Nucleotide Analogues

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N^2-Substituted 2′-Deoxyguanosine Triphosphate Derivatives as Selective Substrates for Human DNA Polymerase κ

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Abstract: *N^2-Alkyl-2′-deoxyguanosine triphosphate (N^2-alkyl-dGTP) derivatives with methyl, butyl, benzyl, or 4-ethynylbenzyl substituents were prepared and tested as substrates for human DNA polymerases. N^2-Benzyl-dGTP was equal to dGTP as a substrate for DNA polymerase κ (pol κ), but was a poor substrate for pols β, δ, η, ι, or ν. In vivo reactivity was evaluated through incubation of N^2-4-ethynylbenzyl-dG with wild-type and pol κ deficient mouse embryonic fibroblasts. CuAAC reaction with 5(6)-FAM-azide demonstrated that only cells containing pol κ were able to incorporate N^2-4-ethynylbenzyl-dG into the nucleus. This is the first instance of a Y-family-polymerase-specific dNTP, and this method could be used to probe the activity of pol κ in vivo.*

Synthetic nucleotide analogues are widely used tools in chemical biology, diagnostics, and therapeutics. Modifications to the Watson–Crick hydrogen-bonding face have been employed to probe the value of Watson–Crick hydrogen bonds in DNA replication[1] and create unnatural base pairs.[2] Minor-groove modifications are used to elucidate critical protein–DNA interactions,[3] while major-groove modifications have proved to be useful in exploring polymerase enzymology[4] and cellular reactivity.[5] Inhibitors of viral reverse transcriptases are in clinical use for treating HIV, hepatitis B, and hepatitus C.[6] Inhibitors of human DNA polymerase are also in use for cancer chemotherapy. Gemcitabine, a cytidine analogue that is incorporated into the DNA but then inhibits DNA synthesis, is used to treat pancreatic cancer, non-small cell lung cancer, breast cancer, and bladder cancer.[6c] Gemcitabine is effective because it affects rapidly growing tumor cells more than normal tissue. More recently, specialized polymerases that are overexpressed in tumors have been the target of inhibition studies.[7] The identification of nucleotide analogues that are selective substrates or inhibitors for specific polymerases is challenging because all polymerases utilize the four canonical dNTPs, and correct base pairing is mostly dependent on the polymerase recognizing the Watson–Crick geometry. Recently, engineered polymerases were utilized to create a modified nucleotide that can recognize a carcinogen-modified DNA template,[8] and size-expanded dNTPs (dxNTPs) have been shown to have some selectivity for human DNA polymerase θ.[9] In this work, we utilized the known reactivity of DNA polymerase κ to rationally design N^2-benzyl-dGTPs that are highly specific substrates for pol κ. These triphosphates are the first reported nucleotide triphosphates that are highly selective substrates for a human Y-family polymerase. These compounds can be utilized to probe the reactivity of pol κ in vivo, and could potentially be modified to be selective inhibitors of pol κ.

The mammalian cell utilizes sixteen DNA polymerases to replicate DNA: the four high-fidelity enzymes that duplicate the bulk of genomic and mitochondrial DNA, together with specialized DNA polymerases that perform roles in the DNA damage response. Translesion DNA synthesis (TLS) polymerases are a subset of the specialized polymerases that are involved in the bypass of DNA damage.[10] TLS polymerases include the Y-family DNA polymerases, pol η, pol ι, pol κ, and REV1; the B-family pol ζ;[10b] and perhaps other pols such as λ, ν, θ, and PrimPol.[11] These polymerases have unique DNA binding sites that enable the polymerases to bypass a variety of DNA damage. However, polymerases that participate in lesion bypass also perform other functions. For example, while DNA polymerase κ (pol κ) is the most active polymerase in the accurate bypass of bulky N^2-dG adducts,[12] pol κ also bypasses the structurally divergent 8-oxo-dG,[13] participates in nucleotide excision repair (NER),[14] and replicates non-B-DNA sequences,[15] and its polymerase activity is involved in initiation of the ATR checkpoint signal.[16] In addition, abnormal expression of pol κ correlates with increased mutations in tumors.[17] Further complicating the matter, many of these function are not unique to pol κ, since both pol ι and pol η[18] can bypass N^2-dG adducts, pol δ is the major NER polymerase, and pol η can also replicate non-B-DNA sequences.[19]

