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Light-activated Binary Nucleotide Reagent For Inactivation Of Dna Polymerase

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LIGHT-ACTIVATED BINARY NUCLEOTIDE REAGENT FOR INACTIVATION OF DNA
POLYMERASE

by

EVAN M. CORNETT
B.S. University of Central Florida, 2009

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ABSTRACT

This work explores a *binary reagent* approach to increase the specificity of covalent inhibitors. In this approach, two ligand analogs equipped with inert pre-reactive groups specifically bind a target biopolymer. The binding event brings the pre-reactive groups in proximity with each other. The two groups react, generating active chemical intermediates that covalently modify and inactivate the target. In the present study we compare the new approach with the traditional single-component reagent strategy using DNA polymerase from bacteriophage T4 as a model target biopolymer. We report the design and synthesis of two analogs of deoxythymidine triphosphate, a natural DNA polymerase substrate. Together, the analogs function as a binary nucleotide reagent which is activated by light with wavelengths 365 nm and longer. However, the active analog functions as a traditional single component reagent when activated by light with wavelengths at 300 nm and longer. The traditional single-component reagent efficiently inactivated DNA polymerase. However, in the presence of non-target protein the inactivation efficiency is greatly diminished. Under the same conditions, the binary nucleotide reagent also inactivated DNA polymerase, and the inactivation efficiency is not affected by the presence of the non-target protein. Our results validate that a binary approach can be employed to design highly specific covalent inhibitors. The binary reagent strategy might be useful as a research tool for investigation of ligand-protein interactions in complex biological systems and for drug design.

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LIST OF ACRONYMS

BR – Binary reagent

BNR – Binary nucleotide reagent

PAGE – Polyacrylamide gel electrophoresis

SR – Single reagent

SCR – Single component reagent

WB – Western blot

dNTPs – Deoxynucleotide triphosphates

dTTP –Deoxythymidine triphosphate

dTMP–Deoxythymidine monophosphate

pol –Polymerase

dPAGE – Denaturing polyacrylamide gel electrophoresis

SDS – Sodium dodecyl sulfate

HPLC– High-performance liquid chromatography

INTRODUCTION

Enzyme Inhibition

Enzymes are essential for nearly all biological functions and malfunction of enzymatic activity is commonly associated with disease[1]. As a result, the inhibition of enzymes has been an important tool for both biological research and drug design[1]. Enzymes compose one of the largest distinct drug classes, with approximately half of all marketed drugs targeting an enzyme[2, 3]. Enzyme inhibitors are delineated into two classes based on their mechanism of action: reversible or irreversible. Reversible inhibitors act through non-covalent mechanisms to bind to a target through high-affinity interactions. High concentrations of inhibitor are required to achieve a desired effect, since the inhibitor and target are at equilibrium. However, increasing the concentration of inhibitor often leads to dose-related adverse effects, which include target related toxicities or off-target inhibitor interactions[4]. Thus, in order to increase the potency of non-covalent reversible inhibitors, medicinal chemistry is employed to increase binding affinity. In order to maintain drug-like properties, there are defined criteria which must be met, and in practice these criteria limit the maximum binding affinity that can be achieved through non-covalent interactions. Traditionally these criteria are known as the 'Lipinski Rule of 5' and establish set values for molecular weight, lipophilicity, and hydrogen bond donors or acceptors that allow a compound to maintain favorable drug like characteristics[5]. Most notably, the molecular weight restriction limits the binding affinity that can be achieved, since it has been shown that a higher binding affinity is directly linked to an increase in molecular weight for inhibitors that function only through non-covalent

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interactions[6, 7]. As a result, the pharmaceutical industry has largely operated to find suitable small molecules that fit these requirements[8]. However, a higher binding affinity can be achieved by using irreversible inhibitors. Irreversible inhibitors usually act through covalent mechanisms to form stable enzyme-inhibitor complexes that permanently destroy enzyme function[9]. Unfortunately, the use of covalent inhibitors has been hampered by off target effects[10].

Binary nucleotide reagent

Here, we suggest a general approach, called the *binary reagent* (BR) approach to increase the specificity of covalent inactivation of biopolymers. The approach uses two ligand analogs conjugated with functional pre-reactive groups (REA and GENT in Fig. 1) that are inert toward biomolecules when separated. However, when brought into close proximity and specific orientation in the biopolymer active site, they interact with each other and form chemically active species (REAGENT). The reagent covalently cross-links to or otherwise covalently modifies the biopolymer. The high specificity of the approach is predetermined by the involvement of two ligands, which may independently
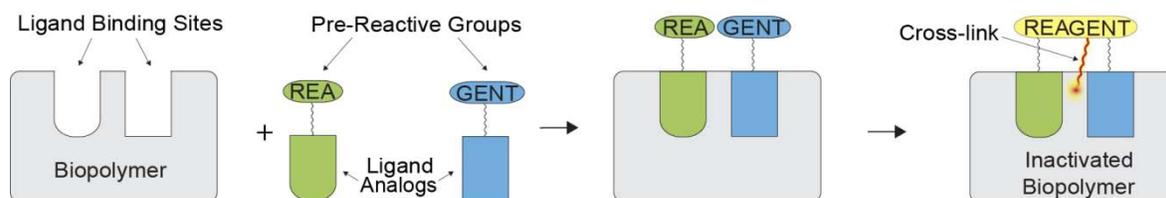

Figure 1: Binary Reagent Approach

or semi-independently interrogate biopolymers. The binary reagent irreversibly inhibits the target due to the covalent nature of modification that is irreparable under

physiological conditions. The binary approach functions in two very distinctly unique ways compared to a traditional covalent inhibitor that contains only a single ligand analog bearing a highly reactive chemical group. First, a binary reagent only generates the highly reactive chemical species at the site of desired modification. Additionally, this species is only formed upon the specific binding of two analogs to the target, rather than one in the case of a single component reagent. Together, these differences allow a binary reagent to operate with higher specificity then a traditional reagent.

