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Nucleotides with Altered Hydrogen Bonding Capacities Impede Human DNA Polymerase η by Reducing Synthesis in the Presence of the Major Cisplatin DNA Adduct

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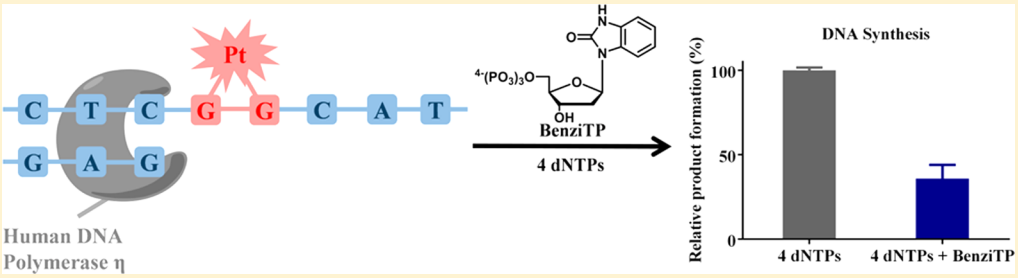
Supporting Information



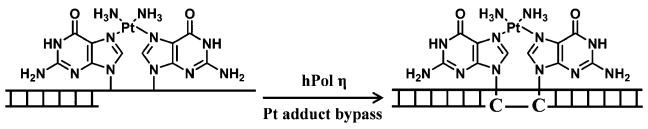
ABSTRACT: Human DNA polymerase η (hPol η) contributes to anticancer drug resistance by catalyzing the replicative bypass of DNA adducts formed by the widely used chemotherapeutic agent cis-diamminedichloroplatinum (cisplatin). A chemical basis for overcoming bypass-associated resistance requires greater knowledge of how small molecules influence the hPol η-catalyzed bypass of DNA adducts. In this study, we demonstrated how synthetic nucleoside triphosphates act as hPol η substrates and characterized their influence on hPol η-mediated DNA synthesis over unmodified and platinated DNA. The single nucleotide incorporation efficiency of the altered nucleotides varied by more than 10-fold and the higher incorporation rates appeared to be attributable to the presence of an additional hydrogen bond between incoming dNTP and templating base. Finally, full-length DNA synthesis in the presence of increasing concentrations of synthetic nucleotides reduced the amount of DNA product independent of the template, representing the first example of hPol η inhibition in the presence of a platinated DNA template.

INTRODUCTION

DNA damage response enzymes process chemically altered forms of DNA. They are essential in preserving cell viability, but therefore contribute to resistance toward DNA-damaging drugs used in chemotherapy.[1−3] The most widely used chemotherapeutics are platinum-based drugs, such as cisplatin [cis-diamminedichloroplatinum(II)],[4] which covalently binds to genomic DNA and forms adducts that stall replicative DNA polymerases (Pols) and induce apoptosis.[5,6] Many cancers, however, acquire resistance toward chemotherapy[7] and cisplatin adducts may be removed by nucleotide excision repair[8] or bypassed during translesion DNA synthesis (TLS).[9]

TLS is catalyzed by specialized Y-family Pols, for example, human DNA polymerase η (hPol η). Mutations in the corresponding gene are linked to a variant form of the sunlight sensitivity-associated disease xeroderma pigmentosum (XP-V).[10,11] Thus, hPol η is specialized for efficient and accurate replication past UV radiation-induced base dimers in DNA.[10] Deficiencies in hPol η increase cellular sensitivity to UV,[12] as well as cytotoxic drugs.[13,14]

HPol η is considered the main polymerase that allows cancer cells to tolerate cisplatin on the basis of TLS. Cisplatin treatment efficacy is inversely correlated to cellular levels of hPol η[15] and hPol η contributes to platinum drug resistance by replicating past platinum-DNA adducts with reasonable efficiency and fidelity.[16−19] Conversely, platinum drug toxicity is enhanced in cells deficient in hPol η (Figure 1).[16,17,20,21] Knockdown of hPol η with siRNA hypersensitizes cells to



	Cellular hPol η status	
	hPol η +	hPol η -
Pt adduct bypass	✓	x
Drug efficacy	x	✓

Figure 1. TLS of the major cisplatin intrastrand cross-link product cis-Pt-1,2-d(GpG) catalyzed by hPol η and the relation between cellular expression of hPol η with platinum adduct bypass and drug efficacy.

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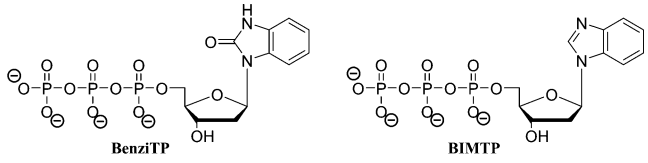
cisplatin.[22] Additionally, compounds that prevent TLS in cancer cells, such as proteasome inhibitors, enhance the action of platinum drugs.[23] Thus, hPol η has been suggested as a drug target for improving cisplatin efficacy by combining it with an hPol η inhibitor.

