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

Yuichi Watanabe [a], Kosuke Kawaguchi [a], Syuken Saito [a], Takayoshi Okabe [b], Kiyoaki Yonesu [b], Shinichiro Egashira [b], Masafumi Kameya [c], Masashi Morita [a], Yoshinori Kashiwayama [a], Tsuneo Imanaka [a,*]

[a] *Department of Biological Chemistry, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan*
[b] *Drug Discovery Initiative, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[c] *Biotechnology Research Center and Department of Biotechnology, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan*

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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

* Corresponding author.
E-mail address: imanaka@pha.u-toyama.ac.jp (T. Imanaka).

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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) × 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

antibody (Medical and Biological Laboratories, Nagoya, Japan).

His-tagged pull-down assay was performed using the same method as the GST pull-down assay for evaluation of His-*Hs*Pex5p interaction with GST-*Hs*Pex14p. 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 *Hs*Pex5p 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: $100 \times \{1-(\text{Compound}_{prey}/\text{Compound}_{bait})/(\text{Control}_{prey}/\text{Control}_{bait})\}$, where the "Compound" is the band intensity of the prey protein (*Tb*Pex5p-His or GST-*Hs*Pex14p) or the bait protein (GST-*Tb*Pex14p or His-*Hs*Pex5p) 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 IC_{50} 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 *Tb*Pex5p and *Tb*Pex14p 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. IC_{50} was obtained by 4-parameter logistic curve.

3. Results and discussion

3.1. Optimization and validation of the HTRF assay

The interaction of GST-*Tb*Pex14p with *Tb*Pex5p-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-*Tb*Pex14p with *Tb*Pex5p-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-*Tb*Pex14p (25 nM) with *Tb*Pex5p-His (25 nM) was confirmed by using *Tb*Pex5p(Δ1,3)-His as the negative control. As *Tb*Pex5p(Δ1,3)-His lacks the first and third WXXXF/Y motif, it is unable to bind *Tb*Pex14p [16]. An increase in the HTRF ratio was observed in the case of wild type *Tb*Pex5p. In contrast, the ratio did not increase in the case of mutant *Tb*Pex5p(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-*Tb*Pex14p with *Tb*Pex5p-His. As shown in Fig. 2(B), the maximum value was observed with the combination of 30 nM of GST-*Tb*Pex14p and *Tb*Pex5p-His. The combination of *Tb*Pex5p(Δ1,3)-His and GST-*Tb*Pex14p did not display any increase in the ratio.

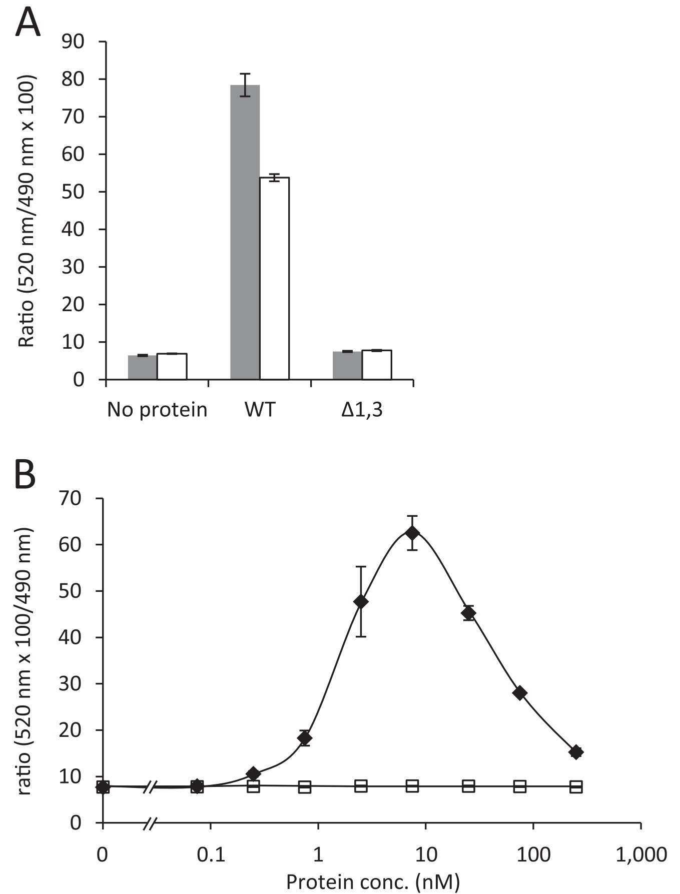

Fig. 2. Evaluation of the interaction of *Tb*Pex14p with *Tb*Pex5p. (A) The increase in the fluorescent ratio by the interaction of GST-*Tb*Pex14p with *Tb*Pex5p-His. WT, wild type *Tb*Pex5p-His; Δ1.3, mutant *Tb*Pex5p-His with the deleted first and third WXXXF/Y motifs. GST-*Tb*Pex14p (25 nM) and *Tb*Pex5p-His or *Tb*Pex5p(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-*Tb*Pex14p and *Tb*Pex5p-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-*Tb*Pex14p and *Tb*Pex5p-His or *Tb*Pex5p(Δ1,3)-His. The closed diamond shows the assay result for *Tb*Pex5p-His and GST-*Tb*Pex14p, and the open square shows the assay result for *Tb*Pex5p(Δ1,3)-His and GST-*Tb*Pex14p.

