BJ www.biochemj.org

BJ Cell

Biochem. J. (2009) **419**, e1–e3 (Printed in Great Britain) doi:10.1042/BJ20090283

COMMENTARY

Drug screening by crossing membranes: a novel approach to identification of trypanocides

Mark C. FIELD[1]

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, UK

Trypanosomes are a group of protozoan parasites that inflict huge health and economic burdens across the globe. The African trypanosome, *Trypanosoma brucei*, the causative agent of sleeping sickness, has a highly sophisticated mechanism of antigenic variation that facilitates chronic survival in the mammalian host, and also all but eliminates any realistic hope for vaccination-based control. However, trypanosomes are also highly divergent organisms, with many biochemical processes setting them apart from their hosts, and there remains great optimism that these features may be exploited for development of new drugs. Unfortunately, the compounds that are in use at present are decades old and resistance has emerged. The article in this issue of the *Biochemical Journal* by Patham et al., a joint team from the universities of Pittsburgh and Georgia, represents one approach to exploiting this divergence. The authors of the study have exploited novel aspects of the biochemistry within the system for translocation of nascent polypeptides across the endoplasmic reticulum membrane to identify three compounds that are able to inhibit the process. They then demonstrate that these same compounds are both trypanocidal, but well tolerated by human tissue culture cells. These observations may present interesting new leads in the fight against trypanosomiasis, and potentially identify a new target that can be explored for therapeutic potential.

Key words: endoplasmic reticulum translocation, microsome, signal recognition particle, transport assay trypanocide, trypanosomiasis, variant surface glycoprotein.

Trypanosomatids constitute a major public health threat across much of the world. Members of this group include the South American trypanosome, *Trypanosoma cruzi* (causative agent of Chagas' disease), the Leishmanias (which invoke several distinct modes of disease pathology) and the African trypanosomes, *T. brucei* (which lead to sleeping sickness in humans and n'agana in cattle). *T. brucei* is transmitted by tsetse flies between mammalian hosts, and is responsible for over 50000 deaths annually and an unquantified impact on agriculture and economic prosperity across much of sub-Saharan Africa. A large wild animal reservoir and high infection rates among domestic cattle provide a major challenge for strategies aimed at alleviating infection of humans. As the disease afflicts countries with some of the lowest gross domestic product per capita in the world, there is a further and serious economic barrier to effective control. The drugs available to treat African trypanosomiasis are old, toxic and becoming ineffective due to resistance [1]. At present, four compounds are in use: suramin and pentamadine for early-stage disease, and eflornithine and melarsoprol.

Recent studies demonstrate that *T. evansi* and *T. equiperdum* are essentially mutant forms of *T. brucei*, with the notable difference that they have lost part, or all, of their mitochondrial genome, rendering them incapable of differentiation within the insect vector. Transmission is still achieved by passive dipteran-mediated mechanisms [2]. This insight increases the impact of the greater *T. brucei* group, as *T. evansi* in particular is an important agricultural parasite of the Middle East and Asia, highlighting both just how large is the disease burden of the *T. brucei* parasite group and our own ignorance of the true impact of these organisms.

To provide some perspective here, only one new agent, eflornithine, has been approved for use against human African trypanosomiasis in the past half century. Suramin, which remains the frontline drug of choice for treatment of early-stage disease, is the only compound against which resistance does not seem to be a major issue at present, and was introduced as early as 1922, predating even the earliest antibiotics. Critically, melarsoprol, the sole agent available for late-stage disease treatment, has the added and tragically shocking feature of killing up to 10% of recipients through an acute encephalitis of incompletely understood mechanism [3]. Tinkering with combination therapy may help to stave off the worst effects of resistance for a while, but the problem is growing, especially with increased mobility and human migration [4,5]. Most concerning has been the failure, in the final stages of development, of a promising lead compound, DB289 [6], which was recently withdrawn from consideration due to toxicity. It should be noted that none of the current drugs would pass Food and Drug Administration safety criteria if they were to be considered for introduction today. In the absence of DB289, there is no trypanocidal compound in any advanced stage of clinical development, despite several ongoing screening efforts around the globe and the potential for a wealth of targets. These efforts however are mainly academic-based, and cannot bring the force of Big-Pharma to bear. Depressingly, prospects for new therapeutic agents entering the clinical pipeline in the near future are very poor [1,5], although progress has been made recently in bringing down the number of cases using existing agents following a concerted effort by the World Health Organization.

T. brucei, and the closely related trypanosomes *T. evansi* and *T. equiperdum*, exhibit antigenic variation [7]. This process

Abbreviations used: GPI, glycosylphosphatidylinositol; HSP, heat-shock protein; SRP, signal recognition particle; VSG, variant surface glycoprotein.

