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

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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 TbPex5p–TbPex14p 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 TbPex5p–TbPex14p interaction. The binding of GST-TbPex14p and TbPex5p-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 coefficient 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 TbPex5p–TbPex14p without any HisPex5p–HisPex14p 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 NH₂-terminus. Each of the receptor proteins TbPex5p and TbPex7p in the cytosol recognizes the newly synthesized enzymes, and subsequently each complex, TbPex5p with the PTS-1 protein or TbPex7p with the PTS-2 protein, is translocated into the glycosome through an interaction with TbPex14p, 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 NH₂-terminal half of low similarity except for multiple pentapeptide WXXXF/Y repeats and a highly conserved COOH-terminal half comprising seven tetrapeptide 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 NH₂-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 NH₂-terminally 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 TbPex5p and TbPex14p with their human counterparts (HsPex5p and HsPex14p) is only 20% and 27%, respectively. The amino acid sequence of Pex5p around the Pex14p binding site is

**2. Materials and methods**

**2.1. Materials**

The Lumig4®-Tb cryptate conjugated anti Glutathione S-transferase (terbium-anti GST) antibody and FITC conjugated anti His (FITC-anti His) antibody were purchased from Cidbio 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 HsPex5p was kindly provided by Dr. Nobuyuki Shimozawa (Gifu University).

**2.2. Plasmid construction**

All of the primers used in this study are listed in Supplemental 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 TbPex5p 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)pLYS 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 (Labyte 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, TbPex5p-His and GST-TbPex14p (16 mM each) in 10 μl of HTS buffer (50 mM Heps-NaOH, pH 7.5, 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 fluoroscent signal detection. The fluorescence intensities were measured at both 520 nm and 490 nm using an excitation wavelength of 373 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) × 100. The accuracy of the assay was evaluated by the Z’-factor and was calculated with the following equation: 1–3 × (SDmax + SDmin)/(Avmax – Avmin), where SD is the standard deviation of the positive control (the maximum HTRF ratio; with TbPex5p-His and GST-TbPex14p, without compound) or the negative control (the minimum HTRF ratio; without TbPex5p-His, GST-TbPex14p, and compound) and Av is the mean of the positive or negative control. The inhibition ratio (Inhibition %) was calculated using the following equation: 100 × (1 – (Rcompound- Avmin)/(Avmax–Avmin)), where Rcompound 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. TbPex5p-His and GST-TbPex14p (64 nM each) were incubated at room temperature for 2 h in 200 μl of pull-down buffer (50 mM Heps-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 TbPex5p-His and GST-TbPex14p upon HTS (the concentration of the compound against TbPex5p-His and GST-TbPex14p 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
His-tagged pull-down assay was performed using the same method as the GST pull-down assay for evaluation of His-HsPex5p interaction with GST-HsPex14p. In the His tagged pull-down assay, cOmpelte His-tag purification resin (Roche Diagnostics GmbH, Mannheim, Germany) was used instead of glutathione-Sepharose 4B resin. 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 HsPex5p antibody.

The amount of each of the proteins binding to the resin was quantified by using the image analysis software Image J [15]. The inhibition % was calculated by using the following equation:

\[
\text{Inhibition} \% = \frac{\text{Compoundprey/Compoundbait} - \text{Controlprey/Controlbait}}{\text{Controlprey/Controlbait}} \times 100
\]

where “Compound” is the band intensity of the prey protein (TbPex5p-His or GST-HsPex14p) or the bait protein (GST-TbPex14p or His-HsPex5p) in the corresponding compound lane. The “Control” is the band intensity of the prey protein or the bait protein in the positive control (8% DMSO without compound) lane.

To calculate IC50 of compound I, at a final concentration of 0.1, 0.3, 1, 3, 10, 30, 100, 300 or 500 μM compound I was incubated with TbPex5p and TbPex14p and GST pull-down assay was performed as described above. Inhibition ratios by various concentration of compound I were calculated by setting 100% inhibition at 500 μM compound I and 0% inhibition at control without inhibitor. IC50 was obtained by 4-parameter logistic curve.

