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Competitive Inhibition of Uracil DNA Glycosylase by a Modified Nucleotide Whose Triphosphate is a Substrate for DNA Polymerase

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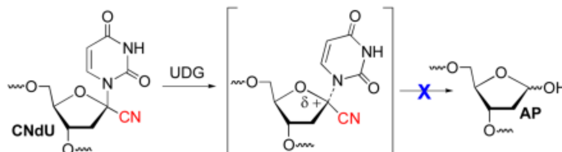
Abstract



Base excision repair (BER) enzymes are attractive targets for antiviral and anticancer agents. A number of nucleotides and nucleotide analogues are potent competitive inhibitors of BER glycosylases when they are incorporated into synthetic oligonucleotides. However, these molecules often are not substrates for DNA polymerases, which limits their utility in cells and as potential therapeutic agents. 1′-Cyano-2′-deoxyuridine (CNdU) is a nanomolar competitive inhibitor of uracil DNA glycosylase. In addition, the respective nucleotide triphosphate is accepted as a substrate by the Klenow fragment (exo^{-}) of DNA polymerase I from *E. coli*. This is the first competitive inhibitor of UDG that is incorporated into DNA by Klenow exo^{-}, a model replicative polymerase. 1′-Cyano-2′-deoxyuridine (CNdU) and related molecules may prove useful as a new family of therapeutic or experimental agents that target DNA repair by using the cells' polymerase(s) to incorporate them into DNA. A potential benefit of such a mechanism is that multiple incorporations can occur for longer DNA molecules leading to amplification of the inhibitory effect beyond that seen here with short DNA duplexes.

DNA base excision repair (BER) is an essential process in prokaryotes and eukaryotes for protecting the integrity of the genome. The first step in BER of modified nucleotides is carried out by glycosylases, such as uracil DNA glycosylase (UDG), which produces abasic sites (AP) from 2′-deoxyuridines that arise from deamination of 2′-deoxycytidine via an oxocarbenium ion (Scheme 1).[1,2] UDG homologues exist in bacteria, viruses (e.g. HIV, HSV-1), as well as humans, and are potential antiviral and anticancer targets.[3,4] Small molecule competitive inhibitors that bind UDG in the micromolar range have been discovered.[5] In addition, synthetic oligonucleotides containing modified nucleotides and nucleotide analogues that inhibit UDG or other BER glycosylases have also been reported.[6–11] However, the most potent of these inhibitors are not incorporated into DNA by polymerases because they lack a nucleobase. In addition, some 2′-deoxynucleotide triphosphates of inhibitors containing 2′-fluoro substituents

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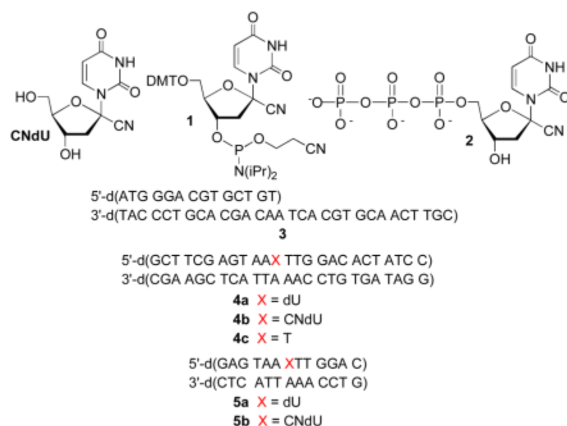
are substrates for thermostable polymerases used in PCR and Pol α, but not Pol III, which limits their potential as therapeutic candidates.[12–14]

We postulated that 1′-cyano-2′-deoxyuridine (**CNdU**) would be a UDG inhibitor whose respective nucleotide triphosphate (**2**) would also be a substrate for DNA polymerase. The strong destabilization of carbocations by α-cyano groups suggested that **CNdU** would be a potent UDG inhibitor in DNA. Furthermore, molecular modeling of the enzyme-DNA complex in which **CNdU** was substituted for pseudouridine suggested that the cyano group would only weakly perturb the protein's structure (Figure 1). The nucleotide triphosphates of various 2′-fluoro substituted nucleotides that inhibit BER glycosylases when incorporated in synthetic oligonucleotides are not accepted as substrates for DNA polymerases because they alter the pucker of the sugar ring. C1′-substituted 2′-deoxyribonucleotide triphosphates (dNTPs) have not been reported as substrates for DNA polymerases. However, the acceptance of C4′-modified dNTPs as substrates by these enzymes encouraged us to investigate **2** in this regard.[15]

