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Structures of a DNA Polymerase Inserting Therapeutic Nucleotide Analogues

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Abstract

Members of the nucleoside analogue class of cancer therapeutics compete with canonical nucleotides to disrupt numerous cellular processes, including nucleotide homeostasis, DNA and RNA synthesis, and nucleotide metabolism. Nucleoside analogues are triphosphorylated and subsequently inserted into genomic DNA, contributing to the efficacy of therapeutic nucleosides in multiple ways. In some cases, the altered base acts as a mutagen, altering the DNA sequence to promote cellular death; in others, insertion of the altered nucleotide triggers DNA repair pathways, which produce lethal levels of cytotoxic intermediates such as single and double stranded DNA breaks. As a prerequisite to many of these biological outcomes, the modified nucleotide must be accommodated in the DNA polymerase active site during nucleotide insertion. Currently, the molecular contacts that mediate DNA polymerase insertion of modified nucleotides remain unknown for multiple therapeutic compounds, despite decades of clinical use. To determine how modified bases are inserted into duplex DNA, we used mammalian DNA polymerase β (pol β) to visualize the structural conformations of four therapeutically relevant modified nucleotides, 6-thio-2'-deoxyguanosine-5'-triphosphate (6-TdGTP), 5-fluoro-2'-deoxyuridine-5'-triphosphate (5-FdUTP), 5-formyl-deoxycytosine-5'-triphosphate (5-FodCTP), and 5-formyl-deoxyuridine-5'-triphosphate (5-FodUTP). Together, the structures reveal a pattern in which the modified nucleotides utilize Watson–Crick base pairing interactions similar to that of unmodified nucleotides. The nucleotide modifications were consistently positioned in the major groove of duplex DNA, accommodated by an open cavity in pol β . These results provide novel information for the rational design of new therapeutic nucleoside analogues and a greater understanding of how modified nucleotides are tolerated by polymerases.

Graphical Abstract

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Notes

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INTRODUCTION

DNA sequence conservation promotes genomic stability, which is vital for the prevention of genomic mutations that can ultimately produce dysfunctional and/or cytotoxic proteins. Reflective of its importance, many enzymatic pathways have evolved to increase genome stability. These include free radical scavenging enzymes to reduce cellular levels of reactive oxygen species (ROS), nucleotide metabolism enzymes to balance nucleotide pools, and DNA repair enzymes to remove and replace DNA damage from the genome.¹ When pathways that conserve genomic integrity are disrupted, potent amounts of cellular DNA damage can result. In some scenarios, disruption of genomic stability can have positive clinical impacts; one situation includes the specific targeting of proliferating cancer cells that have compromised DNA damage responses.² Different methodologies have been established to disrupt genome stability, including radiotherapies, platinum-based antineoplasms, and nucleotide analogue cancer therapies, all of which create cytotoxic effects by inducing high levels of DNA damage.^{3,4} Recent advancements toward increasing the amounts of DNA damage levels in cancer cells have been made by both reducing the cellular capacity to tolerate damage and the generation of additional DNA damage. Some examples of this include poly(ADP-ribose) polymerase (PARP) inhibitors that restrict cells from repairing DNA damage, treatment with nucleoside analogues that cause mutagenesis and cytotoxicity, and promotion of DNA damage through inhibition of the nucleotide cleansing enzyme Mut T Homologue 1, which removes oxidatively damaged nucleotides before they can be inserted into the genome.^{5,6}

Nucleoside analogues have been used clinically as a method of introducing DNA damage into cells for decades. This approach exploits DNA polymerases that will insert modified nucleotides into the genome. These molecules must be structurally similar enough to native nucleotides that DNA polymerases will still insert them while at the same time being structurally different enough from native nucleotides to cause mutagenesis and/or cytotoxicity. Currently, over 30 nucleoside analogues have been FDA approved for intervention against a variety of cancers, viral infections, and other diseases.⁷ One of these compounds, 5-fluorouracil (5-FU), has been used in the clinic for over 60 years and is still the treatment of choice for some cancers.^{8,9} The mechanism of action of 5-FU is complex; one important characterized cellular effect is its action as an antimetabolite, inhibiting thymidine synthase. This starkly decreases the synthesis of thymine nucleotides, causing imbalanced nucleotide pools and promoting mutagenesis.¹⁰ In addition to its role as a thymidine synthase inhibitor, 5-FU is phosphorylated to 5-fluoro-2'-deoxyuridine-5'-triphosphate (5-FdUTP) and inserted into the genome by DNA polymerases, directly generating DNA damage. Once inserted into the genome, the toxicity of genomic 5-FU occurs during DNA repair when uracil-DNA glycosylases (e.g., thymine DNA glycosylase)