3. Results and discussion

3.1. Optimization and validation of the HTRF assay

The interaction of GST-TbPex14p with TbPex5p-His was evaluated by energy transfer of the HTRF pair, using a terbium-anti GST antibody as the donor and an FITC-anti His antibody as the acceptor. When the donor fluorophore was excited at 337 nm, a portion of the emitted energy directly transferred to the acceptor fluorophore in the case where two fluorophores exist in close proximity. This results in a decreased emission of the donor at 490 nm as well as an increase in the acceptor emission at 520 nm (Fig. 1). We thus evaluated the binding of GST-TbPex14p with TbPex5p-His by the ratio of the fluorescence at 520 nm/490 nm × 100. First, a specific increase of the ratio by the interaction of GST-TbPex14p (25 nM) with TbPex5p-His (25 nM) was confirmed by using TbPex5p(Δ1,3)-His as the negative control. As TbPex5p(Δ1,3)-His lacks the first and third WXXXxF/Y motif, it is unable to bind TbPex14p [16]. An increase in the HTRF ratio was observed in the case of wild type TbPex5p. In contrast, the ratio did not increase in the case of mutant TbPex5p(Δ1,3)-His (Fig. 2 (A)). We then fixed the concentration of terbium-anti GST antibody and FITC-anti His antibody at 500-fold dilution and optimized the concentration of GST-TbPex14p with TbPex5p-His. As shown in Fig. 2 (B), the maximum value was observed with the combination of 30 nM of GST-TbPex14p and TbPex5p-His. The combination of TbPex5p(Δ1,3)-His and GST-TbPex14p did not display any increase in the ratio.

Fig. 2. Evaluation of the interaction of TbPex14p with TbPex5p. (A) The increase in the fluorescent ratio by the interaction of GST-TbPex14p with TbPex5p-His. WT, wild type TbPex5p-His; Δ1,3, mutant TbPex5p-His with the deleted first and third WXXXxF/Y motifs. GST-TbPex14p (25 nM) and TbPex5p-His or TbPex5p(Δ1,3)-His (25 nM) were incubated with labeled antibodies against GST and His (250 or 500 dilution; closed or open bar, respectively). (B) Optimization of the GST-TbPex14p and TbPex5p-His concentration for detecting the signal ratio by the interaction. The final concentrations used were 0–250 nM, with a three-fold serial dilution for GST-TbPex14p and TbPex5p(Δ1,3)-His. The closed diamond shows the assay result for TbPex5p-His and GST-TbPex14p, and the open square shows the assay result for TbPex5p(Δ1,3)-His and GST-TbPex14p.

Fig. 1. Principles of the method for detecting the interaction between GST-TbPex14p and TbPex5p-His by HTRF assay. Antibodies against the fused tags GST and His were labeled as the donor and acceptor, respectively. The HTRF signals are generated when each of labeled antibodies exists in close proximity by the interaction of GST-TbPex14p with TbPex5p-His. Tb means terbium in this figure.
Since DMSO was used to solubilize the chemical compounds, the influence of the DMSO concentration on the ratio was tested. Less than 1% (v/v) DMSO exerted little influence on the fluorescence ratio. In addition, the HTRF signal that was measured after the mixing of the antibodies was stable over 5 h (data not shown). The $Z$-factor for the binding of GST-TbPex14p with TbPex5p-His was calculated to be 0.85–0.91 under different concentrations of DMSO. The degree of variability was 11–7.7% in the positive control group and the difference in the fluorescence ratio between the positive group and the control, which lacked GST-TbPex14p and TbPex5p-His ($S/B$ ratio), was ~10. These data suggest that the HTRF-based assay is reliable in HTS and the specific signal induced by the interaction with Pex5p–Pex14p was detectable after 5 h of incubation.

3.2. Screening of compounds that inhibit the interaction of GST-TbPex14p with TbPex5p–His

We screened 11,200 compounds of a Protein-Protein Inhibition library and 9600 compounds of a structurally diverse library provided by the Drug Discovery Initiative (The University of Tokyo). In the assay, the concentration of TbPex5p–His and GST-TbPex14p was fixed at 32 nM (final 8 nM per well), and 10 μl of 500-fold diluted mixtures of antibodies were added in each well. We selected compounds that decreased the value of the ratio to 80% as compared with the positive control.

As shown in Fig. 3(A), 320 compounds (at a final concentration of 20 μM) were assayed in one plate to evaluate inhibition of the binding between TbPex5p–His and GST-TbPex14p. Each of 16 wells in lane 2 and lane 23 were used for the negative and positive controls. Among 20,800 compounds, 139 compounds were selected as hits. We examined the reproducibility of the inhibition among the 139 compounds using 4 different preparations and found that 11 compounds exhibited greater than 20% of the inhibition ratio with a high level of reproducibility (Fig. 3(B)). The structure of the compounds is shown in Fig. 4(C) and Supplementary Fig. 1. Purity of the compounds was determined by LC/MS or HPLC/MS. The purity of compounds except for compound d and i is almost 100% and the purity of compound i is ~91%. The compound d contains mixture of two isomers (51:49) with same molecular weight.

3.3. Pull-down assay for second screening

In terms of inhibition in this assay system, we cannot exclude the possibility that the compounds inhibit the interaction of the antibodies with GST or His-tag. Therefore, we performed a pull-down assay using TbPex5p–His and GST-TbPex14p. In this experiment, we used human His-Pex5p and GST-Pex14p to analyze the specificity.

