

REPORT

DRUG DEVELOPMENT

Inhibitors of PEX14 disrupt protein import into glycosomes and kill *Trypanosoma* parasites

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The parasitic protists of the *Trypanosoma* genus infect humans and domestic mammals, causing severe mortality and huge economic losses. The most threatening trypanosomiasis is Chagas disease, affecting up to 12 million people in the Americas. We report a way to selectively kill *Trypanosoma* by blocking glycosomal/peroxisomal import that depends on the PEX14-PEX5 protein-protein interaction. We developed small molecules that efficiently disrupt the PEX14-PEX5 interaction. This results in mislocalization of glycosomal enzymes, causing metabolic catastrophe, and it kills the parasite. High-resolution x-ray structures and nuclear magnetic resonance data enabled the efficient design of inhibitors with trypanocidal activities comparable to approved medications. These results identify PEX14 as an ‘Achilles’ heel’ of the *Trypanosoma* suitable for the development of new therapies against trypanosomiasis and provide the structural basis for their development.

Human African trypanosomiasis (HAT, sleeping sickness) and Chagas disease are caused by the protists *Trypanosoma brucei* spp. and *Trypanosoma cruzi*, respectively. Animal trypanosomiasis cause huge losses to livestock animals (1–3) and are becoming increasingly resistant to currently available medications (4–6). Chagas disease has been most prevalent in South America but is spreading internationally owing to increased migration (7, 8) and potential climate change-induced vector spreading (9–11). The drug discovery pipeline for trypanosomiasis is thin. Existing medications (Suramin, Pentamidine, Melarsoprol, Benznidazole, and Nifurtimox) have serious side effects, require long treatment schedules, and often fail to eliminate parasitemia. Therefore, new therapeutic

strategies targeting trypanosomiasis are urgently needed.

Glycosomes are peroxisome-related organelles containing enzymes required for glucose metabolism and parts of other intermediary metabolic pathways (12). They are essential and characteristic to a few protists, including *Trypanosoma* spp. (13). The biogenesis of glycosomes depends on proteins called peroxins or PEX (14). The PEX14-PEX5 protein-protein interaction is essential for glycosome biogenesis and glycosomal protein targeting (10, 12). The PEX5 protein is the import receptor for the transport of glycosomal enzymes from the cytoplasm into the organelle (15). The C-terminal tetratricopeptide repeat (TPR) domain of PEX5 recognizes a peroxisomal targeting signal (PTS) peptide motif in cargo proteins (16). The N-terminal half of PEX5 is intrinsically disordered and harbors several diaromatic WxxxF peptide motifs that are recognized by the small globular N-terminal domain of PEX14. PEX14 is associated with the glycosomal membrane and the PEX14-PEX5 interaction is required for cargo translocation (17–21) (fig. S1). PEX14 RNA interference (RNAi) studies have shown that glucose becomes toxic to *T. brucei* when glycosomal import is disrupted (13, 22, 23), thus indicating a potential drug target.

Here, we show that disrupting the PEX14-PEX5 interaction with small molecule inhibitors leads to accumulation of glycosomal enzymes in the cytosol, adenosine triphosphate (ATP) depletion, glucose toxicity, and metabolic collapse resulting in *T. brucei* parasite death.

The N-terminal domain of human PEX14 adopts a small helical fold where two aromatic residues

(Phe³⁵ and Phe⁵²) are engaged in a π -stacking interaction in the center of the binding interface with PEX5 WxxxF ligands. This pair of aromatic side chains (II) separates two hydrophobic pockets, which accommodate tryptophan (III) and phenylalanine (I) in the PEX5 WxxxF peptide motifs (Fig. 1A) (17, 18). To enable structure-based drug design, we determined the solution nuclear magnetic resonance (NMR) structure of the *T. brucei* PEX14 N-terminal domain (fig. S2, B and C, and table S1). The overall fold is very similar to the human PEX14 N-terminal domain but exhibits an additional C-terminal helix $\alpha 5$. The two hydrophobic pockets and the two phenylalanine residues in the binding surface are conserved in *T. brucei*. Characteristic amino acid differences are observed in the PEX5 binding pockets of trypanosome PEX14 (Arg²⁸, Asn³¹, Glu³⁴, and Asp³⁸) compared with human PEX14 (Leu²⁸, Thr³¹, Lys³⁴, and Asn³⁸). This indicates that inhibitors can be designed to selectively target *T. brucei* PEX14 (fig. S2, A and D) using specific, polar interactions.