recognize and excise it, creating an abundance of DNA breaks. This, in turn, causes cytotoxicity.¹¹ The complexity of 5-FU toxicity is also mediated by its insertion into RNA by RNA polymerases.¹²

6-Thioguanine (6-TG) is another clinically relevant nucleoside analogue that has been utilized since the 1950s, and yet its mechanism of action remains unclear.^{13,14} With a similar structure to guanine, one primary mode of action is its metabolic processing to 6-thio-2'-deoxyguanosine-5'-triphosphate (6-TdGTP) and subsequent insertion into genomic DNA by a DNA polymerase. While genomic incorporation alone is not by itself particularly cytotoxic or mutagenic, the toxicity seems to be dependent on DNA repair machinery, in a similar fashion to 5-FU. After the insertion of 6-TG, the molecule is methylated to 6-methylthioguanine, which base pairs with both T and C with similar efficiencies, and targets mismatch repair to the lesion.¹⁵ Because the nondamaged C or T is removed, as opposed to the damage in the templating strand, repair is futile and eventually results in cell cycle arrest.¹⁵ Toxicity of the 6-TG was reduced by the ablation of the base excision repair (BER) protein MUTYH (Mut Y DNA glycosylase), suggesting a role of BER in mediating 6-TG response.¹⁶ Additionally, insertion of 6-TG into the genome at telomere regions was shown to cause telomere dysfunction, suggesting other complex mechanisms of cytotoxicity still undiscovered.¹⁷ Other cellular effects of 6-TG treatment have also been observed, such as a decrease in guanine nucleotide synthesis and disruption of Rac signaling, highlighting the complexity of 6-TG treatment.^{18,19} Importantly, many of these mechanisms of action are dependent on the insertion of 6-TG into the genome via a DNA polymerase.

The treatment potential of additional nucleoside analogues is an active area of investigation. 5-Formylcytosine (5-FoC) treatment has been shown to specifically kill cancer cells that are resistant to treatments with other pyrimidine analogues.²⁰ Unlike the two compounds mentioned above, 5-FoC is produced in the cell under normal physiological conditions when 5-methylcytosine is oxidized to 5-FoC and is used as an epigenetic signal.²¹ The mechanism of 5-FoC cytotoxicity relies on cancer cells overexpressing cytidine deaminase (CDA), which converts cytidine to uracil by removing the amine group. Therefore, upon 5-FoC treatment, 5-FoC is converted to 5-formyluracil (5-FoU) by CDA, which is subsequently metabolically processed to 5-formyl-deoxyuracil-5'-triphosphate (5-FodUTP) and inserted into the genome of cancer cells by DNA polymerases. Noncancerous cells do not overexpress CDA and therefore will not efficiently convert 5-FoC to 5-FoU. Because 5-FoC is not phosphorylated to 5-formyl-deoxycytosine-5'-triphosphate (5-FodCTP), 5-FoC treatment was published to be cancer cell specific via 5-FodUTP insertion based toxicity.^{20,22}

Although the mechanism of action for 5-FU, 6-TG, and 5-FoC treatments universally disrupt genome stability, the structures of these nucleoside analogues are diverse. Table 1 shows the molecular structure of these analogues and describes their clinical uses. Although there is still much to learn about the way nucleoside analogues act, it is known that for some (and in select cases, nearly all) of their cytotoxic and mutagenic effects occur when they are inserted into the genome by a DNA polymerase.^{20,23,24} Therefore, understanding how therapeutic nucleosides are inserted into the genome is an essential part of elucidating their mechanism of action and optimizing future treatments. That being said, there is no reported structural

information regarding the molecular contacts required for 5-FdUTP, 6-TdGTP, 5-FodCTP, or 5-FodUTP being inserted by a DNA polymerase. Obtaining this information is vital to understand why these specific nucleoside analogues perform well therapeutically and for the rational design of future therapeutic nucleoside analogues.