There are limited examples of molecules that inhibit hPol η. The natural product β-sitosteryl (6′-*O*-linoleoyl)-glucoside[24] was reported as the first inhibitor of eukaryotic Y-family polymerases. Recently, ellagic acid[25] and indole thiobarbituric acid derivatives[26] were discovered as potent hPol η inhibitors from high throughput screens. However, there is limited knowledge regarding structure−activity relationships or the physical basis of how small molecule inhibitors may interact with hPol η or the detailed mechanisms by which they inhibit it. Recently published crystal structures[19,27] of hPol η in a complex with platinated DNA and incoming dNTP revealed that the adducted bases are accommodated by hPol η, which undergoes a backbone rearrangement and minimizes the DNA distortion around the lesion, thus allowing the bypass of the major cisplatin-DNA adduct. However, to our knowledge, there are no reported examples of structures with an active site inhibitor. Therefore, the rational design and development of an efficient cotreatment to overcome resistance to existing platinum treatments is hindered.

Complementing structural insight, the strategy of using synthetic nucleotides to probe interactions with damaged sites in DNA has been informative.[28,29] Previously, we have developed adduct-directed nucleoside analogues that vary in size, π surface area, and hydrogen-bonding potential. These modified bases were found to stabilize duplexes containing O^6-alkylguanine DNA adducts.[30−33] With the use of a specialized Pol it has been shown that a synthetic nucleotide can promote full extension past O^6-benzylguanine adduct.[34] However, several of these analogues can be inserted opposite a distortion and block further extension.[35] These results suggest that novel synthetic nucleotides that insert opposite DNA damage but block extension of DNA synthesis could be used to probe hPol η-catalyzed platinum-DNA adduct bypass and serve as a basis for designing active site inhibitors. Yeast Pol η has been shown to incorporate modified nucleotides;[36,37] however, to our knowledge, there are no examples of synthetic nucleotides being accepted as DNA synthesis substrates by hPol η, or of their insertion opposite platinum adducts.

In this study, we characterized the influence of two synthetic deoxynucleoside triphosphates (BenziTP and BIMTP) that vary in hydrogen bonding potential (Chart 1) on hPol η-mediated DNA synthesis and bypass of the main platinum intrastrand cross-link product cis-Pt-1,2-d(GpG). The synthetic nucleotides were incorporated by hPol η opposite unmodified and platinated DNA templates. BenziTP and BIMTP reduced the formation of DNA product in a dose-dependent manner under full-length synthesis conditions. These data provide new insights regarding how chemical properties of nucleotides modulate their influence on platinum adduct bypass by hPol η.

Chart 1. Chemical Structures of the Two Synthetic Nucleotides BenziTP and BIMTP Characterized for Interactions with hPol η



EXPERIMENTAL DETAILS

Materials and Chemicals. Bacterial clone for the expression of hPol η and hPol η mammalian expression vector pJRM56 were a gift from Roger Woodgate (NIH, Rockville, USA). Human recombinant Pol η, Pol δ (hPol δ), and PCNA were expressed and purified as described.[38−40] XP-V patient derived cells transformed with simian virus 40 were a gift from Alan Lehman (University of Sussex, Brighton, UK). All natural dNTPs were obtained from New England Biolabs (Ipswich, USA), [γ-^{32}P] ATP was purchased from PerkinElmer Life Sciences. 1-(α)-Chloro-3,5-di-*O*-(p-toluoyl)-2-deoxy-D-ribose was purchased from Berry & Associates, Inc. (Dexter, USA). 10% Fetal bovine serum, 100 U/mL penicillin−streptomycin, lifofectamine pcDNA 3.1 vector, and SYBR Gold Nucleic Acid Stain were purchased from Invitrogen (Basel, Switzerland). All other reagents were purchased from Sigma-Aldrich (Buchs, Switzerland). Benzi and BIM nucleosides were synthesized by previously reported procedures.[35,41] The nucleosides were converted into the corresponding triphosphates or phosphoramidites as previously reported.[33−35]