The roles of individual polymerases in the cell are difficult to resolve, in part, because all polymerases utilize undamaged DNA and the four dNTPs as substrates. To help elucidate the many roles of pol κ, we have designed a triphosphate that is a highly specific substrate for pol κ. Herein, we describe the synthesis of N^2-benzyl-dGTP (N^2-Bn-dGTP) and show that it is a substrate for purified pol κ but a very poor substrate for pols β, δ, η, ι, and ν. We also show that N^2-*p*-ethynylbenzyl-dG (EBndG), when applied to cells, is incorporated into the DNA only in the presence of pol κ.

The rationale for the design of a dNTP substrate specific to pol κ is illustrated in Figure 1. Pol κ bypasses hydrophobic N^2-alkyl-dG adducts ranging from methyl to (benzo[a]pyren-6-yl)methyl,[20] as well as the carcinogenic N^2-7,8,9-trihy-

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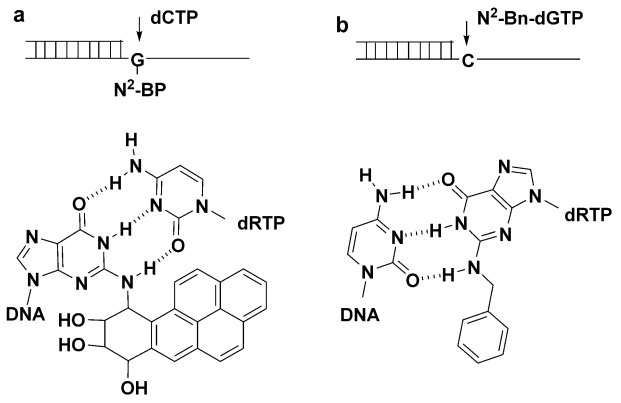

Figure 1. Rationale for the design of a dNTP substrate that is specific for pol κ. a) Incorporation of dCTP opposite N^2-BP-dG. b) Incorporation of N^2-Bn-dGTP opposite dC.

droxy-7,8,9,10-tetrahydrobenzo[a]pyren-10-yl-dG (N^2-BP-dG; Figure 1 a), with kinetic parameters approaching those of undamaged DNA.[21] While high-fidelity polymerases utilize interactions with the minor groove of the DNA to enhance fidelity, pol κ has an opening on the minor-groove side of the DNA that can accommodate bulky alkyl groups attached to the N^2-position of dG in the template base.[22] We hypothesized that this binding pocket could also accommodate N^2-alkyl groups when bound to the incoming dGTP (Figure 1 b). We investigated the specificity of the dNTPs by examining the in vitro reactivity with an array of human polymerases that includes pols κ, β, δ, η, ι, and ν. These enzymes include members of the A-, B-, X-, and Y-family polymerases.

The in vitro activities of purified human polymerases with N^2-alkyl-dGTPs were analyzed by primer-extension assays, as illustrated in Figure 2. The panels on the left show that pols κ, η and ι readily insert dGTP opposite dC. The panels on the right show that only pol κ rapidly incorporates N^2-Bn-dGTP. Even at a higher N^2-Bn-dGTP concentrations and longer time periods, pols η and ι were much less efficient at utilizing N^2-Bn-dGTP. For representative gel images of all the polymerase reactions examined, see Figure S1 in the Supporting Information. The in vitro reactivities of six purified polymerases were examined as described in the Supporting Information. The kinetic parameters are presented in Table S1 in the Supporting Information and the reactivities are shown in Figure 3.

