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Selective Modulation of DNA Polymerase Activity by Fixed Conformation Nucleoside Analogues**

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The human genome encodes multiple enzymes that are capable of synthesizing DNA.[1] In hindsight, it seems obvious that the complex nature of nucleic acid chemistry would necessitate a redundancy of polymerase activity that does not rely upon one single enzyme for nucleotide selectivity.[2,3] Accuracy during replication of the genetic code is vital to multi-cellular organisms, but the molecular constraints that facilitate high-fidelity DNA synthesis often prove inhibitory in the face of adducted (i.e. “damaged”) DNA.[4,5] Evolution has resulted in many non-essential DNA polymerases, including the Y-family, that are conserved as a means of bypassing damaged DNA and/or unusual secondary

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Experimental Section

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structures in the template DNA.[6] The deregulation of Y-family members has been associated with several tumor types, including breast, ovarian, colorectal and non-small cell lung cancers.[7-11] Moreover, germline mutations in the human gene encoding polymerase η result in *Xeroderma pigmentosum* variant type (XPV), which is characterized by a high susceptibility to skin cancer.[12,13]

Some members of the Y-family possess distinctive mechanisms for nucleotide selection including Hoogsteen base pairing modes (e.g. pol ι during insertion opposite template purines) and protein template directed catalysis (e.g. REV1).[6,14,15] As such, the unique properties of these enzymes represent a potential target for specific inhibition/activation by small molecules. Nucleoside analogues such as 3'-azido-2'-deoxythymidine (AZT) have been used successfully to inhibit viral genome synthesis, although the development of resistance to the drug through excision is a major obstacle to long-term efficacy.[16-18] Fixed conformation nucleoside analogues were created in an effort to overcome human immunodeficiency virus type-1 reverse transcriptase (HIV-1 RT)-mediated excision of chain terminating nucleoside analogues.[16,19,20] The bicyclo[3.1.0]hexane scaffold was used to generate nucleosides that are permanently locked in either the North (*N*) (**1**) or South (*S*) (**2**) envelope orientation (Figure 1).[21] The resulting analogues have been designated as methanocarba-2'-deoxynucleoside triphosphates (MC-dNTP), with the *N*- and *S*-template mimicking the most common sugar pucker observed in A- (C3'-endo) and B-form (C2'-endo) dsDNA, respectively.[19] The *North* compound has shown anti-viral activity against herpes simplex virus type 1 and orthopoxviruses and the *South* isomer is inactive.[22] The cytotoxic effect requires viral kinase activity to convert the nucleoside into the monophosphate form.[19,23-25] By locking the cyclopentane ring in either the *N* or *S* conformation, the 3'-OH group of the MC-dNTP is placed in either an equatorial or axial position, respectively. The sugar pucker and the positioning of the 3'-OH group can have important consequences for both the insertion and extension step by DNA polymerases but determining a functional preference for one conformation over the other has been difficult in the absence of the appropriate chemical probes. Previous work has shown that HIV-1 RT only utilizes the *North* versions of AZT and 2',3'-dideoxynucleosides.[26,27] We decided to investigate the ability of several DNA polymerases to incorporate and extend from *N*- and *S*-oriented fixed conformation nucleosides. Here we report that human Y-family DNA polymerases exhibit unique properties during insertion of fixed conformation nucleoside triphosphates and these properties were used to selectively inhibit the growth of breast tumor cells over-expressing one of these error-prone polymerases, namely human pol ι .

First, the ability of different DNA polymerases to insert and extend from *N*- and *S*-oriented fixed conformation nucleosides was tested. Consistent with previous results, HIV-1 RT was able to utilize the *North* template (**1**) but extension is inhibited (Figure S1).[19] HIV-1 RT did not utilize the *S*-template (**2**) under the conditions tested. Three human Y-family DNA polymerases were also tested and showed mixed results for insertion and extension of the fixed conformation nucleosides. Human DNA pol η appears to utilize both (**1**) and (**2**) (Figure 2b). In fact, extension appears to be slightly more favorable for *S*-MC-dATP (Figure 2b, compare 30 minute time points for hpol η). In contrast to hpol η , human pol ι quickly inserted a single dNTP and then slowly added one nucleotide from the first insertion event. Finally, human pol κ was quite efficient at insertion and extension from *N*-MC-dATP (**1**) (Figure 2b, bottom panel). Hpol κ did not utilize *S*-MC-dATP.

Single nucleotide insertion experiments were then performed with *N*-MC-dATP (**1**) and *S*-MC-dATP (**2**) (Figure S2). HIV-1 RT inserted *N*-MC-dATP with only a three-fold decrease in the initial velocity relative to dATP but failed to incorporate *S*-MC-dATP in the time frame tested. In contrast to HIV-1 RT, hpol η inserted both the *N*- and *S*-templates with an initial velocity that was diminished 4- and 14-fold, respectively, relative to dATP insertion.

