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PAIN-less identification and evaluation of small molecule inhibitors against protein tyrosine phosphatase 1B†‡

 Hamid R. Nasiri, §* Philipp Mracek, § Steffen K. Grimm, Janine Gastaldello, Adrian Kolodzik and Dirk Ullmann

A highly miniaturized biochemical assay was set up to test a focused set of natural products against the enzymatic activity of protein tyrosine phosphatase 1B (PTP1B). The screen resulted in the identification of the natural product alkaloids, berberine and palmatine as well as α -tocopheryl succinate (α -TOS) as potential inhibitors of PTP1B. In a second step, several read-out and counter assays were applied to confirm the observed inhibitory activity of the identified hits and to remove false positives which target the enzymatic activity of PTP1B by a non-specific mechanism, also known as PAINS (pan-assay interference compounds). Both, berberine and palmatine were identified as false positives which interfered with the assay read-out. Using NMR spectroscopy, self-association *via* stacking interactions was detected for berberine in aqueous media, which may also contribute to the non-specific inhibition of PTP1B. α -TOS was confirmed as a novel reversible and competitive inhibitor of PTP1B. A concise structure–activity relationship study identified the carboxyl group and the saturated phytyl-side chain as being critical for PTP1B inhibition.

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1. Introduction

Protein tyrosine phosphatase 1B (PTP1B) is a key enzyme in the negative regulation of the insulin receptor and leptin signaling pathway. As an attractive therapeutic target, PTP1B is involved in the pathophysiology of type II diabetes and obesity.^{1,2} PTP1B overexpression causes insulin resistance by constant dephosphorylation of the insulin receptor. In contrast, PTP1B inhibition causes an increase in sensitivity towards insulin as demonstrated in PTP1B knock-out mice.^{3,4} Small molecule inhibitors of PTP1B, which restore the degree of insulin receptor phosphorylation are therefore highly desired due to their potential for treating type 2 diabetes and obesity.^{1,5,6}

Several small molecule inhibitors of PTP1B with competitive^{7–10} and allosteric^{11,12} mechanism of action have been reported (Fig. 1). The majority of these inhibitors were discovered by screening of large libraries using biochemical assays.

In this letter, we present the miniaturization of an *in vitro* biochemical assay to monitor the enzymatic activity of PTP1B enzyme. Testing of a focused set of natural products resulted

in the identification of several primary active compounds. Additional assays were utilized to remove hit compounds acting through unwanted effects such as self-association in aqueous solution or interfering with the assay read-out. As a result, a novel, reversible and competitive inhibitor against PTP1B has been identified.

2. Results and discussion

The assay is centered on difluoromethylumbelliferyl phosphate (DifMUP), an artificial and fluorogenic substrate for

Fig. 1 Chemical structures of a selection of reported inhibitors of PTP1B. Including both fragments 1 (ref. 7 and 8) and 2,⁹ the allosteric inhibitor 3 (ref. 11) and the natural product 4.¹⁰

Evotec AG, Manfred Eigen Campus, Essener Bogen 7, D-22419 Hamburg, Germany. E-mail: info@evotec.com

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§ These authors have contributed equally to this work.

phosphatases.^{13,14} The hydrolysis of the arylphosphate moiety by PTP1B generates a highly fluorescence product. In the presence of a potential inhibitor DifMUP remains phosphorylated and no fluorescent signal is detected. The assay was miniaturized to high density plate format (1536-well plates) and applied as a primary assay to screen a focused set of natural products. The PTP1B enzyme (1–298 aa) was used at an optimized concentration of 5 nM.