DNA dependent DNA polymerase

DNA polymerases are essential enzymes for DNA amplification and repair in all organisms. Development of inhibitors of DNA polymerases is essential for anti-viral[11] and anticancer therapies[12, 13]. Moreover, affinity cross-linking techniques provide invaluable information about interactions of DNA polymerases with DNA during replication and repair[13]. DNA polymerases operate with two substrates: a DNA primer-template complex and nucleoside triphosphates (dNTPs). The enzymes transfer a nucleotide moiety from a dNTP to the 3'-end of a DNA primer. The single stranded 5'-protruding template sequence dictates which nucleotide should be added to the primer. DNA polymerases are a desirable choice to evaluate the efficiency of a binary reagent, since the primer-template complex and dNTPs bind to sites that are in close proximity in the DNA polymerase active site. In this study we chose to use T4 DNA polymerase as a model target. T4 DNA polymerase is the protein that results from gene 43 of T4 bacteriophage[14]. The structure of a similar DNA polymerase from bacteriophage RB69 has been solved and shows that the incoming dNTP is less than 1nm away from

the 3' end of the primer when both substrates are bound to the polymerase (Fig. 2) [15, 16].
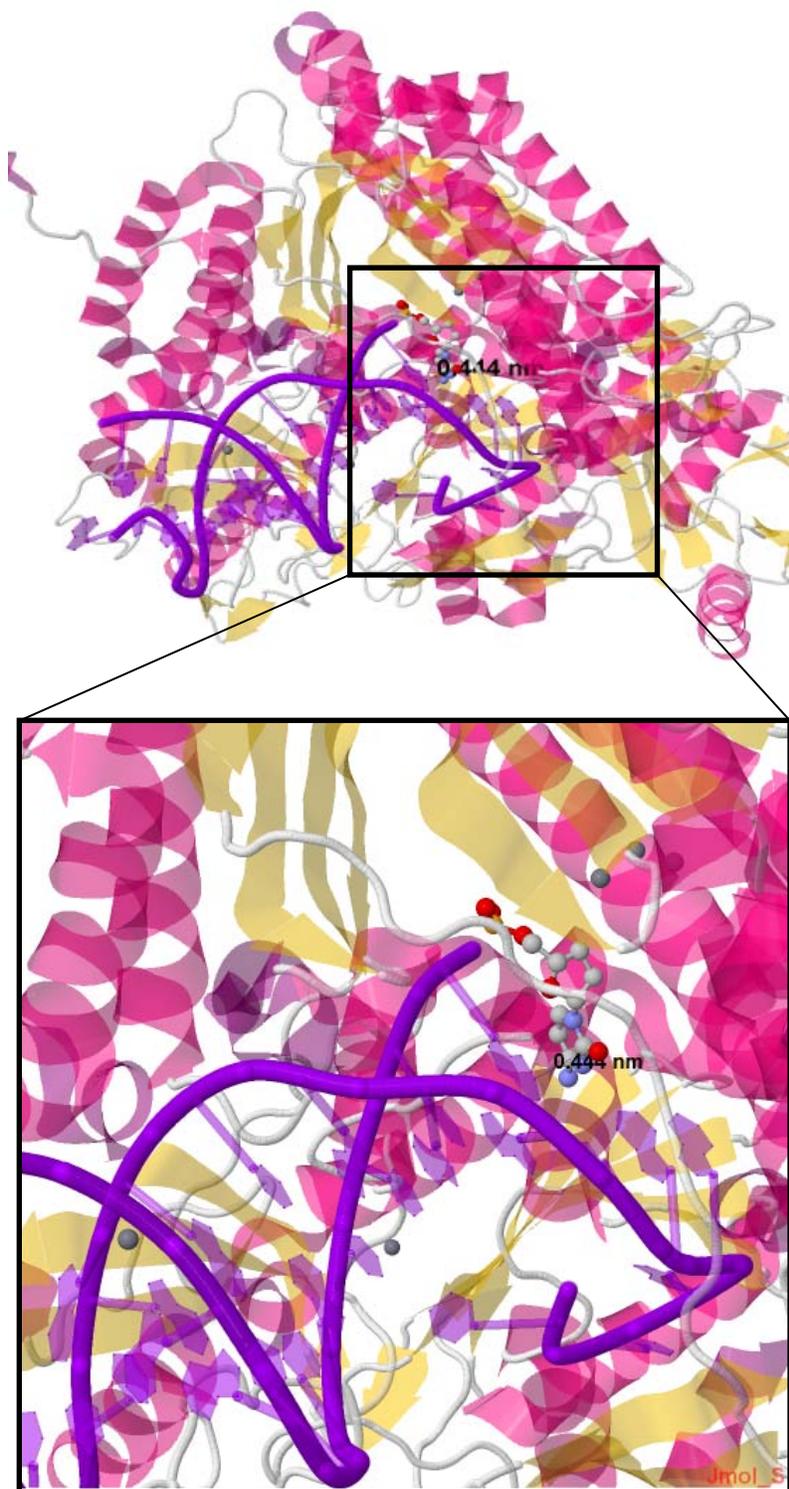
Figure 2: Structure of RB69 polymerase active site [15].

RATIONALE AND APPROACH

The primary objective of this project was to establish and experimentally validate the *binary approach* to covalent inactivation of DNA polymerases. I hypothesized that in the presence of non-specific proteins a binary reagent would inactivate T4 DNA polymerase with higher specificity and potency than a related single-component affinity reagent. To test this hypothesis I designed and synthesized a light-activated binary nucleotide reagent (BNR) that consists of two nucleotide analogs conjugated to two separate pre-reactive groups. The design was such that one of the nucleotide analogs could function as a traditional single-component affinity reagent or a binary reagent depending on two variables: wavelength of light used for activation and presence of cognate nucleotide analog. To evaluate the potency of the binary reagent, the activity of T4 DNA polymerase was determined by denaturing polyacrylamide gel electrophoresis and a radioactive filter binding assay in the presence and absence of a non-target protein. Additionally, the specificity of the BNR was determined by tracking the covalent attachment of a biotin labeled substrate to T4 DNA polymerase or to non-target proteins.

