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A fluorescence-based assay for the apurinic/apyrimidinic-site cleavage activity of human tyrosyl-DNA phosphodiesterase 1

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ABSTRACT

Tyrosyl-DNA phosphodiesterase 1 (Tdp1) catalyzes the hydrolysis of phosphodiester bonds between the DNA 3′-phosphate and tyrosine residues and plays a major role in the repair of stalled topoisomerase I-DNA covalent complexes. Given this role, Tdp1 is of interest as a potential target for anticancer therapy. Inhibiting Tdp1 in combination with clinically used Top1 inhibitors may potentiate the effects of the latter and help to overcome some of the chemoresistance issues currently observed. In addition, Tdp1 can function during DNA repair to remove a variety of other 3′ adducts from DNA such as phosphoglycolates and abasic or apurinic/apyrimidinic (AP) sites. Here we describe a new mix-and-read homogeneous fluorogenic assay for the measurement of the AP-site cleavage activity of Tdp1 that is compatible with high-throughput screening. The application of such an assay will open up further avenues for the discovery of novel Tdp1 inhibitors.

Topoisomerase I (Top1)[1] is an essential enzyme involved in regulating cellular DNA topology, catalyzing the relaxation of supercoiling and the release of torsional strains during DNA replication, transcription, recombination, and chromatin remodeling [1,2]. During these processes, a transient Top1–DNA cleavage complex is formed via the covalent attachment of a catalytic tyrosine of Top1 and a 3′-end phosphate of DNA. Because the nicking–closing activities of Top1 are very fast (up to 6000 cycles/min) [2], the steady-state levels of such Top1–DNA adducts are normally very low. Stalled or trapped Top1–DNA complexes can, however, be generated by endogenous DNA lesions such as strand breaks, abasic sites, base mismatches, and modified bases or by induction with Top1 poisons such as camptothecins [3]. Failure to reseal these normally transient breaks in the DNA can give rise to irreversible double-stranded breaks, resulting from the collision of replication forks with the trapped TopI–DNA complex, and may ultimately lead to cell death. Repair and removal of the stalled Top1 protein from the 3′ ends are via two main mechanisms: by specific cleavage of the tyrosyl-DNA bond by a phosphodiesterase or by endonuclease cleavage and elimination of the DNA strand attached to the topoisomerase. In the former mechanism, tyrosyl-DNA phosphodiesterase 1 (Tdp1), a member of the phospholipase D superfamily, has been identified as the enzyme responsible for the hydrolysis of the phosphodiester bond of this unique protein–DNA linkage in cells [4,5]. Due to its central role in this critical DNA repair pathway, Tdp1 has become of interest as a target for anticancer pharmaceutical intervention. Mutation or genetic inactivation of Tdp1 can hypersensitize cells to camptothecin [6,7], whereas overexpression of the active Tdp1 protein has been shown to result in a significant reduction of camptothecin-induced DNA damage [8]. In non-small cell lung cancer (NSCLC), albeit from a modestly sized cohort, Tdp1 has been shown to be overexpressed in more than 50% of NSCLC tissue [9]. Targeting Tdp1, therefore, may potentiate the effects of Top1 inhibitors and help to overcome some of the intrinsic or acquired resistance associated with current antitumor therapy with camptothecin. Such combination therapy would most likely be suited to those patients with additional defects in the parallel endonuclease pathways (e.g., ERCC1–XPF, Mre11, Mus81–Eme1, CtIP).

Inhibitors of Tdp1 identified to date are, at best, moderately potent (in the μM range and above), and all are far from clinical development. These compounds include the aminoglycoside neomycin [10], furamidine [11], and phosphotyrosine mimetics such as suramin, NF449, and methyl-3,4-dephostatin [12]. More recently, a natural product designated as JBIR-21 was identified as a Tdp1 inhibitor with antitumor activity in a mouse xenograft model [13], whereas Conda-Sheridan and coworkers described a

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[1] *Abbreviations used:* Top1, topoisomerase I; Tdp1, tyrosyl-DNA phosphodiesterase 1; NSCLC, non-small cell lung cancer; AP, apurinic/apyrimidinic; APE1, AP endonuclease 1; HTS, high-throughput screening; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; Tdp2, tyrosyl-DNA phosphodiesterase 2; T3P–pNP, *p*-nitrophenol-thymidine-3′-phosphate; ECL, electrochemiluminescent; ATA, aurintricarboxylic acid.

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subset of indenoisoquinolines as being the first Tdp1/Top1 dual inhibitors [14]. The first sub-micromolar inhibitor of Tdp1 has also just been reported from within a series of arylidene thioxothiazolidinones [15]. Despite these advances, there is still a clear need to identify more potent and promising small molecule inhibitors of Tdp1 that may ultimately develop into effective drug candidates. One challenge is to develop and use suitable high-throughput assays for the identification and characterization of hit matter against this target.

In addition to the cleavage of 3′-phosphotyrosyl bonds, Tdp1 can also hydrolyze other 3′-end DNA alterations, including 3′-phosphoglycolates and 3′-abasic sites, and also possesses limited DNA and RNA 3′-exonuclease activity [16,17]. This would suggest that Tdp1 can function not only as a 3′-tyrosyl-DNA phosphodiesterase but also as a more general DNA repair enzyme. It has recently been postulated that Tdp1 plays an important role in base excision repair of apurinic/apyrimidinic (AP) sites independent from the AP endonuclease 1 (APE1) pathway [17,18]. Assays amenable for high-throughput screening (HTS) against this novel AP-site cleavage activity of Tdp1 have not been described. Here we report the development of a novel fluorescence-based assay against this activity that is suitable for HTS purposes.

