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DEVELOPMENT OF A NOVEL ASSAY FOR HUMAN TYROSYL DNA PHOSPHODIESTERASE 2

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Abstract

Tyrosyl DNA phosphodiesterase 2 (TDP2), a newly discovered enzyme that cleaves 5'-phosphotyrosyl bonds, is a potential target for chemotherapy. TDP2 possesses both 3'- and 5'-tyrosyl-DNA phosphodiesterase activity, which is generally measured in a gel-based assay using 3'- and 5'-phosphotyrosyl linkage at the 3'- and 5'- ends of an oligonucleotide. To understand the enzymatic mechanism of this novel enzyme, the gel-based assay is useful, but this technique is cumbersome for TDP2 inhibitor screening. For this reason, we have designed a novel assay using p-nitrophenyl-thymidine-5'-phosphate (T5PNP) as a substrate. This assay can be used in continuous colorimetric assays in a 96-well format. We compared the salt and pH effect on product formation with the colorimetric and gel-based assays and showed that they behave similarly. Steady-state kinetics studies showed that the 5'-activity of TDP2 is 1000-fold more efficient than T5PNP. Tyrosyl DNA phosphodiesterase 1 (TDP1) and human AP-endonuclease 1 (APE1) could not hydrolyze T5PNP. Sodium orthovanadate, a known inhibitor of TDP2, inhibits product formation from T5PNP by TDP2 (IC₅₀ = 40 mM). Our results suggest that this novel assay system with this new TDP2 substrate can be used for inhibitor screening in a high-throughput manner.

Keywords

DNA Repair; enzyme assay; kinetics; TDP2; high throughput screening

1. INTRODUCTION

DNA topoisomerases I and II (TopI and TopII) are important for maintaining normal DNA topology in cells and are popular targets for the anticancer drugs known as topo-poisons, which generate an irreversible topo-DNA crosslink. [1, 2]. The repair of such lesions requires at least two steps: removal of the trapped enzyme from the 3'- or 5'-DNA end

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followed by DNA religation. The best characterized pathway for removal of TopI-DNA adducts from the 3'-DNA end involves the enzyme tyrosyl-DNA phosphodiesterase 1 (TDP1). Effective inhibition of TDP1 can improve the efficacy of TopI poisons commonly used in anticancer therapy [3, 4].

The TopII family is another important class of topoisomerases that produces 5' DNA-protein crosslinks simultaneously in both strands [5, 6]. Drugs targeting TopII act by preventing the religation of DNA, thereby producing covalent TopII-DNA complexes that lead to double-strand breaks. One major concern is that drugs targeting TopII may lead to secondary malignancies [7, 8]; however, the repair of TopII-DNA complexes is poorly understood. Recently, a human 5'-tyrosyl-DNA phosphodiesterase (named as tyrosyl-DNA phosphodiesterase 2 or TDP2) has been identified for the excision of TopII-DNA adducts [9–10]. Like TDP1, it is likely that effective inhibition of TDP2 can be targeted to improve the efficacy of TopII poisons [10]. It has been shown that the knockdown/knockout of TDP2 in A549 and DT40 cells increased sensitivity to the TopII-targeting agent etoposide, demonstrating that TDP2 can be a target for adjuvant chemotherapy. Therefore, TDP2 inhibitor screening, especially in a high-throughput (HT) manner, should further aid drug discovery.

In the present study, we demonstrate that p-nitrophenyl-thymidine-5'-phosphate (T5PNP), a well-established substrate for snake venom phosphodiesterase, also acts as a substrate for TDP2. The advantage of a T5PNP assay over the currently utilized gel-based assay is that it can be used in a 96-well plate format. Furthermore, when evaluating the effects of salt and pH on enzymatic activity, the 96-well assay showed results comparable to those of similar experiments performed using the gel-based assay. Steady-state kinetics also showed that TDP2 works more efficiently on the traditional 5'-phosphotyrosyl substrate than on T5PNP. TDP1 and human AP-endonuclease 1 (APE1), are the closest relatives of TDP2. None of the enzymes were able to form product from T5PNP, so it is not a non-specific general phosphodiesterase substrate. Sodium orthovanadate, a known inhibitor for TDP2, also inhibits hydrolysis of T5PNP by TDP2. Our results suggest that this assay system with this novel substrate can be useful in HT inhibitor screening.