MATERIALS AND METHODS

Synthesis of dTTP analogs

First, 5-(3-amino)allyl deoxyuridine triphosphate (compound 1) was synthesized as described previously in three steps[17]. <u>Mercuration step</u>: 125mg of dUTP in 22mL of 0.1M sodium acetate, pH 6.0, was treated with 0.446g of mercuric acetate and heated for four hours at 50°C in a wa ter bath. After cooling the solution on ice, 0.108g of Lithium Chloride was added. The solution was extracted with an equal volume of ethyl acetate six times. The aqueous layer(bottom layer) was kept and split into two 50 mL falcon tubes with 10mL of solution in each. Cold 100% ethanol was added and the samples and a white precipitant instantaneously formed. The samples were incubated overnight at -20°C. The precipitant was collected by centrifugation at 3500rpm for 10 minutes. The precipitate was washed twice with 10mL of cold ethanol and once with ethyl acetate. The precipitate was dried under vacuum. <u>Alkylation</u>: The product of the mercuration step was dissolved in 14mL of 0.1M sodium acetate, pH 5.0. Two solutions were prepared: First, 92 mg of palladium(II) chloride was dissolved in 2.25mL of water. Second, 0.3mL of allylamine was diluted in 1.7mL of 4M acetic acid. The allylymine solutions were added to the reaction vessel containing the mercuration product. The solution was incubated for overnight at room temperature (22°C). A black precipitate was visible, and the solution was filtered through P8 filter paper and 0.45 micron filters to get rid of the precipitant. <u>Purification of allylaminodUTP:</u> The product was diluted in water and purified on a column with DE52 (whatman) resin using 0.1-0.5M gradient of triethylammonium bicarbonate buffer. The product (1) eluted off the column in the 0.25M

fraction and was characterized by UV/Vis spectroscopy and by TLC with ninhydrin staining.

Compound 1 was converted to 5-[N-(2,3,5,6-tetrafluoro-4-azido-benzoyl)-amino-trans-propenyl-1]-2'-deoxyuridine-5'-triphosphate (dTTP analog 1) and 5-{N-[4-(1-pyrenyl)-butylcarbonyl]-amino-trans-propenyl-1]-2'-deoxyuridine-5'-triphosphate (dTTP analog 2) as described previously[18]. First, 2x excess of N-hydroxysuccinimide esters of 2,3,5,6-tetrafluoro-4-azido-benzoic and pyrene butyric acids were incubated with compound 1 in the presence of excess 4x excess triethylamine. After incubating four hours at room temperature, 10x excess 2% lithium perchlorate in acetone was added and the precipitant was collected. The precipitant was washed 2X with cold ethanol, dissolved in water, and purified using reverse-phase HPLC.

Substrate properties of dTTP analogs

Samples containing 1 nM T4 DNA polymerase and 2 μM biotin-labeled oligonucleotide substrate (HS) were incubated for thirty minutes at 37°C in the presence of 10 μM dTTP, dTTP analog 1, or dTTP analog 2. A portion of each sample was collected and reaction stopped by addition of an equal amount of formamide containing dPAGE loading buffer. Natural dNTPs 250 μM were added to the remaining samples and incubated for thirty minutes at 37°C. The reaction was stopped by addition of dPAGE loading buffer. The samples were heat denatured for 5 minutes in a 90°C water bath prior to gel analysis of elongation products (15% dPAGE).

Binary Nucleotide Reagent

Samples containing 1 nM T4 DNA polymerase and 2 µM biotin-labeled oligonucleotide substrate (HS) (5' bio-CCT TCG T TCG TTG TTC CCT A GGC TGT ATA GCC CCT ACC TTT TTG GTA GGG GCT ATA CAG CC) were incubated for thirty minutes at 37°C in the presence of 10 µM dTTP analog I. For specificity experiments, 0.1, 1, 2, or 4 µM concentrations of T7 RNA polymerase were used. dTTP analog II was added and the samples were irradiated for 40 minutes in a Spectroline UV crosslinker. Glass filters (Hoya L-37 for λ > 365 nM or standard electrophoresis glass plate for λ > 300 nM) were used to filter the light to allow transmittance of the desired wavelengths. Samples were split and analyzed by urea polyacrylamide gel electrophoresis, blot anlysis, and filter binding assay as detailed below.

Denaturing polyacrylamide gel electrophoresis(dPAGE) analysis

Five microliters of each sample were incubated for 30 minutes in the presence of 250 µM of each dCTP, dATP, dGTP, dTTP. The reaction was stopped by the addition of formamide loading buffer, and heat denatured for 5 minutes in a 90°C water bath. Samples were separated by denaturing polyacrylamide gel electrophoresis (15% 19 acrylamide: 1 bisacrylamide, 1X TBE buffer) using a Thermo Scientific Owl gel apparatus connected to a circulating water bath at 55°C with 1X TBE running buffer. The gel was stained with SYBR gold (Invitrogen) 10,000X dilution in TBE buffer for 5 minutes. Gel images were captured using Syngene U:genius gel documentation instrument.

Blot analysis

Fifteen microliters of each sample were added to 5 µL of 4X SDS-PAGE loading buffer which contained β-mercaptoethanol. The samples were heat denatured for 5 minutes in a 90°C water bath prior to loading 18 µL onto a SDS-PAGE gel (4%stacking/7%resolving Biorad miniprotean apparatus). The gel was run at 75V while the samples were in the stacking gel and was increased to 100V once they entered the resolving gel. 1X Tris glycine was used as the running buffer. The gel was run until tracking dye (xylene cyanol) reached the bottom of the gel electrophoresis plates. The samples were transferred to a PVDF membrane for 30 minutes at 365 mAmps using Biorad miniprotean wet transfer apparatus. The membrane was washed in water 3X, followed by Tris buffered saline with 0.1% Tween (TBS-T) 3X. HS and Streptavidin-HRP conjugate were spotted in the upper left hand corner as controls followed by 1 hour incubation in TBS-T with 5% w/v non-fat dry milk. After washing with TBS-T 3X, a TBS-T solution with a 10,000X dilution of HRP conjugated streptavidin (Pierce) for 1 hour. The membrane was washed 3X in TBS-T followed by 3X in TBS. Next, the blot was developed with a 1X TBS solution with 0.1 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 1% H_2O_2 until staining was sufficient to visualize bands and controls. The membrane was washed with water 3X, dried, and scanned.