Oligonucleotides. The 18mer primer 5′-AGTGTGAGATAG-TGTGAG-3′, the 24mer template 5′-CTACGGCTCACACTAT-CTCACACT-3′, and its 24mer complement 5′-AGTGTGAGAT-AGTGTGAGCCGTAG-3′ were purchased from VbC Biotech (Vienna, Austria). Modified oligonucleotides 24mer 5′-AGTGTGAG-ATAGTGTGAGXCGTAG-3′ (X = Benzi or BIM) were synthesized on a Mermade 4 DNA synthesizer (Bioautomation Corporation) using β-cyanoethyl phosphoramidite chemistry. The synthesis was performed in trityl-off mode and oligonucleotides were purified by reverse phase HPLC on a Zorbax Eclipse XDB-C18 (5 μm, 150 × 4.6 mm) column with a linear gradient of 5−18% (v/v) acetonitrile in 50 mM triethylammonium acetate over 24 min. The 24mer (X = Benzi) eluted at 21.4 min, and the 24mer (X = BIM) eluted at 22.2 min. The eluted fractions containing the desired product were combined and concentrated in a centrifugal evaporator (MiVac, GeneVac). The purity of the desired product was confirmed by HPLC analysis (Figure S1). The presence of the desired products was confirmed by mass spectrometry on an Agilent MSD ion trap mass spectrometer with electrospray ionization, operated in negative ion mode (Figure S2). The ssDNa concentration was determined by UV spectroscopy at 260 nm on a *NanoDrop* 1000 Spectrophotometer.

Thermal Denaturation Studies. UV absorption at 260 nm was monitored with a Varian Cary 100 UV−vis spectrophotometer equipped with Peltier thermal programmer. Duplex DNA solutions at a concentration of 1 μM (in 0.25 M NaCl, 0.2 mM EDTA, 20 mM sodium phosphate, pH 7.0 buffer) in Teflon-stoppered 1 cm path-length quartz cuvettes were annealed by heating to 90 °C (10 °C/min) and then slowly cooling (1 °C/min) to 20 °C. The heating cycle (1 °C/min) then was repeated and UV absorption monitored. Melting temperatures (T_m) were determined by the first-derivative method with the application in the Cary Thermal software. All measurements were performed three times and the mean (±standard deviation) data is reported (Table S1).

Synthesis of Cisplatin Intrastrand Cross-Linked DNA. By adaptation of a previously described procedure,[19] cisplatin was activated by combining $AgNO_3$ (0.125 mL, 625 μM) and cis-diamminedichloroplatinum(II) (0.75 mL, 500 μM in a 0.9% NaCl solution) for 12 h at 24 °C in the dark. The resulting mixture was subjected to centrifugation (2 × 15 000 g, 10 min), where AgCl sedimented and the supernatant containing activated cisplatin was recovered. A 24mer GG-containing DNA strand (5′-CTACGGCT-CACACTATCTCACACT-3′) (77 μL, 15.4 μM) was combined with activated cisplatin (35 μL, 34.6 μM) in ammonium acetate buffer (388 μL, 100 mM, pH 6.0) and allowed to react for 12 h at 37 °C in the dark. The 24mer cross-linked product (cis-Pt-1,2-d(GpG)) was purified by reverse-phase HPLC on a Zorbax Eclipse XDB-C18 (5 μm, 150 × 4.6 mm) column with a linear gradient of 5−15% (v/v) acetonitrile in 50 mM triethylammonium acetate over 60 min. The

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cross-linked product eluted with a retention time of 39.6 min. The eluted fractions containing the desired product were combined and concentrated in a MiVac centrifugal evaporator (GeneVac). The purity of the cross-linked product was confirmed by HPLC analysis (Figure S1). The presence of the desired cross-linked single platination product was confirmed by mass spectrometry on an Agilent MSD ion trap mass spectrometer with electrospray ionization, operated in negative ion mode (Figure S2). That platination occurred at the GG site was further verified by DNA footprinting using dimethyl sulfate (DMS).[42−44] Unmodified and platinated 24mer templates were reacted with DMS, treated with hot piperidine (90 °C), separated by denaturing polyacrylamide gel electrophoresis, and stained with SYBR Gold. For the unmodified template the cleavage of the strand at G's resulted in shortened fragments visible in the gel (Figure S3). For the platinated template, no cleavage bands were observed, confirming the presence of the platinum adduct at the single GG site (cis-Pt-1,2-d(GpG)) and also excluding platination at any of the adenine residues.

Whole Cell Extracts. Xeroderma pigmentosum (XP-V) patient-derived cells transformed with simian virus were grown in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing GlutaMAX-I supplemented with 10% fetal bovine serum and 100 U/mL penicillin−streptomycin. The cells express a truncated and nonfunctional hPol η. To complement XP-V cells, hPol η mammalian expression vector pJRM56 was used. pJRM56 or empty pcDNA 3.1 constructs were transiently transfected into XP-V cells using lipofectamine according to manufacturer's protocol.[45] Cells were harvested 48 h after the transfection and whole-cell extract (WCE) prepared as described previously.[46] Immunoblot analysis of hPol η and tubulin levels has been performed as indicated previously[46] (Figure S4).