2. MATERIALS AND METHODS

The chromogenic substrate T5PNP was bought from Sigma-Aldrich, St. Louis, MO.

2.1 Cloning and Purification of Recombinant human TDP2 (TDP2)

Wild-type (WT) TDP2 was purified as previously described [9].

2.2 Preparation of Oligonucleotide Substrates

3'-phosphotyrosyl- and 5'-phosphotyrosyl-containing 18-mer gel-purified oligonucleotides with the sequence 5'-TCCGTTGAAGCCTGCTTT-Y-3' (oligo 1) or 5'-Y-TCCGTTGAAGCCTGCTTT-3' (oligo 2) were purchased from Midland Certified Reagent Company (Texas, USA). The complementary 18-mer (oligo 3, 5'-AAAGCAGGCTTCAACGGA-3') or 19-mer (oligo 4, 5'-GAAAGCAGGCTTCAACGGA-3') oligonucleotides were purchased from Gene Link (Hawthorne, NY). The 3'-phosphotyrosyl oligonucleotide was labeled at the 5' end using T4 polynucleotide kinase and ^{32}P -ATP and annealed to the complementary oligonucleotide to prepare ^{32}P end-labeled duplex oligonucleotide (3-sub, oligo1 annealed with oligo 3), as described previously [11]. The 5'-phosphotyrosyl oligonucleotide was labeled at the 3' end using [^{32}P]-dCTP and Klenow DNA polymerase. For the 19-bp double-stranded 5'-phosphotyrosyl oligonucleotide, the 5'-phosphotyrosyl substrate was annealed with a 19-bp

complementary oligonucleotide, and the resulting 1-bp 5' overhang was filled in with [^{32}P]-dCTP and Klenow DNA polymerase (5-sub, oligo 2 annealed with oligo 4) [9].

2.3 Development and Optimization of the Chromogenic Assay for TDP2

The enzymatic reactions were performed in 96-well plates in assay buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM Mg^{2+} , 50 mM KCl and 100 g/ml BSA at 37°C. Reactions consisted of a final volume of 60 μl containing different concentrations of TDP2 (0–180 nM), with substrate ranging from 0–7.5 mM in each well. The continuous changes in absorbance at 415 nm were monitored for 20–30 min using a Synergy HT microplate reader (Bio-tek Instruments, Inc, VT). The extinction coefficient (ϵ) of p-nitrophenol was determined to be $15,000 \text{ M}^{-1}\text{cm}^{-1}$ under the assay conditions. The formation of product, i.e., p-nitrophenol, was calculated from the absorbance at 415 nm using the equation $\Delta A = \epsilon \cdot \Delta C \cdot l$ (A, absorbance; ϵ , molar extinction coefficient; C, concentration; l, path length).

2.4 Dependence of TDP2 Activity on Ionic Strength and pH

The purified TDP2 proteins (45 pM) were individually incubated with 40 nM labeled double-stranded 5'-phosphotyrosyl oligonucleotide substrate (5-sub) with buffer that differed in pH (7–10) and salt concentration (0–200 mM KCl). For the 3'-activity assay, 1.4 nM labeled double-stranded 3'-phosphotyrosyl oligonucleotide substrate (3-sub) was incubated with 180 nM TDP2 protein as described above. The reaction mixture was then mixed with 20 μl of loading buffer, containing $1\times$ DNA dye (diluted from blue-orange $6\times$ loading dye; Promega, Madison, WI) and 45% formamide, and was heated at 95°C for 5 min. The samples were then resolved by sequencing gel electrophoresis (Model S2, Life Technologies, Rockville, MD) at 50°C, using gels that contained 20% polyacrylamide (Acrylamide:Bisacrylamide=29:1) and 7 M urea. Radioactivity from the incised oligonucleotides was quantified by exposing the gel to x-ray films and measuring the band intensities using an imager (Chemigenius Bioimaging System) with quantification software (Syngene Inc., San Diego, CA). For the activity assay using T5PNP as a substrate, 1.25 mM T5PNP was incubated with 180 nM TDP2 protein in the presence of different concentrations of salt and pH for 30 min at 37°C in a buffer, as described above.