Filter binding assay

Filter binding assay experiments to determine the activity of T4 DNA polymerase were run in an adapted version from methods described previously[19]. A 15 µL aliquot of FBA master mix (1 mM dCTP, dATP, dTTP, and 10 µM [³H]-dGTP) was added to 15 µL

of each sample and incubated for 30 min at 37°C. The reaction was stopped by the addition of an equal amount of 0.5 M EDTA. The radioactive HS was collected onto DE81 anion exchange paper discs and allowed to dry. All DE81 discs were batch washed in 200 mL of 0.1 M disodium phosphate buffer five times, and the filters were allowed to dry at room temperature. Each disc was added to a vial with 7 mL of scintillation cocktail (Fisher) and radioactivity was counted using a liquid scintillation counter (Beckman LS5000TD). Data collected was analyzed using Microsoft Excel. After subtracting the background, the radioactivity of each sample was compared to the positive control (T4 DNA polymerase alone) to calculate % T4 DNA polymerase activity for three independent experiments.

RESULTS

Design of a binary nucleotide reagent for DNA polymerase

In this study we used commercially available DNA polymerase from bacteriophage T4 as a model enzyme to compare the inactivation efficiency and specificity of a traditional single-component reagent with a binary nucleotide reagent. Figure 2 illustrates the design of single component reagent for T4 DNA polymerase using only dTTP analog 1.
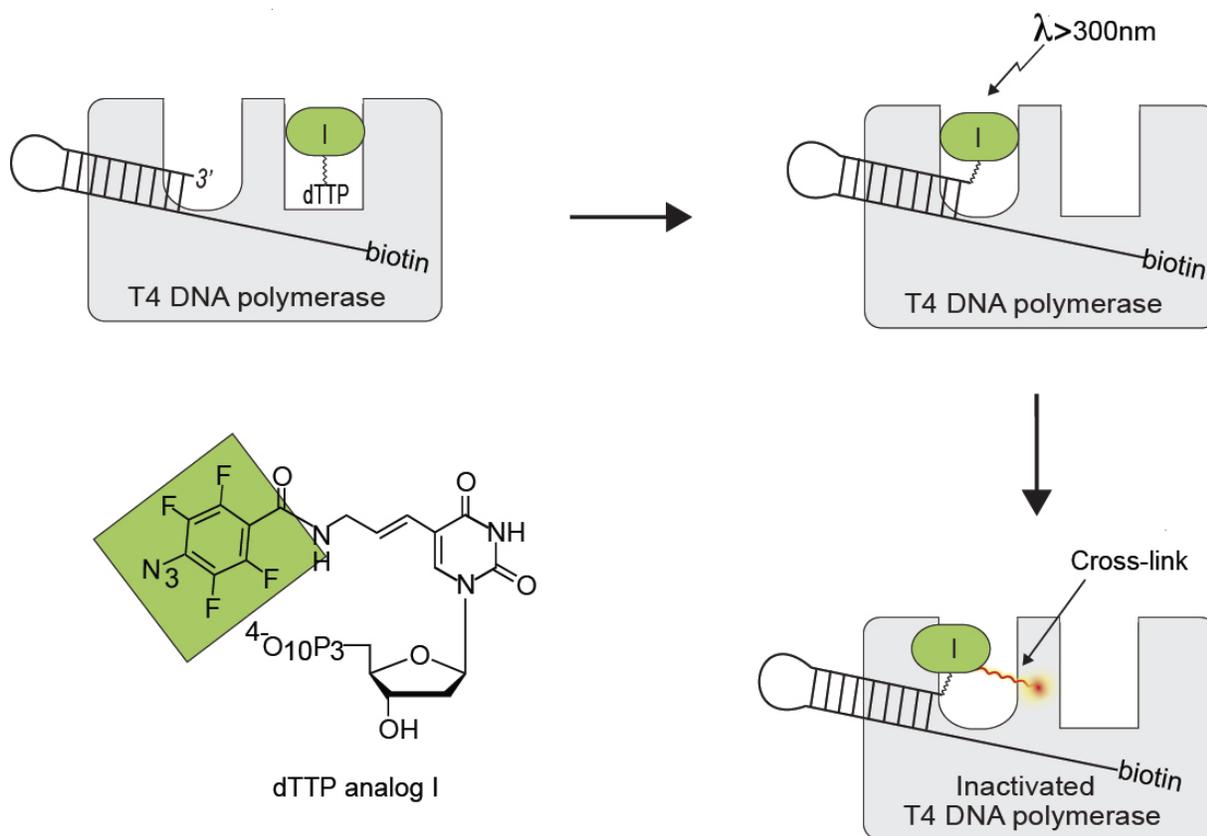

Figure 3: Traditional single-component reagent designed to inactivate T4 DNA polymerase.

The first component of the BNR is a DNA hairpin substrate with an arylazido group on its 3' end (pre-reactive group 1). Pre-reactive group I was introduced at the 3' end of the hairpin substrate (HS, Fig. 3) by DNA polymerase in the presence of dTTP analog I