To have an adequate rate and good assay signal, the concentration of the artificial substrate DifMUP was set at 30 μM , which is twelvefold higher than the reported K_M value (K_M DifMUP = 2.5 μM ¹³). During the assay miniaturization and screening, fragment 1⁸ was used as a positive control. The miniaturization was conducted by using a V-prep automated pipetting station (Agilent Technologies). The miniaturized assay was highly robust with good performance: $Z' = 0.8$ (no enzyme as low control); $Z' = 0.6$ (100 μM fragment 1 as low control). A representative partially filled 1536-well uniformity plate is highlighted in Fig. 2A.¹⁵ Natural products are considered as a diverse source for the discovery of bioactive compounds in hit identification programs.¹⁶ Several molecules derived from natural source have been reported to inhibit the activity of PTP1B¹⁷ and were used as tool compounds in the anti-diabetic drug discovery.¹⁸ Following this approach, we have screened a focused set of natural products in the miniaturized biochemical assay. As a result, we identified several active compounds in the primary assay, among them berberine (5; $\text{IC}_{50} \sim 67 \mu\text{M}$) and the structurally related palmatine (6; $\text{IC}_{50} \sim 234 \mu\text{M}$) as well as α -TOS (7; $\text{IC}_{50} \sim 20 \mu\text{M}$) see Fig. 2B.

Berberine (5) is an alkaloid extracted from various plants used in traditional Chinese medicine. It has been used as an insulin sensitizer and is commercially available as food supplement for its anti-diabetic and anti-inflammatory effects. Berberine has been described in the literature by two independent groups as an inhibitor of PTP1B.^{19–21} α -TOS (7) is a chiral molecule which is used in dietary supplements as a vitamin E dosage form and has anti-cancer activity. The proapoptotic properties of α -TOS (7) is thought to be based on targeting ubiquinone-binding sites in mitochondrial respiratory complex II.²²

Fig. 2 Outcome of the biochemical testing of a focused set of natural products. A) Assay performance in a 1536-well uniformity plate (high control = DMSO only; low control = no enzyme and low control with an inhibitor = in the presence of 100 μM of fragment 1) and B) constitution of the identified primary active molecules.

Fluorescence/luminescence-based high-throughput enzyme inhibition screens often deliver compounds which interfere with the assay readout or inhibit the enzyme of interest in a nonspecific fashion. Identifying such compounds, also known as pan-assay interference compounds (PAINS),²³ represents a challenge for screening campaigns.²⁴ Several assays therefore were put in place to confirm the observed inhibitory effect of the identified primary active molecules and to filter out any non-specific inhibition. These assays were designed to identify promiscuous inhibitors of PTP1B enzyme and compounds prone to aggregation and read-out interference.

First the interference of identified compounds with the readout was investigated. Here the inhibitors were added after the completion of the enzymatic reaction of PTP1B. PTP1B was therefore incubated with DifMUP, and after the conversion of the artificial substrate, the enzyme was deactivated with hydrogen peroxide and the compounds were added. Compounds interfering with the fluorescence readout were identified by a reduction of the fluorescence signal.

The tool compound 1 and α -TOS (7) showed no interference in the read-out counter assay, whereas berberine (5) and palmatine (6), both clearly interfered with the assay read-out (Fig. 3).

Berberine has an absorption maxima $\sim 430 \text{ nm}$, which overlaps with the emission spectra of the product. An effect known as “inner filter effect”. Czaplewski *et al.* also challenged the genuine effect of berberine as a specific inhibitor.²⁵ This paper pointed out that a wide range of activities have been reported for berberine (antibacterial, antifungal, antiviral, anticancer, anti-infective and anti-diabetic activities), which makes a specific mode of action very unlikely.²⁵ Sun *et al.* tested the physicochemical properties of berberine by using light scattering assay in order to rule out unselective inhibition by formation of aggregates. However, no aggregates were found using this assay.²⁶

In this current publication, we used an NMR based approach²⁷ to further study the physicochemical properties of

Fig. 3 Concentration–response curves of identified hits in the primary assay (black circles) and in the read-out counter assay (grey circles).

berberine (5). $1D-^1H$ NMR spectra were recorded for a dilution series of 500 μM to 32 μM compound concentration in aqueous media. As highlighted in Fig. 4, clear shifts of berberine resonances as a function of compound concentration can be observed in the aromatic region. This effect suggests self-association of the compound. Specifically, the reduction of compound concentration shows a corresponding down-field shift of the compound resonances by ~ 22 Hz. This observation is in line with a de-shielding effect of the aromatic protons implicating a loss of stacking interaction. This observed compound self-association may also contribute to an unspecific inhibition of the PTP1B enzymatic activity.²⁸ This hypothesis was confirmed by testing methylene blue, which had the same self-association properties in the NMR assay, in the primary assay. Methylene blue was found to be an inhibitor of the activity of PTP1B (ESI[†]).