Pol κ reacted rapidly with the three dGTP analogues (Figures S2, S3). The methyl and benzyl substitutions did not impact the k_{pol} value, while the K_d rose by a factor of two. N^2-butyl-dGTP was a slightly poorer substrate than dGTP, leading to a 20-fold decrease in k_{pol}. The Y-family pols η and ι, while they were not affected by the methyl substitutions, experienced a 10000-fold reduction in k_{pol} with the butyl substitution (Figures S4–S7). Pol ι reacted poorly with N^2-Bn-dGTP, and the reactivity of pol η further decreased due to a decrease in k_{pol} and an increase in K_d. In spite of the ability of pol η to bypass N^2-(2-naphthyl)methyl-dG in the template, the polymerase is unable to utilize N^2-Bu-dGP or N^2-Bn-dGTP as substrates. Of the Y-family polymerases, only pol κ

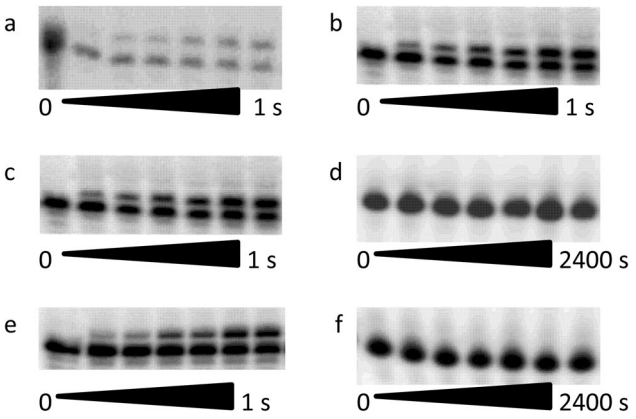

Figure 2. Comparison of the reactivity of pols κ, η, and ι with N^2-Bn-dGTP. In the primer-extension assay, DNA (10 nM) and the polymerase (100 nM) were reacted with 5 μM dGTP (a, c, e) or N^2-Bn-dGTP (b, d, f) at concentrations of 5 μM (b), or 25 μM (d, f) for the indicated amount of time. The substrates and products were separated by polyacrylamide gel electrophoresis (PAGE). The lower bands on the polyacrylamide gels are the 15-mer starting material, while the upper bands are the 16-mer products.

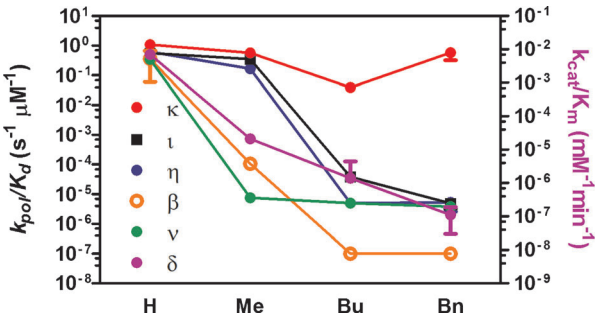

Figure 3. Reactivity of dGTP (H), N^2-methyl-dGTP (Me), N^2-butyl-dGTP (Bu), and N^2-Bn-dGTP (Bn) with DNA polymerases κ, ι, η, β, ν, and δ/PCNA.

reacts with N^2-Bn-dGTP at rates similar to that with unmodified dNTPs.

Next, we examined the reactivity with representatives of the A-, B- and X-family polymerases. Humans have three A-family polymerases: the high-fidelity pol γ, which is responsible for mitochondrial replication, and the low-fidelity pols θ and ν. Pol θ plays a role in alternative non-homologous end joining (NHEJ), while pol ν is implicated in the repair of intrastrand crosslinks and translesion DNA synthesis. Despite the fact that pol ν shows low fidelity, it incorporated the N^2-alkyl-dGTPs very poorly (Figure S10, S11). The k_{pol}/K_d for N^2-Bn-dGTP is reduced more than 10^5 -fold compared to dGTP. The reactivity of N^2-Bn-dGTP with pols γ and θ still needs to be determined. Humans have four B-family polymerases: α, δ, and ε are involved in high-fidelity DNA replication, while pol ζ is involved in TLS. Pol δ is a B-family polymerase that is implicated in lagging-strand replication. We examined the reactivity of pol δ/PCNA under steady-state conditions (Figure S12). The reaction is sensitive to small modifications, as demonstrated by the 300-fold decrease in k_{cat}/K_m for N^2-Me-dGTP relative to dGTP (Table S1).

