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A targeted delivery strategy for the development of potent trypanocides

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Abstract

A new drug delivery strategy was investigated for the development of potent anti-parasitic compounds against *Trypanosoma brucei*, the causative agent of African sleeping sickness. Thus, potent *in vitro* hexokinase inhibitors were rendered cytotoxic by appending a tripeptide peroxosomal targeting sequence that facilitated delivery of the molecular cargo to the appropriate organelle in the parasite.

Graphical abstract

Keywords

African Sleeping Sickness; benzamidobenzoic acid; trypanosomes; Peroxosomal Targeting Sequence

Human African trypanosomiasis (HAT), commonly called African sleeping sickness, is transmitted by the tsetse fly and is caused by trypanosomes of the species *Trypanosoma brucei*.¹ The disease is endemic to sub-Saharan Africa, where millions of people are at risk for infection.^{2,3} Although treatments have existed for a number of years, these therapeutics are marked by serious adverse side-effects, such as acute toxicity, neural disorders, and in some cases, death.^{2,4}

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Bloodstream form (BSF) trypanosomes generate ATP for energy via glycolysis, in which the first step involves the formation of glucose-6-phosphate via the transfer of a phosphoryl group from ATP to the 6' hydroxyl group on glucose.^{1,3,4,5} This process is catalyzed by two hexokinases, TbHK1 and TbHK2, with the former having been genetically and chemically validated for therapeutic design.²⁻⁴ Through a high-throughput screening (HTS) campaign and follow-on small molecule probe development, benzamidobenzoic acid (BABA) derivatives were identified as potent inhibitors of recombinant TbHK1 (*i.e.* *r*TbHK1) *in vitro*.² In this initial screen, BABA **1** (Figure 1) emerged as a promising hit compound,² and represented a convenient starting scaffold for the exploration of structure-activity relationships (SAR) through a medicinal chemistry campaign.⁶ An initial round of SAR study revealed ML205 (*i.e.* **2**) to be a potent inhibitor of *r*TbHK1 ($IC_{50} = 0.98 \pm 0.07 \mu M$). Nonetheless, compound **2** was not an effective trypanocide against whole-cell BSF parasites. Finally, compound **2** had no impact on a mammalian hexokinase, and exhibited no appreciable toxicity toward human cell lines, confirming that this drug scaffold is amenable for the selective inhibition of trypanosomal hexokinases for therapeutic gain.^{2,6}

As an alternative to a conventional medicinal chemistry approach toward lead optimization, we describe herein a relatively underexplored and synthetically simple strategy toward achieving potency against live trypanosomes. Namely, we have successfully improved the trypanocidal activity of a derivative of **2** (*i.e.* compound **3**) by conjugating the drug to a tripeptide peroxisomal targeting sequence (*i.e.* PTS1)⁷⁻¹⁰ that ensures delivery of the agent to the glycosome, the organelle that houses the glycolysis machinery in trypanosomes. Similar to ML205, compound **3** is a reasonably potent *r*TbHK1 inhibitor (*i.e.* $IC_{50} = 3.5 \pm 0.3 \mu M$) that exhibits relatively poor performance against whole-cell BSF *T. brucei* (*i.e.* ~51% growth inhibition at 10 μM). Unlike ML205, compound **3** harbors a second carboxylic acid moiety (highlighted in green) that represented a convenient handle for the installation of a peptide linker and PTS1.

In trypanosomes, molecular cargo bound for glycosomes typically bears a peroxisome targeting sequence (PTS).¹⁰ The PTS type 1 sequence has been employed previously to deliver both proteinaceous cargo⁷⁻⁹ and a pH sensor to the glycosome in trypanosomes,¹⁰ but this appealing strategy has never been exploited for the delivery of a small-molecule drug with the aim of developing potent trypanocides. Because TbHK1 is a glycosome-resident protein, we surmised that a potent TbHK1 inhibitor exhibiting poor whole-cell activity might be rescued by appending a PTS1 sequence through relatively straight-forward synthetic operations. Thus, we designed four derivatives of **3** that incorporate a PTS1 tripeptide via an intervening diglycine or diethylene glycol linker (Figure 1, **4-7**). The synthesis of PTS1-BABA conjugates **4-7** began with the iodination of 2-amino-4-chloromethyl benzoate **8** with molecular iodine in the presence of silver (I) sulfate to afford iodinated aniline derivative **9** in quantitative yield.¹¹ Iodo-aniline **9** was then subjected to Suzuki coupling conditions with 4-*tert*-butyloxycarbonyl phenylboronic acid to afford biaryl aniline derivative **10** in 96% yield.¹² Next, *tert*-butyl benzoic acid was converted to its corresponding acid chloride by action of thionyl chloride and triethylamine, and biaryl aniline derivative **10** was then added to afford the *tert*-butyl ester **11** in 54% yield. The *tert*-butyl ester functionality in **11** was then cleaved by trifluoroacetic acid to afford the mono-