Using our structure and other structural information available (17, 24, 25), we created a three-dimensional (3D) pharmacophore model mimicking the binding mode of the aromatic residues of PEX5 WxxxF to the respective PEX14 pockets (Fig. 1A). Using this model, we performed in silico 3D pharmacophore-based screening followed by 3D docking of the best hits and visual compound inspection. The computationally identified hits were tested experimentally by monitoring binding to ¹⁵N-labeled *T. brucei* PEX14 N-terminal domain using ¹H-¹⁵N heteronuclear correlation NMR spectroscopy. This led to the identification of a druglike pyrazolo[4,3-*c*]pyridine derivative **1** (Fig. 1B). The compound exhibits a moderate affinity to PEX14 as judged by NMR titrations (Fig. 1C) and is able to disrupt the PEX14-PEX5 interaction in an orthogonal, proximity-based AlphaScreen assay (PerkinElmer Life Sciences) with a binding inhibition constant (K_i) = 61.6 μ M (Table 1 and fig. S3). Despite the relatively weak affinity to the target, derivative **1** proved to be toxic to bloodstream-form *T. brucei* cells, with a median effective concentration (EC₅₀) of 21 μ M with low toxicity for murine NIH-3T3 fibroblasts and human HepG2 cells (Table 1 and figs. S4 and S5).

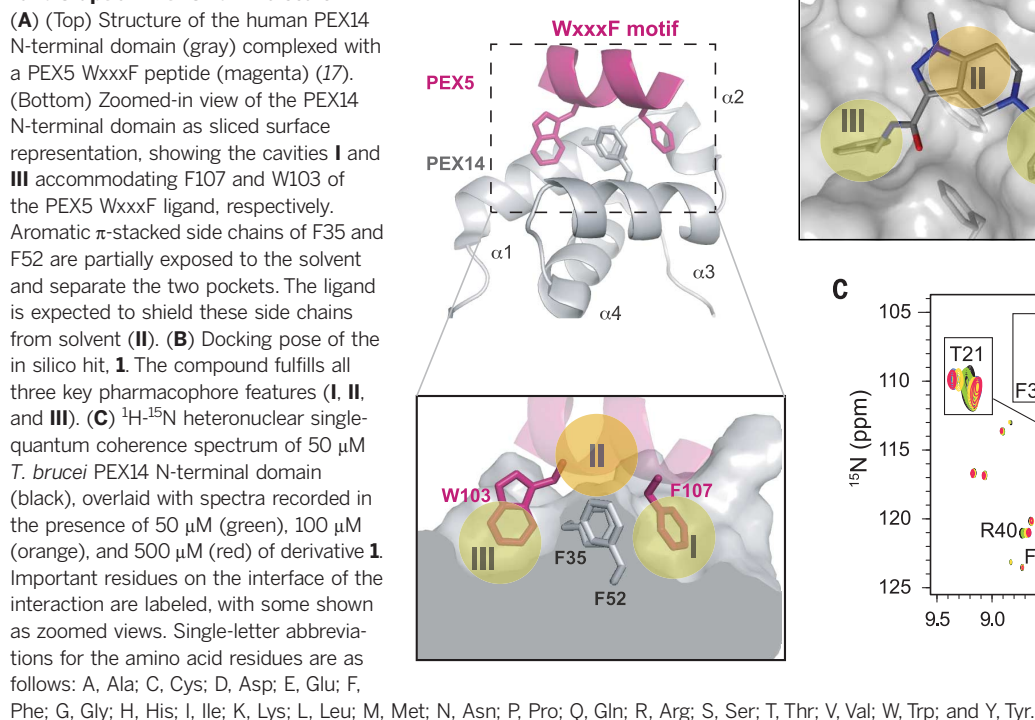
To exclude that potential off-target modes of action and general toxicity contribute to the observed activity, we further optimized the molecule toward higher PEX14 affinity. Initially, we performed an NMR-based fragment screen of 1500 fragments from our in-house library to identify fragment motifs that favorably bind PEX14. We observed a strong preference of PEX14 to bind double-aromatic ring systems (table S2). These chemical groups were directly used to replace the decoration of derivative **1**. This yielded derivatives **2** and **3**, which exhibit increased potency in biochemical assays and enhanced trypanocidal activity (Fig. 2A and Table 1). We solved high-resolution crystal structures for derivatives **2** and **3** in complex with *T. brucei* PEX14 at 0.86- and 1.57-Å resolution, respectively (Fig. 2, B and C, and table S3). Whereas the central element (II in Fig. 1, A and B), present in all compounds,

