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Investigating Mammalian Tyrosine Phosphatase Inhibitors as Potential ‘Piggyback’ Leads to Target *Trypanosoma brucei* Transmission

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Abstract

African trypanosomiasis is a neglected tropical disease affecting humans and animals across 36 sub-Saharan African countries. We have investigated the potential to exploit a ‘piggyback’ approach to inhibit *Trypanosoma brucei* transmission by targeting the key developmental regulator of transmission, *T. brucei* protein tyrosine phosphatase 1. This strategy took advantage of the extensive investment in inhibitors for human protein tyrosine phosphatase 1B, a key target for pharmaceutical companies for the treatment of obesity and diabetes. Structural predictions for human and trypanosome tyrosine phosphatases revealed the overall conservation of important functional motifs, validating the potential for exploiting cross specific compounds. Thereafter, nineteen inhibitors were evaluated; seventeen from a protein tyrosine phosphatase 1B-targeted inhibitor library and two from literature analysis – oleanolic acid and suramin, the latter of which is a front line drug against African trypanosomiasis. The compounds tested displayed similar inhibitory activities against the human and trypanosome enzymes, mostly behaving as noncompetitive inhibitors. However, their activity against *T. brucei* in culture was low, necessitating further chemical modification to improve their efficacy and specificity. Nonetheless, the results validate the potential to explore a ‘piggyback’ strategy targeting *T. brucei* protein tyrosine phosphatase 1 through exploiting the large pharmacological investment in therapies for obesity targeting protein tyrosine phosphatase 1B.

Keywords

biological screening; drug discovery; kinase; phosphatase

Protein tyrosine phosphatase 1B (PTP1B) is one of the most attractive phosphatase targets in the field of drug discovery. PTP1B is a negative regulator of the insulin and leptin pathways,

and mice deficient for this gene show increased sensitivity to insulin and resistance to obesity (1), thus establishing PTP1B as a therapeutic target for the treatment of type 2 diabetes and obesity. Several pharmaceutical companies have been working to develop potent and selective PTP1B inhibitors (2,3) as obesity represents a hugely lucrative market: according to the WHO projections, approximately 2.3 billion adults will be overweight and more than 700 million will be obese by 2015.

Trypanosoma brucei protein tyrosine phosphatase 1 (*TbPTP1*) is 24% identical to human PTP1B at the protein level and possesses 9 of the 10 conserved PTP motifs. *TbPTP1* has been identified as a key regulator of the life cycle of African trypanosomes (4), parasites that are the causative agent of Human African trypanosomiasis and of 'Nagana', a wasting disease affecting cattle. Both the human and livestock disease generate significant public health and economic burdens throughout 36 sub-Saharan African countries (5).

Trypanosoma brucei is transmitted between mammalian hosts by tsetse flies and displays a complex life cycle to survive in these different environmental conditions. In the mammalian bloodstream, 'stumpy' form parasites are responsible for disease transmission, as they are the only life cycle form capable of surviving in the vector (6). Once in the tsetse fly midgut, stumpy forms differentiate into procyclic forms, a response that can be reproduced *in vitro* by a combination of temperature reduction and the addition of citrate/cis-aconitate (7,8). A central component of the signaling pathways contributing to the initiation of differentiation is *TbPTP1*, which prevents the development from stumpy forms to procyclic forms. A downstream substrate for *TbPTP1* is a glycosomal directed phosphatase, *TbPIP39*, which promotes differentiation when it becomes phosphorylated as a consequence of the inhibition of *TbPTP1* (9). Hence, a pharmacological inhibition of *TbPTP1* in the mammalian bloodstream would provoke premature differentiation of stumpy forms to procyclic forms, a response that would prove lethal in the parasite. Targeting *TbPTP1* would therefore eliminate the population of fly-infective parasites and so prevent disease transmission.

Vector control and animal reservoir treatment (10) are strategies currently used to limit the spread of African trypanosomiasis. However, these approaches have limitations that render them unsustainable for the long-term control of disease transmission (11). Consequently, the potential use of a safe and cheap drug to block transmission would be a novel and useful tool for controlling human and animal trypanosomiasis, particularly under epidemic conditions. Moreover, the possibility of 'piggybacking' on the extensive efforts in the pharmaceutical industry to develop PTP1B inhibitors would represent a rapid and cost effective route to implementation where preclinical or clinical studies have already been performed (12). This strategy is particularly attractive for sleeping sickness and for other neglected tropical diseases, where economic considerations and the value of potential markets limits investment by the pharmaceutical industry (13).

Here, we have tested 19 established PTP1B inhibitors against *TbPTP1*, including suramin, a front line therapeutic for early stage trypanosomiasis. In each case, their IC_{50} and K_i values have been calculated, and kinetic parameters determined. Our findings indicate that the PTP1B inhibitors analyzed display similar inhibitory properties against the human and the parasite enzymes, this being consistent with the predicted conservation of their overall 3D structures. Although the low activity of the respective compounds against trypanosomes in

culture indicated further refinement is necessary, these analyses validate the general approach of exploiting 'piggyback' strategies to control African trypanosomiasis transmission.

Methods and Materials

Parasite growth

Bloodstream form trypanosomes were cultured *in vitro* in HMI-9 medium (14) and stumpy-enriched populations were obtained by DEAE-cellulose purification (15) of parasites 6–7 days after infection into cyclophosphamide-treated mice.

