Targeting Peroxisomal Transport in Trypanosoma.

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***Abstract* — Human infection with Trypanosoma parasites (Chagas disease and Human African Trypanosomiasis) affects around 10 million people worldwide resulting in life-threatening disease. Treatment options are limited to historic drugs characterized by significant side effects and decreasing efficacy while new drug development efforts are largely neglected. Here, we review drug discovery effort in human trypanosomiasis undertaken in academia. Peroxisomal (Pex) transport system was validated as a target in Chagas disease and a number of compounds were delivered which have shown promising results in animal experiments. Future perspectives of exploring the Pex system in anti-trypanosoma drug development are discussed.**

***Index Terms* — Chagas disease, *Trypanosoma cruzi*, peroxin, Pex**

I. REVIEW

Trypanosomiasis

Trypanosomiasis refers to a number of diseases in vertebrates which are caused by parasitic protazoans of the genus *Trypanosoma*. *Trypanosoma brucei* is responsible for Human African Trypanosomiasis (HAT), a disease endemic to sub-Saharan Africa. *Trypanosoma cruzi* is responsible for Chagas disease in central and south America. Some estimates of the number of affected individuals reach as high as 10 million people worldwide. Protective vaccines are not available and effective drugs are of limited availability. While HAT was largely eliminated by preventive measures and WHO estimates the total elimination as a public health problem by 2030, Chagas disease is spreading form the endemic areas and is becoming a global threat (Lidani et al., 2019; Montgomery, Starr, Cantey, Edwards, & Meymandi, 2014).

HAT (otherwise known as sleeping sickness) is currently exclusively confined to a number of "foci" in sub-Saharan Africa. The majority of cases are caused by *Trypanosoma brucei gambiense*, while *T.b. rhodesiense* is much less widespread. The parasites are transmitted by bites of tse-tse files (Lejon, Bentivoglio, & Franco, 2013). The disease evolves through two pathological stages. First stage is characterized by intermittent fever related to successive waves of parasite replication in blood. Some patients develop adenopathies, splenomegaly and other symptoms. The second stage develops over months or years and is related to the parasites crossing the blood-brain barrier. The signs associated with the first stage do not completely disappear, while the second stage is characterized by progressive development of neurological symptoms. These include confusion, changes of behavior, bad coordination and other. The major symptom, however, after which the disease derives its name of sleeping sickness is daytime drowsiness. HAT is fatal if untreated. Continued efforts to control HAT transmission were successful over the years. The number of new cases has dropped for the first time below 10,000 in 2009 and in 2018 only 977 cases were reported (WHO, 2019). WHO estimates complete elimination by 2030.

Chagas disease is a chronic human infection with *Trypanosoma cruzi.* The parasites are transmitted by bites of triatomine bugs. Non-vectorial routes include congenital transmission and medical procedures (mainly blood transfusion) (Maguire, 2004; Rassi Jr, Rassi, & Marin-Neto, 2010). The early stage of the disease is usually asymptomatic, though characterized by parasitemia. It is sometimes associated with swelling around the bite site. After the initial phase, the disease transforms into a chronic stage which may be silent for years or even a lifetime. However, up to 30% of affected subjects will develop progressive cardiac or gastrointestinal pathologies. Cardiac degeneration and gastrointestinal megasyndromes significantly affect the quality of life (Montgomery et al., 2014; Nunes et al., 2018) with no available cure. Chagas disease was historically endemic in rural areas of Latin America (Perez-Molina & Molina, 2018). However, the changes in the lifestyle, urbanization and migration have recently transformed Chagas into an urban disease. In consequence of migrations, Chagas has become a global epidemic (Schmunis & Yadon, 2010). Transmissions in non-endemic regions (e.g. North America, Australia, Japan, Spain) are now not uncommon (C. Bern & Montgomery, 2009; Gascon, Bern, & Pinazo, 2010; Lee, Bacon, Bottazzi, & Hotez, 2013; Requena-Mendez et al., 2015). Close to 8-10 million people are estimated to currently suffer from Chagas disease with ~300,000 cases in the nonendemic region of the United States alone (Echeverria & Morillo, 2019; Gascon et al., 2010; Navarro, Navaza, Guionnet, & López-Vélez, 2012).

