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Two-component covalent inhibitor

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Abstract

Inhibitors that covalently damage proteins or nucleic acids offer great potency, but are difficult to rationally design and suffer from poor specificity. Here we outline a general concept for constructing covalent inhibitors, called the two-component covalent inhibitor (TCCI). The approach takes advantage of two ligand analogs equipped with pre-reactive groups. Binding of the analogs to the adjacent sites of a target biopolymer brings the pre-reactive groups in close proximity and causes their interaction followed by covalent damage of the target. In the present study we used light-activated pre-reactive groups to inactivate a DNA polymerase. It was found that the efficiency of a traditional single-component inhibitor was greatly reduced in the presence of a non-target protein, while the TCCI was not significantly affected. Our findings suggest that TCCI approach has advantages in inactivation of biopolymers in complex multi-component systems.

Keywords

Covalent inhibitors; irreversible inhibitors; drug design; DNA polymerase; enzymes; nucleotide analogs; aryl azide; pyrene

Introduction

Efficient and specific inhibition of biopolymers (proteins, DNA or RNA) in complex biological mixtures is used both in drug development and in fundamental studies of complex biologic processes such as metabolic pathways, DNA replication and repair, and signaling cascades [1–7]. Traditional rational approaches for the development of inhibitors focus on the discovery of inert small molecules which reversibly bind a biopolymer. Typically, the non-covalent interaction between inhibitors and targets produces low to moderate affinity and only reversible inhibition [5,6]. Alternatively, inhibitors that interact covalently with their target can achieve higher affinity and irreversible inhibition. The significance of covalent inhibitors is illustrated by the fact that 39 such compounds are currently used as drugs [8]. The most successful covalent inhibitors were, however, discovered serendipitously [8], as there is no straightforward technology that allows design of such agents. There persists a need for a general strategy to develop specific covalent inhibitors which is translatable for use with many target types. Herein, we outline a new concept, named ‘two-component covalent inhibitor’ (TCCI) that meets this need. Furthermore, we

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provide experimental evidence that TCCI has an important advantage of maintaining the inhibition capability even in the presence of non-specific proteins.

The proposed approach takes advantage of two ligands conjugated with two pre-reactive groups (Figure 1a) 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. The reactive intermediates covalently cross-link to or otherwise covalently modify the biopolymer, thus irreversibly inhibiting its biological function. The improved specificity of the approach is achieved due to the involvement of two ligands, which may independently or semi-independently interrogate biopolymers. This hypothesis is quantitatively supported by analysis of the corresponding kinetic schemes (Figure 1b). Traditional single-component covalent inhibitors can be characterized by a kinetic scheme that resembles the classical Michaelis-Menten scheme, where K is the dissociation constant (K_d) for the biopolymer-inhibitor complex (**BI**) and k is the reaction constant between the biopolymer and the reagent. Under the assumption of equilibrium, the relative specificity of the two approaches is expressed by the ratio of the initial rates for specific to nonspecific reactions (v_s/v_n), which we call here a 'specificity factor' σ . It can be expressed by eq. 1 (detailed derivation is in supporting materials, Scheme S1). The interaction of a TCCI with a biopolymer is characterized by two dissociation constants K_1 and K_2 , for binding of the two ligand analogs (**I₁**) and (**I₂**), respectively. The tripartite complex (**BI₁I₂**) undergoes a reaction that is characterized by the rate constant k . The specificity factor σ is defined by eq. 2 (for detailed explanation see Scheme S1). Houk et al. reported that dissociation constants for non-specific ligand-protein binding range from 10^{-1} to 10^{-5} M, while specific interactions range from $\sim 10^{-6}$ – 10^{-16} M [9]. If we assume that the inhibitor concentration is $\sim 10^{-6}$ M (**I₀** = **I₁₀** = **I₂₀** = 10^{-6} M), which is close to our experimental conditions (see below), while $K_s = K_{Is} = K_{2s} = 10^{-9}$ M, and $K_n = K_{In} = K_{2n} = 10^{-5}$ M, parameter σ is estimated to be 11 for the single-component inhibitor (eq.1) and 111 for the TCCI approach (eq.2). Therefore, TCCI might be about 10 times more specific than a conventional single-component inhibitor. This model suggests that TCCI scheme can result in efficient inactivation of a specific target even in the presence of excess amounts of non-targeted molecules. Below we demonstrate that indeed TCCI can inactivate a DNA polymerase even in the presence of a non-targeted protein.