Compound preparation

All the DDP inhibitors tested were dissolved in DMSO (dimethyl sulfoxide) at 25 mM stock concentration of which aliquots were kept at –20 °C in dark 0.2 mL eppendorf tubes. Similarly, oleanolic acid (Sigma O5504, St Louis, MO, USA) was dissolved to have a 20 mM stock solution, of which aliquots were kept at –20 °C. Sodium vanadate (Sigma) was activated by adjusting the pH of a 200 mM solution to pH 10 with 1 M NaOH or HCl (16); the solution was then boiled until it turned colorless, cooled and adjusted to pH 10 again, followed by other cycles of boiling and setting the pH until the solution remained colorless with a stable pH of 10.

The PTP1B inhibitor 3-(3,5-Di-bromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-(thiazol-2-ylsulfamyl)-phenyl)-amide, 'BZ3' (Calbiochem® (Merck KGaA, Darmstadt, Germany), product number 539741) was dissolved in DMSO to give a 67 mM stock solution.

TbPTP1 phosphatase assays

The activity of recombinant *Tb*PTP1 expressed in *E. coli* was assayed by measuring the ability of the phosphatase to catalyze the hydrolysis of *p*-nitrophenylphosphate (pNPP, Sigma N-2765) to *p*-nitrophenol, a chromogenic product with absorbance at 405 nm. Reaction mixtures (50 μ L) contained 2–5 μ g of purified *Tb*PTP1 with 20 mM final concentration of pNPP in 50 mM Tris, 50 mM Bis-Tris, and 100 mM NaAc, pH 5.5. Reactions were incubated at 37 °C for 15–30 min and quenched by addition of 100 μ L of 0.2 M NaOH. The absorbance of the samples was measured at 405 nm in a microplate reader (ELx808 Absorbance Microplate Reader; BioTek, Inc., Winooski, VT, USA).

Human PTP1B phosphatase assay

Recombinant human PTP1B (Sigma, P6244-50UG) was used at 40 ng per reaction and incubated with 10 mM of DiFMUP substrate (6,8-difluoro-4-methylumbelliferyl phosphate, Invitrogen D6567, $e = 17/\text{mM}$) in PTP1B buffer (25 mM Hepes pH 7.2, 50 mM NaCl, 2.5 mM EDTA and BSA 0.01 mg/mL). PTP1B was first incubated with the inhibitor tested for 30 min at 37 °C; then, the substrate DiFMUP was added for 10 min at 37 °C. Changes in fluorescence were read at exc. 360 nm and at em. 460 nm, using a Flx800 Plate Reader (BioTek).

TbPTP1 competition assays

For testing the nature of the inhibitor (competitive, noncompetitive, and mixed inhibitors), competition assays were carried out, in which a constant concentration of enzyme and inhibitor was tested in the presence of different concentrations of substrate (pNPP); TbPTP1 was used, as previously described (4), at 0.3–0.4 μg per reaction, in the same buffer and 96-well plate format used for enzyme kinetic and inhibition assays. The inhibitor to be tested was used at two or three different concentrations lower and higher than the IC_{50} and added to the mix of enzyme and buffer. To start the reaction, 40 μL of substrate was added to a final concentration of 33.3 mM for the first row of triplicate samples and then diluted 1:2 in the subsequent wells. The absorbance obtained was converted into velocity of hydrolysis, as performed for the enzyme kinetic assay, and plotted against the linear molar concentration of substrate. The V_{max} and K_{M} were calculated by GraphPad Prism (Graphpad Software, San Diego, CA, USA) using the Michaelis-Menten equation ($Y = (V_{\text{max}} * X) / (K_{\text{M}} + X)$, where Y is the enzyme velocity, and X is the substrate concentration).

Cell viability assay using Alamar Blue®

Trypanosoma brucei monomorphic single marker cells were grown in normal HMI-9 20% FCS in a 96-well plate with initial cell density of $1 \times 10^5/\text{mL}$ and were treated with serial dilution of the drug to be tested for 48 h in the 37 °C incubator. Following this, 10% v/v Alamar Blue® (Abd Serotec, Raleigh, NC, USA) was added (20 μL in 200 mL) for a further 24 h, and absorbance of the samples was read at 540 and 595 nm, using ELx808 Absorbance Microplate Reader, BioTek and Gen5 software. Samples were run in triplicate and included a blank with the drug in HMI-9 without cells. The average absorbance of the triplicate samples minus the blank was calculated for both the wavelengths and the percentage difference in Alamar Blue® reduction in cells treated with the drug versus untreated cells was calculated using the following formula: $[(A_{540} - (A_{595} \times R_0) \text{ for test well}) / (A_{540} - (A_{595} \times R_0) \text{ for control well})] \times 100$, where R_0 is the correction factor for different filters obtained from: $[(A_{540} \text{ media} + \text{alamar}) - (A_{540} \text{ media})] / [(A_{595} \text{ media} + \text{alamar}) - (A_{595} \text{ media})]$. The percentage of Alamar Blue® reduction was then plotted on the y-axis against the logarithmic concentration of the drug tested using GraphPad Prism, and the LD_{50} was calculated analyzing the data using the sigmoidal dose–response equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{\text{LogIC}_{50} - X})$.

In vivo activity assays of TbPTP1 inhibition

Stumpy form cells were treated with the various inhibitors at 150 and 300 μM or with DMSO alone, in HMI-9 at 27 °C for 4 h. The cells ($2-5 \times 10^6$) were harvested and fixed in 2% formaldehyde/0.05% glutaraldehyde for a minimum of 1 h at 4 °C. Antibody staining was executed according to (17). Flow cytometry analysis was performed using a FACSCalibur FACS machine (BD Biosciences; Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and the data analyzed using the FLOWJO 8.8.6 program (Tree Star, Inc. Ashland, OR, USA).