Only two drugs are currently available in Chagas disease: nifurtimox and benznidazole (Carod-Artal, 2013). The drugs are effective in the acute phase, but their efficacy drops to below 10% in the chronic phase. What is more, long treatment regime is required and the significant adverse effects of these historic drugs often necessitate termination of uncompleted therapy (Castro, de Mecca, & Bartel, 2006). Nifurtimox and benznidazole are discouraged in pregnancy and severe liver and

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kidney disease (Caryn Bern et al., 2007). In many countries, including the United States, nifurtimox is available only within investigational protocols (Meymandi, Hernandez, Park, Sanchez, & Forsyth, 2018). Such situation calls for development of new drugs, but drug development in Chagas disease is largely neglected mostly due to low economic status of affected populations.

Peroxisomes

Peroxisomes are small subcellular compartments which are conserved in evolution and indispensable for normal cell physiology. The peroxisomes number up to several hundreds in mammalian cell. The peroxisome lumen is separated from the cytoplasm by a single lipid bilayer. The functions of peroxisomes (and what follows the spectrum of contained enzymes) differ between organisms and tissues. Mammalian peroxisomes are responsible for fatty acid α- and β-oxidation, degradation of purines, polyamines and D-amino acids and biosynthesis of bile acids (Wanders & Waterham, 2006). In plants, the peroxisomes mediate photorespiration, hormone biosynthesis and glyoxylate cycle. In yeast peroxisomes are responsible for methanol degradation and glyoxylate cycle. In fungi, the peroxisomes are involved in biosynthesis of penicillin. Other functions are known in different species (Wanders & Waterham, 2006). Of interest for this review, in *Trypanosoma* the peroxisomes compartmentalize glycolysis (Szoor, Haanstra, Gualdron-Lopez, & Michels, 2014), what is unique among eucaryotes. Moreover, peroxisomes of *Trypanosoma* are responsible for purine salvage. As such, the organelles are essential for the protist survival.

PEX transport system

Peroxisomes lack protein synthetic abilities. Therefore all the enzymes necessary for peroxisomal function must be imported. Cellular organelles commonly import proteins synthesized by ER-associated ribosomes *via* routes involving vesicular transport. Uniquely, the proteins directed to the peroxisome are produced on free-ribosomes (Goldman & Blobel, 1978) and imported to the organelle using a dedicated PEX translocon. The cargo proteins are tagged for peroxisomal transport by two types of peroxisomal targeting sequences (PTS). These short stretches of amino acids are recognized by relevant PEX receptors in the cytoplasm. Cargos tagged with PTS1, three C-terminal amino-acids with a consensus sequence SKL-COOH, are recognized directly by C-terminal tetratricopeptide repeat (TPR) domain of Pex5. The cargo-Pex5 complex docks at the peroxisomal membrane by virtue of Pex5/Pex14 interaction, where Pex14 constitutes a peroxisome membrane embedded receptor of Pex5 (Lanyon-Hogg, Warrin`er, & Baker, 2010). Less common than PTS1, PTS2 involves Pex7/Pex5 complex for cargo recognition and Pex13/Pex14 for docking at the peroxisomal membrane. Following docking, the recognition complex (PTS1-cargo/Pex5 or PTS2-cargo/Pex7/Pex5) translocate into the peroxisome by poorly understood mechanism. What is known is that a transient pore forms in the peroxisomal membrane (Erdmann & Schliebs, 2005) and the pore is large enough to translocate a folded cargo, or even more – the entire docking complex. The cargo is released in the peroxisomal lumen while the cargo receptor is recycled into the cytoplasm. More than a dozen Pex proteins participate in recognition, membrane docking, translocation and regeneration of the transport machinery (Kalel, Maser, Sattler, Erdmann, & Popowicz, 2018).