Materials and methods

Production of recombinant Tdp1 protein

Tdp1 (NM_060789.2) was cloned into a pHis expression vector (proprietary construct, University of Manchester) to generate an N-terminal His-tagged construct and the protein expressed in *Escherichia coli* JM109(DE3) cells. Purification was performed on an Ni^{2+}–IDA metal chelate affinity resin, followed by imidazole elution. The protein was then buffer-exchanged using a PD10 column against 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 10% glycerol and was stored aliquoted at −80 °C.

Oligonucleotides

The oligonucleotides 5′-GAG TCG TAC GAG GGT GA-[BHQ2]-3′, where BHQ2 is Black Hole Quencher-2, and 5′-[TAM]-TCA CCΦ TCG TAC GAC TC-3′, where TAM is TAMRA and Φ is dSpacer (a tetrahydrofuran derivative used to mimic an abasic site), were synthesized by Sigma–Aldrich. To create a double-stranded DNA substrate, equimolar concentrations of each oligonucleotide were mixed together in a buffer of 10 mM Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM NaCl and were heated at 95 °C for 5 min. The annealing mixture was then allowed to cool for at least 60 min at room temperature before use. Aliquots of annealed oligonucleotide could be stored at −20 °C for future use.

Tdp1 assay

The assay was developed to be performed in 384-well black plates in a 25-μl final volume with a reaction buffer composed of 50 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 25 mM NaCl, 1 mM dithiothreitol (DTT), 0.01% Tween 20, and 1% dimethyl sulfoxide (DMSO). During assay development, both enzyme and substrate were titrated individually and reaction progress was monitored over time to determine the optimal conditions. A final concentration of 5 nM Tdp1 and 50 nM labeled double-stranded AP-site-containing DNA substrate, as described above, was used for routine screening assays. Under standard conditions, the reaction was initiated by the addition of the DNA substrate and the assay was allowed to proceed at 25 °C for 30 min before stopping by the addition of 0.08% sodium dodecyl sulfate (SDS). The fluorescent signal generated during the assay was quantified with excitation at 540 nm and emission at 590 nm in a BioTek Synergy 2 multi-mode microplate reader.

APE1 screening

APE1 enzyme was obtained from New England Biolabs. The assay was performed in 384-well black plates in a 25-μl final volume with a reaction buffer composed of 50 mM Tris–HCl (pH 7.5), 2 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT, 0.01% Tween 20, 1% DMSO, 50 nM labeled double-stranded AP-site-containing DNA substrate (as described above), and 0.03 nM APE1. The assay was allowed to proceed for 30 min at 25 °C before stopping by the addition of 18 mM EDTA and reading fluorescence with 540 nm excitation and 590 nm emission.

Tdp2 screening

The assay was performed as described previously [19] in 384-well clear plates in a 25-μl final volume with a reaction buffer composed of 25 mM Hepes (pH 8.0), 10 mM $MgCl_2$, 130 mM KCl, 1 mM DTT, 0.03% bovine serum albumin (BSA), 1% DMSO, and 36 nM tyrosyl-DNA phosphodiesterase 2 (Tdp2) protein. The reaction was initiated by the addition of 20 mM 4-nitrophenyl phenylphosphonate, and the assay was run for 60 min at 25 °C before stopping by the addition of 33 mM EDTA. The signal generated during the assay was quantified by absorbance at 405 nm in a BioTek Synergy 2 multi-mode microplate reader.

Results and discussion

The development of Tdp1 assays that would be compatible with performing HTS has, to date, focused exclusively on the enzyme's ability to hydrolyze the 3′-phosphotyrosyl-DNA linkage by using substrates that mimic this phosphodiester bond. Chromogenic substrates such as *p*-nitrophenol-thymidine-3′-phosphate (T3P–pNP) have been proposed [20], whereas synthetic oligonucleotides containing 3′-phosphotyrosine surrogates such as 4-methylumbelliferone and fluorescein have been exploited for fluorescence intensity readout [3,13] and fluorescence polarization assays [21], respectively. Other nonphysiological substrates that have been employed include 5′-biotinylated oligonucleotides containing a tag coupled to the 3′-tyrosyl moiety, which have been used as the basis for both electrochemiluminescent (ECL) [11] and AlphaScreen [12] assays. There have been no published reports describing HTS-amenable assays for the recently identified AP-site cleavage activity of Tdp1.

Current approaches for the measurement of the AP-site cleavage activity of Tdp1 rely on the use of a radiolabeled oligonucleotide substrate, containing an abasic site or analogue, and the subsequent quantification of the products of the cleavage reaction via denaturing polyacrylamide gel electrophoresis (PAGE) and autoradiography [17,18]. Such an approach is not suited to an HTS campaign for the identification of novel inhibitors of Tdp1, and this led us explore an alternative assay format.

A recent publication on APE1 highlighted the potential of a fluorogenic donor/quencher assay for the identification of inhibitors against this particular endonuclease [22], and using this approach we investigated applying this format to Tdp1. A previously identified DNA substrate for APE1 [22] was tested as a potential substrate for the AP-site cleavage activity of Tdp1. This substrate was a double-stranded oligonucleotide in which one strand contains both an internal abasic site and a 5′-TAMRA fluorophore donor, whereas the complementary strand contains a quenching 3′-BHQ2 moiety. When the donor and quencher are in close