General Conditions for Primer Extension Assays. T4 polynucleotide kinase (Promega Corp., Madison, USA) and [γ-^{32}P] ATP were used to label the 18mer primer strand at the 5′ end following the manufacturer protocol.[47] The labeled primer strands were annealed to either the unmodified or platinated template; annealing was performed by heating the primer:template (1:1) mixture in the presence of 20 mM Tris−HCl (pH 8) and 150 mM NaCl at 95 °C for 5−10 min and subsequently cooling over 12 h. Standard 10 μL reaction mixtures contained 40 mM Tris−HCl (pH 8), 10 mM DTT, 1 mM $MgCl_2$, and 250 mg/mL bovine serum albumin, 5 nM 5′[^{32}P]primer:template DNA, 5 nM hPol η and 0, 1, 2, 5, 10, 25, and 50 μM dNTP(s). For the reactions with hPol δ, 2 nM hPol δ, 2 nM 5′[^{32}P]primer:template DNA, 5 mM $MgCl_2$, and 100 nM PCNA were used, the other components were used as described above. The reaction mixture was incubated at 37 °C for 15 min and terminated by adding 10 μL stop buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue). Reaction mixtures (4 μL) were subjected to 15% polyacrylamide/7 M urea gel electrophoresis and replication products were visualized from the phosphorimaging screen using a phosphorimager (BioRad, Hercules, CA). Product band intensities were quantified by densitometry using Quantity One Software (Bio-Rad).

Primer Extension Assays with Cell Extract. Reaction mixtures (10 μL) containing 2 nM primer:template DNA were prepared as described above, contanining 40 mM Tris−HCl (pH 8), 10 mM DTT, 1 mM $MgCl_2$, 250 mg/mL bovine serum albumin, and 4 μg of WCE. dNTPs (dCTP, BenziTP, or BIMTP) were titrated with final concentration being 0, 50, 100, or 200 μM. After 30 min incubation at 37 °C, products were separated by 20% polyacrylamide/7 M urea gel electrophoresis and replication products visualized by auto-radiography.

Steady-State Kinetics Assays. Steady-state kinetics parameters for single nucleotide incorporation opposite unmodified or platinated templates were determined by measuring the amount of extended products over a range of dNTP concentrations and times. The parameters were adjusted to result in extents of reaction of 20% or less to maintain initial velocity conditions. Enzymatic reactions were carried out at 37 °C and contained 40 mM Tris−HCl (pH 8), 10 mM DTT, 1 mM $MgCl_2$, and 250 mg/mL bovine serum albumin, 50 nM 5′[^{32}P]primer:template DNA, and 5 nM hPol η. hPol η was preincubated with DNA and reactions were initiated by adding dNTP(s). Aliquots were quenched with stop buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and bromophenol blue) at varying times. Product band intensities were quantified by densitometry with Quantity One Software (Bio-Rad). The initial portion of the velocity curve was fit into a liner equation with Microsoft *Excel*. The resulting velocity was plotted as a function of dNTP concentration and fit to a hyperbola using the Michaelis−Menten equation to obtain an estimate of k_{cat} and K_M with *SigmaPlot 12* (Systat Software, Inc.). Reactions were performed three times and the mean (±standard deviation) of K_M and k_{cat} are reported.

Computational Modeling Studies. Structures were computed with the *Molecular Operating Environment* software suite (Chemical Computing Group, Montreal, Canada).[48] Crystal structures of hPol η containing incoming dCMPNPP paired opposite unmodified or platinated template were used (PDB codes: 4DL2 and 4DL4).[19] The incoming dCMPNPP was replaced by BenziTP or BIMTP. Energy minimizations were performed by fixing the potential energy of the protein and the solvent and using the Amber99 Force Field. The results of the energy minimization are shown in Figures 3 and S6.

Full-Length Extension in the Presence of Synthetic dNTPs. Full-length extension reactions were carried out using the same conditions described above for primer extension assays, but including all four natural dNTPs (10 μM) and either BenziTP or BIMTP at increasing concentrations (0, 10, 50, 100, 200, 500, 1000, 2000, and 4000 μM). As a control the same experiment was carried out increasing the concentration of each of the natural dNTPs individually from 0 to 4000 μM and maintaining the other three constant (10 μM). The amount of product formation was calculated using eq 1 in which n is the position of the band (from +0 to +6).

$$\sum_{n=1}^{6} n\left(\frac{\mathrm{Int}(n)}{\sum_{n=0}^{6}\mathrm{Int}(n)}\right)\cdot[\mathrm{DNA}] \quad (1)$$

The percent of product formation was normalized to the case where only the four natural dNTPs were present (Lane 2 of the gels, Figures 4, 5, and S7a, S8a) and plotted as a function of the concentration of the nucleotide used in increasing concentrations (Figures 4b, 5b, and S7b, S8b). Experiments were performed three times and the mean (±standard deviation) value of product formation is reported. Statistical analysis was performed by using the unpaired t test.

