G	T	C
5'- 3'-	KF(exo-) and pol η																-3'- -5'
	G	A	T	G	A	G	T	A	C	T	G	A	T	C	G	T	C

Table 2
Steady State Kinetic Parameters for the Hyperbolic Mixed-type Inhibition of DNA Polymerase by HNL^a

pol	k_{cat} (min^{-1})	K_m (nM)	K_i (μM)	α	β
β	28 (26, 30)	220 (180, 260)	4.0 (2.4, 5.6)	0.55 (0.31, 0.79)	0.20 (0.15, 0.25)
λ	5.2 (4.8, 5.5)	274 (230, 310)	8.3 (4.8, 11.9)	0.51 (0.24, 0.78)	0.07 (0.02, 0.12)
η	7.4 (6.7, 8.0)	390 (330, 450)	20 (7, 34)	0.49 (0.12, 0.85)	0.03 (-0.10, 0.15)
Kf	18 (17, 20)	76 (63, 88)	26 (14, 38)	0.48 (0.23, 0.74)	0.16 (0.09, 0.23)

^aInhibition experiments were conducted in triplicate. All data points were used to fit the data to eq 3. The data are reported as the values with the lower and upper 95% confidence limits.

Table 3Toxicity of HNL, TMZ, and BLM with Select Combinations with Cancer Cell Lines^a

		-log(IC ₅₀) (95% CI)	
tested compound	supplemented with	24 h	72 h
GM12878			
TMZ		>3	3.5 (3.4, 3.7)
TMZ	HNL	>3	3.9 (3.8, 4.1)
HNL		>3	4.7 (4.6, 4.9)
HNL	TMZ	>3	4.9 (4.8, 5.1)
BLM		4.1 (3.91, 4.3)	4.1 (4.0, 4.3)
BLM	HNL	4.2 (4.0, 4.3)	4.20 (4.0, 4.4)
HNL		3.9 (3.7, 4.0)	4.0 (3.8, 4.1)
HNL	BLM	3.9 (3.8, 4.1)	4.0 (3.9, 4.1)
A549			
TMZ		3.4 (3.3, 3.5)	3.6 (3.4, 3.7)
TMZ	HNL	3.6 (3.5, 3.7)	4.1 (4.0, 4.2)
HNL		4.6 (4.4, 4.7)	4.0 (3.9, 4.0)
HNL	TMZ	4.9 (4.7, 5.0)	4.1 (4.0, 4.2)
BLM		4.8 (4.7, 5.0)	5.2 (5.0, 5.3)
BLM	HNL (10 μM)	5.30 (5.18, 5.43)	5.6 (5.5, 5.7)
HNL		4.6 (4.5, 4.8)	4.8 (4.6, 4.9)
HNL	BLM (10 μM)	5.0 (4.8, 5.1)	5.3 (5.2, 5.4)
MCF7			
TMZ		3.3 (3.2, 3.4)	3.7 (3.6, 3.8)
TMZ	HNL (10 μM)	3.7 (3.6, 3.8)	3.9 (3.7, 4.0)
HNL		4.7 (4.6, 4.8)	4.8 (4.7, 4.9)
HNL	TMZ (250 μM)	4.9 (4.8, 5.0)	5.0 (4.9, 5.1)
BLM		4.9 (4.8, 5.0)	5.3 (5.2, 5.4)
BLM	HNL (10 μM)	6.0 (5.7, 6.2)	6.3 (6.1, 6.6)
HNL		4.8 (4.7, 4.8)	4.9 (4.8, 5.0)
HNL	BLM (10 μM)	5.6 (5.4, 5.7)	5.6 (5.4, 5.8)
PANC1			
TMZ		3.5 (3.4, 3.7)	3.8 (3.7, 3.9)
TMZ	HNL (10 μM)	3.9 (3.8, 4.0)	4.3 (4.2, 4.4)
HNL		4.7 (4.5, 4.9)	5.1 (5.0, 5.3)
HNL	TMZ(250 μM)	5.0 (4.9, 5.1)	5.3 (5.2, 5.4)
BLM		4.9 (4.7, 5.1)	5.2 (5.0, 5.3)
BLM	HNL (10 μM)	5.9 (5.7, 6.2)	6.09 (5.9, 6.3)
HNL		4.6 (4.5, 4.8)	4.8 (4.7, 4.9)
HNL	BLM (10 μM)	5.5 (5.3, 5.7)	5.6 (5.4, 5.8)
UACC903			
TMZ		3.2 (3.1, 3.3)	3.5 (3.4, 3.7)

tested compound	supplemented with	-log(IC ₅₀) (95% CI)	
		24 h	72 h
TMZ	HNL (10 μ M)	3.6 (3.5, 3.8)	3.9 (3.8, 4.1)
HNL		4.6 (4.5, 4.7)	4.8 (4.7, 4.9)
HNL	TMZ(250 μ M)	4.8 (4.7, 4.9)	5.0 (4.9, 5.1)
BLM		4.9 (4.7, 5.0)	5.22 (5.1, 5.4)
BLM	HNL (10 μ M)	5.5 (5.4, 5.7)	6.3 (6.0, 6.5)
HNL		4.6 (4.47, 4.8)	4.8 (4.65, 4.9)
HNL	BLM (10 μ M)	5.1 (5.0, 5.2)	5.4 (5.2, 5.5)

^aThe -log(EC₅₀) values were determined for the tested compound alone and in the presence of 10 μ M HNL, 10 μ M BLM, or 250 μ M TMZ. The experiments were conducted in triplicate. All data points were used to fit the data to eq 3. The data are reported as the value with the lower and upper 95% confidence limits.