(Fig. 3), which contained the 2,3,5,6-tetrafluoro-4-azido benzoyl group(green highlight in Fig. 3). This aromatic azide is known to form highly reactive intermediates after photolysis, which can cross-link to a broad variety of functional groups including amino acid side chains [20, 21]. Importantly, direct photolysis of pre-reactive group I occurs efficiently upon irradiation by light with λ >300 nm, but only at a low rate under irradiation by light with λ>350 nm. It was shown earlier that pyrene (pre-reactive group II, Fig. 2C) excited by UV light within 350-400 nm can transfer energy or an electron to pre-reactive group I facilitating its photolysis.
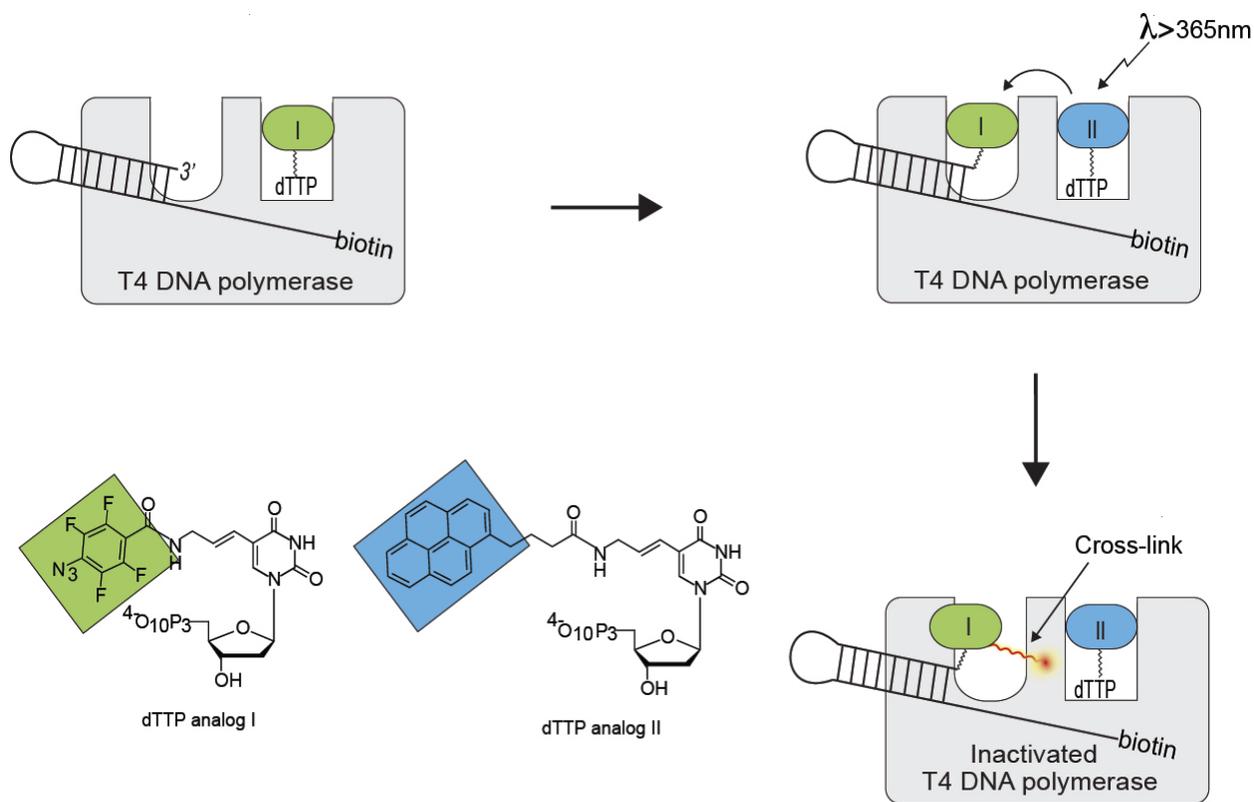

Figure 4: Design of a binary nucleotide reagent for inactivation of T4 DNA polymerase

When dTTP analog II binds in the dNTP-binding site, the two pre-reactive groups are situated within close proximity, allowing them to react. Upon irradiation by light λ > 350

nm, the photolysis of pre-reactive group I occurs predominantly in the tertiary complex DNA polymerase-HS-dTTP analog II, which corresponds to the BNR scheme (Fig. 4). Additionally, HS with dTMP analog I at the 3' end can function as a single component reagent with the use of irradiation λ>300 nm, allowing for a direct comparison between the efficiency of single-component and two-component reagents. Earlier, it was shown that pyrene-dependent activation of arylazides can be used for the detection of contacts between DNA substrates and DNA processing proteins, including DNA polymerases [22]. However, the approach was never applied for inactivation of DNA polymerases. This study is the first to experimentally demonstrate the inactivation of a DNA polymerase by a BNR.

Substrate Properties of dUTP analogs

After synthesis and purification of the dTTP analogs was complete, it was important to investigate whether the dTTP analogs could be used by T4 DNA polymerase as a substrate. Each analog was incubated with HS and T4 DNA polymerase (Fig. 5). HS was elongated by analog I (compare lanes 1 and 4) with slightly lower efficiency than by natural dTTP (lane 2). The addition of excess amount of four natural dNTPs resulted in the efficient elongation of both the HS with natural dTMP (lane 3) and the HS containing dTMP analog I on the 3' end (lane 5). dTTP analog II was also found to be a non-terminating substrate of T4 DNA polymerase (lanes 6, 7).
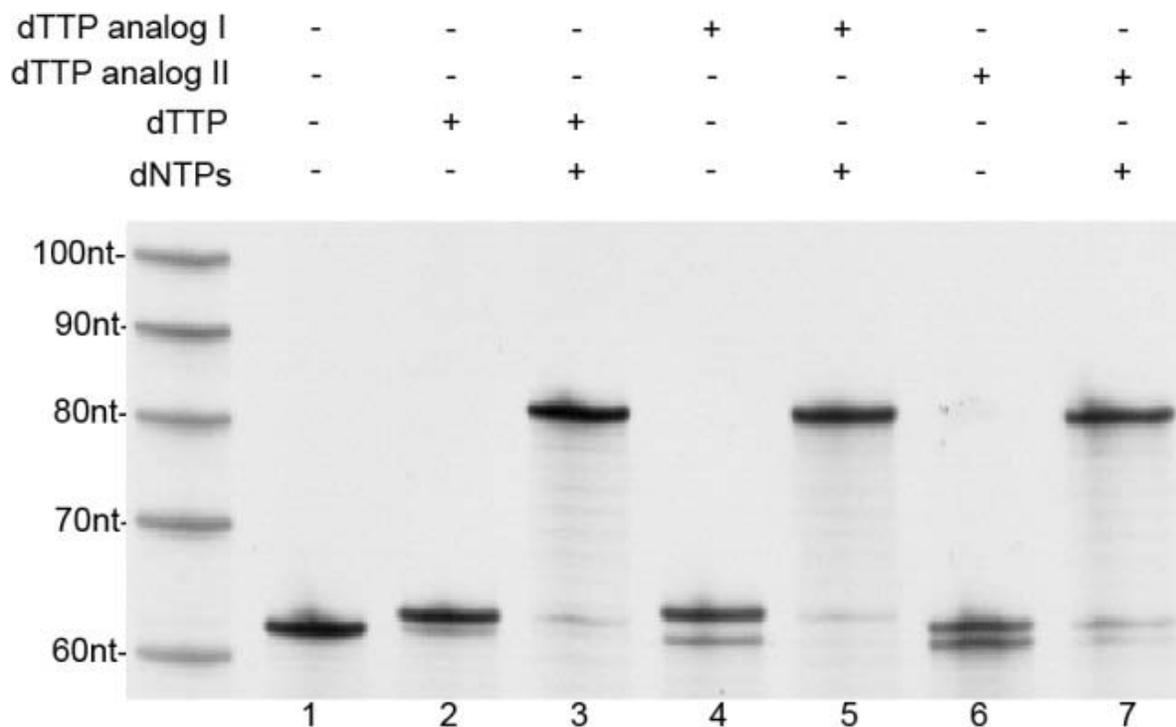
Figure 5: Urea PAGE analysis of the elongation products of DNA hairpin substrate (HS) by dTTP and dTTP analogs.