carboxylic acid **12**, poised for coupling with the linker and PTS1. Mono-acid **12** was saponified with aq. LiOH in THF to provide the previously evaluated¹³ BABA diacid **3**.

The PTS1-BABA conjugates **4–7** were prepared by coupling the appropriate linker region and the LKA PTS1 sequence onto mono-acid **12** by routine Solid Phase Peptide Synthesis (SPPS) using the ultra-reactive uronium reagent, HCTU¹⁴, along with conventional deprotection and resin cleavage protocols¹⁵ (See ESI for details). The SPPS protocol returned the PTS1-BABA methyl esters **4** and **6**, which could be subsequently converted to the PTS1-BABA carboxylic acids **5** and **7** by saponification.

Next, we evaluated PTS1-BABA conjugates **4–7** as TbHK1 inhibitors and as trypanocides against BSF *T. brucei* cultures. Our biological screening returned four parameters: % TbHK1 inhibition at 10 μ M drug concentration, IC₅₀ values for TbHK1 inhibition (*i.e.* drug concentration capable of effecting 50% inhibition of TbHK1 activity), % growth inhibition of BSF *T. brucei* at 10 μ M drug concentration, and BSF EC₅₀ values (*i.e.* drug concentration capable of killing 50% of trypanosomes in culture). BABA derivatives **2**, **3**, **11**, and **12** along with the peptide-drug conjugates **4–7** were initially evaluated against both *r*TbHK1 and BSF *T. brucei* at 10 μ M (See ESI for details). Based on the results of these initial screens, the compounds were evaluated in a dose-response format to calculate the IC₅₀ and EC₅₀ values for *r*TbHK1 and BSF *T. brucei* inhibition, respectively. The results of these assays are collected in Table 1.

We first tested the BABA analogs **2**, **3**, **11**, and **12** against both the enzyme (*r*TbHK1 isolated from *Escherichia coli* or yeast) and live parasites (tests run in triplicate). Recall that ML205 **2** is a potent inhibitor of *r*TbHK1 (IC₅₀ = 0.98 $\pm$ 0.067 μ M), but exhibits poor efficacy against live BSF parasites (6.9 $\pm$ 3.0 % inhibition of BSF *T. brucei* growth) (entry 1).⁶ Similarly, diacid analog **3** inhibits *r*TbHK1 with an IC₅₀ of 3.5 $\pm$ 0.3 μ M, but only marginally inhibits the growth of BSF parasites (51.0 $\pm$ 14.3%) (entry 2).¹³ The mixed diester analog **11** was ineffective against the *r*TbHK1 enzyme and in the assay with live BSF parasites (entry 3). Monoacid **12**, our target molecule for PTS1 conjugation, was effective against the *r*TbHK1 enzyme (87.1 $\pm$ 3.5% inhibition at 10 μ M), yet showed weak activity against the BSF parasite (24.5 $\pm$ 6.8% growth inhibition).

Thus, most of the BABA derivatives prepared in this study are reasonably active against the purified *r*TbHK1 enzyme but lack efficacy against the whole-cell BSF parasite. Next, we evaluated the PTS1-BABA conjugates **4–7** against *r*TbHK1 and against BSF *T. brucei*. PTS-1 BABA methyl ester **4** and acid **5** (both attached to the PTS1 via an intervening diglycine linker) were essentially inactive. Conjugate **4**, did not inhibit *r*TbHK1 or live BSF trypanosomes at 10 μ M (entry 5). Similarly, the corresponding acid **5** was a weak inhibitor of *r*TbHK1 (26.3 $\pm$ 3.8% at 10 μ M) and was ineffective against BSF trypanosomes at 10 μ M (entry 6).