EXPERIMENTAL PROCEDURES

DNA Sequences.

The following DNA sequences were used to generate the 16-mer DNA duplexes used in crystallization studies (the templating base is underlined): template, 5'-CCGACGGCGCATCAGC-3', 5'-CCGACAGCGCATCAGC-3', and 5'-CCGACCGCGCATCAGC-3'; primer, 5'-GCTGATGCGC-3'; downstream, 5'-GTTCGC-3'. The downstream sequence was 5'-phosphorylated. Each oligonucleotide was suspended in 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, and the concentration was determined from their ultraviolet absorbance at 260 nm. The annealing reactions were performed by incubating a solution of primer with downstream and template oligonucleotides (1:1.2:1.2 molar ratio, respectively) at 95 °C for 5 min, followed by 65 °C for 30 min, and finally cooling 1 °C min⁻¹ to 10 °C in a thermocycler.

Expression and Purification of Pol β .

Human wild-type DNA pol β was overexpressed from a pET-28 vector in the BL21-CodonPlus-(DE3)-RP *Escherichia coli* strain. Purification of pol β was carried out as described previously and briefly written here.²⁵ Cell lysate containing pol β was run over GE HiTrap Heparin HP, GE Resource S, and HiPrep 16/60 Sephacryl S-200HR columns, and fractions containing pure pol β were concentrated and stored at -80 °C in 20 mM BisTris propane, pH 7.0, for crystallization. Pol β was determined to be pure by SDS-PAGE, and the final concentration was determined by A280 using a NanoDrop One UV-vis Spectrophotometer ($\epsilon = 23\,380\text{ M}^{-1}\text{ cm}^{-1}$).

Crystallization of Pol β .

Pol β was complexed with 1-nt gapped DNA to form binary complex crystals containing the desired corresponding templating base. The binary complex crystals were grown as previously described in a solution containing 50 mM imidazole, pH 7.5, 13–19% PEG3350, and 350 mM sodium acetate.²⁶ Binary pol β /DNA crystals were then soaked in a cryosolution containing 25% ethylene glycol, 50 mM imidazole, pH 7.5, 19% PEG3350, 70 mM sodium acetate, 2 mM nucleoside analogue (6-TdGTP, 5-FdUTP, 5-FodCTP, or 5-FodUTP), and 50 mM CaCl₂ for 20 min. This resulted in ground state ternary pol β /DNA/nucleoside analogue crystals for collection.

Data Collection and Refinement.

Data were collected at 100 K on a Rigaku MicroMax-007 HF rotating anode diffractometer equipped with a Dectris Pilatus3 R 200K-A detector system at a wavelength of 1.54 Å. Data were processed and scaled using the HKL3000R software package.²⁷ Initial models were determined using molecular replacement with the previously determined closed (PDB: 2FMS) structure of pol β as a reference. All R-free flags were taken from the starting model.

Refinement was performed using PHENIX and model building using Coot.^{28,29} The metal ligand coordination restraints were generated by ReadySet (PHENIX). The figures were prepared in PyMOL (Schrodinger LLC). For Figure 5, residue R61 (placed above the nucleotide triphosphate) in pol η is highly mobile, sampling multiple rotamers with the same occupancy; therefore, rotamer A is displayed.³⁰ Ramachandran analysis determined that 100% of nonglycine residues lie in the allowed regions and at least 96% in favored regions.

RESULTS

To determine the molecular mechanism by which 6-TdGTP, 5-FdUTP, 5-FodCTP, and 5-FodUTP are inserted into the genome, we utilized human pol β . Pol β fills gaps in the DNA following DNA damage removal during BER and is an established model for both kinetic and structural analysis of DNA synthesis.^{31–33} Additionally, treatment with any of these nucleoside analogues generates DNA damage, creating additional opportunities for pol β to insert modified nucleotides during DNA repair. Pol β is also upregulated in breast and colon adenocarcinomas.³⁴ Furthermore, ovarian cancer cell lines with overexpressed pol β exhibited increased sensitivity to nucleoside analogue treatment.³⁵ This suggests that pol β may be even more involved in inserting nucleotide analogues in certain cancers and contribute to the selective toxicity of therapeutic nucleoside analogues.