Results

Analysis of the predicted structures of TbPTP1 and PTP1B

To investigate the potential for inhibitors to cross target PTP1B and *Tb*PTP1B, we initially carried out a comparison of the predicted structures of each protein. Our analyses involved *in silico* comparison of the catalytic domain of *Tb*PTP1 with the structural parameters from previous crystallization analyses of the equivalent regions of PTP1B [i.e., aa 1–298 (18) and 1–321] (19) using the online workspace Swiss Model (20). Subsequently the *Tb*PTP1 crystal structure has been characterized (21), this providing very good agreement with our predicted model (245/288 aa shared the same secondary structure; the superimposition of the predicted and observed structure is shown in Figure S1). Indeed, the models differed only in the absence of two short stretches of beta sheet in the predicted model (residues 60–62 and 165–167) and in the residues defining the precise limits of the three helices (aa 12–13 and aa 43–47). This similarity provided the reassurance that our analysis of the model prediction was valid.

Analysis of the conserved motifs in PTPs

As previously reported, the alignment of the amino acid sequence of PTP1B and *Tb*PTP1 showed that nine of ten landmark PTP motifs (M1–M10) (22), except for motif 2 (M2), are present in the parasite enzyme (4). Indeed, more detailed analysis of the respective motifs demonstrated that all the invariant residues of the nine landmark PTP motifs (underscored bold letters in Figure 1) were perfectly conserved in *Tb*PTP1 with the overall identity of the motifs varying between 38 and 100%, with seven of eight motifs scoring more than 50%.

Outside the landmark motifs, seven residues have been reported to be conserved among PTPs (22), of which five were not identical in *Tb*PTP1. Specifically, Glu19, Arg156, Arg169, Leu192, and Arg254 of PTP1B are replaced by Phe25, Asp167, His188, Glu211, and Lys267 in the parasite enzyme. Of these five PTP residues, four are considered to be important for the definition of particular helices/sheets in the enzyme (22). Nonetheless, the parasite-specific residues are predicted to fold into the same secondary structure in *Tb*PTP1. Specifically, Phe25 defines the conserved α '2 helix, Asp167 and His188 the β 10 and β 11-sheet, and Glu211 the α 3 helix.

Of more interest was the fifth substitution (Arg254 → Lys267) in *Tb*PTP1. In PTP1B, Arg254 forms hydrogen bonds with the PTP loop of the enzyme and has been hypothesized to promote the interaction with the phosphorylated tyrosine (pTyr) of the substrate and/or in stabilizing the thiolate form of the nucleophilic cysteine (22). However, mutations of Arg254 to either Lys or Gln in PTP1B have been shown to have an inhibitory effect on the dephosphorylation of the artificial substrate pNPP (23). Hence, the presence in *Tb*PTP1 of Lys267 could indicate a weaker nucleophilic activity of the catalytic Cys residue.

In addition, Arg254 has been shown to form a second phosphate-binding site, which has lower affinity for the substrate, but that could be targeted, together with the main active site, to develop inhibitors with increased specificity (24). Indeed, a peptide possessing two adjacent phosphorylated tyrosine residues able to interact at both phosphate-binding sites has been shown to have a 70-fold higher binding affinity for PTP1B compared with the

monophosphorylated tyrosine peptide (23). Therefore, the observation that Arg254 is substituted by Lys267 in *TbPTP1* indicates that the parasite enzyme might not prefer substrates containing tandem pTyr over mono pTyr. Also, the absence of the conserved residue may assist the design of specific *TbPTP1* inhibitors.

Analysis of kinetoplastid motifs in *TbPTP1*

In addition to the PTP conserved motifs, orthologues of *TbPTP1* in *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma cruzi*, and *Leishmania major* possessed six conserved kinetoplastid-specific motifs. Two of these were located in the precatalytic region (PcT1 and PcT2), and four were distributed within the catalytic core (T1–T4) (4). Superimposition of the predicted *TbPTP1* secondary and tertiary structure on to the available mammalian PTP1B structure showed that most of the trypanosome-specific motifs align with the PTP1B backbone (Figure 2A,B). However, these motifs are arranged in random coils or U-turns, rather than α -helices or β -sheets, and are positioned on the predicted surface of the enzyme. Exceptions were motif T3, found within the core of the enzyme, and motif T4, which is positioned beyond the region represented in the 3-D structure prediction (aa 1–288).

In terms of alignment to the structural model, the trypanosome-specific precatalytic motif PcT1 (aa 20–29) was predicted to be found on the enzyme surface and fold into an α -helix, which aligns well with the corresponding PTP1B $\alpha 2'$ helix, a motif that is conserved structurally but not at the sequence level among PTPs (22). In contrast, PcT2 (aa 32–43), which is predicted to form a coil, partly deviates (residue 33–35) from the backbone structure of PTP1B and protrudes toward the outside of the enzyme. Similarly, the trypanosome-specific motif T1 (aa 55–63), which substitutes for the conserved PTP1B M2 motif, is predicted to fold into a loop, in contrast to M2, which forms a β -sheet. Moreover, Tyr62 of T1 is predicted to map on the edge of the loop, facing away from the central core of the enzyme. Similarly to T1, T2 (aa 164–166) also forms a U-turn on the surface of the enzyme.

The most interesting trypanosome-specific motif is probably T3 (aa 239–244), as it is found in the vicinity of the active site of the enzyme, although none of the corresponding residues in PTP1B have been shown to participate in catalysis. Motif T3 aligns well with the PTP1B backbone and, similarly to the human sequence, is predicted to fold into an α -helix. The only non conserved residue between T3 and the corresponding region of PTP1B is Ala242. This residue, together with Tyr243 of *TbPTP1*, is found only in one of thirty-seven PTPs (22).