In contrast to the diglycine linked PTS1-BABA conjugates, the biological evaluation of the diethylene glycol linked PTS1-BABA ester **6** and acid **7** yielded an interesting dichotomy. The methyl ester PTS1-BABA conjugate **6**, while exhibiting little activity toward *r*TbHK1 (10.5 $\pm$ 3.6% inhibition at 10 μ M), proved to be a potent inhibitor of BSF *T. brucei* (100%

inhibition at 10 μM ; BSF $\text{EC}_{50} = 6.8 \pm 1.5 \mu\text{M}$) (entry 7, highlighted in blue). In sharp contrast, the corresponding acid **7** was reasonably effective at inhibiting *rTbHK1* ($67 \pm 4.6\%$ inhibition at 10 μM ; $\text{IC}_{50} = 6.8 \pm 0.2 \mu\text{M}$), yet exhibited significantly poorer efficacy against BSF parasites in culture ($38.9 \pm 11.1\%$ inhibition at 10 μM) (entry 8). In addition to the compounds denoted in Table 1, we also assayed a PTS peptide sequence that lacked a drug moiety to demonstrate that the targeting sequence itself was non-toxic. Thus a PTS tripeptide bearing a fluorescein isothiocyanate moiety instead of the BABA drug conjugate was found to exhibit no toxicity to trypanosomes at 10 μM nor did it detectably inhibit the *TbHK1* enzyme *in vitro* (see ESI for structure and data), despite effectively localizing into the glycosomes as judged by fluorescence imaging as previously described.¹⁰ Finally, we sought to benchmark our new PTS-BABA conjugates to a well-known and clinically relevant anti-trypanosomal drug, pentamidine (see ESI for structure). In our hands, pentamidine returned an EC_{50} of $0.003 \pm 0.001 \mu\text{M}$ against BSF parasites. This value is in reasonable agreement with results reported previously by others for pentamidine against the two human-infective subspecies of *T. brucei*,¹⁶ and points to the potency we aim to achieve through the further development and optimization of future PTS tripeptide-drug conjugates.

Several conclusions from these results warrant emphasis. First, the data depicted in entry 7 demonstrates the success of our approach toward rescuing the trypanocidal potency of the potent *rTbHK1* inhibitor **3** by appending a peroxisomal targeting sequence to the drug scaffold. Nonetheless, it is interesting that the enhanced efficacy of **6** against live BSF parasites is accompanied by a significant reduction in potency against *rTbHK1* *in vitro*. This seemingly strange dichotomy may in fact indicate that the PTS1-BABA methyl ester conjugate **6** acts as a pro-drug, whose biologically active form (PTS1-BABA acid **7** perhaps) is only revealed upon arrival, or *en route* to, the glycosomal target after PTS-1 mediated passage across the cell membrane. The data for PTS1-BABA acid **7** supports this hypothesis (entry 8). The carboxylic acid of the benzamidobenzoic acid core in conjugate **7** allows for reasonably potent *rTbHK1* inhibition, but may preclude passage across the cell membrane, despite the presence of the PTS-1 tripeptide. The data presented in Table 1 highlights the potential utility of this synthetically simple approach to further lead optimization of potent *in vitro* enzyme inhibitors by ensuring proper targeted delivery to the appropriate cellular target *in vivo*.

In an effort to demonstrate that localization to the trypanosome glycosome is facilitated by appending a PTS-1 tripeptide to a drug candidate, we prepared conjugate **13** (Figure 2). This molecule is essentially identical to the active conjugate **6**, except that the side-chain amine of the lysine residue in the PTS sequence has been modified with a FITC fluorophore that facilitated cellular imaging.

Thus, we imaged live procyclic form (PF) and BSF *T. brucei* expressing PTS2-mCherry from the tubulin locus¹⁷ (in the presence of **13**). The majority of the FITC signal co-localized with the glycosome marker protein (PTS2-mCherry). These findings suggest that in living parasites, the PTS-1 modified cargo was delivered to the appropriate subcellular compartment. The activity of conjugate **6** against enzyme and the distribution of its fluorescent analog **13** in live trypanosomes, suggests that the toxicity observed in the parasites for **6** may be in part due to inhibition of the essential hexokinase activity *in vivo*.