RESULTS AND DISCUSSION

To test the propensity for hPol η to use BenziTP or BIMTP as a substrate, standing-start primer extension assays were performed with an unmodified or platinated DNA template (Figure 2a). A range of dNTP substrate concentrations ranging from 1 to 50 μM was used; the natural substrate dCTP was tested as a control. The single nucleotide insertion data (Figure 2b) show that both synthetic nucleotides BenziTP and BIMTP were incorporated by hPol η opposite the unmodified and the platinated DNA template, with BenziTP being better incorporated than BIMTP. On both unmodified and platinated template, dCTP was incorporated at both positions +1 and +2, whereas the synthetic nucleotides were incorporated only once and less efficiently than natural nucleotide dCTP. The percentage of product formation resulting from reactions with 5 μM dNTP is depicted in Figure 2c.

To verify that hPol η incorporates BenziTP and BIMTP opposite the platinated DNA template even in the presence of other Pols, we compared the incorporation in the extract from XP-V patient cells (WCE XP-V) deficient for hPol η and the hPol η complemented XP-V whole cell extract (WCE o.e. hPol η). Complementation of XP-V cells was achieved by hPol η overexpression (Figure S4a). Activity of WCEs was compared in the primer extension assays with the platinated DNA template. Very little bypass could be observed for the cell

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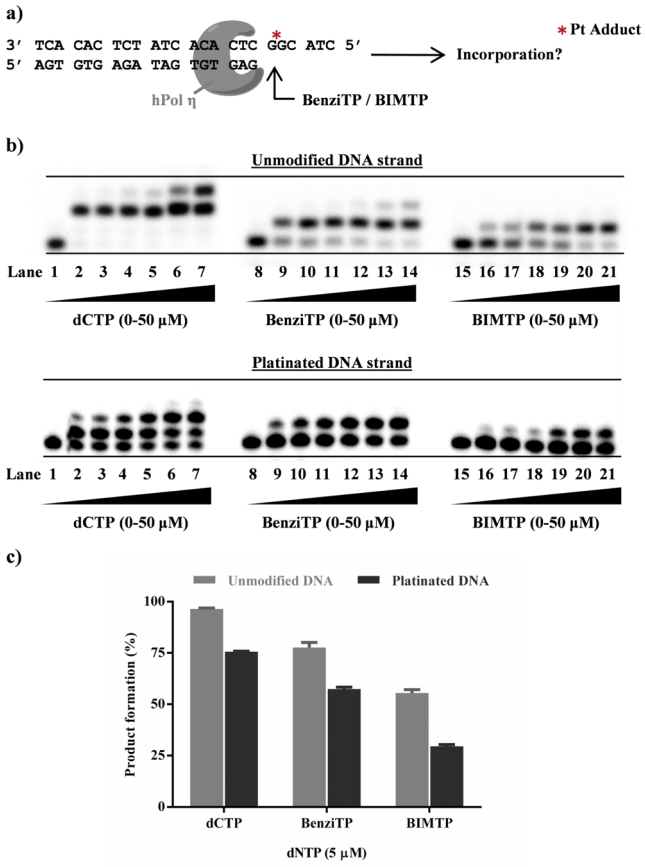

Figure 2. (a) Illustration of the primer extension experiments. (b) Single nucleotide incorporation by hPol η with dCTP and synthetic nucleotides BenziTP and BIMTP opposite unmodified or platinated templates. Conditions: 37 °C for 15 min. Concentrations: hPol η, 5 nM; DNA, 5 nM; dCTP, BenziTP, and BIMTP, 0, 1, 2, 5, 10, 25, and 50 μM. (c) Percentage of product formation with 5 μM dNTP (lanes 4, 11, and 18 of the gels). Error bars represent the standard deviation from three independent experiments.

extracts lacking hPol η, whereas in the presence of the enzyme dCTP was inserted opposite the adduct (Figure S4b and c). These results confirmed the need of hPol η to bypass the platinum lesion.[49] Furthermore, BenziTP was observed to be incorporated in the reaction with a cell extract overexpressing hPol η (Figure S4c). These data indicate that from Pols present in the cell, BenziTP is specifically utilized by hPol η. As observed for the purified enzyme (Figure 2b and c), dCTP incorporation was favored over the synthetic nucleotides.

To further test whether the synthetic nucleotides are exclusively hPol η substrates, we tested the incorporation of BenziTP and BIMTP by the B-family human replicative polymerase hPol δ. Standing-start primer extension assays were performed with an unmodified or platinated DNA template (Figure S5) and neither BenziTP nor BIMTP were incorporated regardless of the template.

