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Stoking the drug target pipeline for human African trypanosomiasis

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Abstract

Trypanosoma brucei is the causative agent of African sleeping sickness, putting at risk up to 50 million people in sub-Saharan Africa. Current drug therapies are limited by toxicity and difficult treatment regimes and as the development of vaccines remains unlikely, the identification of better drugs to control this deadly disease is needed. Strategies for the identification of new lead compounds include phenotypic screening or target-based approaches. Implementation of the latter has been hampered by the lack of defined targets that are both essential and druggable. In this issue of *Molecular Microbiology*, Jones *et al*. report on the characterization of *T. brucei* pyridoxal kinase (PdxK), an enzyme required for the salvage of vitamin B6, an essential enzymatic cofactor. Genetic knockdown and small molecule inhibitor studies were used to demonstrate that PdxK is essential for parasite growth both *in vitro* and in a mouse model, providing both genetic and chemical validation of the target. An enzyme assay compatible with high throughput screening (HTS) was developed and the X-ray crystal structure solved, showing the potential for species selective inhibition. These studies add a greatly needed additional target into the drug discovery pipeline for this deadly parasitic infection.

The protozoan pathogen, *Trypanosoma brucei*, is the causative agent of human African trypanosomiasis (HAT), which is endemic to sub-Saharan Africa (Brun *et al*., 2010, Kennedy, 2008, Stuart *et al*., 2008). HAT is transmitted via the tsetse fly by two sub-species: *Trypanosoma brucei gambiense* in West Africa, representing 95% of the cases and *Trypanosoma brucei rhodesiense* in East Africa, representing 5% of the cases. The parasite remains entirely extracellular throughout its life cycle, and the course of the disease is divided into two stages: 1) early blood stage infection that leads to a febrile illness with flu-like symptoms; and 2) late stage infection in which parasites cross the blood brain barrier and are found in the cerebral spinal fluid. This latter stage of the disease leads to neurological symptoms, including disruption of the sleep/wake cycle and psychological effects. If untreated HAT is usually fatal. The World Health Organization (WHO) estimates that up to 50 million people are at risk for infection, and additionally the effected regions are plagued by protein malnutrition due in part to difficulty in raising live-stock as cattle are also susceptible to the parasite. Human cases have been declining in the past decade and in 2009 it was reported by the WHO that fewer than 10,000 people were infected (http://www.who.int/mediacentre/factsheets/fs259/en/). However lack of full scale screening programs of the at risk populations, coupled with poor diagnostic tools (Wastling & Welburn, 2011) leads to under reporting of the case-load, which is likely to be at least 3-fold higher than the measured value.

The global effort to control HAT is based on a combination strategy of vector control and drug treatment of infected patients (Brun et al., 2010, Kennedy, 2008, Stuart et al., 2008).

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Vaccines are not available and are unlikely to be developed as an extensive program of antigenic variation over the course of the infection allows the parasite to avoid the immune response (MacGregor *et al.*, 2012). Currently there is no drug that is effective against both stages of the disease or both subspecies, and all therapies require parenteral administration (Table 1). Early stage disease is treated with pentamidine (*T. b. gambiense*) or suramin (*T.b. rhodesiense*), and while both compounds show some toxicity they are typically tolerated well enough for successful treatment outcomes (Barrett, 2010, Brun *et al.*, 2011, Burri, 2010, Jacobs *et al.*, 2011a). Treatment of late stage disease is more problematic. Historically the highly toxic arsenical compound melarsoprol was used to treat both subspecies of the disease, causing 5–10% fatality in treated patients. However in 2009 a new nifurtimox/eflornithine combination therapy (NECT) was advanced for the treatment of late stage *T.b. gambiense* after showing equivalent to better efficacy than eflornithine alone in clinical trials (Yun *et al.*, 2010). NECT has the advantage of a much simpler treatment regime that lead to fewer adverse events and for these reasons has been registered on the WHO's Essential Medicines List. NECT has not yet been tested against *T.b. rhodesiense*, and despite improvements over eflornithine alone, administration still requires 7 days of twice daily i.v. infusions of eflornithine along with oral nifurtimox administration. Thus despite recent efforts a safe and effective treatment for all stages and forms of HAT is still lacking, and there is a clear need for the development of new anti-trypanosomal agents that can overcome these short comings.