Increasing the size of the substitution further decreased reactivity, with a 10^5-fold decrease in k_{cat}/K_m for pol δ with N^2-Bn-dGTP. Similarities among B-family polymerases suggest that both pol α and pol ε will be equally poor at utilizing N^2-Bn-dGTP as a substrate. It was previously shown that N^2-butylphenyl-dGTP was not a substrate for pols α, γ, or β, but was an inhibitor of pol α.[23] Humans have three X-family polymerases: β, λ, and μ. Pol β is the primary polymerase involved in base excision repair, while pols λ and μ are involved in NHEJ. The preferred substrate for pol β is a duplex DNA with a single-nucleotide gap. Pol β was very inefficient at incorporating nucleotides opposite N^2-BP-dG.[24] Unsurprisingly, we found that pol β did not accommodate steric bulk at the N^2-position of the dNTP (Figures S8, S9). Methyl substitution reduced the k_{pol}/K_d values by over 1000-fold. Further increases in the size of the alkyl group lead to a decrease in k_{pol}/K_d of more than six orders of magnitude.

The fidelity of pol κ with N^2-Bn-dGTP is slightly better than with dGTP. We investigated the fidelity of the incorporation of N^2-Bn-dGTP opposite dA, dC, dG, and dT as the template base with Michaelis–Menten kinetics. As summarized in Table S2, the preference for dC is higher with N^2-Bn-dGTP than with dGTP. The increased selectivity for dC as a template is due to a reduced rate of mispair formation. The f_{inc} value for dGTP was 0.07 for each nucleotide, while f_{inc} averaged 0.015 for N^2-Bn-dGTP.

The in vitro reactions indicated that N^2-Bn-dGTP is a significantly better substrate for pol κ, than for pols β, δ, η, ι, or ν. To determine whether this reaction occurs in vivo, we synthesized N^2-(4-ethynylbenzyl)-dG (EBdG) as described in the Supporting Information. Attachment of the ethynyl moiety to the benzyl group enables the use of copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) to attach a fluorescent group to the nucleoside and thus determine the amount and location of EBdG in the cell.[5] Labelling of the nuclei is shown in Figure 4a–c, in which mouse embryonic fibroblasts (MEFs) were incubated with 10 μM EBdG for 24 h. The cells were fixed and the ethynyl groups were reacted with 5(6)-FAM-azide, and the nuclei were identified by 4′,6-diamidino-2-phenylindole (DAPI) staining of the DNA. The complete overlap of the DAPI (Figure 4a) and FAM (Figure 4b) signals indicates that the cells incorporated EBdG into the nucleus.

To determine whether pol κ is the polymerase that incorporates EBdG into DNA in mammalian cells, we incubated MEF cells deficient in pol κ with the nucleoside and then analyzed incorporation by visualization after the click reaction. Figure 4d–f shows that the pol κ deficient MEF cells were unable to incorporate EBdG into the nucleus. The relative intensities of FAM signal and the DAPI signal were measured for each nucleus. The first lane of Figure 4g shows the background level of FAM fluorescence in the nucleus. At 10 nM EBdG, approximately half of the wild-type cells incorporated a small amount of the nucleoside. Above this concentration, all cells incorporated EBdG. In cells without pol κ, EBdG is not incorporated into the DNA. These results indicate that EBdG diffuses into the nucleus, is converted into the triphosphate, and is incorporated into the DNA. In addition, incorporation into the DNA is dependent on the presence of pol κ.