To quantify the insertion efficiencies of the two synthetic nucleotides, steady-state kinetic measurements were carried out. By directly comparing the synthetic nucleotides it was observed that independent of the template, BenziTP was incorporated over 10-fold more efficiently than BIMTP (Table 1). This increase in efficiency is due to a combination of better binding affinity (K_M of 52.8 μM vs 194 μM and 47 μM vs 161 μM, for unmodified and platinated templates, respectively) and

Table 1. Steady-State Kinetic Parameters for Single Nucleotide Incorporation Opposite Unmodified vs Platinated Template by hPol η

base pair	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})	rel. efficiency[a]
C:G	2.96 ± 0.32	44.3 ± 1.3	14.97	4400
Benzi:G	52.8 ± 5.6	2.33 ± 0.09	0.044	13
BIM:G	194 ± 20	0.66 ± 0.03	0.0034	1
C:PtG	2.01 ± 0.15	29.6 ± 0.5	14.73	10000
Benzi:PtG	47.0 ± 5.0	1.16 ± 0.04	0.025	17
BIM:PtG	161 ± 13	0.24 ± 0.01	0.0015	1

[a]Describes the ratio of $(k_{cat}/K_{M,dCTP/BenziTP})/(k_{cat}/K_{M,BIMTP})$.

higher turnover values (2.33 min^{-1} vs 0.66 min^{-1} and 1.16 min^{-1} vs 0.24 min^{-1}, for unmodified and platinated templates, respectively). The insertion efficiency of dCTP was also measured and hPol η was confirmed to incorporate dCTP opposite unmodified and platinated template with measured efficiencies similar to published values.[27,50] Compared with BenziTP, dCTP insertion was 330-fold more efficient opposite unmodified template and 590-fold more efficient opposite platinated template. For BIMTP, dCTP was inserted 4400-fold more efficiently on the unmodified template and 10 000-fold more efficiently on the platinated template. Moreover, by comparing the two templates, it was observed that the synthetic nucleotides were incorporated twice more efficiently opposite the unmodified DNA strand than on the platinated DNA strand. The presence of the adduct did not influence K_M but significantly decreased the turnover number. Therefore, the difference in incorporation efficiencies results exclusively from the k_{cat}, which were lower on the platinated template (1.16 min^{-1} vs 2.33 min^{-1} for BenziTP and 0.24 min^{-1} vs 0.66 min^{-1} for BIMTP).

To test whether this pattern was reflected in the stability of DNA duplexes containing the synthetic nucleotides paired opposite unmodified vs platinated DNA, thermal denaturation studies were carried out. The results (Table S1) indicate an increase in stability of the Benzi:G pair compared to BIM:G (ΔT_m = 2.0 °C) and of the Benzi:PtG pair compared to BIM:PtG (ΔT_m = 1.7 °C). The duplexes with base pair C:G and C:PtG were the most stable. These data parallel the trends observed for the enzymatic reaction.

To visualize a possible structural basis for the observed higher incorporation of BenziTP compared to BIMTP, computational modeling was performed. Molecular pairing interactions of BenziTP and BIMTP with guanine and platinated guanine in the active site of hPol η were investigated by molecular mechanics simulations (Molecular Operating Environment (MOE) software). The published crystal structures of hPol η containing an incoming dCMPNPP opposite unmodified and platinated template were used as starting points (PDB codes: 4DL2 and 4DL4).[19] The incoming dCMPNPP was replaced by BenziTP or BIMTP, and the resulting complexes were energy minimized with the Amber 99 force field. When the incoming modified bases Benzi and BIM were placed in a syn conformation, a steric clash between the base (substituent at position 2) and the oxygen of the sugar moiety was evident (Figure S6). Additionally, no favorable binding interactions were identified. When Benzi and BIM were placed in an anti conformation, however, the unfavorable interactions were avoided and, additionally, favorable binding interactions were observed. BIM formed one H-bond with the

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templating G in both the unmodified and platinated examples (Figure 3 top, calculated distance 1.9 Å), and for Benzi, two H-