Our experimental design involved crystallizing pol β in complex with a double stranded DNA (dsDNA) substrate containing a 1-nt gap.³⁶ This binary complex (pol β /DNA) has been previously characterized with the pol β N-subdomain in an open position, generating a highly accessible active site for nucleotide binding. These binary crystals were transferred to a cryoprotectant solution containing the crystallization conditions, 25% ethylene glycol, CaCl₂, and a nucleotide analogue. Calcium ions allow for nucleotide binding but prevent nucleotide insertion by causing a minor change in the coordination distance between the primer terminus and the α phosphate of the incoming nucleotide.^{26,31} This allows the use of a natural primer terminus and nucleotide analogue, rather than a dideoxy terminated primer or nonhydrolyzable nucleotide analogue version. For each modified nucleotide described below, the soak promoted the N-subdomain shift from the open to closed conformation, placing each analogue in position for catalysis in a precatalytic ternary complex (pol β /DNA/nucleotide).³⁷

5-Fluorouracil.

To obtain a pol β precatalytic ternary complex with 5-FdUTP as the incoming nucleotide opposite adenosine (A), we transferred a binary pol β crystal bound to 1 nucleotide (nt) gapped DNA into a cryosolution with 5-FdUTP and CaCl₂. The crystal diffracted to 2.10 Å (Table 2), the polymerase was in the closed conformation, and the structure showed atomic contacts that were virtually identical to that of unmodified uracil. Figure 1A shows a close up of the pol β active site with 5-FdUTP base pairing with a templating A through its Watson–Crick face; with N1 of A acting as a proton acceptor for N2 of 5-FdUTP; and the amine group branching off of N6 from A acting as a proton donor for the carbonyl group at the C4 position of 5-FdUTP. This is identical to structures of unmodified uracil with the base pairing interaction remaining planar (Figure 1B). The distances between the heavy atoms in

the two Watson–Crick faces (2.6 and 3.1 Å, see Figure 1C) and the position of the pyrimidine ring itself are not altered between uracil and 5-FdUTP (see Figure 1C). Both metal binding sites were occupied by Ca^{2+} .

The fluoro group that modifies dUTP into 5-FdUTP was observed to be pointing into the major groove of the double stranded DNA helix. This placement puts it in an isolated position, with no direct atomic contacts to either the enzyme or dsDNA. Because the fluoro group is isolated to the major groove, it does not require any major structural rearrangements of either the protein, DNA, or its triphosphate group. Previous findings show that 5-FdUTP will be incorporated into DNA by pol β at rates similar to those of native nucleotides and are consistent with our structural snapshot showing the enzyme in a position to support catalysis if it were in the presence of magnesium.³⁸

6-Thioguanine.

To determine the structure of 6-TdGTP being inserted opposite a templating C, we soaked a binary pol β crystal bound to dsDNA with a 1-nt gap (templating C) in a cryosolution containing CaCl_2 and 6-TdGTP. The crystal diffracted to 2.55 Å, and the polymerase was in the closed position with 6-TdGTP bound (Table 2). Figure 2A shows a close-up of the active site, with the incoming 6-TdGTP base pairing with C through the Watson–Crick face, and the incoming nucleotide is poised for insertion. The structure obtained was nearly identical to that of pol β inserting unmodified dGTP opposite C, as indicated by an overlay of the two nucleotides in Figure 2B,C. The major difference between the base pairing contacts of 6-TdGTP compared to unmodified dGTP is that a thio group is substituted for the carbonyl group at the C6 position. This sulfur substitution has the same number of valence electrons and can act as a hydrogen bond acceptor. However, the difference in bond lengths alters the position of the incoming nucleotide analogue slightly.³⁹ The distance between the modified thio group and the C2 carbonyl group of the templating C is 3.5 Å, which is slightly longer than typical hydrogen bond distances (Figure 2C). For comparison, the hydrogen bond from the oxygen on C6 of unmodified dGTP to the C2 carbonyl of C is 2.98 Å.