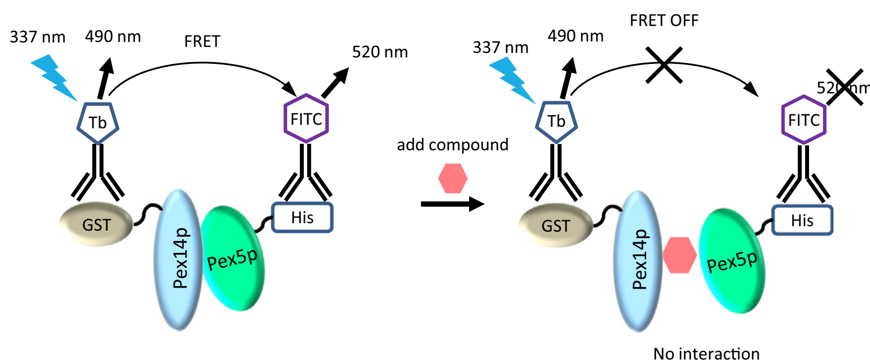

Fig. 1. Principles of the method for detecting the interaction between GST-*Tb*Pex14p and *Tb*Pex5p-His by HTRF assay. Antibodies against the fused tags GST and His were labeled as the donor and acceptor, respectively. The HTRF signals are generated when each of labeled antibodies exists in close proximity by the interaction of GST-*Tb*Pex14p with *Tb*Pex5p-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-*Tb*Pex14p with *Tb*Pex5p-His was calculated to be 0.85–0.91 under different concentrations of DMSO. The degree of variability was 1.1–7.7% in the positive control group and the difference in the fluorescence ratio between the positive group and the control, which lacked GST-*Tb*Pex14p and *Tb*Pex5p-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 *Tb*Pex5p-His and GST-*Tb*Pex14p 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 *Tb*Pex5p-His and GST-*Tb*Pex14p. 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.

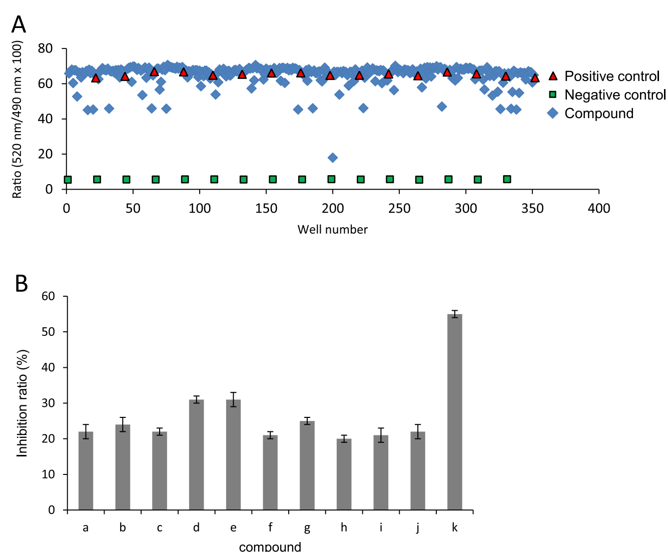

Fig. 3. Screening of chemical compounds by HTRF-based HTS. (A) An example of the screening on one plate. The abscissa is the number of the compounds used for screening and the ordinate is the fluorescence ratio. The triangle, square and diamond indicate the positive control, negative control and compound, respectively. (B) Inhibition % of 11 compounds (20 μM). The data are the mean ± S. D. of three experiments.

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 *Tb*Pex5p-His and GST-*Tb*Pex14p. 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 *Tb*Pex5p-His and GST-*Tb*Pex14p, we examined their effect by pull-down assay. As a positive control the *Tb*Pex5p-His was co-precipitated with GST-*Tb*Pex14p 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 *Tb*Pex5p and *Tb*Pex14p the same as in the HTRF assay. Almost the same amount of *Tb*Pex5p-His was co-precipitated with GST-*Tb*Pex14p in the presence of 8% or 1.6% DMSO (Fig. 4 upper panel, lanes 4 and 5). However, no *Tb*Pex5p-His was co-precipitated in the absence of GST-*Tb*Pex14p (lane 3). Under these conditions, the amount of *Tb*Pex5p-His that co-precipitated was strongly reduced by compound **i** (lane 14). In addition, the amount of *Tb*Pex5p-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-*Tb*Pex14p to the resin along with the binding of *Tb*Pex5p-His to *Tb*Pex14p. Furthermore, we investigated the effect of the compounds on the binding between *Hs*Pex5p and *Hs*Pex14p. In the case of GST-*Hs*Pex14p, a certain amount of His-*Hs*Pex5p and *Hs*Pex5p-His 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 *Tb*Pex5p-His with *Tb*Pex14p. 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 *Tb*Pex5p with *Tb*Pex14p 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 *Tb*Pex5p and *Tb*Pex14p 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 *Tb*Pex14p 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 carbonic 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, WXXXF/Y, are different between the trypanosome and humans (Supplementary Fig. 2). The amino acid sequences of Pex14p around Pex5p binding site are also different