This result demonstrates that both dTTP analog I and II are substrates of T4 DNA polymerase, and that DNA synthesis is not terminated by the addition of the modified nucleotides to the 3' end. This data is consistent with the previous observations that C5-substituted pyrimidine triphosphates are good non-terminating substrates for other DNA polymerases [23].

BNR inactivates T4 DNA polymerase

To compare the performance of the traditional single-component inactivation scheme with the binary approach, T4 DNA polymerase was inactivated under two different conditions as shown in figures 3 and 4, respectively. First, the complex of T4 DNA polymerase with HS carrying pre-reactive group I was exposed to UV light with λ>300

nm. Under this condition, direct activation of pre-reactive group I corresponds to the classical single-component scheme. These conditions resulted in 96% loss of the polymerase activity (Fig. 6B, bar 7). The efficient inactivation of the DNA polymerase was also evident by the absence of the 81-nucleotide product of complete HS elongation (Fig 6A, lane 7). This result demonstrates that the single-component reagent designed in this study is able to efficiently inactivate DNA polymerase *in vitro*, when other biopolymers are not present. In an alternative inactivation setup, the binary scheme was used. After synthesis of photo-reactive HS, the second component of the binary nucleotide reagent, dTTP analog II, was added to the reaction mixture, followed by irradiation by light with $\lambda > 365$ nm. Under these conditions, pre-reactive group I is predominantly activated in the tertiary complex of DNA polymerase-HS-dTTP analog II (Fig. 4). These conditions resulted in an 88% loss of polymerase activity (Fig. 6B, bar 6), which was also observed as a loss in full elongation product of HS (Fig. 6A, lane 6). The lower inactivation efficiency of the BNR in comparison with the single-component reagent is a result of incomplete photolysis of pre-reactive group I, and may be improved by increasing the irradiation time. However, longer irradiation time cannot be used without compromising the BNR scheme by directly activating pre-reactive group I. Indeed, even at the irradiation time used, we observed a noticeable loss in polymerase activity even in the absence of dTTP analog II which reflects direct photolysis of pre-reactive group I by light $\lambda > 365$ nm (Fig. 6B, bar 4).
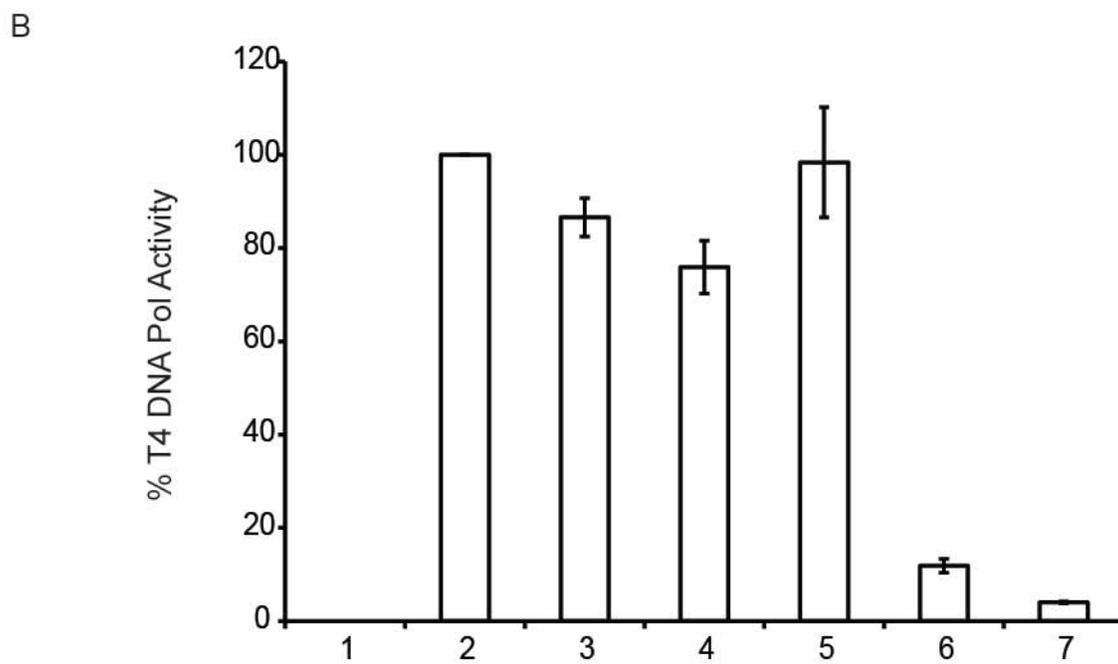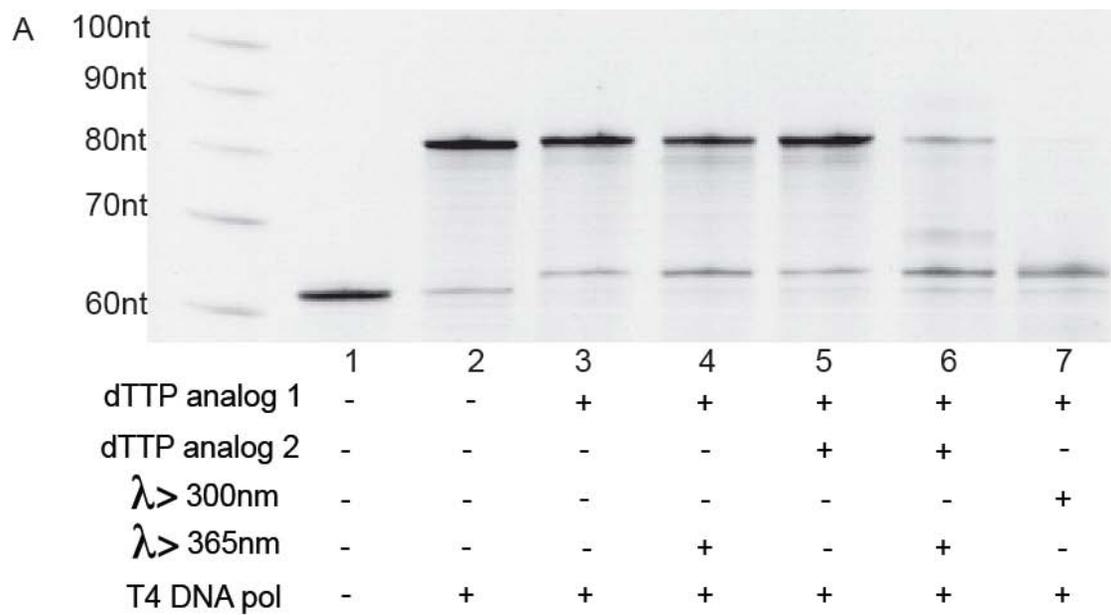
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Figure 6: Inactivation of T4 DNA polymerase by traditional single-component reagent and the binary nucleotide reagent.