2.5 Steady-State Kinetic Study

The TDP2 enzyme (45 pM) was incubated with 5-sub (40–140 nM) for 3 min to measure 5'-phosphotyrosyl steady-state parameters. TDP2's 3'-phosphotyrosyl activity was extremely low. In a gel-based assay, it was not possible to determine reliable steady-state parameters under conditions that follow the Michaelis-Menten equation for TDP2. For the T5PNP substrate steady-state parameters determination, 30 nM TDP2 enzyme was incubated with T5PNP (0–80 mM) for 15 min. The formation of the reaction product p-nitrophenol was monitored at 415 nm and quantified as described in the activity assay section.

2.6. Specificity of the chromogenic substrate

To check the specificity of the T5PNP as a substrate we have incubated different phosphodiesterases [TDP2 (60 nM), APE1 (180 nM) and TDP1 (120–240 nM)] individually with T5PNP (25 mM) for 20 minutes at 37°C. Also we have incubated sodium orthovanadate (0–134 mM), a known TDP2 inhibitor, with T5PNP and TDP2 under the conditions described above. The formation of the reaction product p-nitrophenol was monitored at 415 nm and quantified as described in the activity assay section.

3. RESULTS

3.1 Identification of the Chromogenic Substrate

To develop a chromogenic assay for TDP2, we chose T5PNP as the substrate. This compound contains a phosphodiester bond between the phosphate group at the 5' position of thymidine and the hydroxy group of the p-nitrophenol, that mimics the phosphodiester bond in the topoisomerase II–DNA complex. Hydrolysis of the phosphodiester bond in T5PNP releases free p-nitrophenol that absorbs light at 415 nm (Fig. 1). This compound is sold by Sigma-Aldrich as a potent substrate for snake venom phosphodiesterase.

3.2 Development and Optimization of a Chromogenic Assay for TDP2

Previously, the substrate used for TDP2 activity was an oligonucleotide whose 5'-phosphate was conjugated to the TopII active site tyrosine [9, 10]. Substrate and product were subsequently separated by denaturing gel electrophoresis. We chose to develop a chromogenic enzymatic assay for TDP2 using T5PNP (molecular weight 465 Da) as a substrate. The TDP2 enzymatic activity was continuously monitored as an increase in absorbance at the wavelength of 415 nm during chromophore (i.e., p-nitrophenol) release upon hydrolysis of the 5'-phosphodiester bond (Fig. 1 & Fig. 2). This chromogenic assay was easily adapted to a 96-well plate format to facilitate the high-throughput screening of inhibitors. When enzyme reactions were conducted with varying protein concentrations (0–180 nM TDP2) with a fixed amount of T5PNP (1.25 mM) or varying T5PNP concentration (0–7.5 mM) with a fixed amount of TDP2 (180 nM) in Tris buffer at pH 7.5, the changes in absorbance at 415 nm showed a linear relationship in a time-dependent manner (Fig. 2A, B).

3.3 Dependence of TDP2 activity on pH and salt

The TDP2 activity was then measured in buffers adjusted to different pH levels (6.7–10) (Fig. 3A) and different salt concentrations (0–200 mM KCl) (Fig. 3B). Similar results were obtained for all of the substrates. Maximum activity was obtained for 0–25 mM of KCl at a pH of 6.7 to 7. Higher salt concentrations reduced the activity of TDP2 towards different substrates.