Incorporation of **2** by the Klenow fragment of *E. coli* DNA polymerase I that lacks proofreading capability (Klenow exo$^-$) was examined quantitatively under steady-state conditions using primer-template complex **3** and dNTP **2** that was synthesized from **CNdU** via standard methods.[16,17] The dNTP was accepted as a substrate by Klenow exo$^+$ (apparent K_m = 14.8 ± 1.1 μM, V_{max} = 6.5 ± 1.2 %•min^{-1}, 0.67 nM), which contains proofreading ability (Figure 2A). **CNdU** was also incorporated slightly less efficiently by Klenow exo$^-$ (apparent K_m = 15.6 ± 1.1 μM, V_{max} = 6.7 ± 1.0 %•min^{-1}, 2 nM), albeit ~350-fold less efficiently than dT is (Figure 2B). **CNdU** was not incorporated opposite dG or dC, and only weakly opposite dT in the presence of high concentrations (0.3 mM) of **2**. In addition to selective incorporation opposite dA, complete extension was achieved when the primer-template complex (50 nM) was in excess of enzyme (5 nM), indicating that multiple molecules of **CNdU** can be incorporated.[17]



Having established that **CNdU** can be incorporated into DNA we turned our attention to its ability to inhibit UDG when present in the biopolymer. Oligonucleotides containing **CNdU** were synthesized by solid-phase synthesis from the respective phosphoramidite (**1**). DNA containing **CNdU** (**4b**) was not a substrate for UDG. Hydrolysis of **CNdU** by UDG would produce 2-deoxyribonolactone, which like **AP** is cleaved under mild alkaline conditions.[17,18] However, no evidence of reaction of 5′-^{32}P-**4b** was observed even upon prolonged exposure (24 h, 37 °C) to *E. coli* UDG. In contrast, **4b** was a potent inhibitor. Measurement of the apparent K_m (K'_m) of *E. coli* UDG acting on **4a** as a function of concentration of an otherwise identical duplex containing **CNdU** (**4b**) yielded K_i = 1.4 ± 0.1 nM (Figure 3A). The K_i of **4b** was lower than the K_m (10.7 ± 0.2 nM) for the substrate. The strength of the inhibition was independently verified using a method in which the ratio of observed rate constants at various concentrations

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of inhibitor (**4b**) relative to that in the absence of inhibitor were measured when the substrate is present at a concentration much lower than its K_m (Table 1).[19] The K_i (4.6 ± 1.2 nM) very close to that determined from the plot of $K_{m,app}$ versus **4b** concentration. Control experiments using duplex DNA containing thymidine (**4c**) in place of **CNdU** showed that inhibition was not due to nonspecific binding. For instance, addition of 20 nM **4c** (> 4 × the K_i of **4b**) diminished the hydrolysis of the substrate by <15%. Finally, **4b** also effectively inhibits human UDG (Table 1).

The importance of the ability of DNA polymerase to incorporate **CNdU** into DNA is illustrated by inhibition studies using the free nucleoside. Although the free nucleoside of **CNdU** inhibited UDG, its K_i was more than 10,000 times higher than when it was present in DNA (Table 1). In addition, UDG inhibition by dU and **CNdU** monomers are comparable, indicating that the uracil ring of the inhibitor is bound within the same enzyme active site as the substrate. We propose that a portion of the improved inhibition is attributable to the inherent electrostatic attraction between the protein and DNA. The 1′-cyano substituent may also indirectly contribute to the ability of DNA containing **CNdU** to bind to UDG by destabilizing the duplex. Van't Hoff plots of otherwise identical duplexes containing dU (**5a**) or **CNdU** (**5b**) show that the modified nucleotide decreases the enthalpy of melting and decreases the increase in entropy (Table 2). The thermodynamic differences are consistent with a destabilized duplex, which would be expected to make binding to UDG more favorable by decreasing the energy required to flip the base out of the helix.