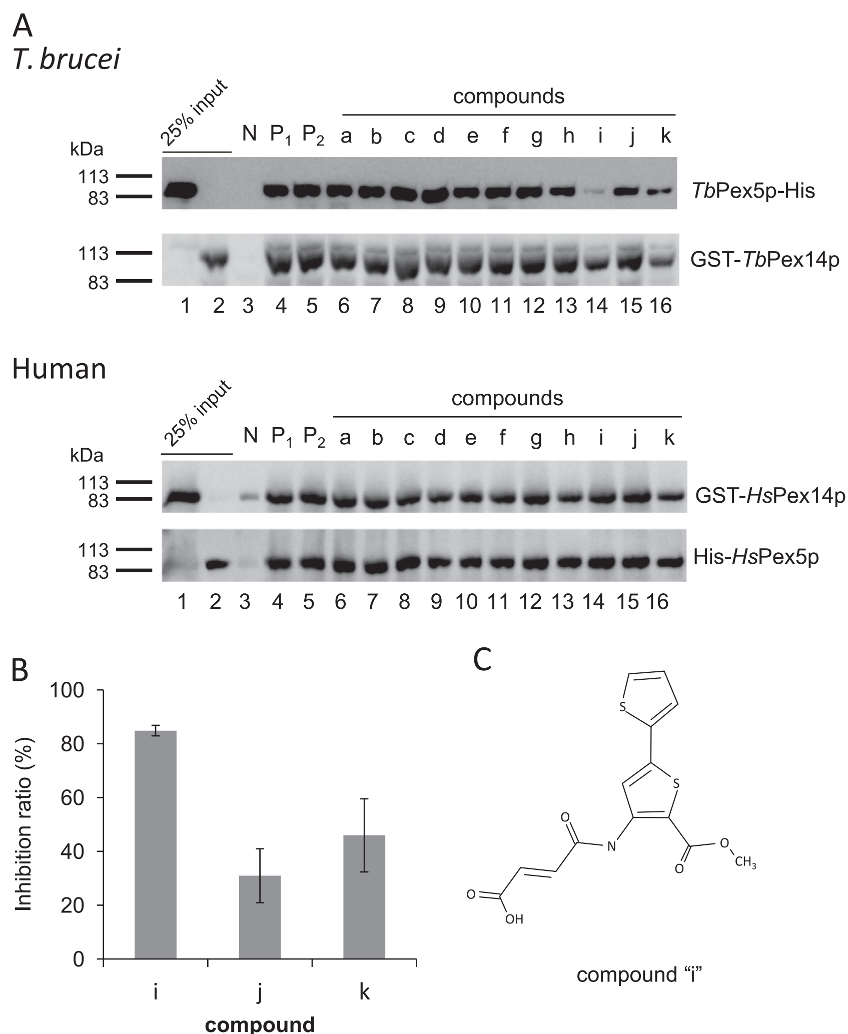

Fig. 4. Effect of 11 compounds on the interaction of both *T. brucei* and human Pex5p and Pex14p. (A) Effect of 11 compounds on the interaction of *Tb*Pex5p with *Tb*Pex14p (upper panel), and *Hs*Pex5p with *Hs*Pex14p (lower panel). The *Tb*Pex5p–*Tb*Pex14p interactions were assessed by GST pull-down assay and the *Hs*Pex5p–*Hs*Pex14p interactions were assessed by His tag pull-down assay. In the GST pull-down assay, the bait protein was GST-*Tb*Pex14p and the prey protein *Tb*Pex5p-His. In the His tag pull-down assay, the bait protein was His-*Hs*Pex5p and the prey protein GST-*Hs*Pex14p. Lane 1 and 2: 25% input of the bait and prey proteins, respectively; lane 3: negative control (N; without bait protein); lane 4 and 5: positive control (P_1; 8% and P_2; 1.6% DMSO, respectively); lane 6~15: compounds with 1.6% DMSO; lane 16: a compound with 8% DMSO. (B) The reproducibility of 4 compounds. The data are the mean $\pm$ S. D. of three experiments. The pull-down assay was performed as indicated in Fig. 3(A). The inhibition ratio was judged from the quantification of each band by the image analysis software Image J. (C) The chemical structure of the compound **i** was 4-{[2-(methoxycarbonyl)−5-(2-thienyl)−3-thienyl]amino}−4-oxo-2-butenoic acid.

(Supplementary Fig. 3). 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 *Tb*Pex14p and/or *Tb*Pex5p at present. However, *Tb*Pex14p and/or *Tb*Pex5p may have different binding site (s) for compound **i** since *Tb*Pex14p and *Tb*Pex5p 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 *Tb*Pex5p with *Tb*Pex14p is important for survival of *T. brucei*. In this study we developed the assay system designed for screening chemical compounds that inhibit the *Tb*Pex5p–*Tb*Pex14p interaction. By using this assay system, we found one compound that specifically inhibits *Tb*Pex5p–*Tb*Pex14p 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.

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