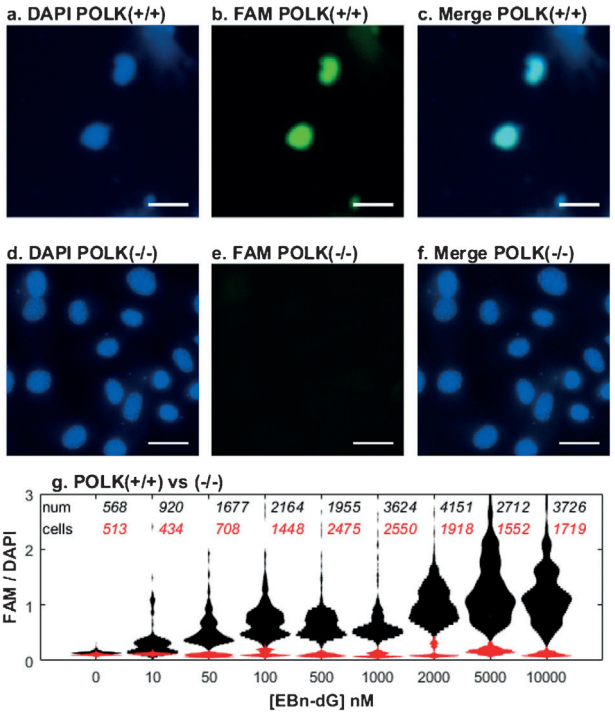

Figure 4. Detection of EBdG in the nuclei of pol κ containing cells. Wild-type (a–c) and POLK(−/−) (d–f) cells were treated with 10 μM EBdG for 24 h. The DNA is visualized with DAPI-staining (a,d) and EBdG incorporation is visualized by click reaction with FAM-N_3 (b,e). Scale bars: 40 μm. g, The levels of FAM–EBdG conjugate in nuclei of wild-type MEF [POLK(+/+); black] and POLK(−/−) (red) cells are shown. The incubation with EBdG was performed three times. The total number of cells are shown in violin plots for wild-type (black) and POLK(−/−)(red) cells.

In conclusion, we have found that N^2-alkyl-dGTPs are substrates for DNA pol κ. The kinetic parameters with N^2-Bn-dGTP are only slightly slower than with dGTP. The fidelity of the pol κ catalyzed incorporation is slightly better for N^2-Bn-dGTP than it is for dGTP. We also found that N^2-Bn-dGTP reacted slowly with representatives from the A-family (ν), B-family (δ), or an X-family (β) polymerases; the k_{pol}/K_d or k_{cat}/K_m values are 10^5 times lower that of pol κ. We also found that the ethynyl nucleoside N^2-EBn-dG is incorporated into DNA in cells by pol κ. These reagents could prove very useful for elucidating the cellular activities of pol κ, and could from the basis for selective inhibition of pol κ.

Acknowledgements

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Conflict of interest

Thomas Spratt and AS Prakasha Gowda have applied for a provisional patent on the compounds.

Keywords: DNA · DNA polymerases · fluorescent labelling · nucleotide analogues · nucleotides

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Communications

Nucleotide Analogues

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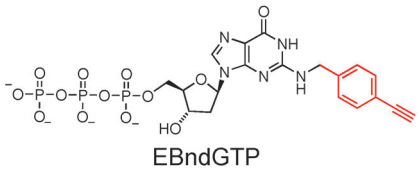
N^2-Substituted 2′-Deoxyguanosine Triphosphate Derivatives as Selective Substrates for Human DNA Polymerase κ



Heading for the pols: N^2-Alkyl-2′-deoxyguanosine triphosphate (N^2-alkyl-dGTP) derivatives with methyl, butyl, benzyl, 4-ethynylbenzyl substituents were prepared and tested as substrates for human DNA polymerases. N^2-Benzyl-dGTP was equal to dGTP as a substrate for DNA polymerase κ (pol κ), but was a poor substrate for pols β, δ, η, ι, or ν. In vivo reactivity was evaluated, and only cells containing pol κ were able to incorporate N^2-4-ethynylbenzyl-dG into the nucleus.