To evaluate the inhibitory effect of the 11 compounds against the interaction of TbPex5p–His and GST-TbPex14p, we examined their effect by pull-down assay. As a positive control the TbPex5p–His was co-precipitated with GST-TbPex14p in the presence of a final concentration of 1.6% or 8% DMSO, since the compounds were dissolved at the concentration of 2 mM or 10 mM in DMSO, and the final concentration of the compounds was fixed at 160 μM in order to keep the molar ratio of the compounds to TbPex5p and TbPex14p the same as in the HTRF assay. Almost the same amount of TbPex5p–His was co-precipitated with GST-TbPex14p in the presence of 8% or 1.6% DMSO (Fig. 4 upper panel, lanes 4 and 5). However, no TbPex5p–His was co-precipitated in the absence of GST-TbPex14p (lane 3). Under these conditions, the amount of TbPex5p–His that co-precipitated was strongly reduced by compound i (lane 14). In addition, the amount of TbPex5p–His that co-precipitated was reduced by compounds j and k. However, GST-TbPex14p was also reduced, suggesting the compounds inhibit the binding of GST-TbPex14p to the resin along with the binding of TbPex5p–His to TbPex14p. Furthermore, we investigated the effect of the compounds on the binding between HsPex5p and HsPex14p. In the case of GST-HsPex14p, a certain amount of His-HsPex5p and HisPex14p was associated with glutathione-resin. Therefore, we performed His-tag pull-down assay. As shown in the Fig. 4 (A) lower panel, compound i exerted no effect on the binding, suggesting that compound i selectively inhibits the interaction of TbPex5p–His with TbPex14p. The reproducibility of the effect of compound i–k was confirmed by using 3 different preparations (Fig. 4(B)). As a result, compound i inhibited the interaction of TbPex5p with TbPex14p approximately 80%. $IC_{50}$ of compound i was determined 150 ± 88.2 μM by the pull down assay of 3 different experiments.

The compound i is 4-[(2-(methoxycarbonyl)-5-(2-thienyl)-3-thienyl]amino]-4-oxo-2-butenoic acid (Fig. 4(C)). The mechanism by which the compound inhibits the interaction between TbPex5p and TbPex14p is unknown at present. It is unlikely the compound directly inhibits the interaction of the WXXXF/Y motif with the NH$_2$-terminal binding site of TbPex14p since the structure of the NH$_2$-terminus of Pex14p is highly conserved between T. brucei and humans [16–19]. The compound i possesses two carboxic acids and an amide bond. In addition, the compound i possesses heteroatoms and becomes a donor or acceptor of a protein hydrogen bond. The properties of the compound suggest it may interact with several proteins that have a cavity-like structure. There is no similarity of the structure among compounds i, j and k. However, the nitro group and α-amino ether in compound j and the phenolic hydroxyl group in compound k make generally hydrogen bond. The compounds might be possible to become incorporated in the pocket of proteins.

The binding mode between Pex5p and Pex14p is very similar between trypanosome and human [16], but the overall homology between the human and trypanosome Pex5p is 20% and Pex14p is 27%. In addition, the amino acid sequences of Pex5p around the Pex14p binding motif, WXXXXF/Y, are different between the trypanosomes and humans (Supplementary Fig. 2). The amino acid sequences of Pex14p around Pex5p binding site are also different.
Therefore, it is likely that the compound $i$ has a specific interaction with $T. brucei$ Pex5p and/or Pex14p. Unexpectedly, the compound was found to be an inhibitor of adipocyte fatty acid binding protein (AFABP/aP2). The compound is suggested to bind in a structurally similar manner to the long chain fatty acid in AFABP/aP2 based on the X-ray crystal structure of AFABP/aP2 with the compound [20]. On the other hand, the three-dimensional structure of trypanosome and human Pex5p and Pex14p has not yet been determined. We cannot reveal the binding site(s) of compound $i$ to TbPex14p and/or TbPex5p at present. However, TbPex14p and/or TbPex5p may have different binding site(s) for compound $i$ since TbPex14p and TbPex5p do not seem to possess a ligand-binding cavity like fatty acid binding proteins. The compound $i$ is expected to become a seed compound for the development of anti-trypanosomal drugs. Whether the compound inhibits the growth of $T. brucei$ and whether this inhibition is followed by the death of the parasite will be taken up in future research.

In addition, this HTRF-based HTS system is highly sensitive and has the capacity to evaluate rapidly protein-protein interactions. As a result, the system may prove to be useful in the development of a screening system based on the protein-protein interactions involved in the biogenesis of glycosomes, such as those of Pex7p-Pex14p, PTS-1 protein-Pex5p and PTS-2 protein-Pex7p.

4. Conclusions

The interaction of TbPex5p with TbPex14p is important for survival of $T. brucei$. In this study we developed the assay system designed for screening chemical compounds that inhibit the TbPex5p-TbPex14p interaction. By using this assay system, we found one compound that specifically inhibits TbPex5p-TbPex14p interaction from 20,800 compounds.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at [http://dx.doi.org/10.1016/j.bbrep.2016.05.004](http://dx.doi.org/10.1016/j.bbrep.2016.05.004).

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