Hpol κ , on the other hand, inserted only *N*-MC-dATP with an initial velocity that was diminished 3.5-fold compared with dATP. The most striking result was obtained with hpol ι where the velocity of *N*-MC-dATP insertion was increased 5-fold over that of unmodified dATP.

The concentration dependence of dNTP insertion was determined for the fixed conformation nucleosides in order to compare the steady-state catalytic efficiencies of the human Y-family DNA polymerases to that of HIV-1 RT in a quantitative manner (Table 1). HIV-1 RT was not inhibited during insertion of *N*-MC-dATP opposite thymidine. Both hpol η and hpol κ showed a four-fold decrease in catalytic efficiency when utilizing the *N*-template. The reduction in efficiency by hpol κ could be attributed to a much reduced k_{cat} , whereas hpol η showed both a slower k_{cat} and a slightly higher $K_{\text{m,dNTP}}$ (Table 1). Hpol η inserted *S*-MC-dATP 80-fold less efficiently than dATP insertion opposite template thymidine, but the ability of hpol η to utilize *S*-MC-dATP is notable because it is different from the other enzymes tested here. The available crystal structures of both human and yeast pol η consistently show B-form dsDNA.[28-31] In these structures the distance between phosphate groups near the nascent base pair is B-form in nature (6.5 to 7.5 Å). The incoming dATP remains in the more A-form C3'-endo form when paired opposite either dT or CPD.[29] The dsDNA helical structure in the pol η structures are distinct from those observed in many DNA polymerase ternary structures where the double-helix near the active site is more A-form in nature with phosphate distances closer to 5.5 to 6.5 Å.[32-34] Notably, the DNA in the binding cleft of yeast pol δ is also B-form in the crystal structure.[35]

Consistent with the single concentration time course the specificity constant of hpol ι insertion of *N*-MC-dATP was five-fold greater than that for dATP insertion and was nearly as efficient as hpol ι -catalyzed insertion of dGTP opposite thymidine. Hpol ι has the unusual proclivity to insert dGTP opposite template dT more efficiently than any other dNTP.[36] The active site of hpol ι shortens the C1'-C1' distance from the normal 10 or 11 Å to 8 or 9 Å. These constraints result in the preferential formation of Hoogsteen base pairs when the template base is a purine.[15,37] The exact molecular reason for the increased hpol ι activity with *N*-MC-dATP may be related in part to the fact that unmodified adenine adopts the *syn* orientation in the active site of hpol ι , whereas dGTP maintains the typical *anti* orientation through an interaction between the exocyclic amino group of guanine and Gln59 (Figure S3).[34] Maintaining dGTP in the *anti* orientation causes template dT to shear out of plane with the other template bases, while the purine ring of the incoming dGTP maintains base-stacking interactions with the nascent base pair. The purine ring system for *syn*-oriented adenine, on the other hand, is tilted out of plane with the nascent base pair. The glycosyl torsion angle χ determines the orientation of the base relative to the sugar. Unrestricted furanose moieties allow the purine base to adopt both the *syn* and *anti* orientations with a small energy barrier (~1 kcal/mol).[38,39] The bicyclo[3.1.0]hexane scaffold places a greater energy barrier on the interconversion between *syn* and *anti* orientations of the thymidine analogue (10-15 kcal/mol).[22] The same value has not been measured for the adenosine analogue but in the solid state *N*-MC-dATP is always in the *anti* orientation while *S*-MC-dATP adopts both *anti* and *syn* orientations.[20] It is possible that a more stable *anti*-oriented *N*-MC-dATP forces template dT to adopt a conformation similar to the one observed in the hpol ι /dGTP:dT ternary complex, which contributes to greater catalytic activity.

The increased catalytic activity of hpol ι with *N*-MC-dATP was striking. Given the fact that *N*-MC-dATP is an inhibitor of replication we hypothesized that the growth of cells showing an over-abundance of or increased reliance upon pol ι activity might be more inhibited by the presence of *N*-MC-dATP than the proliferation of "normal" cells. Previous reports have shown that hpol ι is over-expressed in several breast tumor-derived cell lines, as well as in

tumor biopsies.[7,11] Two syngeneic breast cell lines were treated with *N*-MC-dATP in order to test the hypothesis that *N*-MC-dATP might prove more effective at inhibiting the growth of cells over-expressing hpol ι than cells with normal hpol ι levels. The malignant Hs578T cell line is derived from a triple negative metastatic invasive ductal carcinoma of the breast and has been shown to express hpol ι at levels three-fold higher than the non-malignant Hs578Bst cell line.[11] We reaffirmed over-expression of hpol ι in Hs578T cells relative to Hs578Bst cells (Figure S4). The cells were exposed to varying concentrations of *N*-MC-dATP by nucleofection and plated in 96-well plates. It was important to plate the cells at a density of 1000 cells/well or less in order to see effective inhibition of growth (Figure 3a). After allowing the cells to grow in culture for one week we then measured cell viability. The tumor-derived Hs578T cells were found to be 16-fold more sensitive to growth inhibition by *N*-MC-dATP than Hs578Bst cells based on IC₅₀ values of 6 ± 1 and 94 ± 10 for Hs578T and Hs578Bst, respectively (Figure 3b). Treatment with the electrophile 4-hydroxynonenal (HNE) resulted in IC₅₀ values of 27 ± 3 and 38 ± 24 for Hs578T and Hs578Bst, respectively (Figure 3c). HNE is a relatively non-specific electrophile that damages proteins, lipids, and DNA.[40] The similar IC₅₀ values observed following exposure of the cells to HNE indicates that the sensitivity observed with *N*-MC-dATP is not due to an inherent difference in sensitivity to cytotoxic agents between the two lines.