Finally, to rule out another source of false positives in search for inhibitors against cysteine-containing enzymes, the intrinsic redox activity of α -TOS (7) was tested. Redox active compounds interfere with PTP1B enzymatic activity directly or indirectly *via* hydrogen peroxide mediated protein inactivation. The inactivation is caused by the oxidation of the active cysteine in the catalytic center of PTP1B.^{29,30} The non-enzymatic redox activity of α -TOS (7) in reducing environment was tested by using an assay reported by Lor *et al.* from GlaxoSmithKline.³¹ As expected, no redox activity was observed for α -TOS (data not shown). This finding is in line with the commonly used description of α -TOS (7) as a redox-silent vitamin E analogue.

A concise structure–activity relationship (SAR) study was performed by testing some close analogues of α -TOS (7) (compounds 8, 9 and 11; see Fig. 5). In order to investigate the contribution of the hydrophobic tail of α -TOS (7), analogue 11 was synthesized. Starting from 2,2,5,7,8-pentamethyl-6-chromanol

Fig. 4 Aromatic region of a series of 1H -NMR spectra superimposed from various concentrations of berberine (5) (dilutions from 500 μM (black spectrum) to 32 μM (orange spectra) from top to bottom). The buffer employed for these studies was 5 mM TRIS pH 7.4, 50 mM NaCl in 5% DMSO- d_6 .

Fig. 5 SAR study and mode of inhibition of α -TOS (7). A) Constitution of the α -TOS analogues, B) synthesis route for the preparation of the truncated analogue (11). C) Testing of analogues 7, 8, 9 and 11 in the primary assay. D) Inhibitory activity of α -TOS (7) at different DifMUP concentrations.

10 as a precursor and following the succinic anhydride route *via* ring opening reaction³² the product 11 was formed in excellent yield³³ (Fig. 5B).

Loss of inhibitory activity was observed when: i) the carboxyl-group was removed (8) and replaced by an acetyl group (9), or ii) when the phytyl side chain was removed (11) (Fig. 5C). This observation implies that both groups, the carboxyl group and the hydrophobic tail, are necessary for the inhibitory activity of α -TOS (7). The observed SAR outcome is also in line with the chemical constitution of reported PTP1B inhibitors shown in Fig. 1, both fragments (1 and 2) have the carboxyl moiety and the natural product RK682 (compound 4) has a hydrophobic aliphatic chain contributing to the inhibition. Notably, other reported PTP1B inhibitors such as corosolic acid, oleanolic acid and ursolic acid have similar structural features consisting of a polar carboxy-group and a hydrophobic moiety.^{34,35} We postulate that the hydrophilic carboxyl group probably acts as a phosphate surrogate, whereas the phytyl tail potentially forms attractive hydrophobic interaction with the protein. It has been reported that the hydrophobic side chain of bioactive molecules can indeed participate in specific interactions and forms distinct contacts with the target protein.³⁵

In a final experiment, the competitive mode of action of this molecule was confirmed by testing the inhibitory activity in the presence of different concentrations of the substrate (Fig. 5D). The reversibility of the inhibition of PTP1B by α -TOS (7) was also confirmed by running time course experiments, following the enzymatic inhibition and subsequent addition of 10-fold excess of DifMUP, which resulted in the recovery of the enzymatic activity (data not shown).