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Fig. 1. PEX14-PEX5 interface is suitable for disruption with small molecule.

efficiently shields the partially exposed Phe³⁵ and Phe⁵² in the PEX14 interface, the two aromatic groups fill the hydrophobic cavities. Notably, the indole ring of **2** addresses the phenylalanine pocket (**I**), whereas the naphthyl ring fills the tryptophan pocket (**III**). In the PEX14-**3** complex, the phenylalanine and tryptophan pockets are filled by the methoxynaphthyl and phenyl moieties, respectively. One bound water molecule forms two important hydrogen bonds with the ligands. Remarkably, this water is found in all the complex structures of the inhibitors tested, indicating that this interaction is critically important (Fig. 2, B to D, and fig. S6). This interaction has not been predicted by docking of derivative **1**, which explains its docking pose being shifted toward pocket (**I**) when compared with the crystal structures (Fig. 2E). Our attempts to displace this water molecule with a polar element of the inhibitor yielded inactive compounds, indicating that the water is very strongly bound to the protein. Importantly, the water molecule interacts with the side chain of Asn³¹, a residue that is specific to *Trypanosoma* and distinct from humans (fig. S2A). Therefore, this interaction can contribute to ligand specificity. Both derivatives **2** and **3** offer a significantly extended hydrophobic interface when compared to the in silico hit **1**, which results in the improved binding affinity. Notably, the lower K_i leads to significant improvement of in vitro trypanocidal activity, with EC_{50} values being lower than K_i (Table 1). Molecular hybridization of derivatives **2** and **3** followed by $-\text{OH}$ to $-\text{NH}_2$ replacement yielded compound **5** (Fig. 2A), a more soluble and potent PEX14-PEX5 interaction inhibitor ($K_i = 207$ nM). The structure

of derivative **5** bound to PEX14 (Fig. 2D) shows that the 5-NH_2 group is in proximity to the negatively charged carboxyl side chains of Glu³⁴. This interaction improves compound specificity because in human PEX14 the positively charged side chain of the corresponding Lys³⁴ would introduce electrostatic repulsion (fig. S2). Overall, PEX5 binds PEX14 primarily by hydrophobic interactions. NMR titrations of peptide ligands derived from human and *T. brucei* PEX5 to ^{15}N -labeled trypanosome PEX14 show different chemical-shift perturbations (fig. S7A). This indicates that distinct features in the binding interface contribute to the ligand specificity of PEX14. Consistently, derivatives **4** and **5** containing hydrophobic features identified as preferred by *T. brucei* PEX14 by fragment screen show enhanced selectivity with much weaker binding to the human ortholog (fig. S7B).