Materials and Methods

Analog of deoxythymidine-5'-triphosphate (dTTP) were synthesized from dUTP (Sigma-Aldrich, Missouri) and characterized as described previously [10–12]. For inactivation experiments, samples containing 1 nM T4 DNA polymerase and 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 fifteen minutes at 37°C in the presence of 10 μ M dTTP analog I. For specificity experiments, 0.1, 1, 2, or 4 μ M T7 RNA polymerase was added to the reaction mixture. dTTP analog II (200 μ M) was added, and the samples were irradiated for 40 min in a Spectroline UV crosslinker. Glass filters (Hoya L-37 for $\lambda > 365$ nm or standard electrophoresis glass plate for $\lambda > 300$ nm) were used to allow transmittance of the desired wavelengths. Samples were split and analyzed by urea denaturing polyacrylamide gel electrophoresis and filter binding assay, after incubation with natural dNTPs. For the filter binding assay [3H]-dGTP (Moravek) was also added, and the HS was collected using DE81 anion exchange paper followed by counting in a liquid scintillation counter (Beckman LS5000TD). Data collected was analyzed using Microsoft Excel. T4 DNA polymerase activity was calculated as the mean % activity from three independent experiments. The % activity was calculated by comparing each sample to the positive control, after subtracting background radioactivity.

Results

Development of inhibitors for DNA polymerases is essential for anti-viral and anticancer therapies as well as for the study of DNA replication and repair [13–16]. DNA polymerases operate with two substrates: a DNA primer-template complex and nucleoside triphosphates (dNTPs), and, therefore, represent an ideal target for TCCI approach. In this study we used commercially available DNA polymerase from bacteriophage T4 as a model enzyme to compare a single-component covalent inhibitor with the TCCI strategy. The overall idea was to introduce a pre-reactive group at the 3' end of the harping substrate (HS) as shown in Fig. 2a. The group can be directly activated to inactivate DNA polymerase (Fig. 2a, right) or it can be activated by the presence of pre-reactive group II (Fig. 2b), which corresponds to TCCI scheme. Second pre-reactive group can be delivered as a part of dNTP analog, which binds to DNA polymerase. The two dTTP analogs used to create a TCCI are shown in Fig. 2c. dTTP analog I contained 2,3,5,6-tetrafluoro-4-azido benzoyl group (pre-reactive group I, Fig. 2c, green). 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 [17–19]. dTTP analog I can be used as a substrate of T4 DNA polymerase (Fig. S1), which introduces pre-reactive group I at the 3' end of the substrate (Fig. 2a, HS). Only one dTMP residue can be incorporated in 3' end of HS, as dictated by the template sequence. Direct photolysis of group I occurs efficiently upon irradiation by light with $\lambda > 300$ nm, but only at a low rate upon irradiation by light with $\lambda > 350$ nm. dTTP analog II contained pyrene (pre-reactive group II, Fig. 2C). When excited by UV light with λ 350–400 nm group II can transfer energy or an electron to group I facilitating its photolysis [19]. Thus, when dTTP analog II binds in the dNTP-binding site, the two pre-reactive groups are situated within close proximity. Importantly, group II can activate group I only when the groups are in close proximity to each other. Upon irradiation by light $\lambda > 350$ nm, the photolysis of pre-reactive group I occurs predominantly in the ternary complex of DNA polymerase-HS-dTTP analog II, which corresponds to the TCCI scheme (Fig. 2b). Alternatively, pre-reactive group I can be directly activated in the absence of group II upon irradiation by UV light with $\lambda \sim 300$ –350 nm (Fig. 2a). These conditions constitute the activity of a single component covalent inhibitor. DNA polymerase itself was not affected by light irradiation under the experimental conditions used (data not shown).