The C6 thio group also points toward the major groove of the DNA helix and is located at the Watson–Crick face of the modified nucleotide. In contrast to 5-FdUTP, where the modification is isolated from any atomic contacts, the thio modification was within weak hydrogen bonding distance of the templating C (3.5 Å). That being said, this structure also revealed no perturbation to the position of any residues of pol β (relative to the insertion of an unmodified G) and the triphosphate of the incoming nucleotide (see Figure 2). Additionally, both metal binding sites were occupied by calcium, with the nucleotide binding in a catalytically competent position. This agrees with previous kinetic results that suggest several human DNA polymerases insert 6-TdGTP at comparable efficiency to dGTP and that pol β also can insert it at a reduced efficiency.^{40,41}

5-Formylcytosine.

To determine the structure of 5-FodCTP being inserted opposite a templating guanine (G), we soaked a binary pol β crystal bound to 1-nt gap DNA with a templating G in a cryosolution containing CaCl_2 and 5-FodCTP. The crystal diffracted to 2.20 Å and pol β was

in the closed precatalytic conformation, poised for insertion with two calcium ions bound (Figure 3A and Table 2). Because the adducted formyl group does not alter the Watson–Crick face of 5-FodCTP, hydrogen bonding likely occurs between the Watson–Crick face of 5-FoC and G, with 5-FodCTP binding in a highly similar position to unmodified dUTP. Also, the pyrimidine ring is planar with respect to the templating G (see Figure 3B,C). Even though the atomic contacts involved in mediating the hydrogen bonding distances with the templating G are similar for both the dUTP and 5-FodCTP, the oxygen of the adducted formyl group is positioned within hydrogen bonding distance of the amine group at the C4 position on the pyrimidine ring (Figure 3C). This second proton acceptor pulling from the amine group changes the hydrogen bonding character along the Watson–Crick edge and may act to reduce the stability of base pairing.⁴² This interaction also places the formyl group in a position to prevent clashing with the nearest phosphate group and is stabilizing enough that clear electron density (Figure 3D) was seen for this group with a *B*-factor of 34.8 Å².

As with 5-FU and 6-TG, 5-FoC is positioned with its altered group in the major groove of the DNA helix. This positioning allows for the group to be isolated from any protein contacts and because of its resemblance to a native nucleotide, no shifting was observed for any amino acid residues in comparison to a native nucleotide. Previous kinetic studies of other pyrimidine analogues with highly similar structures to that of 5-FodCTP, such as 5-fluorodeoxycytidine triphosphate, 5-fluorodeoxyuracil triphosphate, and 5-formyldeoxyuracil triphosphate, show that pyrimidines modified at the C5 position are tolerated well by polymerases.^{38,43} These previous kinetic results corroborate our structural result that 5-FodCTP minimally disrupts the active site of pol β , implying that insertion is expected to proceed efficiently.

5-Formyluracil.

To acquire precatalytic structures of pol β inserting 5-FodUTP into dsDNA containing a 1-nt gap with A in the templating position, we soaked binary pol β crystals in a cryosolution with CaCl₂ and 5-FodUTP. The resulting crystal diffracted to 1.60 Å with pol β in the closed position, 5-FodUTP bound, and two Ca²⁺ ions present in the active site (Table 2 and Figure 4A). 5-FodUTP binds in a nearly identical fashion to its unmodified counterpart dUTP, with the pyrimidine ring planar and within hydrogen bonding distance to the templating A through the Watson–Crick face (Figure 4B,C).