A multiple sequence alignment (fig. S2A) indicates a high degree of conservation of PEX14 among trypanosomatid protists. Therefore, we tested derivative **5** in cell-based assays against different *Trypanosoma* species. The inhibitor exhibits remarkable cellular potency against the livestock pathogen *T. brucei brucei* ($\text{EC}_{50} = 186$ nM). Moreover, the compound is even more potent against the human-infecting *T. brucei rhodesiense* subspecies ($\text{EC}_{50} = 21$ nM). The activity of derivative **5** against the intracellular form (amastigote) of *T. cruzi* ($\text{EC}_{50} = 570$ nM) is approximately twofold greater than the reference drug Benznidazole (Table 1 and fig. S9). Mammalian cell toxicity of derivative **5** from a potential interference with the human PEX14-PEX5 interaction is not expected because there is no correlation between mamma-

lian cell toxicity and *Hs*PEX14 affinity (Table 1). Collectively, these data show that targeting glycosome import by disrupting the PEX14-PEX5 interaction is a promising strategy for the development of broad-spectrum trypanocidal agents.

The PEX14 inhibitors based on the pyrazolo-[4,3-*c*]pyridine scaffold show a correlation between inhibition in biophysical assays (K_i) and trypanocidal activity in cellular assays (EC_{50}) (Fig. 3A). This indicates that the compounds indeed act by targeting the PEX14-PEX5 interaction. Notably, for most of the inhibitors, we observe that the cell-based activities (EC_{50}) on *T. brucei* are significantly lower than inhibition (K_i) in vitro. This results from the fact that the trypanosomal hexokinase (HK) and phosphofructokinase (PFK) enzymes lack feedback regulation. Therefore, their compartmentation inside glycosomes with a limited pool of ATP is necessary to control their activities. Their mislocalization to the cytosol leads to unregulated glucose phosphorylation (thus amplifying the effect) causing ATP depletion (fig. S1), which results in the accumulation of glucose metabolites to toxic levels and eventually induces cell death (22, 23). Consistent with this, we observe significantly higher trypanocidal activity of derivative **5** in the presence of glucose compared with cells grown on amino acid-rich medium lacking glucose. This confirms PEX14 RNAi studies reported previously (23). In contrast, the presence of glucose in the medium was protective when cells were treated with the general cytotoxic agent blasticidin (Fig. 3B).

Immunofluorescence microscopy using antibodies against glycosomal enzymes confirms that derivative **5** prevents compartmentation of the

