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New fluorescence-based high-throughput screening assay for small molecule inhibitors of tyrosyl-DNA phosphodiesterase 2 (TDP2)

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ABSTRACT

Tyrosyl-DNA phosphodiesterase 2 (TDP2) repairs topoisomerase II (TOP2) mediated DNA damages and causes resistance to TOP2-targeted cancer therapy. Inhibiting TDP2 could sensitize cancer cells toward TOP2 inhibitors. However, potent TDP2 inhibitors with favorable physicochemical properties are not yet reported. Therefore, there is a need to search for novel molecular scaffolds capable of inhibiting TDP2. We report herein a new simple, robust, homogenous mix-and-read fluorescence biochemical assay based using humanized zebrafish TDP2 (14M_zTDP2), which provides biochemical and molecular structure basis for TDP2 inhibitor discovery. The assay was validated by screening a preselected library of 1600 compounds ($Z' \geq 0.72$) in a 384-well format, and by running in parallel gel-based assays with fluorescent DNA substrates. This library was curated via virtual high throughput screening (vHTS) of 460,000 compounds from Chembridge Library, using the crystal structure of the novel surrogate protein 14M_zTDP2. From this primary screening, we selected the best 32 compounds (2% of the library) to further assess their TDP2 inhibition potential, leading to the IC_{50} determination of 10 compounds. Based on the dose-response curve profile, pan-assay interference compounds (PAINS) structure identification, physicochemical properties and efficiency parameters, two hit compounds, **11a** and **19a**, were tested using a novel secondary fluorescence gel-based assay. Preliminary structure-activity relationship (SAR) studies identified guanidine derivative **12a** as an improved hit with a 6.4-fold increase in potency over the original HTS hit **11a**. This study highlights the importance of the development of combination approaches (biochemistry, crystallography and high throughput screening) for the discovery of TDP2 inhibitors.

1. Introduction

Tyrosyl-DNA phosphodiesterase 2 (TDP2), also known as TNF receptor associated factor (TRAF) and TNF receptor associated protein (TTRAP) (Pype et al., 2000) and ETS1-associated protein 2 (EAPII) (Pei et al., 2003), was the first human 5′-tyrosyl DNA phosphodiesterase identified (Cortes Ledesma et al., 2009). TDP2 plays a major role in DNA repair by specifically cleaving the 5′tyrosyl-DNA phosphodiester bond of stalled topoisomerase II (TOP2) cleavage complexes [reviewed in (Menon and Povirk, 2016; Pommier et al., 2014)].

TOP2 resolves topological problems with double-stranded DNA during normal physiological processes, such as transcription and replication. Mechanistically, TOP2 acts by generating TOP2-DNA cleavage complexes (TOP2cc) featuring a covalent phosphotyrosine linkage between its active site tyrosine and the 5′ phosphate end at the site of DNA cleavage. These complexes are transient as TOP2 reseals cleaved DNA at the end of its catalytic cycle (Pommier et al., 2016). However, under certain conditions, such as exposure to TOP2 poisons, TOP2cc become abortive (Fortune and Osheroff, 2000; Nitiss, 2009; Pommier, 2013). Repair of abortive TOP2cc by cellular DNA repair machinery, particularly TDP2, can lead to cancer resistance to TOP2 poisons. By inhibiting TDP2, cancer cells could be sensitized toward treatment with TOP2 poisons, resulting in therapeutic efficacy with much lower doses of TOP2 poisons (Marchand et al., 2016).

Several lines of evidence support the potential clinical benefits of specifically targeting TDP2 in cancer therapy: (*i*) TDP2-deleted cells show hypersensitivity to TOP2 poisons (Cortes Ledesma et al., 2009; Zeng et al., 2011); (*ii*) TDP2 knockout vertebrate cells show normal cell growth and knockout mice are viable without noticeable pathology, suggesting that TDP2 inhibitors could be well tolerated (Gomez-

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Herreros et al., 2013); (*iii*) the oncogenic role of overexpressed TDP2 has been established in several non-small-cell lung carcinoma cells (Li et al., 2011), and TDP2 up-regulation attained by p53 gain-of-function mutation has been linked to TOP2 poison resistance in human lung cancer (Do et al., 2012); and (*iv*) tumor cells frequently lack parallel DNA repair pathways, which makes them more vulnerable to certain cancer chemotherapy (principle of synthetic lethality) (Curtin, 2012), and more specifically to TOP2 inhibitors (Hoa et al., 2016; Maede et al., 2014; Pommier et al., 2014).

In addition, TDP2 is also implicated in picornavirus and hepatitis B virus (HBV) infections during steps where cleavage of a 5′-phosphotyrosine covalent bond occurs. In particular, picornavirus uses TDP2, also known as VPg-unlinkase, to release the viral genomic RNA from VPg protein necessary during RNA replication (Maciejewski et al., 2016; Virgen-Slane et al., 2012). HBV uses the DNA repair machinery of the infected cells to convert viral genomic relaxed circular DNA (RC-DNA) into covalently closed circular DNA (cccDNA) where TDP2 could play a role in releasing the viral P-protein from the 5′end of RC-DNA (Koniger et al., 2014).