Conclusions

In conclusion, we have successfully prepared a series of PTS1-*BABA* conjugates and evaluated their potency as inhibitors of recombinant *T. brucei* hexokinase 1 (*rTbHK1*) and as trypanocides against live BSF *T. brucei*. In doing so, we have demonstrated that appending the PTS1 tripeptide can rescue the poor trypanocidal efficacy of an otherwise potent inhibitor of *rTbHK1*. To the best of our knowledge, these results represent the first example of the successful delivery of a drug candidate to the glycosome of a kinetoplastid parasite facilitated by the incorporation of a simple targeting sequence through straight-forward synthetic operations. This strategy represents a complementary strategy for the development of potent trypanocides that does not rely on conventional medicinal chemistry derivitization of the parent scaffold. Further, we have shown that the PTS1-*BABA* conjugates are efficiently localized within the glycosome of PF and BSF parasites by means of fluorescence microscopy. We have confirmed for the first time that PTS1 mediated drug delivery is possible in trypanosomatids. Further, we suspect that the PTS1 conjugation strategy for drug delivery described herein could be applicable to other members of the family, including *T. cruzi* and *Leishmania major*, the causative agents of Chagas disease and leishmaniasis, respectively. Efforts to demonstrate the broader efficacy of this strategy with other trypanosomatids and to develop molecular probes to understand the cellular mechanisms for PTS1 mediated uptake in trypanosomes are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
BABA TbHK1 inhibitors and PTS1-BABA conjugates.

Figure 2.

Localization of conjugate **13** into the glycosome of BSF *T. brucei*. Top row from left to right: DIC image of live procyclic form (PF) *T. brucei*; PTS2-mCherry localized into glycosomes of PF *T. brucei*; Green fluorescent PTS1-FITC-BABA conjugate **13** localized into glycosomes of PF *T. brucei*; merged images of PTS2-mCherry and **13** localized into glycosomes of PF *T. brucei* (orange hue indicates overlap). Bottom row from left to right: DIC image of live bloodstream form (BSF) *T. brucei*; PTS2-mCherry localized into glycosomes of PF *T. brucei*; Green fluorescent PTS1-FITC-BABA conjugate **13** localized into glycosomes of BSF *T. brucei*; merged images of PTS2-mCherry and **13** localized into glycosomes of BSF *T. brucei* (orange hue indicates overlap). Scale bar = 5 μ m

Scheme 1.
Synthesis of PTS1-*BABA* conjugates **3–6**.

Table 1

Biological data for *BABA* derivatives and PTS1-*BABA* conjugates.^[a]

Entry	Compound	TbHK1% inhibition (10 μ M)	TbHK1 IC ₅₀ (μ M)	%BSF Growth Inhibition (10 μ M)	BSF EC ₅₀ (μ M)
1	2	–	0.98 $\pm$ 0.067	6.9 $\pm$ 3.0	> 10
2	3	93.6 $\pm$ 2.8	3.5 $\pm$ 0.3	51.0 $\pm$ 14.3	> 10
3	11	0	> 10	9.6 $\pm$ 9.7	> 10
4	12	87.1 $\pm$ 3.5	> 10	24.5 $\pm$ 6.8	> 10
5	4	0	> 10	0	> 10
6	5	26.3 $\pm$ 3.8	> 10	0	> 10
7	6	10.5 $\pm$ 3.6	> 10	100	6.8 $\pm$ 1.5
8	7	67.5 $\pm$ 4.6	6.8 $\pm$ 0.2	38.9 $\pm$ 11.1	> 10

^aTrypanosome cultures were assayed in triplicate in a total volume of 40 μ L, and the DMSO vehicle was used as a negative control. Percent inhibition was calculated by comparison to the growth of parasites grown with the DMSO controls from each plate. SID 17387000[21] was included in each assay as a positive control. Dose-response curves performed in triplicate for compounds that elicited > 50% growth inhibition at 10 μ M were pursued in a 384-well plate format, with 50% effective concentrations (*i.e.* EC₅₀) determined using GraphPad Prism (version 6.0) software (GraphPad Software, Inc., La Jolla, CA).