In conclusion, our results illustrate two important points. First, different DNA polymerases (even those within a sub-family) show substantial differences in the preferences for furanose geometry during catalysis, with hpol η showing greater tolerance than the other two Y-family polymerases tested here. The increased catalytic efficiency by hpol ι is fascinating when considering the structural determinants that influence polymerase dNTP selectivity. Besides placing different “sugar” geometries in the polymerase active site, the *N*- and *S*-templates also influence the glycosyl torsion angle χ in different ways. The influence of the cyclopropane ring upon χ most likely stabilizes the *anti*-orientation of the purine in *N*-MC-dATP, thereby increasing the activity of hpol ι . Additionally, the results with hpol ι show that the 7'-carbon does not necessarily perturb polymerase catalysis in a negative fashion. The second major conclusion derived from our work is related to targeting non-essential DNA polymerases for modulation within cells to alter biological outcomes. Other reports have illustrated that nucleoside analogues can inhibit the growth of cells over-expressing non-essential DNA polymerases with some specificity[41] and a number of inhibitors specific to certain polymerase sub-families have been identified.[42-45] The results presented here are consistent with the idea that targeted inhibition of specialized DNA replication machinery can slow the growth of cells that have an over-abundance of these enzymes. The specialized DNA polymerases β and η are known to alter the cell-killing effect of platinum-based chemotherapeutics.[46,47] Experiments are underway to investigate the potential use of *S*-MC-dATP to target cells that exhibit reliance upon pol η for survival. Compounds that modulate the activity of these and perhaps other non-essential polymerases may therefore represent a means of reducing the dose of toxic anti-cancer agents needed to achieve therapeutic benefits.

Supplementary Material

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Figure 1.

Chemical structure of *N*-MC-dATP **1** and *S*-MC-dATP **2**, which mimic the sugar pucker observed in A- and B-form double-stranded nucleic acid helices, respectively.

Figure 2.

DNA polymerase-catalyzed insertion and extension from fixed conformation nucleoside analogues. a) The primer-template DNA sequence is shown in schematic form. b) Full-length extension products for three human Y-family DNA polymerases. Catalysis was performed in the presence of dATP, *N*-MC-dATP, or *S*-MC-dATP and the other three dNTPs and the products were analyzed by PAGE.

Figure 3.

Selective growth inhibition of cells over-expressing human DNA polymerase ϵ using fixed conformation nucleosides. a) Hs578T cells were exposed to varying concentrations of *N*-MC-dATP and then plated at a density of ($\square$) 25,000, ($\circ$) 10,000, ($\blacksquare$) 1,000, and ($\bullet$) 500 cells/well. Hs578Bst (blue) and Hs578T (red) were exposed to varying concentrations of *N*-MC-dATP (b) or (c) HNE in order to measure the IC_{50} value.

Table 1

Steady-state kinetic analysis of polymerase-catalyzed insertion of fixed conformation nucleoside triphosphates

Polymerase	k_{cat} ($\text{s}^{-1} \times 10^3$)	$K_{\text{m,dNTP}}$ (μM)	$k_{\text{cat}}/K_{\text{m,dNTP}}$ ($\text{s}^{-1} \times 10^3 \mu\text{M}^{-1}$)	Δ efficiency relative to dATP
HIV-1 RT				
dATP	91 $\pm$ 15	10.2 $\pm$ 6.0	9	-
N-MC-dATP	59 $\pm$ 7	6.9 $\pm$ 2.9	8	unchanged
hpol η				
dATP	89 $\pm$ 4	0.36 $\pm$ 0.13	247	-
N-MC-dATP	39 $\pm$ 2	0.68 $\pm$ 0.21	57	4-fold less
S-MC-dATP	30 $\pm$ 2	10 $\pm$ 4	3	80-fold less
hpol ι				
dATP	121 $\pm$ 8	27 $\pm$ 9	4.5	-
N-MC-dATP	185 $\pm$ 12	8.0 $\pm$ 2.0	23	5-fold greater
dGTP	630 $\pm$ 47	17 $\pm$ 5	37	8-fold greater
hpol κ				
dATP	686 $\pm$ 14	0.76 $\pm$ 0.12	900	-
N-MC-dATP	172 $\pm$ 10	0.73 $\pm$ 0.27	240	4-fold less