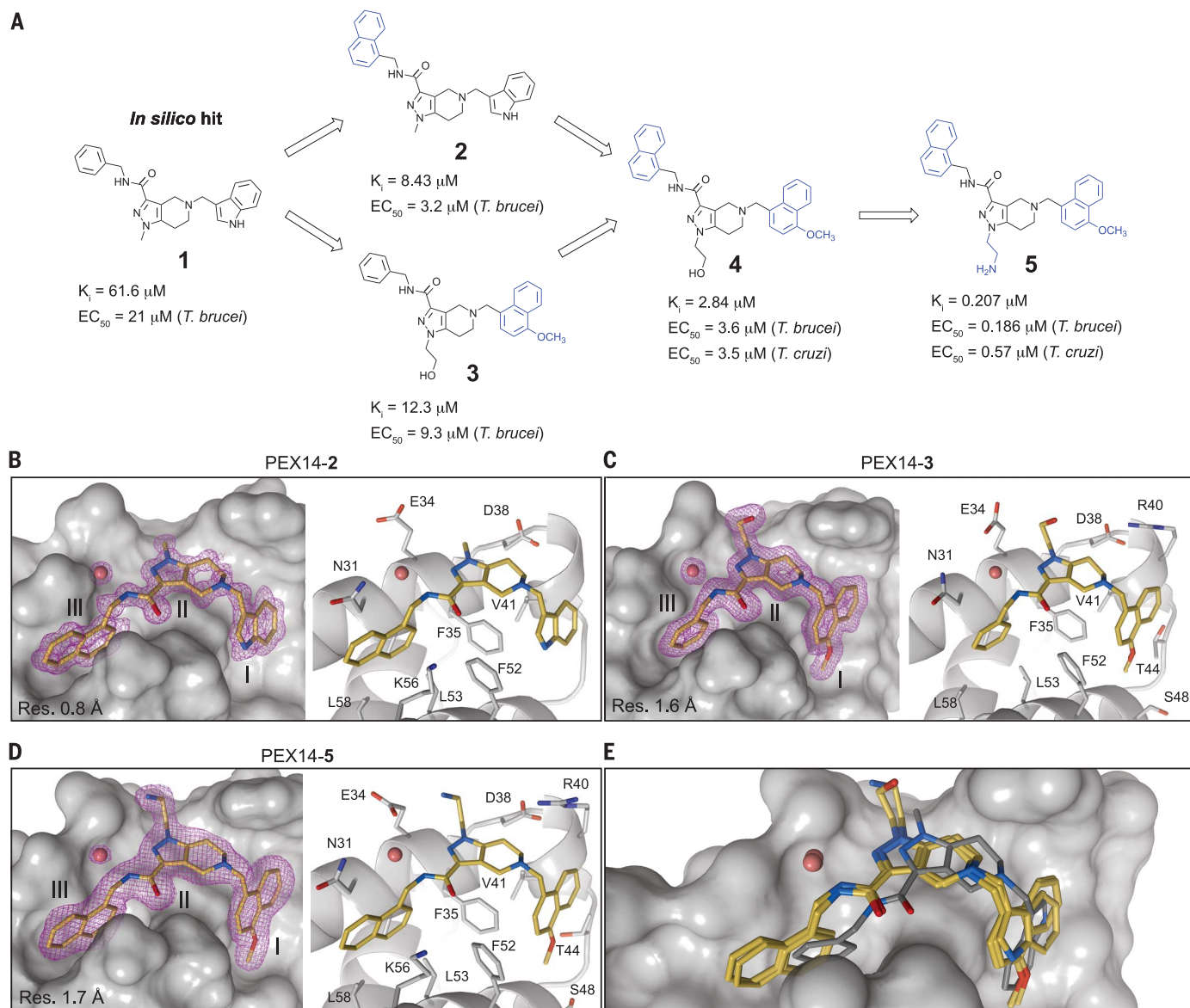


Fig. 2. Structure-based optimization of PEX14-PEX5 interaction inhibitors. (A) Optimization of the hydrophobic residues of the initial in silico hit **1** to better address the Trp and Phe binding pockets in PEX14, followed by an $-\text{OH}$ to $-\text{NH}_2$ replacement, leads to the potent PEX14-PEX5 binding inhibitor **5**. (B to D) Crystal structures of the PEX14-2 (B), PEX14-3 (C), and PEX14-5 (D) complexes. (Left) Ligand electron densities contoured at 1σ ($2F_o - F_c$ map).

(Right) Cartoon representations of the complexes; residues contacting the ligands are annotated. Structurally conserved water molecules that mediate hydrogen bonds between PEX14 N31 and the inhibitor amide group are shown as red spheres. (E) Overlay of crystallography-derived binding conformations of derivatives **2**, **3**, and **5** (yellow) with the docking pose of in silico hit **1** (gray).

glycolytic enzymes in glycosomes of *T. brucei brucei* (Fig. 3C). Glycosomal cargo is designated by one of two distinct localization motifs: PTS1 and PTS2 (26, 27). When exposed to derivatives **5**, all tested glycosomal enzymes—phosphofructokinase (PTS1), glycosomal glyceraldehyde-3-phosphate dehydrogenase (PTS1), hexokinase (PTS2), and aldolase (PTS2)—were mislocalized to the cytosol (Fig. 3, C and D, and fig. S8). Complete mislocalization was not expected, but even its partial defect is toxic to trypanosomes, consistent with the trypanocidal activity observed in our cell-based assays. Trypanosome cells round up when exposed to PEX14 inhibitors. This could be an indirect effect

of metabolic/osmotic imbalance caused by glycosome disruption, as has been shown previously (28). Defective compartmentation of glycolytic enzymes is accompanied by a significant reduction in ATP levels (Fig. 3E).