Although the formyl group of 5-FodUTP was placed in the major groove of the dsDNA, its position is slightly changed compared to that of 5-FodCTP; this altered placement is presumably driven by the change from a positively charged C4 amine in 5-FodCTP to a negatively charged C4 carbonyl in 5-FodUTP, which would eliminate hydrogen bonding potential with the proton accepting formyl modification. Even without this stabilization, clear density was seen for the formyl group with a *B*-factor of 28.2 Å² (Figure 4D). The formyl group is placed in the major groove of the dsDNA in both cases. For 5-FodUTP, the formyl group is isolated from any DNA or protein contacts, allowing the polymerase to adopt a catalytically competent conformation to support efficient insertion into the DNA. This is consistent with kinetic studies of other DNA polymerases (DNA polymerase I, T7 DNA polymerase, and Taq DNA polymerase) that show efficient insertion of 5-FodUTP.⁴³

DISCUSSION

In this article, we present novel X-ray crystal structures of four nucleotide analogues (5-FdUTP, 6-TdGTP, 5-FodCTP, and 5-FodUTP) base pairing to their respective complementary base and poised for insertion by pol β . These structures reveal a trend that therapeutic nucleotides are inserted with the modified portion of the nucleobase placed in the major groove of the DNA helix and do not require a shift in the DNA or polymerase active site. Prior to this study, structural characterization of these modified nucleotides was largely limited to structures of the analogues in dsDNA with no enzyme present and never with any polymerase.^{21,44–50} Of note, all of these previous structures show identical base pairing orientation as observed here, within the pol β active site. This implies that after catalysis occurs, the structure of the modified nucleotide remains in the same conformation in which a DNA polymerase inserted it. This is in contrast to previous reports of noncanonical base pairs (such as mismatches) that adopt alternative conformations in DNA alone and in protein/DNA complexes.⁵¹ This underscores the importance of determining the molecular structures of insertion to understand how nucleotide analogues are accommodated in the genome and mediate their biological impact.

It is important to note that, although insertion of these modified nucleotides by pol β is physiologically relevant during DNA repair, it is likely that many insertion events occur during DNA replication or translesion synthesis.^{52,53} Additionally, some of the cytotoxic effects of these compounds result from their insertion by RNA polymerases.¹² Therefore, cytotoxicity may be mediated by insertion events from many polymerases with diverse structures, which may interfere with the nucleobase modification. For example, other polymerases may have additional domains or alternate conformations that result in contacts with the DNA major groove. To probe this possibility, we compared our structures with ternary (protein/DNA/nucleotide) crystal structures of polymerases representing DNA replication (polymerase δ), translesion synthesis (polymerase η), and transcription (N4 RNA polymerase), presented in Figure 5. This was done by overlaying the incoming nucleotide with our modified analogue and inspecting the major groove for clashes. There was no obvious clashing that would interfere with the insertion of each analogue, suggesting that our results for pol β insertion of these modified nucleotides may also be relevant during DNA replication, translesion synthesis, and transcription. Consistent with this, recent structures of KlenTaq DNA polymerase inserting nonphysiological nucleobases, such as dyes, affinity tags, and other groups with high steric demand were shown to be tolerated in the major groove.⁵⁴

Multiple rationales would explain why modification placement in the major groove has been selected for in prevalent nucleoside analogue therapies. One being placement of modifications in the major groove (such as the C5 and N7 positions of pyrimidines and purines, respectively), which minimizes the structural perturbation to the DNA polymerase during insertion. Large structural disruptions of the polymerase active site have been shown to reduce activity, and placements of modifications in the major groove side evade this potential problem.^{36,55} A second rationale is that placement of modifications in the major groove causes minimal disruption to Watson–Crick base pairing interactions, which act to stabilize the incoming nucleotide in the active site and increase insertion efficiency. A third

rationale is that placement of modifications in other positions may cause structural disruptions. For example, a modification positioned near the nucleotide triphosphate may clash with catalytically key groups (e.g., α phosphate) and require additional structural rearrangements for insertion.⁵⁵ Alternately, large modifications in the DNA minor groove would be sterically hindered by enzyme contacts, as DNA polymerases from many families have been observed to contact the minor groove.^{55,56}

Furthermore, many of these compounds, in the state in which they are inserted into the genome, have minimal cytotoxicity and mutagenicity. The full cellular effects of insertion do not occur until other enzymes recognize them and excise them, causing their cytotoxicity.³⁹ The placement of the adducted group in the major groove could also aid in postinsertion processing. For example, 6-TG is not cytotoxic until the sulfur group is methylated because the methylated version of 6-TG base pairs with T, promoting mutagenesis.⁵⁷ Thus, placement of the sulfur group in the major groove of the helix could make it more accessible to agents that methylate it, such as *S*-adenosylmethionine. Additionally, it is possible that placing the modifications in the major groove not only assists in the insertion into the genome but also creates a more detectable lesion for DNA repair machinery.¹⁶ Increasing detectability of lesions could lead to higher rates of excision and therefore would cause more cytotoxic DNA repair intermediates to be generated.