Since the discovery of TDP2 in 2009 (Cortes Ledesma et al., 2009), only a few scaffolds have been identified possessing TDP2 inhibitory activity (Laev et al., 2016): compound **1** (Ro 08-2750, Fig. 1) (Thomson et al., 2013), deazaflavins (e.g. **2**) (Hornyak et al., 2016; Marchand et al., 2016; Raoof et al., 2013), isoquinoline-1,3-diones (e.g. **3**) (Kankanala et al., 2016), the triple inhibitors TOP1/TDP1/TDP2 indenoisoquinolines (e.g. **4**) (Beck et al., 2016; Wang et al., 2017), compounds **5** (NSC375986), **6** (NSC114532) (Kossmann et al., 2016) and **7** (NSC111041) (Kont et al., 2016). Deazaflavin derivatives are the only described TDP2 inhibitors with activities in the nanomolar range. However, their use as molecular probes in studying cellular functions and their potential as drug candidates are severely hindered by the poor cell permeability. (Hornyak et al., 2016). Therefore, new scaffolds with good potencies and desirable physicochemical properties are highly desired.

The first reported methods developed to biochemically measure TDP2 activity employed gel-based assays (Cortes Ledesma et al., 2009; Gao et al., 2012; Zeng et al., 2011). In addition to using radiolabeled-substrates, these assays can be expensive and time-consuming, and thus may not be suitable for high-throughput screening (HTS). Colorimetric assays using T5NPP (Adhikari et al., 2011) or NPPP (Raoof et al., 2013; Thomson et al., 2013) as substrate have been developed, though difficulties in achieving enzyme inhibition above 75% were observed when NPPP was employed, and high enzyme concentration (30–36 nM) was required for both chromogenic assays. Since TDP2 prefers more physiologically relevant 5′-phosphotyrosyl oligonucleotides substrates over the small compound surrogates (Gao et al., 2012), a new colorimetric assay using a DNA substrate was reported (Thomson et al., 2013). However, this particular assay required the addition of calf intestinal alkaline phosphatase (CIP) to cleave the phosphate group necessary for the reaction development. Recently, Hornyak et al. reported a fluorescence-based assay using a 13-mer oligonucleotide substrate with a 5′-tyrosine conjugated with FITC fluorophore and an enzyme concentration much lower than the chromogenic assays (50 pM) (Hornyak et al., 2016). However, the TR-FRET nature of this assay required the addition of trivalent metal ion sensor (Gyrasol technologies) to quench the fluorescence of the substrate while stopping the reaction, resulting in increased assay costs, and allowing only end-point quenched readings.

We report herein a new fluorescence-based assay allowing reading in both continuous and quenched modality. With quenched reaction protocol this new assay is amenable for HTS and requires low enzyme concentration. In addition, the continuous reaction reading allows easy detection of false positives due to the presence of fluorescent compounds, as well as kinetic data collection (Acker and Auld, 2014). By employing a humanized zebrafish protein (14M_zTDP2) developed by our group, and whose crystal structure is included in this report, we screened a library of 1600 compounds preselected via virtual high-throughput screening (vHTS).

2. Materials and methods

2.1. Chemistry

All commercial chemicals were used as supplied unless otherwise indicated. Flash chromatography was performed on a Teledyne Combiflash RF-200 with RediSep columns (silica) and indicated mobile phase. All moisture sensitive reactions were performed under an inert atmosphere of ultrapure argon with oven-dried glassware. ^{1}H and ^{13}C NMR spectra were recorded on a Varian 600 MHz or Bruker 400 spectrometer. Mass data were acquired on an Agilent 6230 TOF LC/MS spectrometer capable of ESI and APCI ion sources. All tested compounds have a purity ≥95%.

2.1.1. General procedural for synthesis of 2, 2, 4-trimethyl dihydroquinolines (**10**)

To a solution of corresponding aniline **8** (10 mmol) in acetone (15 mL), was added catalytic $InCl_3$ (5 mol%) and the resulting mixture

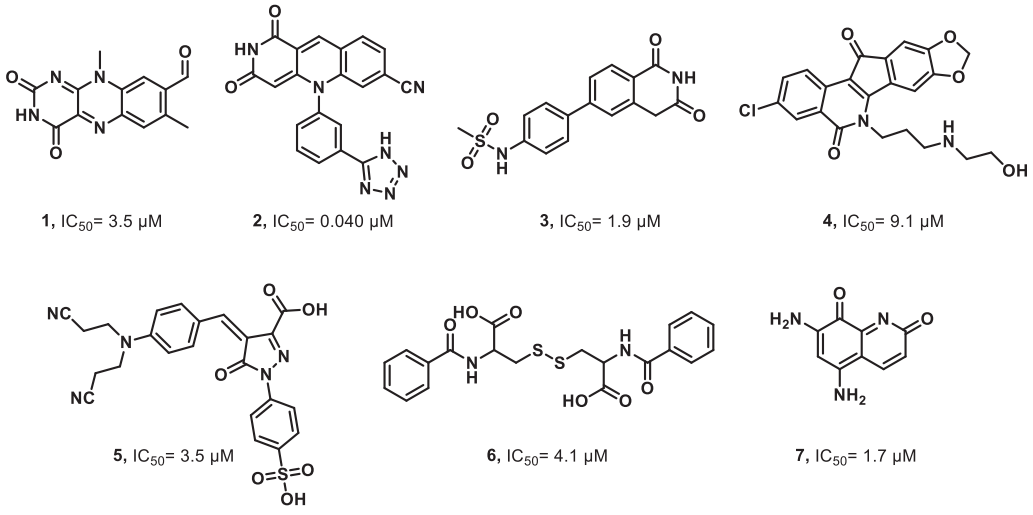

Fig. 1. Representative reported TDP2 inhibitors.