BNR covalently modifies T4 DNA polymerase

In order to investigate if the inactivation of T4 DNA polymerase by both the traditional and BNR was indeed due to a covalent modification, we used denaturing SDS-PAGE and a blot procedure to analyze whether the HS was covalently attached to T4 DNA polymerase. The HS contains a biotin label on the 5' end, which allowed us to take advantage of the streptavidin-biotin interaction to determine the location of the HS. We found that there was a stable covalent product present both in the sample that corresponds to the binary reagent and traditional reagent (lanes 5 and 6, respectively). The crosslink product migrated near the 130kDa marker, which corresponds to the 112kDa T4 DNA polymerase with the ~19kDa HS covalently attached (Fig. 7). This data confirms that both the binary and single component reagent likely function through a covalent mechanism to inactivate T4 DNA polymerase.
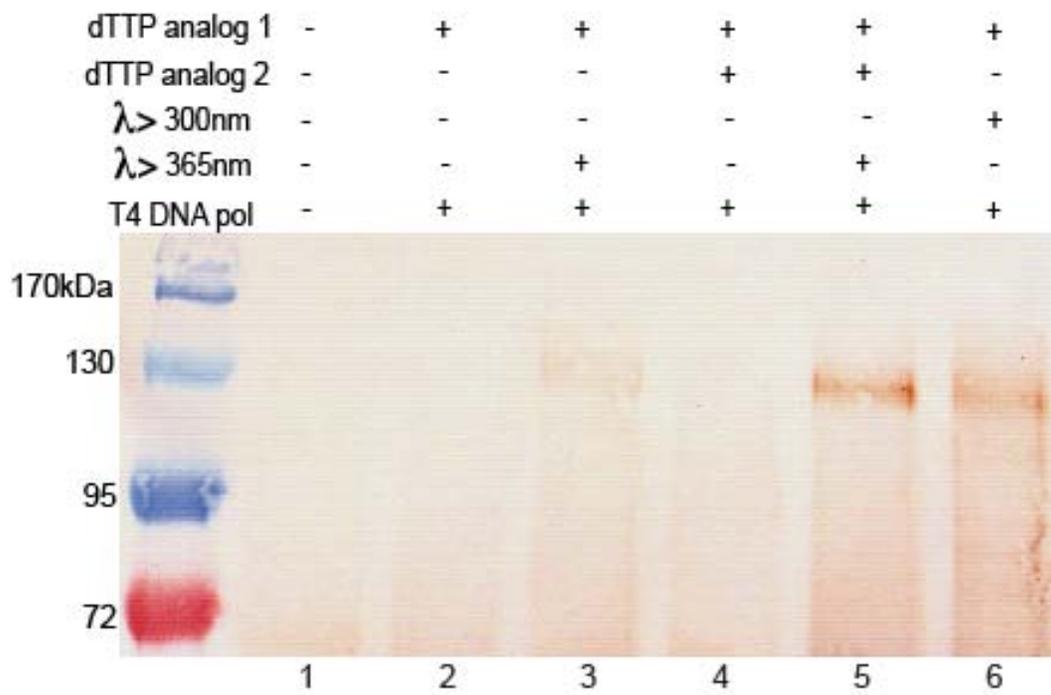
Figure 7: Blot analysis of HS-T4 DNA polymerase crosslink product.