In summary, we have described the first competitive inhibitor of UDG that is incorporated into DNA by the Klenow fragment of DNA polymerase I, a replicative polymerase. The presence of the molecule within the DNA scaffold contributes significantly to its potency. Nucleosides are often useful as therapeutic agents. 1′-Cyano-2′-deoxyuridine (**CNdU**) and related molecules may prove useful as a new family of therapeutic or experimental agents that target DNA repair by using the cells' polymerase(s) to incorporate them into DNA. In order to be useful in this way, **CNdU** or a pro-drug of it will need to be a substrate for cellular kinases, which at this time is unknown. A potential benefit of such a mechanism is that multiple incorporations can occur for longer DNA molecules leading to amplification of the inhibitory effect beyond that seen here with short DNA duplexes. The in vivo effectiveness of such a strategy has been validated for the inhibition of cytosine 5-methyl DNA methyltransferases by the nucleoside prodrugs 5-azadeoxycytidine and deoxyzebularine.[20]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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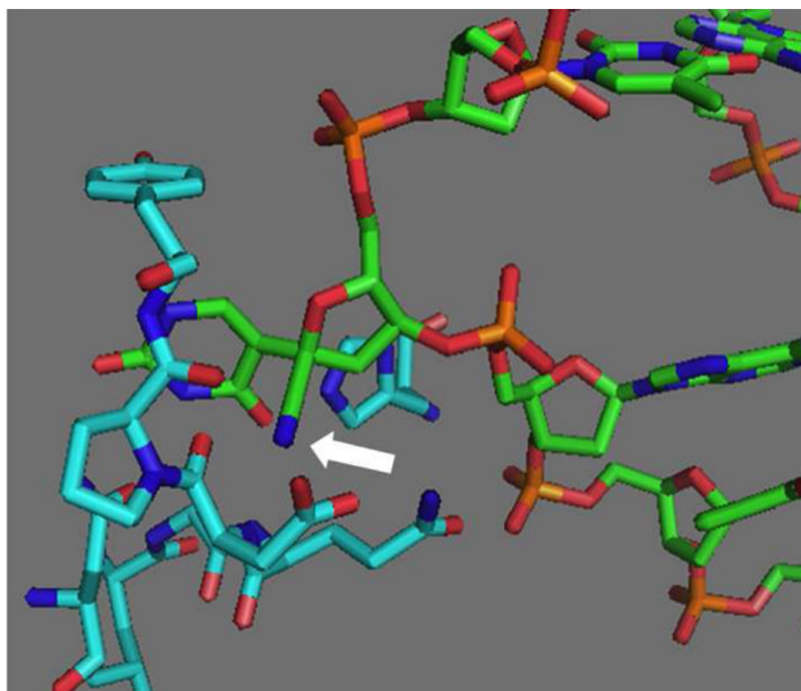

Figure 1.

Molecular modeling showing the accommodation of a 1′-cyano substituent on pseudo-2′-deoxyuridine containing DNA co-crystallized with human UDG (PDB ID: 1EMH). The arrow points towards the cyano substituent.

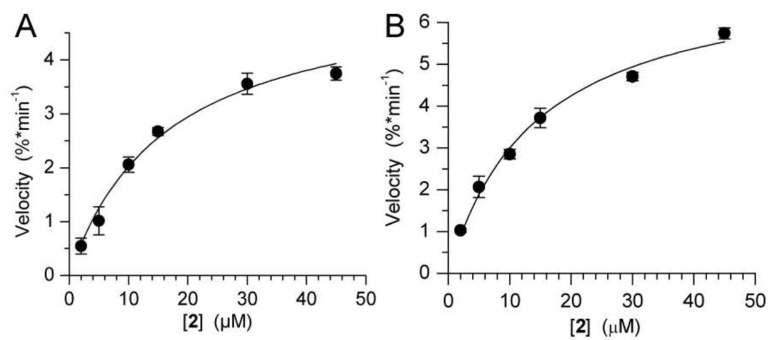

Figure 2.
Acceptance of **2** as a substrate by (A) Klenow exo^{+} (B) Klenow exo^{-} using **3** as a template under steady-state conditions.