Here, we have reported the first atomic resolution structures of pol β inserting multiple therapeutically relevant nucleotide analogues into dsDNA. The cellular impacts of these therapeutic compounds are complex, and there is still much to learn about their biological mechanism of action.⁵⁸ That being said, much of the mechanism of action of these compounds is dependent on their insertion into the cellular genome, and understanding the mechanism of insertion is vital to understanding why these drugs are effective. From drugs that have been in the clinic for six decades, to recently discovered compounds currently under investigation for therapeutic relevance, similarities can be seen in the way these structures are inserted into DNA. These themes include Watson–Crick base pairing similar to unmodified nucleotides, minimal alterations to the positioning of triphosphate groups, and positioning of the adducted modification into the major groove of the DNA during insertion. These themes can serve to guide rational optimization of future drug structures for novel nucleoside analogue therapies.

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ABBREVIATIONS

5-FU	5-fluorouracil
5-FdUTP	5-fluorodeoxyuridine triphosphate
6-TG	6-thioguanine

6-TdGTP	6-thiodeoxyguanine triphosphate
5-FoC	5-formylcytidine
5-FodCTP	5-formyl-deoxycytidine triphosphate
5-FoU	5-formyluracil
5-FodUTP	5-formyldeoxyuridine triphosphate
pol β	DNA polymerase β
dsDNA	double stranded DNA
pol	polymerase
nt	nucleotide
A	adenosine
C	cytosine
T	thymine
G	guanine

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Figure 1.

Pol β ternary ground state with incoming 5-FdUTP base pairing to A. (A) Key active site residues of pol β (gray) inserting 5-FdUTP (yellow) are shown in stick format with the N-subdomain in cartoon. Calcium ions are shown in orange. The fluoro group is shown in cyan. (B) An overlay of the 5-FdUTP (gray) with an incoming dUTP (salmon) nucleotide base pairing to A (PDB 2FMS). (C) A 90° rotation of B, showing the Watson-Crick edge of the overlay, with distances labeled in Å. H-bonds are shown as dashed lines. (D) The omit map (green), contoured at 2.0σ .

Figure 2.

Structural features of the precatalytic pol β ternary ground state with incoming 6-TdGTP. (A) A close-up view of the pol β active site (gray), with the incoming 6-TdGTP shown in cyan and the sulfur modification in yellow. The N-subdomain is shown in cartoon, DNA in gray, and calcium ions in orange. (B) A superposition of 6-TdGTP with a structure of an incoming dGTP nucleotide base pairing to C (in salmon, PDB 4UB4). (C) A view of the Watson-Crick edge of the incoming 6-TdGTP, with H-bonds shown as dashed lines and distances in Å. (D) An omit map (green) for the 6-TdGTP, contoured at 2.0σ .

Figure 3.

Structure of incoming 5-FodCTP, primed for insertion by pol β . (A) 5-FodCTP (green) bound in the active site of pol β (gray), with key residues shown as sticks. DNA bases are labeled, with calcium ions shown in orange. The N-helix is shown in cartoon form, and the formyl modification is labeled. (B) Close-up of the incoming 5-FodCTP and its templating base, overlaid with a structure of an incoming pyrimidine base (in salmon, PDB 2FMS). (C) A 90° rotation of B, highlighting the Watson–Crick edge of the nucleotide, with distances displayed in Å. (D) A green omit map, contoured at 2.0σ for the incoming 5-FodCTP.

Figure 4.

Structure of pol β bound to gapped DNA and 5-FodUTP. (A) A close-up view of 5-FodUTP (purple) in the active site of pol β (gray). Calcium ions are shown in orange, and the N-subdomain is displayed as a cartoon. DNA bases (gray) are labeled, along with the adducted formyl group. (B) A structural overlay of 5-FodUTP with a structure of an incoming dUTP base in the active site of pol β (in salmon, PDB 2FMS). (C) Rotated view of B, showing the hydrogen bonding of the bases as dashed lines and selected distances shown in Å. (D) Omit map for the incoming 5-FodUTP in green, contoured at 2.0σ .