3.4 Steady-State Kinetic Study

We further determined the steady-state parameters for TDP2 towards T5PNP and for the 5' substrate. T5PNP steady-state parameters were measured in a 96-well plate in a 60 μ l volume during the linear increase period of product formation. The steady-state experiments showed an apparent K_m of 54 mM and k_{cat} of 35 sec^{-1} for T5PNP. The k_{cat}/K_m , an approximate measurement of enzyme efficiency, was 0.002 $\text{hr}^{-1} \text{ nM}^{-1}$ for T5PNP. Our results indicated that TDP2 has a higher efficiency towards 5-sub than T5PNP (Table 1) (Supplementary Fig S1 and S2). We did not determine steady state parameters for 3-sub because of TDP2's extremely weak activity towards 3-sub (Supplementary Fig S3).

3.6 Specificity of the chromogenic substrate

We further checked if other phosphodiesterases can hydrolyze T5PNP. Our results showed that APE1 and TDP1 could not produce p-nitrophenol by hydrolyzing T5PNP and thus, it has specificity towards TDP2 (Fig. 4A). Sodium orthovanadate, a known inhibitor of TDP2 can effectively inhibit the product formation from T5PNP by TDP2 with an IC_{50} of 40 mM (Fig. 4B).

4. DISCUSSION

TopII has become an enzyme of major interest because it is targeted by anticancer drugs that are routinely used in the clinic, such as etoposide and doxorubicin [12]. Most clinically active drugs that target TopII generate protein-DNA covalent complexes [13–15]. The generation of TopII–DNA complexes has profound effects on cell physiology, such as blocking transcription and replication. Following treatment with TopII poisons, DNA strand breaks are rapidly detected, and most are covalently attached to proteins [16, 17]. Cells subsequently undergo apoptosis [18].

The repair mechanism of TopII-DNA complexes is not very clear, although it is likely that blocking the repair of the TopII-DNA complex can increase the efficacy of drugs like etoposide and doxorubicin. Recently, it was shown that TDP2 can cleave TopII-DNA adducts [9–10]. It was proposed that TDP2 functions in conjunction with components of the non-homologous end-joining machinery. Ku and DNA ligase IV probably control events at the double strand breaks (DSBs) before and/or after TDP2 activity because *Lig4*^{−/−} and *Ku70*^{−/−} DT40 cells have exhibited high levels of sensitivity to etoposide [19].

Like TDP1, it is likely that effective inhibition of TDP2 can be targeted to improve the efficacy of TopII poisons [10]. DT40 cells with a targeted deletion of TDP2 are hypersensitive towards etoposide but not to methyl methane sulfonate (MMS) or the TopI poison camptothecin. Thus,

TDP2 inhibitors may have therapeutic utility in treating cancers that are refractory to TopII-poison treatment. To facilitate inhibitor screening in a high throughput manner, we developed an efficient assay system and studied the kinetic properties of TDP2 using chromogenic T5PNP as a substrate in a 96-well format. We also compared those properties with the established TDP2 substrates.

TDP2 displayed a fast reaction rate for cleavage of the 5'-phosphotyrosyl adduct, although the overall efficiency of TDP2 towards 5-sub was much higher than towards T5PNP. Our study indicates the 3'-phosphotyrosyl phosphodiesterase activity of TDP2 is not very efficient. A recent study showed that DT40 cells lacking TDP2 were not hyper-sensitive to camptothecin but that they were to etoposide. This result indicates that the 3' enzymatic activity of TDP2 is not very prominent in the presence of TDP1 *in vivo* [10]. The fast 5'-phosphotyrosyl phosphodiesterase activity of TDP2 is quite unique among human repair enzymes. To our knowledge, the only known comparable human repair enzymes are APE1 and TDP1. APE1 requires such high reactivity for cell survival because the spontaneous production of abasic sites is lethal [20, 21]. It has been shown that deficiency in the TDP1-mediated repair pathway in humans causes spinocerebellar ataxia with axonal neuropathy (SCAN1) by affecting large, terminally differentiated, non-dividing neuronal cells [22]. TDP2 is another fast enzyme in the repair field. The biological need for such a highly efficient enzyme to remove 5'-phosphotyrosyl adducts will obviously be interesting to study in the future. The K_m value of TDP2 towards T5PNP was determined to be 54 mM (Table 1). This value is much higher than the K_m for 5-sub used in the gel-based assay, which offered a more extensive surface for binding although the turnover actually is higher for T5PNP compare to 5-sub (Table 1). Salt and pH effects were similar towards all 3 substrates. Increasing the salt concentration actually decreased TDP2 activity, possibly indicating the presence of more charged amino acids in the catalytic pocket. The exact nature of the macromolecular substrate, i.e., the TopII–DNA covalent complex, required by TDP2 for efficient catalysis remains unknown. Sodium orthovanadate is known to inhibit TDP2, shown in gel based assay (10). Our result showed sodium orthovanadate also inhibits product formation from T5PNP (IC_{50} =40 mM). TDP1 and APE1, the closest relatives of