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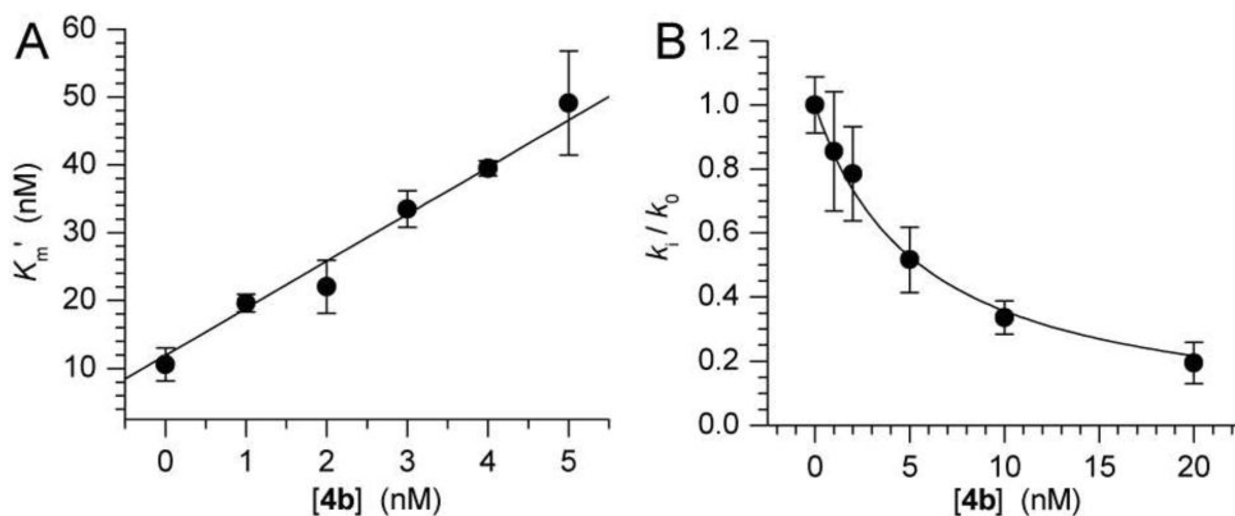

Figure 3.

Determination of K_i of *E. coli* UDG by **4b** by (A) determining the apparent K_m (K'_m) of **4a** as a function of inhibitor concentration and (B) measuring the rate constant ratio in the presence of varying [**4b**] (k_i) versus no inhibitor (k_0) at [**4a**] $\ll K_m$.

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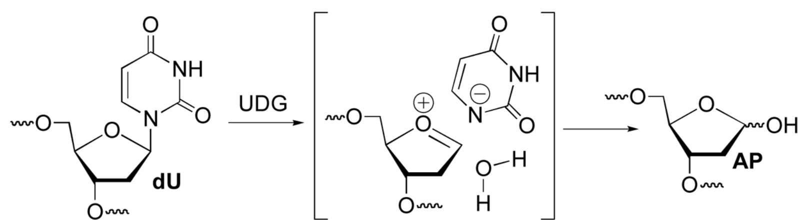

Scheme 1.

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Table 1

UDG inhibition by 1′-cyano-2′-deoxyuridine (**CNdU**).

Inhibitor	**UDG**	**K_i (μM)**[a]
4b	*E. coli*	$4.6 \pm 1.2 \times 10^{-3}$
4b	Human	$13.8 \pm 1.7 \times 10^{-3}$
CNdU[b]	*E. coli*	245.7 ± 19.9
CNdU[b]	Human	131.5[c]
dU[b]	Human	86.4[c]

[a] Data are the average of at least three experiments. Each experiment consists of 3 replicates.

[b] Free nucleoside.

[c] Result of a single experiment.

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Table 2

Melting thermodynamics of DNA containing **CNdU** and dU.

Duplex	T_M (°C)[a]	ΔH (kcal/mol)	ΔS (cal/mol•deg)	ΔG_{298} (kcal/mol)
5a	48.9 ± 0.1	92.9 ± 0.7	260.8 ± 0.1	15.5
5b	44.7 ± 0.3	85.2 ± 4.8	240.2 ± 0.1	13.6

[a] [Duplex] = 2.5 μM