[1] email mcf34@cam.ac.uk

serves to prevent recognition of the parasite by the host immune system, which remarkably is exclusively extracellular and invades the bloodstream, lymphatic system and tissue spaces. In late-stage disease, the parasite also gains access to the central nervous system. The parasite surface is dominated, at approx. 90 % of total surface protein, by 5×10^7 copies of the GPI (glycosylphosphatidylinositol)-anchored variant surface glycoprotein (VSG). The protein is expressed from a single telomere-proximal expression site, ensuring homogeneity of the surface coat. Recombination of the telomere-proximal VSG gene with a repertoire of chromosomal internal copies facilitates the generation of novel sequence within the expression site VSG. The rapid switching of the expressed VSGs produces a huge potential range of distinct VSG sequence variants, and the immune system does the rest. While VSG is highly antigenic, and parasites are efficiently eliminated via antibody-dependent complement killing and other mechanisms, parasites that have undergone a VSG switch and possess distinct antigenic surface profiles escape. This process is clearly highly efficient as in many animals trypanosome infection can continue for many months, or even years, often in the absence of overt symptoms. In humans, where co-evolution is presumed to have been rather more brief, disease progression is usually more rapid and severe, and trypanosomiasis is invariably fatal. True clinical recovery in the absence of therapeutic intervention is unknown. As one would expect, ongoing expression of VSG is essential for infectivity [8], and most workers consider its presence as an insurmountable barrier to vaccination. Attempts to vaccinate against *Plasmodium*, for example, which has a more limited antigenic variation potential, have met with limited success so far (discussed in [9]), which does not bode well for controlling the more sophisticated African trypanosomes; most authorities consider that vaccination against trypanosomes is unlikely to produce therapeutic gain.

Clearly, VSG represents the major virulence determinant in *T. brucei*, and any mechanism interfering with the expression of the protein or the switching between antigenically distinct VSG genes could, in principle, deliver a fatal blow to the parasite. VSG is a predominantly α-helical 58-kDa glycoprotein, bearing two or three N-linked glycans and a GPI-anchor [10,11]. Biosynthesis of VSG appears conventional; the nascent polypeptide is translocated across the endoplasmic reticulum (ER) membrane, followed rapidly by addition of the GPI-anchor via a GPI-8-dependent *trans*-amidation reaction, N-glycosylation and Golgi complex-mediated processing of both the GPI- and asparagine-linked glycans [12]. Biosynthesis and delivery to the surface is efficient. Following completion of sequencing the trypanosome genome it also emerged that *T. brucei* possesses a mainly conventional Sec61 translocon, a signal recognition particle (SRP), an SRP receptor and many of the expected ER chaperones, including BiP (immunoglobulin heavy-chain-binding protein), other HSPs (heat-shock proteins) and protein disulfide isomerases. Beyond the rather unusual exclusive presence of dimyristoylglycerol (C14:0) as a component of the VSG GPI, there is little here that appears unique, despite the great evolutionary distance between trypanosomes and mammals.

The study in this issue of the *Biochemical Journal* by Patham et al. [13], which reconstitutes VSG translocation into ER-membranes *in vitro*, represents a potential double advance. First, at the biochemical level, attempts to reconstitute protein processing, and in particular ER translocation, in trypanosomatids have previously met with only limited success. Attachment of GPI-anchors to endogenous polypeptide acceptors has been achieved in isolated membrane systems, but not complete reconstitution of translation, translocation and glycosylation [14]. The failure to achieve this previously has been explained away as being due to high divergence between the parasite signal sequence and the heterologous biochemical systems being used. This was never an entirely satisfactory answer as, for example, trypanosome signal sequences can function well *in vivo*, such as during expression in mammalian cells. This suggests that heterologous expression is possible and that the issue has been technical, rather than reflecting a true biochemical incompatibility.

Patham et al. [13] returned to this issue and, using a classic three-step *in vitro* translocation assay (transcription/translation/translocation), demonstrate that the missing requirement is for homologous (i.e. trypanosome) cytosol. This also demonstrates a potential divergence in mechanism. For example, while recent work has shown good conservation of many factors involved in ER-targeting and protein folding, trypanosome ER translocation does not necessarily require SRP54, despite the presence of the factor in the trypanosome SRP itself [15,16], a central factor in translocation systems in most taxa where this has been analysed. Furthermore, the SRP receptor β-subunit is apparently absent from the trypanosome genome; SRβ has been demonstrated as a central player in higher eukaryote translocation mechanisms, and this may suggest a fundamental difference in the manner in which trypanosomes co-ordinate the delivery of ribosome-bound nascent chains to the ER translocon [17]. The ability to reconstitute this step in trypanosomes provides an excellent model from which greater detail of the workings of trypanosome ER translocation systems will hopefully now emerge, potentially to provide deeper insight into this mechanism in additional deeply divergent cellular systems.