Combined these analyses predicted that *TbPTP1* showed a general structural conservation with PTP1B that could enable its inhibition by compounds identified through their effect on the mammalian protein. However, the presence of kinetoplastid-specific motifs also generated the potential for differences in the inhibitor profile of the enzyme with respect to its mammalian counterpart.

Inhibitor analyses of PTP1B and TbPTP1

Seventeen PTP1B inhibitors, derived from the Drug Discovery Portal (DDP) at the University of Strathclyde, UK (25), were tested against human PTP1B and *Tb*PTP1. Also, two additional reported PTP1B inhibitors were tested: oleanolic acid and suramin (26). Oleanolic acid is a natural compound discovered from traditional Chinese medicinal herbs (27), and its affordability, commercial availability, and safety are valuable properties for a potential drug, particularly for the control of a neglected tropical disease. Suramin is a drug already in use for the treatment of sleeping sickness (28). The *in vitro* inhibitory activities of these compounds were determined against human PTP1B and *Tb*PTP1, and their IC_{50} and K_i values were calculated to compare their activities (Table 1, Figure 3, S2 and S3). Among the DDP inhibitors, inhibitor 2, 5, 7, and 12 possessed IC_{50} between 50 and 100 μM (with 95% confidence interval between 10 and 300 μM); DDP inhibitor 11, 15, and 17 showed an IC_{50} between 100 and 200 μM , and DDP inhibitor 1, 6, and 13 showed IC_{50} between 200 and 350 μM (Table 1, Figure 3). The respective dose–response curves for the most potent inhibitors (In. 2, 5, 7, and 12) are shown in Figure 4, whereas the equivalent information for all inhibitors is shown in Figures S2 (*Tb*PTP1) and 3 (PTP1B). For the remaining seven DDP inhibitors (In. 3, 4, 8, 9, 10, 14, and 16), it was not possible to calculate accurate values, probably due to very low inhibitory activities or sometimes to precipitation of the compound. Suramin showed K_i and IC_{50} values of less than 100 μM , whereas oleanolic acid was found to have a K_i and IC_{50} between 100 and 200 μM . BZ3 and vanadate represented positive controls, as their inhibitory activity on *Tb*PTP1 had already been tested (4) (Table 1). The comparative analysis of the inhibitory activities of the same compounds against *Tb*PTP1 and human PTP1B revealed no significant differences in K_i and IC_{50} values for most of them; only DDP inhibitor 6 and oleanolic acid showed a significantly greater inhibition of the human enzyme compared with the parasite homolog (Table 1). These findings demonstrate that a ‘piggyback’ strategy to target *Tb*PTP1 using this type of human PTP1B inhibitors is feasible, because the compounds tested displayed similar inhibitory activities against the two phosphatases. Moreover, oleanolic acid and suramin were identified as *Tb*PTP1 inhibitors for the first time.

The PTP1B inhibitors are mainly noncompetitive inhibitors of both human PTP1B and *Tb*PTP1

To further characterize the mechanism of action of the inhibitors tested, competition assays were performed between the compounds and the synthetic substrate pNPP. Of the nine DDP inhibitors tested, five (In. 5, 11, 12, 15, and 17) showed a noncompetitive type of inhibition, indicated by a decreased V_{max} but constant K_M in presence of inhibitors, compared with control assays containing enzyme and substrate alone (Table 2 and Figure S4). The K_M and V_{max} values for DDP inhibitors 2, 7, and 13 were not confidently determined due to poor fitting of the data to the Michaelis-Menten equation for unknown reasons. DDP inhibitor 1 did not significantly affect V_{max} and K_M at half IC_{50} concentration, and inhibitor 6 was excluded from the competition assays, being a very weak *Tb*PTP1 inhibitor ($IC_{50} > 1$ mM). Among the other PTP1B inhibitors, oleanolic acid was found to be a noncompetitive inhibitor, similarly to most of the DDP inhibitors, whereas suramin was found to be a competitive inhibitor of *Tb*PTP1 (Table 2). The comparative analysis between the data

obtained with *Tb*PTP1 and with the human PTP1B is shown in Figure S5. Most of the DDP inhibitors showed the same type of inhibitory behavior for the two enzymes. Of the other inhibitors oleanolic acid, which has been shown to be a competitive PTP1B inhibitor (29), was found not to bind the *Tb*PTP1 catalytic site. Contrasting with this, suramin was found to be a competitive inhibitor for both enzymes (26) (Table 1). These findings confirm an overall similar type of inhibition of the compounds toward the human and parasite phosphatases, suggesting that they may bind to similar sites.

The PTP1B inhibitors lack in vivo activity on stumpy form *Trypanosoma brucei*

After the *in vitro* characterization of the different PTP1B inhibitors, it was important to test the compounds on trypanosome cells. The specificity of the compounds for *Tb*PTP1 *in vivo* was assessed by determining whether they induced differentiation of stumpy forms to procyclic forms, the phenotype that was observed in *Tb*PTP1 RNAi lines and in cells treated with the PTP1B inhibitor, BZ3 (4). The eight most potent DDP PTP1B inhibitors, together with inhibitor 4, as a negative control, were tested at 150 and 300 μ M, concentrations that have been shown to be effective for BZ3 (4). However, none of the DDP inhibitors specifically inhibited the *Tb*PTP1, as judged by lack of induction of the procyclic surface protein EP, a hallmark of differentiation (Figure 5). In contrast, the differentiation trigger cis-aconitate was able to stimulate the expression of procyclin, as was treatment of the cells with the *Tb*PTP1 inhibitor BZ3, these acting as positive controls. Similarly, oleanolic acid and suramin did not induce stumpy to procyclic form transition (Figure 5). It is likely that the compounds do not accumulate in the cells at a concentration sufficient to inhibit *Tb*PTP1 to the extent required to induce differentiation, or that their specificity is insufficient. The comparative analysis of the inhibitory activities of the same compounds against *Tb*PTP1 and human PTP1B revealed no significant differences in K_i and IC_{50} values for most of them; only DDP inhibitor 6 and oleanolic acid showed a significantly greater inhibition of the human enzyme compared with the parasite homolog (Table 1).

