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An HTRF based high-throughput screening for discovering chemical compounds that inhibit the interaction between *Trypanosoma brucei* Pex5p and Pex14p

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ABSTRACT

The glycosome, a peroxisome-related organelle, is essential for the growth and survival of trypanosomatid protozoa. In glycosome biogenesis, Pex5p recognizes newly synthesized glycosomal matrix proteins via peroxisome-targeting signal type-1 (PTS-1) and transports them into glycosomes through an interaction with Pex14p, a component of the matrix protein import machinery on the glycosomal membrane. Knockdown of the *PEX5* or *PEX14* with RNAi has been shown to inhibit the growth of *Trypanosoma brucei.* Thus, compounds that inhibit the interaction of *Tb*Pex5p–*Tb*Pex14p are expected to become lead compounds in the development of anti-trypanosomal drugs. Here, we report a homogenous time-resolved fluorescence (HTRF) assay for the screening of compounds that inhibit the *Tb*Pex5p–*Tb*Pex14p interaction. The binding of GST-*Tb*Pex14p and *Tb*Pex5p-His with or without additional compounds was evaluated by measuring the energy transfer of the HTRF pair, using a terbium-labeled anti GST antibody as the donor and an FITC-labeled anti His antibody as the acceptor. The assay was performed in a 384-well plate platform and exhibits a Z'-factor of 0.85–0.91, while the coefficiency of variation is 1.1–7.7%, suggesting it can be readily adapted to a high-throughput format for the automated screening of chemical libraries. We screened 20,800 compounds and found 11 compounds that inhibited energy transfer. Among them, in a pull-down assay one compound exhibited selective inhibition of *Tb*Pex5p–*Tb*Pex14p without any *Hs*Pex5p–*Hs*Pex14p interaction.

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1. Introduction

Trypanosoma brucei is responsible for the fatal human disease of sleeping sickness in tropical and subtropical parts of the world. Many attempts to develop therapeutic agents have been carried out and currently there are a few drugs available for treatment. However, these drugs possess severe side effect and emerging resistant parasite has been reported [1]. Therefore, the development of new, effective and safe anti-trypanosomal drugs is urgently needed.

The trypanosomatid possesses certain unique organelles called glycosomes. These are peroxisome-related organelles that contain the majority of the enzymes involved in the glycolytic pathway [2]. It has been shown that *T. brucei* grows in a manner entirely dependent on aerobic glycolysis in order to generate ATP in the bloodstream form, since glycolysis is the sole source of energy [3]. In contrast, in the insect midgut, glucose is only available briefly after the fly has taken a blood meal, so the procyclic form of the trypanosome has to expand the metabolic pathway in order to metabolize amino acids such as proline and threonine in mitochondria [4]. Although ATP is synthesized in mitochondria in the procyclic form of the trypanosome, impairment of glycosome biogenesis is lethal in a medium containing glucose [5]. Thus, the proteins involved in the biogenesis of the glycosome are

Abbreviations: HTRF, homogenous time-resolved fluorescence; PTS-1, peroxisome-targeting signal type-1; GST, glutathione *S*-transferase; HTS, high-throughput screening; FRET, fluorescence resonance energy transfer

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considered potentially valuable targets for drug development.

The glycolytic enzymes possess either peroxisome-targeting signal type-1 (PTS-1) at the COOH-terminus or PTS-2 at the NH_2-terminus. Each of the receptor proteins *Tb*Pex5p and *Tb*Pex7p in the cytosol recognizes the newly synthesized enzymes, and subsequently each complex, *Tb*Pex5p with the PTS-1 protein or *Tb*Pex7p with the PTS-2 protein, is translocated into the glycosome through an interaction with *Tb*Pex14p, a component of the matrix protein import machinery on the glycosomal membrane. Recently, depletion of Pex5p, Pex7p or Pex14p with RNAi has been shown to cause mislocalization of the enzymes to the cytosol and to exert a profound effect on the growth of *T. brucei*, in most cases followed by the death of the parasite [6,7].

Pex5p is composed of two distinct parts: an NH_2-terminal half of low similarity except for multiple pentapeptide WXXXF/Y repeats and a highly conserved COOH-terminal half comprising seven tetratricopeptide repeat (TPR) motifs [8]. The WXXXF/Y motif has been shown to be essential for the interaction with Pex14p [9], while the TPR region was shown to mediate the binding to PTS-1 containing proteins [10]. Pex14p, a membrane-anchored protein, is a central component in the glycosomal protein import machinery [7]. The NH_2-terminal region is composed of 21–70 amino acids that are highly conserved among species and bind with Pex5p, Pex13p and Pex19p [11,12]. Recently, the binding mode of the WXXXF/Y motif with the NH_2-teminal conserved domain of Pex14p was reported in mammal and *T. brucei* [13]. The binding mode between Pex5p and Pex14p is comparable in the trypanosome and humans. However, the overall amino acid sequence identity of *Tb*Pex5p and *Tb*Pex14p with their human counterparts (*Hs*Pex5p and *Hs*Pex14p) is only 20% and 27%, respectively. The amino acid sequence of Pex5p around the Pex14p binding site is also different between the trypanosome and humans (See Results and discussion). This difference may afford a target for anti-trypanosomal drugs.