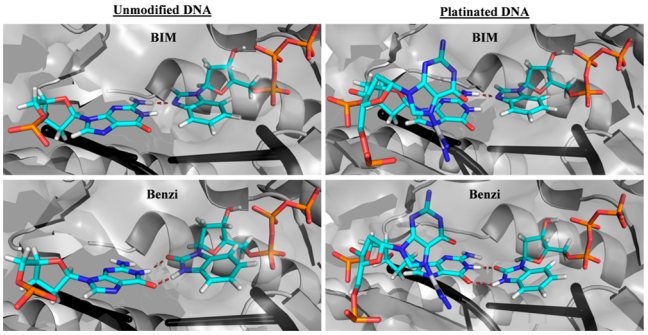

Figure 3. Computational modeling (molecular mechanics) of modified nucleotide probes BIM (top) and Benzi (bottom) in an anti conformation opposite unmodified (left) and platinated template (right) within the active site of hPol η. The enzyme surface is represented in gray; template and primer DNA are represented in black. The incoming dNTP and the templating base are represented as stick models. Light blue: carbon; red: oxygen; blue: nitrogen; orange: phosphate, white: hydrogen and platinum. Hydrogen bonds are shown as dashed red lines. Figures were prepared using PyMOL.[51]

bonds were predicted (Figure 3 bottom, calculated distances: for unmodified template: 1.7 and 2.0 Å; for platinated template: 1.8 and 2.2 Å). The model highlighting the importance of the hydrogen bonding interaction is consistent with and complements the observation that when the yeast variant of Pol η inserts base analogues opposite canonical bases, selection is dependent on H-bonding capacities.[37]

With the propensity for the synthetic nucleotides to be incorporated opposite the platinated template established, we tested whether they also inhibited hPol η-mediated synthesis under full-length synthesis conditions. This experiment was carried out in the presence of a fixed concentration of all four natural dNTPs and unmodified DNA template. BenziTP or BIMTP was added in increasing concentrations (from 10 to 4000 μM) and the extent of product formation was examined (Figure 4a). In both cases the amount of product formation was reduced; however, the reduction was more pronounced for BenziTP (Figure 4a, left gel). When BenziTP was present, the reduction in product formation was significant ($p < 0.01$) starting from 50 μM BenziTP (Figure 4a, left gel, lanes 4 to 10), whereas 10-fold higher concentration of BIMTP was needed to achieve a significant decrease ($p < 0.01$) in product formation (Figure 4a, right gel, lanes 7 to 10).

The percentage of product formation was normalized to the case where all four natural dNTPs were present at the same concentration (10 μM) (lane 2 of each gel), and plotted as a function of dNTP concentration (Figure 4b). To ensure that the reduction of product formation was specific to BenziTP and BIMTP, the same experiments were carried out, but each of the natural dNTP concentration was increased instead (Figure S7, representative data for dATP only shown for comparison in Figure 4b). It has been previously reported that hPol η is inhibited by dATP at concentrations greater than 0.5 mM.[19] In our reaction conditions, a significant decrease in product formation was observed starting from 2 mM dATP.

To address whether the observed reduction in product formation occurred also when the DNA template was platinated, we tested the ability of the synthetic nucleotides to inhibit full-length extension templated by a platinated DNA

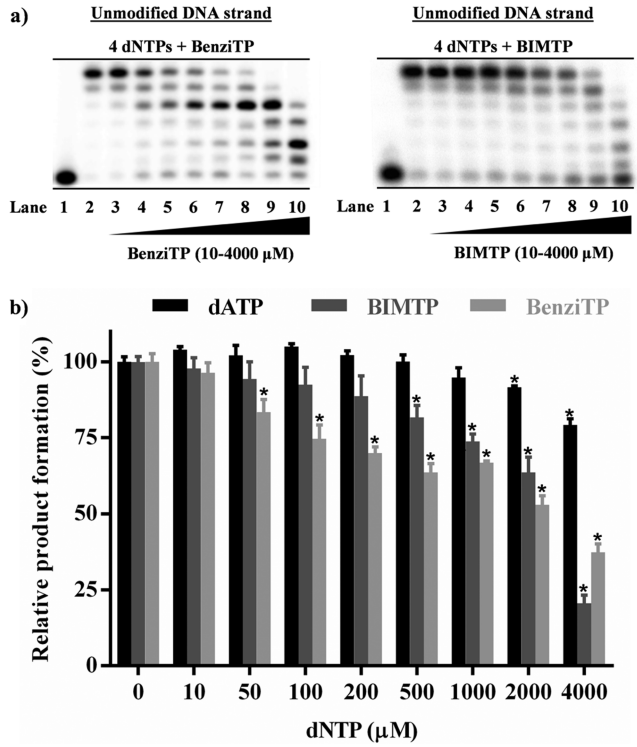

Figure 4. (a) Human DNA polymerase η-mediated full length DNA synthesis with an unmodified template and increasing concentrations of BenziTP (left) or BIMTP (right). Conditions: 37 °C for 15 min; 5 nM hPol η; 5 nM DNA; 10 μM dNTPs; increasing concentrations of BenziTP or BIMTP. (b) Relative percentage of product formation as a function of BenziTP (light gray) and BIMTP (dark gray) concentration in comparison with dATP (black). Error bars represent the standard deviation from three independent experiments. * represents significant decrease in product formation: $p < 0.01$.