These findings demonstrate that a ‘piggyback’ strategy to target *Tb*PTP1 using this type of human PTP1B inhibitors is feasible, because the compounds tested displayed similar inhibitory activities against the two phosphatases. Moreover, oleanolic acid and suramin were identified as *Tb*PTP1 inhibitors for the first time although other targets may be more important for antiparasitic activity. Indeed, oleanolic acid has been reported to affect a wide variety of physiological functions (30), and suramin has been shown to bind different enzymes probably because of its six negative charges (28,31). RNAi-mediated screening for mechanisms of suramin resistance has also identified a number of genes in bloodstream forms (32).

Discussion

Currently, research targeting sleeping sickness transmission has not been considered a priority, because other strategies, such as vector control or animal reservoir treatment, are already in use (10), and safer drugs to cure the disease are still under developed (33). Nevertheless, in a scenario where the incidence of African trypanosomiasis has been reduced and the focus is on its elimination, the use of a drug targeting disease transmission

would be beneficial. Sleeping sickness elimination has already been considered a reasonable aim in countries reporting <100 cases per year (34); in these countries, the removal of the vector altogether has been chosen as the main solution for disease elimination although tsetse reinvasion remains a constant threat (11). Furthermore, tsetse control has been successful only where transmission sites are localized, such as in the savanna and along forest galleries, whereas vector control has proven to be more difficult in forest habitats where *Glossina* spp. are more dispersed (35). For the above reasons, in areas with low sleeping sickness incidence, the possibility of treating the human host with a safe oral drug able to eliminate transmissible forms of the parasite could provide a novel and valuable approach to limit disease spread, complementing therapeutic strategies.

To investigate the potential to target *Tb*PTP1 using identified inhibitors for PTP1B, we initially carried out an *in silico* comparison of the parasite protein with the derived crystal structure of the mammalian enzyme. Subsequently, *Tb*PTP1 has itself been crystallized, and its structure analyzed with respect to the mammalian protein [21]. Both analyses supported a strong overall conservation of the structure of the parasite protein with respect to the PTP1B core, increasing the potential to use inhibitors that target both enzymes as a route to identify *Tb*PTP1 inhibitors. In addition to the common domains between *Tb*PTP1 and PTP1B, there also exist in *Tb*PTP1 a number of conserved kinetoplastid-specific domains. Both *in silico* analyses and crystallization studies highlight that these regions locate to the external and surfaces of the enzyme perhaps rendering them accessible to parasite-specific inhibitors.

In the present study, nineteen PTP1B inhibitors were tested against recombinant *Tb*PTP1, including suramin – an established therapy for trypanosomiasis. Although suramin apparently gains entry to the trypanosomes through a surface protein, ISG75, and is thereafter trafficked through a lysosomal pathway (32), the targets of this inhibitor are unknown. Interestingly, a biochemical study has revealed that suramin and close derivatives can target tyrosine phosphatases (26), and our study supports this concept, with *Tb*PTP1 being inhibited at similar concentrations to human PTP1B. Similarly, for most of the compounds tested here, the inhibitory activity and mode of inhibition of the compounds were consistent between the human and parasite proteins. However, in contrast to a previously tested inhibitor, BZ3, none of the compounds exhibited activity against stumpy forms sufficient to elicit differentiation to the procyclic form. Moreover, unlike BZ3, one of the DDP compounds (Inhibitor 12) caused death of bloodstream form parasites (Figure 6), likely reflecting its targeting of other molecules in the parasite due to the high concentrations used. Indeed, the calculated LD₅₀ for DDP inhibitor 12 was found to be 9.4 ng/mL or 40 μ M (11–74 μ M, 95% confidence interval) (Figure 6), which is at least 1000 fold higher than the 0.2 μ g/mL or 10 μ M threshold set by the current drug discovery program for HAT (<http://www.dndi.org>) (36). Therefore, future studies will need to focus more on the identification of the chemical modifications needed to achieve efficacy and specificity in the parasite itself. However, specificity for the parasite enzyme over the mammalian protein phosphatase is less important than for many other targets, because PTP1B knockout mice do not show any deleterious effects beyond their reduced susceptibility to obesity. This finding opens the possibility to exploit compounds that are not only identified by primary high throughput screens, but also those that have gone through the considerable additional