TDP2, were not able to form product, so T5PNP is not a nonspecific substrate for any phosphodiesterase.

In this study, the synthetic chromogenic substrate T5PNP was used to determine the kinetic parameters of TDP2, which hydrolyzes the phosphodiester bond linking the TopII enzyme and its DNA substrate in the presence of TopII poisons, such as etoposide. Inhibitors of TDP2 might potentiate or synergize the cytotoxic effect of TopII poisons used as cancer therapeutic agents. The 96-well format developed for this assay should facilitate drug screening in a HT manner, and we obtained a signal to noise ratio of 5 when we used only 90 nM protein. However, one probable limitation of this assay may be the spectrophotometric detection of p-nitrophenol because of p-nitrophenol's relatively poor extinction coefficient ($15,000 \text{ M}^{-1}\text{cm}^{-1}$). TDP2 reaction kinetics are rapid, which explains why it needs a minimum protein concentration of 90 nM to obtain the signal in only 30 min. Also, because this assay does not require any washing steps, the signal to noise ratio is high. Although fluorescence-based assays may be more sensitive, the background signal is typical of fluorescent methods. Furthermore, auto-fluorescence of some compounds may increase this background and limit the utility of this assay. Overall, our assay offers a quick, simple, and straightforward method that can be used in a HT manner for inhibitor screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DSB repair	Double strand Break repair
TDP1	Tyrosyl-DNA phosphodiesterase 1
TDP2	Tyrosyl-DNA phosphodiesterase 2
TopI	Topoisomerase I
TopII	Topoisomerase II
T5PNP	p- nitrophenyl-thymidine-5'-phosphate

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Figure 1. Designing a new substrate for TDP2: T5PNP

As a substrate for TDP2 in this study, T5PNP was used to mimic the TopII–DNA complex. TDP2 cleaves the phosphodiester bond in T5PNP, and the chromogenic *p*-nitrophenol group is released.

A

B

Figure 2. TDP2-mediated T5PNP cleavage reaction

Varying amounts of TDP2 (0–118 nM) were reacted with T5PNP (1.25 mM) at 37°C (**A**), and varying amounts of T5PNP (0–7.5 mM) were reacted with TDP2 (180 nM) (**B**). The details of the reaction conditions are described in “Materials and Methods.” Data represent mean values with the standard error derived from three independent experiments.

Figure 3. Comparison of basic enzymatic properties of the purified TDP2 with different substrates

Dependence of TDP2 activity on pH (**A**) and KCl concentrations (**B**). The relative activity is defined as the ratio of the cleavage activity at the optimum pH or KCl concentrations to the similar activity at a different pH or KCl concentrations. Data represent mean values with the standard error derived from three independent experiments.

Figure 4. Specificity of T5PNP as a substrate

Kinetics of product formation by APE1, TDP1 and TDP2 (A). APE1 and TDP1 did not show any product formation. Inhibition of TDP2 – T5PNP reaction by sodium orthovanadate (B).

Table 1

Steady-state parameters of TDP2 towards different substrates.

Substrate	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($\text{hr}^{-1} \text{nM}^{-1}$)
5'-phosphotyrosyl oligonucleotide	0.089 ± 0.0023	3.6 ± 1.0	145
T5PNP	$54,000 \pm 7,568$	35 ± 2.8	0.002