strand (Figure 5a). In this case, the full-length synthesis potential of hPol η was already limited by the presence of the adduct and a major stall after incorporating two dNTPs was observed (Figure 5a). Increasing the concentration of the synthetic nucleotides reduced product formation. For both BenziTP and BIMTP, this decrease was significant ($p < 0.01$) starting from concentrations of 200 μM (Figure 5a, left gel, lanes 6 to 10). The data were evaluated in the same manner as for the unmodified template described above and the same controls performed with the natural dNTPs (Figure 5b and Figure S8). As in the case of the unmodified template, increasing the amount of natural dNTPs did not result in a strong reduction of product formation (representative data for dATP only shown for comparison in Figure 5b). Most importantly, even when a reduction in product formation was observed, this reduction was smaller than what was observed by increasing the synthetic nucleotides BenziTP or BIMTP.

CONCLUSION

We investigated how synthetic nucleotides behave as subtrates of human DNA polymerase η during standard DNA synthesis and bypass of the major cisplatin DNA adduct. This report is the first example of synthetic nucleotides being incorporated by hPol η. The adduct-directed dNTPs (BenziTP and BIMTP) had a significant influence on platinum adduct bypass by hPol η. Furthermore, it was found that BenziTP had a larger influence and reduced the amount of product formation to a

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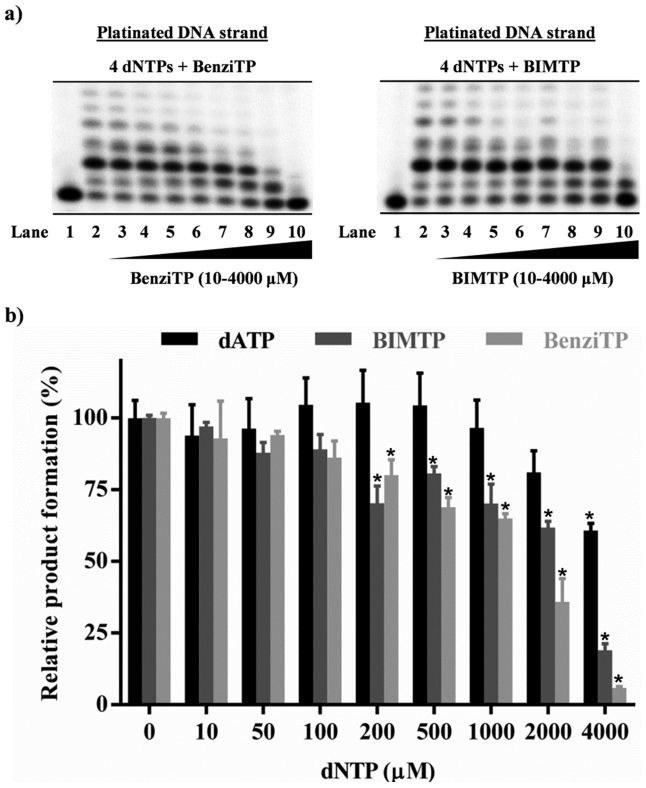

Figure 5. (a) Human DNA polymerase η-mediated full length DNA synthesis with a platinated template and increasing concentrations of BenziTP (left) or BIMTP (right). Conditions: 37 °C for 15 min; 5 nM hPol η; 5 nM DNA; 10 μM dNTPs; increasing concentrations of BenziTP or BIMTP. (b) Relative percentage of product formation as a function of BenziTP (light gray) and BIMTP (dark gray) concentration in comparison with dATP (black). Error bars represent the standard deviation from three independent experiments. * represents significant decrease in product formation: $p < 0.01$.

greater extent than BIMTP, indicating that a modification in the hydrogen binding potential of the synthetic base resulted in an increase in insertion efficiency of the probe. Inhibitors of hPol η[25,26] have been shown to inhibit the enzyme when catalyzing DNA synthesis with an unmodified DNA template, while here we report the first example of inhibition of hPol η with a platinated DNA template. With these kinetic data and mechanistic insight, BenziTP and BIMTP provide starting points for generating novel structures with improved insertion efficiency and inhibition of hPol η. Together with further anticipated structural data, the new information could lead to novel therapeutics developed to overcome platinum drug resistance in cancer therapy.

■ ASSOCIATED CONTENT

Supporting Information

HPLC trace and mass spectrometry analysis of platinated DNA strand, and Benzi and BIM containing 24mers, DNA footprinting of DNA template, whole cell extracts experiments, melting temperatures of duplexes, modeling results of Benzi and BIM base in syn conformation, and control experiments of full length DNA synthesis (Figures S1 to S8, Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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