hurdles of efficacy and safety testing en route to the development of antiobesity therapies. In the case of PTPs, therefore, the benefits of a ‘piggyback’ approach can extend much further along the drug development pipeline and so offer considerable advantages for efforts focused on the control of diseases of the developing world.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Elchebly M, Payette P, Michaliszyn E, Cromlish W, Collins S, Loy AL, Normandin D, Cheng A, Himms-Hagen J, Chan CC, Ramachandran C, Gresser MJ, Tremblay ML, Kennedy BP. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science*. 1999; 283:1544–1548. [PubMed: 10066179]
2. van Montfort RL, Congreve M, Tisi D, Carr R, Jhoti H. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature*. 2003; 423:773–777. [PubMed: 12802339]
3. Zasloff M, Williams JJ, Chen Q, Anderson M, Maeder T, Holroyd K, Jones S, Kinney W, Cheshire K, McLane M. A spermine-coupled cholesterol metabolite from the shark with potent appetite suppressant and antidiabetic properties. *Int J Obes Relat Metab Disord*. 2001; 25:689–697. [PubMed: 11360152]
4. Szoor B, Wilson J, McElhinney H, Tabernero L, Matthews KR. Protein tyrosine phosphatase TbPTP1: a molecular switch controlling life cycle differentiation in trypanosomes. *J Cell Biol*. 2006; 175:293–303. [PubMed: 17043136]
5. Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, Krishna S. The trypanosomiasis. *Lancet*. 2003; 362:1469–1480. [PubMed: 14602444]
6. Vickerman K, Tetley L, Hendry KA, Turner CM. Biology of African trypanosomes in the tsetse fly. *Biol Cell*. 1988; 64:109–119. [PubMed: 3067793]
7. Czichos J, Nonnengaesser C, Overath P. *Trypanosoma brucei*: cis-aconitate and temperature reduction as triggers of synchronous transformation of bloodstream to procyclic trypomastigotes in vitro. *Exp Parasitol*. 1986; 62:283–291. [PubMed: 3743718]
8. Engstler M, Boshart M. Cold shock and regulation of surface protein trafficking convey sensitization to inducers of stage differentiation in *Trypanosoma brucei*. *Genes Dev*. 2004; 18:2798–2811. [PubMed: 15545633]
9. Szoor B, Ruberto I, Burchmore R, Matthews K. A novel phosphatase cascade regulates differentiation in trypanosomes via a glycosomal signaling pathway. *Genes Dev*. 2010; 24:1306–1316. [PubMed: 20551176]
10. Welburn SC, Fevre EM, Coleman PG, Odiit M, Maudlin I. Sleeping sickness: a tale of two diseases. *Trends Parasitol*. 2001; 17:19–24. [PubMed: 11137736]
11. Hargrove JW, Torr SJ, Kindness HM. Insecticide-treated cattle against tsetse (Diptera: Glossinidae): what governs success? *Bull Entomol Res*. 2003; 93:203–217. [PubMed: 12762862]
12. Ashburn TT, Thor KB. Drug repositioning: identifying and developing new uses for existing drugs. *Nat Rev Drug Discov*. 2004; 3:673–683. [PubMed: 15286734]
13. Trouiller P, Torreele E, Olliaro P, White N, Foster S, Wirth D, Pecoul B. Drugs for neglected diseases: a failure of the market and a public health failure? *Trop Med Int Health*. 2001; 6:945–951. [PubMed: 11703850]

14. Hirumi H, Hirumi K. Continuous cultivation of animal-infective bloodstream forms of an East African *Trypanosoma congolense* stock. *Ann Trop Med Parasitol*. 1984; 78:327–330. [PubMed: 6486939]
15. Lanham SM. Separation of trypanosomes from the blood of infected rats and mice by anion-exchangers. *Nature*. 1968; 218:1273–1274. [PubMed: 5656665]
16. Gordon JA. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. *Methods Enzymol*. 1991; 201:477–482. [PubMed: 1943774]
17. Dean SD, Marchetti R, Kirk K, Matthews K. A surface transporter family conveys the trypanosome differentiation signal. *Nature*. 2009; 459:213–217. [PubMed: 19444208]
18. Sun JP, Fedorov AA, Lee SY, Guo XL, Shen K, Lawrence DS, Almo SC, Zhang ZY. Crystal structure of PTP1B complexed with a potent and selective bidentate inhibitor. *J Biol Chem*. 2003; 278:12406–12414. [PubMed: 12547827]
19. Jia Z, Barford D, Flint AJ, Tonks NK. Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B. *Science*. 1995; 268:1754–1758. [PubMed: 7540771]
20. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*. 2006; 22:195–201. [PubMed: 16301204]
21. Chou S, Jensen BC, Parsons M, Alber T, Grundner C. The *Trypanosoma brucei* life cycle switch *TbPTP1* is structurally conserved and dephosphorylates the nucleolar protein NOPP44/46. *J Biol Chem*. 2010; 285:22075–22081. [PubMed: 20444707]
22. Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, Jansen PG, Andersen HS, Tonks NK, Moller NP. Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol Cell Biol*. 2001; 21:7117–7136. [PubMed: 11585896]
23. Salmeen A, Andersen JN, Myers MP, Tonks NK, Barford D. Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. *Mol Cell*. 2000; 6:1401–1412. [PubMed: 11163213]
24. Puius YA, Zhao Y, Sullivan M, Lawrence DS, Almo SC, Zhang ZY. Identification of a second aryl phosphate-binding site in protein-tyrosine phosphatase 1B: a paradigm for inhibitor design. *Proc Natl Acad Sci U S A*. 1997; 94:13420–13425. [PubMed: 9391040]
25. Clark RL, Johnston BF, Mackay SP, Breslin CJ, Robertson MN, Harvey AL. The Drug Discovery Portal: a resource to enhance drug discovery from academia. *Drug Discov Today*. 2010; 15:679–683. [PubMed: 20547242]
26. McCain DF, Wu L, Nickel P, Kassack MU, Kreimeyer A, Gagliardi A, Collins DC, Zhang ZY. Suramin derivatives as inhibitors and activators of protein-tyrosine phosphatases. *J Biol Chem*. 2004; 279:14713–14725. [PubMed: 14734566]
27. Zhang XY, Chen VL, Rosen MS, Blair ER, Lone AM, Bishop AC. Allele-specific inhibition of divergent protein tyrosine phosphatases with a single small molecule. *Bioorg Med Chem*. 2008; 16:8090–8097. [PubMed: 18678493]
28. Fairlamb AH, Bowman IB. *Trypanosoma brucei*: suramin and other trypanocidal compounds' effects on sn-glycerol-3-phosphate oxidase. *Exp Parasitol*. 1977; 43:353–361. [PubMed: 598447]
29. Zhang YN, Zhang W, Hong D, Shi L, Shen Q, Li JY, Li J, Hu LH. Oleanolic acid and its derivatives: new inhibitor of protein tyrosine phosphatase 1B with cellular activities. *Bioorg Med Chem*. 2008; 16:8697–8705. [PubMed: 18707891]
30. Liu J. Pharmacology of oleanolic acid and ursolic acid. *J Ethnopharmacol*. 1995; 49:57–68. [PubMed: 8847885]
31. Wierenga RK, Kalk KH, Hol WG. Structure determination of the glycosomal triosephosphate isomerase from *Trypanosoma brucei brucei* at 2.4 Å resolution. *J Mol Biol*. 1987; 198:109–121. [PubMed: 3430602]
32. Alsford S, Eckert S, Baker N, Glover L, Sanchez-Flores A, Leung KF, Turner DJ, Field MC, Berriman M, Horn D. High-throughput decoding of antitrypanosomal drug efficacy and resistance. *Nature*. 2012; 482:232–236. [PubMed: 22278056]
33. Brun R, Blum J, Chappuis F, Burri C. Human African trypanosomiasis. *Lancet*. 2010; 375:148–159. [PubMed: 19833383]