A homogenous time-resolved fluorescence (HTRF)-based assay has been developed for the purpose of high-throughput screening (HTS) for drug targets [14]. This method combines fluorescence resonance energy transfer (FRET) with time-resolved measurement that allows the elimination of short-lived background phenomena. In the case of an analysis of protein-protein interaction, a pair of antibodies against target proteins that are labeled with the respective HTRF donor and acceptor fluorophores is used. In this study, we developed an HTRF-based HTS assay specifically designed for screening chemical compounds that inhibit the *Tb*Pex5p–*Tb*Pex14p interaction. The screening of a chemical library was performed in a 384-well plate platform. We found one compound that inhibited the interaction of *Tb*Pex5p–*Tb*Pex14p, but not *Hs*Pex5p–*Hs*Pex14p.

2. Materials and methods

2.1. Materials

The Lumi4®-Tb cryptate conjugated anti Glutathione *S*-transferase (terbium-anti GST) antibody and FITC conjugated anti His (FITC-anti His) antibody were purchased from Cisbio Bioassay (Codolet, France) and Abcam (Cambridge, MA), respectively. The rabbit anti GST antibody was prepared by immunization of rabbits with purified recombinant GST. Rabbit anti *Hs*Pex5p was kindly provided by Dr. Nobuyuki Shimozawa (Gifu University).

2.2. Plasmid construction

All of the primers used in this study are listed in Supplementary Table 1. The details on the construction of the expression vectors for GST tagged *T. brucei* and human Pex14p and His-tagged *T. brucei* and human Pex5p are provided in the Supplemental Methods. To prepare the expression plasmids for the mutated *Tb*Pex5p(Δ1,3)-His, a KOD -Plus- Mutagenesis Kit (TOYOBO, Osaka, Japan) was used according to the manufacturer's instructions.

2.3. Purification of recombinant proteins

All of the recombinant proteins in this study were expressed in *E. coli* BL21(DE3)pLysS and purified using tag-affinity resins. The details of the purification procedures are provided in the Supplemental Methods section.

2.4. HTRF-based HTS

Two hundred nl of the compounds in DMSO (2 mM each) were applied to 384-well plates (Greiner-Bio-One, Austria) using POD™ Automation (Labcyte Inc., Sunnyvale, CA). The final concentration of each compound was fixed at 20 μM. The same amount of DMSO was applied to the negative and positive control wells in lane 2 and lane 23, respectively. Then, *Tb*Pex5p-His and GST-*Tb*Pex14p (16 nM each) in 10 μl of HTS buffer (50 mM Hepes-NaOH, pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.02% Triton X-100, 10% glycerol, and 0.1% BSA) were applied to the wells in lane 23 (positive control) and lanes 3–22 (compounds), and 10 μl of HTS buffer were applied to the wells in lane 2 as a negative control using Multidrop Combi reagent dispenser (Thermo Fisher Scientific, Waltham, MA). After the plates were incubated at room temperature for 1 h, 10 μl of HTS buffer containing the terbium-anti GST antibody and FITC-anti His antibody were added. The plates were then incubated at room temperature for 2 h before fluorescent signal detection. The fluorescence intensities were measured at both 520 nm and 490 nm using an excitation wavelength of 337 nm with PHERAstar Plus (BMG LABTECH, Offenburg, Germany).

The HTRF ratio was calculated using the following equation: (Intensity of 520 nm)/(Intensity of 490 nm) $\times$ 100. The accuracy of the assay was evaluated by the Z'-factor and was calculated with the following equation: $1–3 \times (SD_{max} + SD_{min})/(Av_{max} - Av_{min})$, where SD is the standard deviation of the positive control (the maximum HTRF ratio; with *Tb*Pex5p-His and GST-*Tb*Pex14p, without compound) or the negative control (the minimum HTRF ratio; without *Tb*Pex5p-His, GST-*Tb*Pex14p, and compound) and Av is the mean of the positive or negative control. The inhibition ratio (Inhibition %) was calculated using the following equation: $100 \times \{1 - (R_{compound}–Av_{min})/(Av_{max}–Av_{min})\}$, where $R_{compound}$ is the HTRF ratio of the compound in the assay well.

2.5. Pull-down assay

GST pull-down assay was performed as described below. *Tb*Pex5p-His and GST-*Tb*Pex14p (64 nM each) were incubated at room temperature for 2 h in 200 μl of pull-down buffer (50 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.02% Triton X-100, 10% glycerol and 0.01% BSA) with 3.2 μl or 16 μl of compound in DMSO (10 mM or 2 mM, respectively). The final concentration of each compound was fixed at 160 μM in order to examine the effect at the same ratio of the compound to *Tb*Pex5p-His and GST-*Tb*Pex14p upon HTS (the concentration of the compound against *Tb*Pex5p-His and GST-*Tb*Pex14p was 2500 fold). After this incubation, the mixture was further incubated with 20 μl of glutathione-Sepharose 4B resin equilibrated with pull-down buffer at room temperature for 1 h. The resins were washed four times with 250 μl of pull-down buffer. The bound proteins were eluted in SDS-sample buffer and analyzed by SDS-PAGE followed by immunoblot analysis with an anti GST antibody or anti His

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