It is interesting to note, that *T. cruzi* intracellular amastigotes are also very susceptible to PEX14 inhibitors (Table 1 and fig. S9). We confirmed that our inhibitors bind to *T. cruzi* PEX14 (fig. S10). Although intracellular *T. cruzi* amastigotes do not express glucose transporters (29), inhibitors of *T. cruzi* hexokinase (30), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (31), or squalene synthase (32) kill intracellular amasti-

gotes, indicating that glycosomes are also essential in this stage. Therefore, unlike *T. brucei*, where glucose toxicity kills the parasites (13), the mechanism of killing *T. cruzi* intracellular amastigotes by PEX14 inhibitors could be due to disruption of other essential glycosomal metabolic pathways, which is a subject for future investigations.

The PEX14-PEX5 interface is largely hydrophobic. This results in unfavorable absorption, distribution, metabolism, and excretion (ADME) parameters of the PEX14 inhibitors tested here and poses some expectable challenges for drug development that will be addressed by further

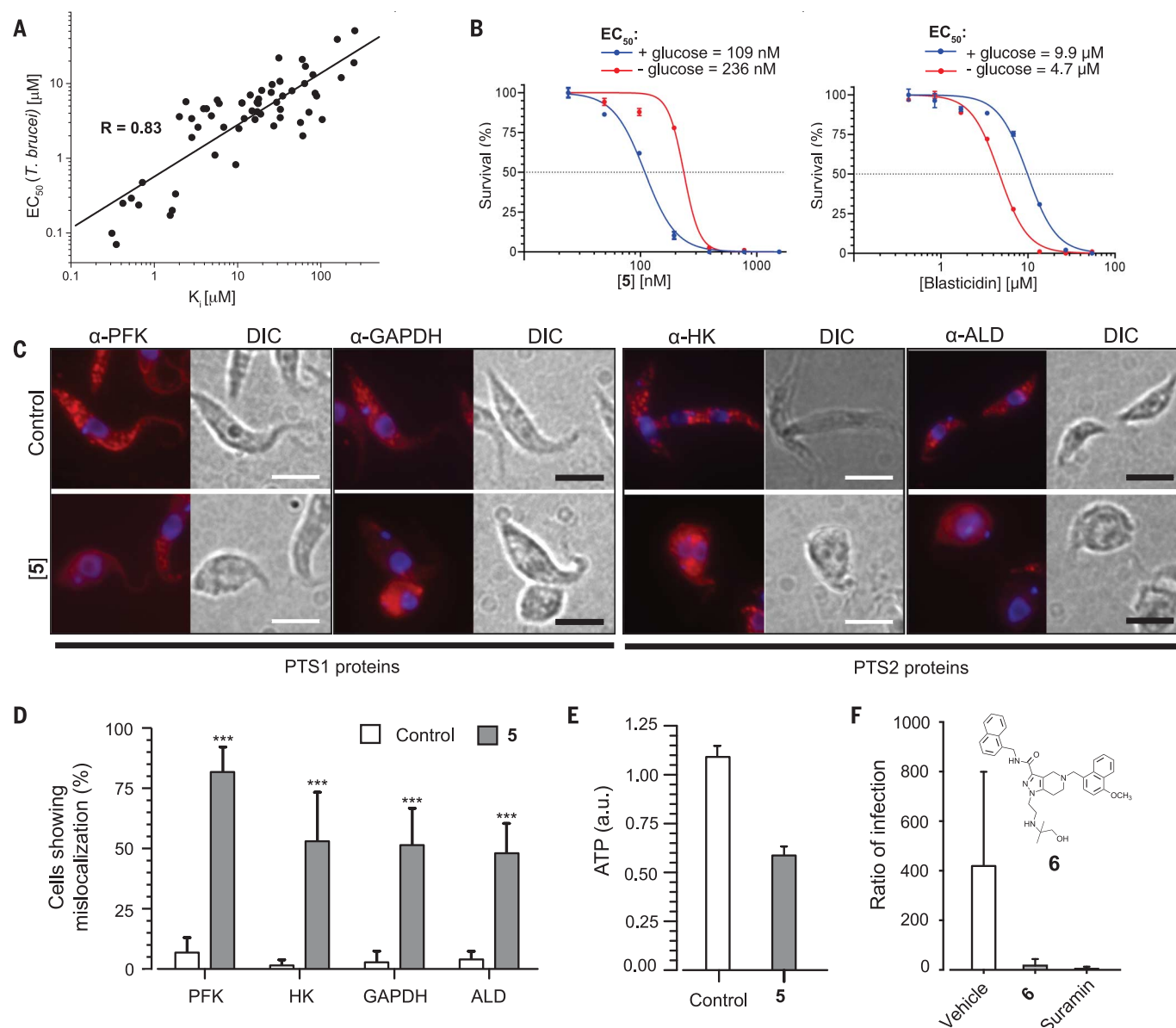


Fig. 3. PEX14 target validation in *T. brucei*. (A) Correlation between in vitro PEX14-PEX5 interaction inhibition and *T. brucei* toxicity. (B) EC_{50} analysis of derivative **5** (left) and blasticidin (control, right) against the procyclic form of *T. brucei* grown in medium with glucose (blue line) or without glucose (red line). Glucose boosts the toxic effect of derivative **5** but protects trypanosomes against the general cytotoxic agent blasticidin. (C) Immunofluorescence microscopy analysis of subcellular localization of endogenous glycosomal enzymes in the procyclic form of trypanosomes, expressing green fluorescent protein-PTS1 to stress the import. PFK, phosphofructokinase; HK, hexokinase; GAPDH, glycosomal glyceraldehyde-3-phosphate dehydrogenase (red, Alexa Fluor 594); ALD, aldolase; DIC, differential inter-