34. Welburn SC, Maudlin I, Simarro PP. Controlling sleeping sickness – a review. *Parasitology*. 2009; 136:1943–1949. [PubMed: 19691861]
35. Fevre EM, Picozzi K, Jannin J, Welburn SC, Maudlin I. Human African trypanosomiasis: epidemiology and control. *Adv Parasitol*. 2006; 61:167–221. [PubMed: 16735165]
36. Aguero F, Al-Lazikani B, Aslett M, Berriman M, Buckner FS, Campbell RK, Carmona S, et al. Genomic-scale prioritization of drug targets: the TDR Targets database. *Nat Rev Drug Discov*. 2008; 7:900–907. [PubMed: 18927591]
37. Wiesmann C, Barr KJ, Kung J, Zhu J, Erlanson DA, Shen W, Fahr BJ, Zhong M, Taylor L, Randal M, McDowell RS, Hansen SK. Allosteric inhibition of protein tyrosine phosphatase 1B. *Nat Struct Mol Biol*. 2004; 11:730–737. [PubMed: 15258570]
38. Huyer G, Liu S, Kelly J, Moffat J, Payette P, Kennedy B, Tsaprailis G, Gresser MJ, Ramachandran C. Mechanism of inhibition of protein-tyrosine phosphatases by vanadate and pervanadate. *J Biol Chem*. 1997; 272:843–851. [PubMed: 8995372]

Figure 1.

Comparative analysis of the residues conserved between *TbPTP1* and mammalian PTPs. (A) *TbPTP1* structure highlighting the nine conserved landmark PTP motifs (22) and the trypanosome-specific ones. (B) The consensus sequences of the PTP motifs are compared with the residues found in *TbPTP1* (4). The percentage of amino acid identity is also shown (identity); invariant amino acids are underscored and bold, and bold letters are conserved >90% and non bold >80% [from (22)].

Figure 2.

TbPTP1 structure. (A) Schematic diagram of *TbPTP1* highlighting the PTP motifs (M1–10) and the trypanosome-specific motifs (PcT1–2, T1–4). The nine conserved PTP motifs are shown (M1–M10) and colored according to the degree of conservation among PTPs (red: high, orange: medium, blue: low); the trypanosome-specific motifs are colored in pink (PcT1, PcT2, T1–T4). The secondary structure prediction for *TbPTP1* (α -helix, β -sheet and coil, predicted obtained using Swiss Model (20)) is also shown and labeled according to (22). (B) The 3-D structure of *TbPTP1* and PTP1B is shown, as predicted by the Swiss Model server and visualized using Swiss pdb-Viewer. The PTP motifs are labeled and colored as for A.

A Inhibitors showing IC_{50} or K_i 50-100 μM against *Tb*PTP1 **B** Inhibitors showing IC_{50} or K_i 100-200 μM against *Tb*PTP1 **C** Inhibitors showing IC_{50} or K_i 200-350 μM against *Tb*PTP1 **D** Inhibitors lacking inhibitory activity against *Tb*PTP1 **Figure 3.**

Summary of the inhibitory properties and chemical structures of the different inhibitors tested on *Tb*PTP1. (A) Chemical structures of the inhibitors that showed an IC_{50} or K_i values between 50 and 100 μM , together with the corresponding DDP ID and calculated IC_{50} ; suramin and vanadate are grouped together because they are both competitive inhibitors, whereas all the other compounds were noncompetitive inhibitors, with the exception of DDP inhibitor 7, which was found to be competitive also. (B) Chemical structures of the inhibitors that showed an IC_{50} or K_i values between 100 and 200 μM . (C) Chemical structures of the inhibitors that showed an IC_{50} or K_i values between 200 and 350 μM . (D) Chemical structures of four of the inhibitors that lacked activity against *Tb*PTP1.

Figure 4.

Dose–response curves for DDP inhibitors 2, 5, 7 and 12 against *TbPTP1*. A constant concentration of enzyme (0.3–0.4 $\mu\text{g}/$ reaction) and substrate (0.175 mM) was incubated with serial dilutions of inhibitors (from 200 to 0 μM). The percentage of substrate hydrolysis in presence of inhibitor compared with control without inhibitor (Hydrolysis % of control) is plotted against the logarithmic concentration of inhibitor (Log[In]); The calculated IC_{50} values for inhibitor 2, 5, 7, and 12 were 90, 56, 57, and 66 μM , respectively (A, B, C, and D).

Figure 5.