Further, we evaluated the efficiency and specificity of T4 DNA polymerase inactivation by these two approaches in the absence of non-target proteins. Under these conditions, the single-component scheme efficiently inactivated DNA polymerase. We observed almost complete loss of the full elongation product of HS (Fig. 3a, lane 7), which correlated with the loss of 96% of the polymerase activity measured by the filter binding assay (Fig. 3, bar 7). The observed inhibition is a result of the covalent modification of T4 DNA polymerase within its active site. Indeed, under experimental conditions a stable cross-link of HS to the protein was detected (Fig. S2).

TCCI scheme resulted in less efficient inactivation. After synthesis of photo-reactive HS, dTTP analog II was added to the reaction mixture, followed by irradiation by light with $\lambda > 365$ nm. Under these conditions, only 87% of polymerase activity was inhibited (Fig. 3b, bar 6), which correlated with the observed significant loss of the HS elongation product (Fig. 3a, sample 6). The lower inactivation efficiency of the TCCI in comparison with the single-component inhibitor is likely due to the incomplete photolysis of pre-reactive group I.

The perceived potency advantage of the single-component inhibitor disappears in the presence of a non-target protein, a situation more relevant to *in vivo* conditions. The experiments described above were repeated with the addition of another DNA-binding protein, T7 RNA polymerase, at various concentrations. Under these conditions, the single

component inhibitor was less effective as the concentration of the non-target protein increased (Fig. 4). The dramatic loss of the inactivation efficiency observed for the single-component inhibitor was likely due to the competitive non-specific binding of the HS to T7 RNA polymerase, which reduced the availability of the available inhibitor. In contrast, the presence of even 4000 times excess of non-specific T7 RNA polymerase had little or no effect on TCCI scheme (Fig. 4, red line). This is likely due to the preferential activation of the group I 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 reactive HS for reaction in the specific tripartite complex (photo-reactive HS-DNA polymerase-dTTP analog II). The results demonstrate that TCCI maintains the inactivation potential even in the presence of great excess of a non-target protein, an advantage over single component reagent that is significant for application of TCCIs in complex mixtures of biopolymers.

Discussion

In this study, we propose a general approach for rational design of covalent inhibitors. Covalent inhibitors of proteins and nucleic acids are used as tools to elucidate the details of biological processes as well as to treat human diseases [8,20,21]. The major drawback of covalent inhibitors is their reactivity towards nonspecific biopolymers, which are normally present at higher concentrations than a specific target. This may result in high toxicity of covalent drugs. For example, due to high toxicity, the DNA alkylating N-mustards (chlorambucil, bendamustine and cyclophosphamide) are only prescribed for patients with terminal diseases such as chronic leukemia [22,23]. On the other hand, there are at least 39 covalent inactivators that are currently used as drugs [8]. However, the covalent mechanism of action of most of these drugs was discovered after they showed favorable clinical outcomes. A general strategy for rational design of specific covalent inhibitors may provide impetus for the discovery of new drugs.

In this study, we propose a general approach for rational design of covalent inactivators that has a potential to address the problematic low specificity of covalent inhibitors. In the TCCI scheme binding of two ligands is required to produce a reagent that inactivates a target. In this case if one of the ligands is non-specifically bound to a non-target protein there is only negligible chance of binding the second ligand to the same protein at an adjacent position to the first ligand. Therefore, binding of HS by T7 RNA polymerase II by individual ligand to a non-target molecule is reversible, which allows the inhibitor to re-bind the specific target.