ference contrast. Nucleus and kinetoplast are stained blue with 4',6-diamidino-2-phenylindole. Scale bars, 5 μ m. The enzymes are localized in glycosomes in the control images (granular distribution pattern). This localization is disturbed in the presence of derivative **5**. (D) Statistical analysis of the percentage of cells showing mislocalization of glycosomal enzymes in the presence of a sublethal concentration of derivative **5**. (E) ATP levels in equal numbers of control (white bar) and derivative **5**-treated (gray bar) cells, determined after 28 hours. (F) Murine HAT model. Bar plot represents the ratio of post-treatment (day 5) to initial (day 0) parasitemia, measured as in vivo parasite luminescence. Derivative **6** was administered orally at 2 x 100 mg/kg, and Suramin was given intraperitoneally at 40 mg/kg daily.

optimization (33). To verify the utility of our inhibitors for applications in disease models in vivo, we performed a panel of ADME experiments (table S4). The PEX14 inhibitors exhibit plasma stability, microsomal stability and, as expected, relatively high plasma protein binding. In a murine HAT model, administration of inhibitor **5** did not significantly affect parasitemia. To increase the available concentration of free

PEX14 inhibitor in serum, we synthesized derivative **6**, with significantly reduced plasma protein binding (95.7% versus 99.4% for **5**). Derivative **6** has excellent in vitro trypanocidal properties (Table 1) and retains on-target activity similar to derivative **5** (fig. S11). To test the in vivo activity of derivative **6**, we monitored parasitemia levels in a *T. brucei brucei* animal model. Oral application [100 mg per kg of weight (mg/kg) twice a

day for 5 days] of compound to infected mice reduced or stalled the parasitemia levels. Averaged parasitemia levels rose 400, 17, and 4 times for untreated, derivative **6**- and Suramin-treated mice, respectively (Fig. 3F and fig. S12B). No adverse effects on the mice were observed at these doses.

When protein import into glycosomes is defective, the trypanosomes' essential energy sources become lethal. Here, we have exploited this

Table 1. Optimization of PEX14-PEX5 binding inhibitors. EC₅₀, median effective concentration; IC₅₀, median inhibitory concentration; K_i, inhibition constant. Values are given in μM. Reference compounds: Suramin, *T. b. brucei* EC₅₀ = 26 nM; Benznidazole, *T. cruzi* EC₅₀ = 1.17 μM; *T. b. brucei* Lister 427; *T. b. rhodesiense* strain STIB 900; *T. cruzi* strain Tulahuen C4.