Peroxisomal transport system is a valid target in trypanosomiasis

A number of targets have been evaluated for drug discovery in trypanosomiasis. Among metabolic pathways, glycolysis was validated as a relevant target. For example, McNae and colleagues demonstrated recently that allosteric inhibition of phosphofructokinase cures acute HAT in mice (McNae et al., 2021). Such studies are of great interest as they establish glycolysis as a relevant drug target in trypanosomiasis. Nonetheless, inhibition of metabolic enzymes is difficult due to high concentration in the cell.

In *Trypanosoma*, glycolysis is uniquely compartmentalized within the peroxisomes. Different than in other eucaryotes, the glycolytic flux is regulated by glucose transport into the peroxisomes rather than negative feedback in the glycolysis pathway. It was demonstrated that mis-localization of glycolytic enzymes into the cytoplasm results in metabolic catastrophe – an uncontrolled glucose metabolism leading to parasite cell death (Furuya et al., 2002; Haanstra et al., 2008; Kessler & Parsons, 2005). This information allowed to hypothesize that inhibition of peroxisomal transport may constitute a relevant target in trypanosomiasis. By targeting peroxisomal transport one could affect not only a single metabolic enzyme, but with a single Pex inhibitor one should be able to deregulate the entire glycolytic pathway turning it form an energy source into a metabolic catastrophe within a pathogen cell. However, the brilliant idea required validation.

Dawidowski and collaborators selected the Pex14/Pex5 interaction for the validation of peroxisomal transport inhibition hypothesis (Dawidowski et al., 2017). Pex14/Pex5 interaction is structurally well characterized. A short motif within Pex5 characterized by a consensus sequence WxxxF folds into an amphipathic α-helix which is recognized by a small N-terminal domain of Pex14. The hydrophobic residues within the WxxxF motif occupy two shallow pockets at the surface of Pex14. The pockets are formed within the cleft between the adjacent Pex14 helices. Two characteristic phenylalanine residues of Pex14 contributed by two adjacent helices and positioned by their ring stacking interactions border the two pockets providing further stacking interaction to the hydrophobic residues of the Pex5 motif (Neufeld et al., 2009). As many protein-protein interaction surfaces, the Pex5 accommodating pocket at the surface of Pex14 is not ideal for drug design. Nevertheless, utilizing a pharmacophore model based on available structural data for *in silico* screening, Dawidowski and colleagues succeeded in targeting the interface with pyrazolo[4,3-c]pyridine derivatives. The compounds were able to disrupt the Pex5/Pex14 interaction *in vitro* and were toxic to Trypanosoma cells with low toxicity towards human cells. To optimize the initially identified compounds, a fragment

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screening approach was utilized, which allowed to identify double aromatic ring systems as Pex14 binders. Incorporation of the identified chemical groups in decoration of the original scaffold afforded increased affinity for Pex14 while crystallographic analysis revealed the mode of interaction. In brief, the two hydrophobic moieties decorating the central scaffold occupy the tryptophane and phenylalanine pockets at the surface of Pex14 while the scaffold pyrazolo[4,3-c]pyridine shields the phenylalanine rings of Pex14 involved in the stacking interaction. Further optimization afforded a compound characterized by high nanomolar K_i and trypanocidal activity characterized by EC_{50} of 0.19 µM towards *T. brucei* and 0.57 µM towards *T. cruzi* matching the activity of a reference drug benznidazole. Because of the physicochemical characteristics of the Pex5/Pex14 interaction surface, the reported inhibitors are characterized by unfavorable absorption, distribution, metabolism, and excretion (ADME) which requires further optimization. Despite the drawback, one of the most active inhibitors reduced parasitemia levels in an animal model of *T. brucei brucei* infection upon oral administration, while no adverse effects were observed. This seminal study (Dawidowski et al., 2017) validates the concept of therapeutic targeting of the peroxisomal transport system in trypanosomiases.