Figure 5.

Other polymerases have minimal contacts with the major groove side of incoming nucleotides. We modeled an incoming 5-FdUTP as sticks, the DNA as a cartoon, and each enzyme as a surface representation. A purple sphere is placed where the fluoro group at the C5 position of the ring would rest. (A) A white surface representation of a structure from this work of pol β with incoming 5-FdUTP. (B) A blue surface of N4 RNA polymerase, with less restriction along the major groove, and nothing contacting a modification at the C5 position (PDB 3Q23). (C) A green surface representation of DNA polymerase η , with no contacts to a modification in the major groove (PDB 3MR2). (D) A dark gray surface of the replicative DNA polymerase δ , also with minimal contacts along the major groove of DNA (PDB 3IAY).

Table 1.

Selected Nucleoside Analogues and Their Clinical Use

Structure	Name	Use
5-Fluorouracil (5-FU)	5-FU is used in the clinic for colon, rectal, pancreas, breast, stomach, and ovarian cancer interventions, and has been used in the clinic for around 60 years. ⁹
6-Thioguanine (6-TG)	6-TG is used clinically for acute myeloid leukemia, acute lymphatic leukemia, chronic myeloid leukemia, and also used as an immunosuppressant. 6-TG has also been used in the clinic for around 60 years. ¹⁴
5-Formylcytidine (5-FoC)	5-FoC is currently under investigation for use with cancers resistant to cytidine analog treatment. It is thought that 5-FoC is converted to 5-FoU before it can be inserted into the genome. ²⁰
5-Formyluracil (5-FoU)	5-FoU is the deaminated form of 5-FoC, a reaction is specific to cancer cells overexpressing cytidine deaminase. 5-FoC deamination presumably must occur before phosphorylation and subsequent genomic insertion. ²⁰

Table 2.

Data Collection and Refinement Statistics of Ternary Pre-catalytic Pol β :DNA Co-complexes with Incoming Nucleotide Analogues

	5-FdUTP	6-TdGTP	5-FodCTP	5-FodUTP
	Data Collection			
space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
cell dimensions				
a, b, c (Å)	50.5, 79.7, 55.6	50.2, 79.2, 55.6	50.6, 79.6, 55.6	50.7, 79.6, 55.3
α, β, γ (deg)	90, 107.2, 90	90, 107.4, 90	90, 107.2, 90	90, 107.3, 90
resolution (Å)	25–2.10	50–2.55	25–2.20	50–1.60
R_{sym} or R_{merge}^a (%)	13.7 (45.9)	13.3 (76.6)	9.7 (43.0)	6.5 (74.0)
$I/\sigma I$	15.3 (2.3)	15.1 (2.1)	12.7 (1.8)	20.6 (1.4)
completeness (%)	99.8 (97.4)	99.9 (99.7)	98.0 (94.5)	99.9 (99.5)
redundancy	4.5 (2.6)	7.0 (5.3)	3.4 (2.0)	4.2 (2.8)
	Refinement			
resolution (Å)	2.10	2.55	2.20	1.60
no. of reflections	113357	96895	71630	232652
$R_{\text{work}}/R_{\text{free}}$	17.1 (23.3)	20.6 (28.9)	18.6 (25.7)	20.3 (22.8)
no. of atoms	3536	3273	3378	3664
protein	2626	2600	2618	2641
DNA	659	661	662	661
water	243	7	90	354
B -factors (Å ²)				
protein	33.38	43.84	41.61	25.04
DNA analogue	42.14/25.74	55.74/34.57	52.16/32.16	30.82/19.27
water	38.78	32.93	42.01	32.58
rms deviations				
bond length (Å)	0.015	0.014	0.013	0.007
bond angles (deg)	1.241	1.386	1.297	0.907
PDB ID	5WNY	5WNX	5WNZ	5WOO

^aThe highest resolution shell is shown in parentheses.