In vivo effect of the DDP inhibitors, oleanolic acid and suramin on differentiation of stumpy form to procyclic forms *Trypanosoma brucei*. Differentiation of stumpy to procyclic form cells, after treatment with the different inhibitors for 4 h at 27 °C, was quantified as the percentage of cells expressing EP-procyclin protein using FACS. The DDP inhibitors were tested at 0.15 and 0.3 mM whereas oleanolic acid (Ol.Ac) and suramin at lower concentrations. Cis-aconitate-treated cells, established procyclic form (Pcf), and BZ3-treated cells were included as positive controls.

Figure 6.

Trypanocidal activity of DDP inhibitor 12 and suramin. DDP inhibitor 12 and suramin were tested in triplicate on bloodstream form S16 cells, and their effect on cell viability was quantified as LD₅₀ using the Alamar Blue[®] assay and found to be 40 μM (95% CI: 1.5–851) and 0.02 μM (95% CI: 0.005–0.13), respectively (A and B); the percentage of Alamar Blue[®] reduction in treated versus untreated cells is shown on the y-axis and the logarithmic of concentration in μM is shown on the x-axis.

Table 1Summary of the IC_{50} and K_i calculated for the different inhibitors against *Tb*PTP1 and PTP1B

Inhibitor	K_i <i>Tb</i> PTP1	K_i PTP1B	IC_{50} <i>Tb</i> PTP1	IC_{50} PTP1B
In. 1	294 (32–2688)	51 (13–205)	319 (34–2922)	36 (0.3–3549)
In. 2	83 (26–269)	61 (37–103)	90 (28–292)	53 (41–67)
In. 5	51 (21–127)	30 (11–137)	56 (22–138)	32 (28–38)
In. 6	n.d.	39 (12–131)	1786 (246–12963)	33 (28–38)
In. 7	53 (13–126)	16 (7–39)	57 (14–234)	16 (14–19)
In. 11	116 (49–294)	n.d.	126 (49–319)	n.d.
In. 12	62 (36–106)	196 (22–1755)	66 (54–80)	113 (7–1734)
In. 13	306 (12–7495)	55 (34–89)	330 (13–8151)	70 (40–124)
In. 15	118 (49–284)	204 (140–299)	128 (53–308)	199 (168–236)
In. 17	102 (31–331)	42 (13–138)	111 (34–360)	35 (29–42)
BZ3	40 (7–234)	n.d.	43 (7–254)	8
Oleanolic ac.	120 (54–266)	n.d.	111 (60–206)	24
Suramin	6 (3–14)	n.d.	7 (3–15)	12
Vanadate	54 (14–200)	0.4 58 (15–217)	n.d.	

*Tb*PTP1, *Trypanosoma brucei* protein tyrosine phosphatase 1; PTP1B, protein tyrosine phosphatase 1B.

The number of the DDP inhibitors tested (In. 1–17), together with the other four PTP1B inhibitors are listed with the corresponding K_i and IC_{50} values for *Tb*PTP1 and PTP1B. Values are expressed in μM , and the 95% confidence interval is shown in parenthesis. The values listed for BZ3, oleanolic acid (Olean. acid), suramin and vanadate against PTP1B are the published ones [26,29,37,38]; n.d. is not determined. See Figures S1 and S2 for *Tb*PTP1 and PTP1B dose–response curves.

Table 2Competition experiments performed with *Tb*PTP1

	[In.]	$V_{\max}$	K_M	Inhibition
<i>Tb</i> PTP1 (batch 1)		2.0–4.0	0.1–5.0	
In. 5	11	1.78 (1.3–2.1)	3.3 (0.9–5.6)	Noncomp
	56	0.35 (0.3–0.6)	2.5 (0.6–8.3)	Noncomp
	111	0.25 (0.02–0.3)	2.23 (0.6–3.8)	Noncomp
In. 11	11	0.68 (0.4–0.8)	3.71 (0.2–7.2)	Noncomp
In. 12	43	0.19 (0.05–0.33)	1.3 (0.2 to 4.9)	Noncomp
	217	0.34 (0.19–0.48)	2.40 (0.1 to 5.9)	Noncomp
In. 15	11	1.86 (1.4–2.2)	2.15 (0.4–3.8)	Noncomp.
In. 17	27	0.48 (0.3–0.56)	7.42 (3.0–11.4)	Noncomp
BZ3	43	0.27 (0.0–0.65)	80 (0.0–238)	Mixed
Oleanolic ac.	3	0.41 (0.2–0.5)	3.28 (0.2–6.3)	Noncomp.
	18	0.40 (0.3–0.5)	1.92 (0.2–3.6)	Noncomp.
<i>Tb</i> PTP1 (batch 2)		1.8–3.1	0.2–4.4	
Suramin	3	1.7 (0.7–2.6)	12.6 (0.0–28.2)	Competitive
<i>Tb</i> PTP1 (batch 3)		1.1–1.7	0.7–4.0	
Vanadate	25	1.43 (1.2–1.6)	7.7 (4.3–11.0)	Competitive

*Tb*PTP1, *Trypanosoma brucei* protein tyrosine phosphatase 1.

The name of the inhibitor tested is listed in the first column from the left-hand side, together with the concentration of the inhibitor used in the assay in the second column ([In.] in μM); the third and fourth columns show the calculated $V_{\max}$ and K_M for the compounds (including the corresponding 95% confidence interval), and the last column summarizes the type of inhibition, according to the changed in $V_{\max}$ and K_M ; all the concentrations are in μM units. Note that BZ3 has been classified as 'mixed' because of its clear increase in K_M , despite the wide 95% confidence interval. Inhibitors were tested against distinct batches of recombinant *Tb*PTP1 and were tested against the respective K_M and $V_{\max}$ values for that batch.