Traditionally, neglected tropical diseases have not been the focus of robust efforts to identify new drugs due to lack of a profitable market and effective strategies to implement control programs. However the recent emergence of public-private partnerships has begun to fill this gap, with organizations such as Medicines for Malaria Venture, focused on malaria drug discovery, (Burrows *et al.*, 2011b) and Drugs for Neglected Diseases Initiative (DNDi), which emphasizes development of drugs for the treatment of trypanosomiasis and leishmaniasis (Chatelain & Ioset, 2011), taking a leading role in this area. Within DNDi's portfolio, two new promising compounds for the treatment of HAT that are orally active and effective against both early and late stage disease are currently undergoing clinical trials (Chatelain & Ioset, 2011, Maser *et al.*, 2012). These include the oxaborole SCYX-7158 that was discovered in a phenotypic screen against cultured parasites by Scynexis and Anacor (Jacobs *et al.*, 2011b), and fexinidazole, rediscovered from literature searches, mining of pharmaceutical company databases, and phenotypic screening of nitroheterocyclics (Kaiser *et al.*, 2011).

With the exception of eflornithine, the current pipeline of existing and potential new drugs for the treatment of HAT all resulted from phenotypic screens. Phenotypic screening has been an effective strategy for the identification of new antimicrobial drugs, including for HAT and malaria (Maser et al., 2012, Burrows et al., 2011b, Chatterjee & Yeung, 2012). However, as a result, the mechanism of action of these therapies remains largely unknown. The lack of mechanistic understanding complicates lead optimization of novel scaffolds, and hinders an effective understanding of toxicological mechanisms. For HAT therapy the only exceptions are eflornithine, for which the target has been shown to be ornithine decarboxylase, an essential enzyme in the biosynthesis of polyamines (Jacobs et al., 2011a) and nifurtimox, where there is good evidence that activation by a type I nitroreductase leading to production of intracellular free radicals, is key to its efficacy (Hall *et al.*, 2011, Wilkinson *et al.*, 2008). Suramin has been reported to inhibit a number of glycolytic enzymes (Barrett *et al.*, 2007), including a recent report that it inhibits pyruvate kinase by binding the ATP site as shown by X-ray structure analysis (Morgan *et al.*, 2011). However, no follow up studies to confirm the potential mechanism of action have been reported for any of the glycolytic enzymes. Analogs of pentamidine have been shown to collapse the mitochondrial membrane potential but the protein targets that mediate these effects are

unknown (Lanteri *et al.*, 2008). Recent efforts to utilize genome-wide RNAi approaches have led to the identification of genes involved in suramin uptake, and to the identification of several other lysosomal proteins that contribute to its action (Alsford *et al.*, 2012). However these studies also showed that for pentamidine, suramin and melarsoprol, a number of genes were identified that modulate their function, suggesting that clear identification of their molecular targets will not be straight-forward.

An alternative approach to the identification of new lead molecules for drug discovery is a target-based approach (Jacobs et al., 2011a), and indeed in the field of anti-protozoal drug discovery this approach has been successfully exploited for the development of eflornithine(Jacobs et al., 2011a), for the identification of dihydrofolate reductase inhibitors (Burrows *et al.*, 2011a) and more recently for the identification of a preclinical candidate targeting dihydroorotate dehydrogenase for the treatment of malaria (Coteron *et al.*, 2011, Phillips & Rathod, 2010). The lack of clinically validated targets for the treatment of HAT has hindered the use of the target-based approach. However a number of important tools are available that facilitate the search for targets including: 1) the *T. brucei* genome sequence published in 2005 (Berriman *et al.*, 2005); 2) robust genetic tools (RNAi, homologous recombination, and regulated gene expression) that make it possible to determine if a gene is essential in *T. brucei* (Kolev *et al.*, 2011, Wirtz *et al.*, 1999, Alsford *et al.*, 2011); and 3) a growing understanding of factors that govern gene expression in the parasite (Siegel *et al.*, 2011). Tools to prioritize genes based on likely druggability are also available, with druggability defined as the likelihood that a target will bind small drug like molecules that are disease modifying with high affinity (Aguero *et al.*, 2008, Magarinos *et al.*, 2012). Recently, efforts to utilize these resources have led to the identification of a number of genes that have been shown to be essential using genetic strategies, and for which chemical inhibitors with anti-trypanosomal activity have been described, including enzymes involved in polyamine and trypanothione biosynthesis, energy metabolism, purine and pyrimidine metabolism, DNA modification, fatty acid and sphingolipid biosynthesis and protein modification (Jacobs et al., 2011a). Ultimately, the key to the target-based approach is to identify genes that are both essential and druggable, and to develop the tools needed for hit identification and lead optimization programs, including heterologous expression systems, high throughput screening (HTS) compatible enzyme assays, and X-ray structures to help guide the medicinal chemists during the lead optimization program.