The TCCI used in this study was designed based on the azide-pyrene photo-reactive pair characterized earlier [12,19]. Up to the best of our knowledge, there are no published reports of using this photo-reactive or any other pair of pre-reactive groups for inactivation of proteins. In this study, we have converted a photo-reactive azide-pyrene pair into a covalent inhibitor and demonstrated that the TCCI approach has an important advantage as compared to traditional single-component inhibitors in ability to inactivate a target protein when 4000 times excess of a non-target protein is added. We explain this observation by improved specificity of TCCI approach as illustrated by the kinetic scheme in Fig. 1a.

The light-dependent TCCI used in this study can be triggered at any desired time or metabolic stage. This chemical reaction initiation method is advantageous in studying complex molecular interplay in vitro or in cell culture [23,24]. Light-dependent reagents are also known to be used in photodynamic therapy of cancer [25–28]. Light dependence, however, is not particularly useful in the context of TCCI, since this approach takes advantage of reagent formation on a target and does not require additional spatio-temporal control provided by light. Light-independent chemical pre-reactive groups would be beneficial for therapeutically significant TCCIs. Such functional groups should satisfy the

following criteria. (i) They should be inert toward water and biological molecules under physiological conditions. (ii) They should efficiently react with each other when brought in close proximity and properly oriented. (iii) Finally, the reaction should produce highly reactive intermediates, which are capable of cross-linking or otherwise damaging the functional groups of biopolymers (amino acid side chains, peptide bonds, nucleotides or phosphodiester bonds). Future development of TCCI should be focused on discovery of new light-independent pairs of pre-reactive groups for TCCI approach, on targeting the proteins and nucleic acids, as well as testing the approach in cells cultures and in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
TCCI	two-component covalent inhibitor
dTTP	deoxythymidine-5'-triphosphate
HS	hairpin substrate

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b) Single-component Irreversible Inhibitor:

Two-component Irreversible Inhibitor:

$$\sigma \approx \frac{I_{1o} \times I_{2o} + K_{2n} \times I_{1o} + K_{1n} \times K_{2n}}{I_{1o} \times I_{2o} + K_{2s} \times I_{1o} + K_{1s} \times K_{2s}} \quad (\text{eq. 2})$$

Figure 1.

Principle scheme of 'two-component covalent inhibitor' (TCCI). a) General scheme of inactivation of a biopolymer by two ligand analogs conjugated with pre-reactive groups (blue and green). Jagged contour of the biopolymer on the right indicates the loss of its biological activity. b) Kinetic schemes for single component and two-component covalent inhibitors. I_o , I_{1o} and I_{2o} are initial concentrations of the inhibitors; K_s , K_{1s} and K_{2s} are dissociation constants for specific, while K_n , K_{1n} and K_{2n} for non-specific complexes. Equations 1 and 2 express the relative inhibition specificity factor (σ) for single-component and TCCIs, respectively.

Figure 2. Single-component and two-component irreversible covalent inhibitors for DNA polymerase. a) Single-component covalent inhibitor scheme. b) TCCI scheme. c) dTTP analogs I and II. Pre-reactive group I (2,3,5,6-tetrafluoro-4-azido benzoyl) and pre-reactive group II (pyrene) are shown in green and blue, respectively.

Figure 3.

Inactivation of T4 DNA polymerase by the single covalent inhibitor or by TCCI in the absence of a non-target protein. a) Urea-PAGE analysis of HS elongation. The length of the starting hairpin substrate (HS) was 61 nucleotides (plus biotin), while the product of its full elongation contained 81 nucleotides (plus biotin). b) T4 DNA polymerase activity based on filter binding assay. Bars represent average values of three independent experiments with a single standard deviation.

Figure 4. Inactivation of T4 DNA polymerase (1 nM) by a traditional single-component covalent inhibitor (black line) or by TCCI (red) in the presence of different concentrations of non-target T7 RNA polymerase. The data represents the average of three independent experiments (see Figure S3) with a single standard deviation.