Due to the charged and polar function of the hit molecule 7, we assumed that the molecule occupies the active site, deep within the catalytic A site of PTP1B. The crystal structure of PTP1B (PDB ID: 2HNQ)³⁶ from protein data bank was used for the docking study (Fig. 6).

Fig. 6 A proposed binding mode of **7** bound to PTP1B.

The docking study³⁷ revealed that the carboxyl-group of compound **7** forms several hydrogen bonds, among them also with Arg221. This attractive interaction was also found in the co-crystal structures of fragments **1** and **2** bound to PTP1B,^{7–9} and explains the observed SAR results highlighted in Fig. 5.

In summary, a fully developed highly miniaturized biochemical primary assay was used to monitor the enzymatic activity of the PTP1B phosphatase. Testing of a focused set of natural products resulted in the identification of several hits. A selection of assays was applied to remove hit compounds acting through unwanted effects. This in-depth evaluation, identified berberine (**5**) and palmatine (**6**) as false positives, interfering with the assay read-out and self-associate in aqueous solution. α -TOS (**7**), in contrast appears to be a novel, reversible and competitive inhibitor against PTP1B.

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- 15 Miniaturized fluorescence intensity assay: the measurements were performed in 1536-well Greiner microplates (Greiner Bio-One, Monroe, NC, USA, cat # 782076). All reagents used in the assay were diluted in the assay buffer (50 mM citrate buffer; 100 mM NaCl; 1 mM EDTA; 0.1 mM DTT; 0.01% Brij35 pH 6.0) except for H₂O₂ (0.5% final assay concentration) which was diluted in ddH₂O and was used as a reaction-stop-solution at the end of the assay workflow. The PTP1B assay workflow-procedure is as following: first, 0.3 μ l of compound (diluted in 100% DMSO) was incubated with 3 μ l of the 1–298 aa long PTP1B enzyme (5 nM final concentration) for 30 min at RT in the dark. Then 3 μ l of DifMUP (DifMUP-substrate at 30 μ M final concentration) was added and the reaction was incubated for 30 min at RT. Afterwards, 1.5 μ l H₂O₂ stop-solution (0.5% H₂O₂ final concentration) was added and the plate incubated for 30 min at RT. Subsequently, the plate was measured on a Tecan Safire 2 Multimode Reader (Tecan). Fluorescence intensity values were detected by using an excitation wavelength at 360 nm and an emission wavelength at 450 nm.
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- 33 Synthesis of **11**: 1.1 g (5 mmol), of 2,2,5,7,8-pentamethyl-6-chroman-1-ol (**10**) and 0.75 g (1.5 eq., 7.5 mmol) succinate anhydride were dissolved in 10 mL toluene. To this solution 0.2 mL NEt_3 was added. The mixture was heated at 80 °C for 6 h. After the completion of the reaction water was added and organic phase extracted by CH_2Cl_2 . The organic phase was repeatedly washed with water and diluted acetic acid and dried over MgSO_4 . The crude product was purified by column chromatography using cyclohexane:ethyl acetate (3:1) as eluent to afford 1.56 g (98% yield) of the desired product (**11**) as a white powder $^1\text{H-NMR}$ (600 MHz, CDCl_3) δ : 2.94 (t, J = 6.8 Hz, 2H); 2.84 (t, J = 6.8, 2H); 2.59 (t, J = 6.8, 2H); 2.09 (s, 3H); 1.928 (s, 3H); 1.92 (s, 3H); 1.72 (t, J = 6.8 Hz, 2H); 1.21 (s, 6H). $^{13}\text{C-NMR}$ (62.9 MHz, CDCl_3) δ : 177.9, 170.8, 149.5, 140.4, 126.7, 124.9, 123.0, 117.2, 73.0, 32.7, 28.9, 28.6, 26.7, 20.9, 12.9, 12.0, 11, 8. ESI calculated for $\text{C}_{18}\text{O}_5\text{H}_{24}$: 320.38 g mol^{-1} ; found: 321.17 MH^+ (purity > 99%).
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