In this current issue a paper by Alan Fairlamb's group at the University of Dundee, “Chemical, genetic and structural assessment of pyridoxal kinase as a drug target in the African trypanosome” describes the identification of an additional potential enzymatic target, pyridoxal kinase (PdxK), to feed the drug discovery pipeline for the treatment of HAT. PdxK is required for the salvage of pyridoxal and pyridoxamine from the environment in order to generate pyridoxal-5'-phosphate (PLP) (vitamin B6), an essential cofactor in many enzymatic reactions including the decarboxylation, racemization and transamination of amino acids. Notably the target of eflornithine, ornithine decarboxylase, is a PLP-dependent enzyme. In this paper the authors demonstrate that PdxK is an essential enzyme for the growth of blood form parasites using a genetic knock out approach where the two wild-type alleles of the gene were replaced through homologous recombination with antibiotic selection markers, the second of which was deleted only after the insertion of a tetracycline regulated copy of PdxK into the rRNA tandem gene array. Thus in the presence of tetracycline the parasite growth rate is similar to wild-type levels, and upon withdrawal of tetracycline PdxK was no longer expressed leading to growth arrest. While many studies in the field focus on the effects of genetic knockdown of a gene on cultured blood form parasites, the current study also demonstrates that loss of PdxK function leads to parasite clearing in a mouse model. This result is particularly important for establishing the essentiality of this gene, as the *in vitro* growth arrest caused by gene knock down was

attenuated by the addition of pyridoxal or pyridoxamine to the media. However, the concentrations required to attenuate the growth effect were 20-fold higher than present in human serum suggesting it is unlikely that pyridoxal levels *in vivo* would be sufficient to overcome PdxK inhibition. This result was confirmed by the inability of the PdxK knockdown cells to establish an infection in mice, clearly showing that the enzyme is an essential protein to sustain *T. brucei* infection. This work demonstrates the importance of extending the study of genetic models to an animal model of *T. brucei* infection. In addition to utilizing a genetic approach the authors of the paper also demonstrated that a known inhibitor of PdxK, ginkgotoxin, also inhibits the growth of blood form parasites, and that parasite cell lines missing either a single PdxK allele or both alleles are more sensitive to growth inhibition, suggesting ginkgotoxin is likely to exert its anti-parasitic effects by an on-target mechanisms. These studies thus both genetically and chemically demonstrate the essential nature of *T. brucei* PdxK, providing evidence that the target is both essential and druggable.

In the second part of the study the authors focus on developing the necessary tools to ready PdxK for the drug discovery pipeline by characterizing the steady-state kinetic profile of the *E. coli* expressed recombinant enzyme, developing a spectrophotometric enzyme assay that is suitable for HTS and solving the X-ray structure of the *T. brucei* enzyme. Comparison of the *T. brucei* PdxK X-ray structure with that of the human enzyme shows that several amino acids in the pyridoxal and ATP binding sites differ between the species, providing the potential for species selective inhibitors of the *T. brucei* enzyme to be identified. This latter point is key to the potential of the target to be exploited, as PdxK is also essential in human cells.

In summary, the described PdxK studies have comprehensively addressed the issues needed to set the stage for the exploiting PdxK in a drug discovery program, demonstrating that the target is essential and druggable, and providing the tools necessary to prosecute a hit-identification program. Additionally, the X-ray structure provides a valuable asset to guide any future lead optimization programs. What is needed next is to identify novel and species-selective inhibitors of *T. brucei* PdxK, and to determine if they can be progressed to have the necessary *in vivo* properties, including pharmacokinetic and toxicological profiles, for advancement as a potential new therapy.

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Table 1

HAT drugs in clinical use

Compound	Indication/limitations/status	Dosing method	Mechanism of Action
Suramin	Used for the treatment of early stage *T. b. rhodesiense;* does not cross the blood brain barrier	IV injection	Unknown; binds to pyruvate kinase (Morgan et al., 2011) contribution to toxicity unknown; lysosomal proteins contribute to activity (Alsford et al., 2012)
Pentamidine	Used for the treatment of early stage *T. b. gambiense;* does not cross the blood brain barrier	IM injection	Unknown; analogs collapse the mitochondrial membrane potential but protein target unknown(Lanteri et al., 2008); P-type ATPases contribute to uptake (Alsford et al., 2012)
Melarsoprol	Late stage, all strains; currently recommended only for late stage *T. b. rhodesiense;* highly toxic	IV infusion	Unknown; Forms a stable adduct with trypanothione, role in toxicity unknown
Eflornithine	Late stage, *T. b. gambiense* recommended therapy in combination with nifurtimox	IV infusions	Inhibitor of ornithine decarboxylase(Jacobs et al., 2011a)
Nifurtimox	Late stage, *T. b. gambiense* in combination with eflornithine	oral	Activation by a type I nitroreductase required (Hall et al., 2011, Wilkinson et al., 2008)
SCYX-7158	Phase I started. Target profile, both stages and strains	oral	Unknown
Fexinidazole	Phase I complete; Phase II/III scheduled. Target profile, both stages and strains	oral	Unknown, but activation by a type I nitroreductase is required in leishmania(Wyllie *et al.*, 2012)