BNR is more efficient then single reagent in the present on non-target protein

The perceived advantage of the single-component reagent observed in the absence of interfering biopolymers disappears in the presence of another non-specific protein, a situation more relevant to in vivo conditions. The experiments described above were repeated with the addition of various concentrations of another DNA-binding protein, T7 RNA polymerase. For the single component reagent (Fig. 8, black line), high concentrations of T7 RNA polymerase almost completely recovered the activity of the target T4 DNA polymerase, which reflects a decrease in inactivation efficiency. At the highest concentration of T7 RNA polymerase, the single component reagent reduced the activity of the target enzyme by only 14%. The dramatic loss of the inactivation efficiency by single-component reagent is presumably a result of the competitive non-specific binding of the photo-reactive HS to T7 RNA polymerase, which reduces the availability of the active reagent for the target T4 DNA polymerase.
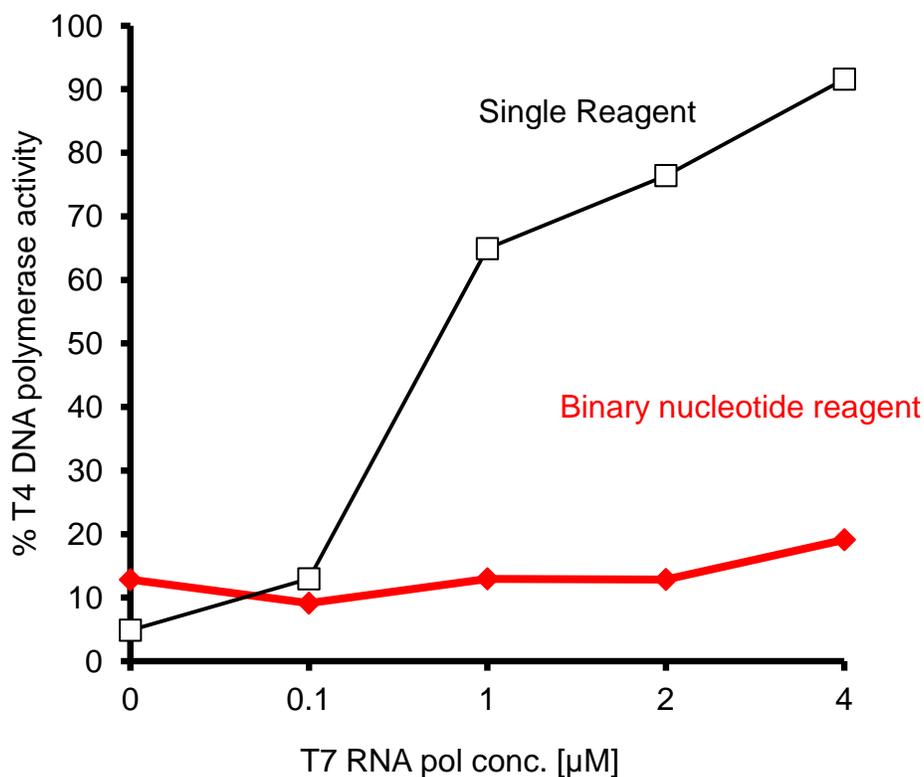
Figure 8: Effect of non-target T7 RNA polymerase concentration on T4 DNA polymerase inactivation by binary reagent or single reagent.

In contrast, the presence of the non-specific protein had almost no effect on the BNR (Fig. 8, red line). This is likely due to the preferential activation of the photo-reactive HS only in the complex with the specific target (T4 DNA polymerase), but not in solution or in complex with T7 DNA polymerase. Such conditions preserve the photo-reactive HS to be utilized in only the specific tripartite complex (photo-reactive HS-DNA polymerase-dTTP analog II), an effect which is the basis for the superior performance of the BNR.

DISCUSSION

Covalent inhibitors of proteins and nucleic acids are used as tools for elucidation of the details of biological processes as well as for treatment of human diseases [8, 10, 20, 24, 25]. The significance of covalent inhibitors is illustrated by the fact that 39 such compounds are currently used as drugs [26]. Most of the known covalent drugs, however, were identified not by rational design but by serendipity [26]. Covalent inhibitors can dramatically increase the inhibitor-target residency time allowing for a lower effective dose. The lower concentration of the drug in serum reduces side effects. However, the major drawback of covalent inhibitors is the reactivity towards nonspecific biopolymers, which are normally present at higher concentrations than a specific target. This results in high toxicity for covalent drugs. For example, due to high toxicity, the DNA alkylating N-mustards (chlorambucil, bendamustine and cyclophosphamide) are only prescribed against terminal diseases such as chronic leukemia [27, 28].

In this study, we propose a general approach for rational design of covalent inhibitors that addresses the problematic low specificity of current approaches. In the binary approach, both ligand analogs independently (or semi-independently in the case of cooperative ligand binding) interrogate a biopolymer of interest. Therefore, nonspecific binding of each individual ligand to a non-target molecule is reversible, thus allowing the reagent to re-bind the correct target. However, the additional requirement does decrease the efficiency in a pure in vitro system as shown in Fig. 6, but the improved

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specificity allows the binary reagent to function more efficiently in the presence of non-target protein (Fig. 8).

The binary nucleotide reagent used in this study was activated by light irradiation. The reaction can be triggered at a desired time, which corresponds to a certain phase of a metabolic process. This chemical reaction initiation method is advantageous for the study of complex molecular interplay in vitro or in cell culture[29-31]. Therefore, the binary reagent described here can be applied for the study of DNA replication and repair processes that involve DNA polymerases, such as DNA replication and repair. Light dependence, however, is not beneficial for drug development purposes. Light-independent chemical pre-reactive groups are required to create therapeutically significant BRs.

The application of the binary approach is not limited to targeting DNA polymerases. For example, a BR that targets a specific mRNA or microRNA can be envisioned. In this approach two oligonucleotides complementary to the abutting positions of the target nucleic acid are conjugated with the pre-reactive groups. Hybridization of this BR to the specific fragment of a target RNA or DNA will result in activation of BR and covalent modification of the nucleic acid. Additionally, proteins that bind only one ligand can be targeted using a derivative of the natural ligands as one component and an aptamer bearing the second pre-reactive group as the second component of BR. Therefore, the approach is potentially applicable to a broad variety of biological targets.

CONCLUSION

The results presented here demonstrate the potential of BRs to maintain inactivation of a targeted enzyme even in the presence of a non-targeted protein. The light activated reagent described in this study might be useful for studying DNA replication and repair machinery by affinity cross-linking techniques. Generally, this study shows the binary reagent approach holds promise to become a general strategy for designing highly specific covalent inhibitors.

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