Compound	<i>T. b. brucei</i> PEX14 affinity (K _i /IC ₅₀)	<i>T. b. brucei</i> toxicity (EC ₅₀)	<i>T. b. rhodesiense</i> toxicity (EC ₅₀)	<i>T. cruzi</i> toxicity (EC ₅₀)	Human PEX14 affinity (IC ₅₀)	Mammalian cells toxicity (EC ₅₀)
1	61.6/173	21	–	–	1912	95* (51) [†]
2	8.43/23.4	3.2	–	–	119	6.4 [†]
3	12.3/34.5	9.3	–	–	581	130 [†]
4	2.84/799	3.6	8.8	3.5	120	51* (>100)
5	0.207/1.34	0.186	0.021	0.57	37.8	8.6* (1.21) [†]
6	0.72/4.66	0.278	0.045	2.67	47	>100* (1.43) [†]

*NIH/3T3 fibroblasts used as host cells for *T. cruzi* assay.

†HepG2 cells.

phenomenon to explore the potential of blocking glycosome assembly by inhibiting PEX14 as a drug target for trypanosomiasis. The relatively weak initial in silico hit **1** was decorated with PEX14-binding fragments identified by NMR-based screen. Subsequent rational compound optimization was efficiently guided by high-resolution crystal structures. The PEX14 inhibitors have strong trypanocidal activity, with favorable safety profiles that are comparable or better than those of currently approved therapeutics in cell-based assays. The in vivo evaluation of derivative **6** shows that—upon further balancing of ADME properties, especially the volume of distribution and plasma protein binding—our compounds have the potential to become clinical candidates against trypanosomiasis. Our proof-of-concept study shows that trypanosomal protein-protein interactions are attractive drug targets and open the way for further clinical development of PEX14 inhibitors.

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Helmholtz Zentrum München has a patent application (WO2016038045) describing chemical matter presented in the manuscript. The NMR data and atomic coordinates of the *T. brucei* PEX14 N-terminal domain are deposited in the Protein Data Bank (www.rcsb.org) with accession code 5MMC and the Biological Magnetic Resonance Data Bank (www.bmrb.wisc.edu) with accession code 34073. Structures and restraints of the PEX14-**2**, PEX14-**3**, and PEX14-**5** complexes are deposited under Protein Data Bank accession codes 5L87, 5L8A, and 5N8V, respectively. We thank V. Napolitano for help in protein crystallization. We are grateful to M. Brönstrup, J. Kelly, and G. Klebe for discussions and critical reading of our manuscript. We thank the European Synchrotron Radiation Facility for beamtime on ID23 and ID30 and the Bavarian NMR Centre (BNMRZ) for NMR measurement time. This work was supported by the Deutsche Forschungsgemeinschaft (FOR1905 to M.S. and R.E. and ER178/4-1 to R.E.), by the EU Horizon 2020 (2014–2020) framework program under the Marie Skłodowska-Curie Grant Agreement no. 675555, AEGIS to G.M.P. and M.S., and by the Helmholtz portfolio and cross-program activities “Drug discovery” and “Metabolic Dysfunction.” M.K.’s work was supported by Wrocław Centre of Biotechnology program, The Leading National Research Centre (KNOW) for years 2014–2018. K.T. acknowledges support from the Ministry of Education, Youth and Sport of Czech Republic under the project CEITEC 2020 (LQ1601). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S12
Tables S1 to S4
References (34–58)
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Small molecules to target parasite organelle

The glycosome is a peroxisome-like organelle that packages glycolytic enzymes of the parasites that cause sleeping sickness, Chagas disease, and leishmaniasis. Dawidowski *et al.* designed small-molecule inhibitors to disrupt interactions between two of the proteins involved in peroxisome biogenesis (PEX5 and PEX14), which permit import of glycosomal matrix proteins from the cytoplasm. The small peptide-mimicking molecules kill the trypanosome parasites by causing metabolic collapse without interfering with human PEX homologs. Preliminary studies in mice confirmed an antiparasitic effect.

Science, this issue p. 1416

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