Progress in Pex14 inhibitor development

Following the seminal discoveries, in depth characterization and optimization of the pyrazolo[4,3-c]pyridine based compounds was reported (Dawidowski et al., 2020). The initial compound (EC_{50} = 265 µM and 539 µM for Tb and TcPex14, respectively) was divided into four regions for subsequent optimization. The regions included the moieties addressing phenylalanine and tryptophane pockets, the pyrazole N substituent, and the central scaffold. Because Phe and Trp pocket addressing moieties are responsible for the major compound-protein interactions it was expected that optimization in these regions will allow highest improvement in affinity. Indeed, substitution of the phenyl moiety initially targeting the tryptophane pocket with naphthyl substituent afforded compounds with 5-fold increased affinity. Optimization of the Phe pocket proved more challenging. Initial efforts of substituting the original indole were unsuccessful, however with the help of structural information on intermediate compounds it was possible to arrive at substituted naphthyl derivatives which improved the affinity by another 4-fold factor. Pyrazole N substituent directs the solvent-exposed region with possibility of improving electrostatic interactions. Optimization at that position yielded further 5-fold improvement in affinity reaching single digit micromolar values towards tbPex14 and low double digit micromolar values towards tcPex14. Limited optimization of the central scaffold afforded further improvement in affinity with pyrrolo[3,4-c]pyrazole derivative affording the first submicromolar inhibitor.

Apart from few outliers, the trypanocidal activity of tested compounds correlated with Pex14 affinity. Interestingly, compounds with aminoalkyl chain attached to the N-1 position of the pyrazole exhibited unexpectedly high trypanocidal activities in the nanomolar range. The selectivity index for trypanocidal activity vs. toxicity against human cells increased with compound efficacy in killing trypanosoma. The authors concluded that submicromolar trypanocidal activities and fair therapeutic indices may have practical implications in human disease and merit further development.

Optimization efforts of pyrazolo[4,3-c]pyridine compounds have lead to interesting findings regarding the influence of water envelope in medicinal chemistry driven optimization (Ratkova et al., 2020). The potency of protein-protein interaction inhibitors may change by orders of magnitude upon manipulation of the water envelope. So called "happy" water molecules interact tightly with polar surface residues and are interconnected within the hydration shell. Such waters may support the protein-ligand complementarity while the potential benefit of displacing such waters is a matter of dispute. Additionally, in case of PPI inhibition, the newly formed water network requires optimization. The investigation of solvation effects of Pex14/inhibitor complexes demonstrated that one particular water molecule, mediating the interaction with Asn13 was conserved in all Pex14 / inhibitor structures. The water molecule provided favorable interface with the inhibitor molecule and interconnections within the surface water envelope. Modifications of the favorable water envelope resulted in significantly decreased inhibitor affinity which was explained by disruption of the water envelope and displacement of the "happy" water molecule documented by relevant crystal structures. The above findings guide further optimization of Pex14 interaction inhibitors.

We have recently completed another study where we identified a completely novel scaffold for Pex5/Pex14 inhibition which affords trypanocidal compounds. This development further validates the concept of pharmacological peroxisome transport inhibition in trypanosomiasis and provides a new chemical entity for further development (to be published).

Future perspectives

The peroxisomal transport system was convincingly demonstrated a relevant target in trypanosomiases which opens the road to development of therapeutically valid approaches. However, the Pex system involves more than a dozen of factors, some of which may offer a potential of more druggable pockets compared to Pex14. Identification of such targets remains the current challenge. Once identified, inhibitor development campaigns have to be initiated and successfully completed, which will possibly identify clinical candidates. The road is still long and difficult, but the initial findings summarized in this review allow to expect significant progress in the forthcoming years.

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