However, in an interesting choice, Patham et al. have made an oblique move. Rather than pursue the further dissection of their *in vitro* system, they opted to exploit it for the identification of potential compounds that may block the translocation reaction. The logic here is good: it is clear that ongoing VSG synthesis is an essential process for viability of the trypanosome, and furthermore, as the basis of the immune evasion strategy even a small defect in VSG biosynthesis is likely to cost the parasite dearly. The authors tested three compounds in their system: MA3-101, which inhibits activation of HSP70, together with two *Escherichia coli* SecA inhibitors CJ-21,058 and equisetin, which are highly related compounds differing by a single methyl group. All three caused significant inhibition of *in vitro* translocation activity, which was taken as potential evidence for trypanocidal activity, although the biochemical roles of putative HSP70 or Sec A-related or analogous factors in trypanosome translocation is not known. Regardless, in this prediction, Patham et al. were proven to be correct, and indeed all three compounds offer trypanocidal activity with an IC_{50} of 1–5 to 3.3 μM, while being well tolerated by mammalian culture cells.

Is this a breakthrough in the identification of new drugs against trypanosomes? Even at low micromolar concentrations, these compounds are not in themselves likely treatment agents, and further development is needed. The road to new therapeutics in any context is a long one, and made doubly challenging for parasitic disease by the lack of available financial incentives, although the interest of many non-governmental organizations is beginning to show some promise. In the present case, there are also some very important issues that remain. First, Patham et al. [13] have developed a potentially complex assay, and one that may not readily translate to a high-throughput format. Secondly, there is no formal proof that the putative targets suggested for the *in vitro* assay and the *in vivo* growth effect are the same, and in neither setting has a target been validated. For example, the putative molecular mechanism for all three compounds is related to ATPase activity, and when applied to whole cells the possibility of an effect on ATP levels or high energy phosphate metabolism

might be anticipated. Before these observations can be turned into a potential route forward, it is essential that the mode of action of these compounds be verified, and that effects on VSG synthesis and processing are investigated directly prior to more downstream exploitation of the assay. Thirdly, a vexing issue common to all drug development, is pharmacokinetics: a group of parameters that encompasses serum accessibility, clearance rate and susceptibility to hydrolase turnover, in addition to the all-crucial ability to enter the parasite cell. This has led some investigators to consider abandoning the *in vitro* target-based screening strategy altogether and to address the parasite directly; it remains to be seen if this is a wise position that could speed up identification of effective trypanocides. Nevertheless, two new lead compounds may have been identified, and a new biochemical system is now available as a potential therapeutic target. We can hope that this does indeed yield therapeutically useful compounds.

This study also highlights another serious issue. The trypanosome research community is not short of targets, and in one systematic study it was estimated that up to 35 % of genes may be essential, representing several thousand possible points within the genome for intervention [18]. Even a quick scan of the relevant literature reveals a number of promising targets, including GPI biosynthesis, trypanothione reductase and cysteine proteases. These all fulfil the criterion of essentiality, are sufficiently distinct in the parasite compared to the host for exploitation of a therapeutic window, and have been studied extensively and characterized by many highly talented workers in the field; however, none of these has yet to progress to the clinic. The present paper highlights a new potential target, and an interesting one as there is the possibility of multiple points of attack, with obviously increased opportunity for therapeutic gain. The major problem has been in taking such observations forward, and difficulty remains even when there are potential lead compounds, as here. Experience with DB289 underscores the need to deal with bigger numbers of targets and compounds if reliable progress against the disease can be made, and a more concerted push into functional post-genomics in trypanosomes is sorely needed. It is sobering that treating trypanosomiasis essentially relies on technology that is over 50 years old. There are probably few items of technology that we in the developed world use that are that old and have not seen a significant upgrade since their initial introduction. The individuals who presently use this technology to combat trypanosomiasis must do so or face loss of life and livelihood.

REFERENCES

1 Barrett, P., Boykin, D. W., Brun, R. and Tidwell, R. R. (2007) Human African trypanosomiasis: pharmacological re-engagement with a neglected disease. Br. J. Pharmacol. **152**, 1155–1171

2 Lai, D. H., Hashimi, H., Lun, Z. R., Ayala, F. J and Lukes, J. (2008) Adaptations of *Trypanosoma brucei* to gradual loss of kinetoplast DNA: *Trypanosoma equiperdum* and *Trypanosoma evansi* are petite mutants of *T. brucei*. Proc. Natl. Acad. Sci. U.S.A. **105**, 1999–2004

3 Pépin, J., Milord, F., Khonde, A. N., Niyonsenga, T., Loko, L., Mpia, B. and De Wals, P. (1995) Risk factors for encephalopathy and mortality during melarsoprol treatment of *Trypanosoma brucei gambiense* sleeping sickness. Trans. R. Soc. Trop. Med. Hyg. **89**, 92–97

4 Priotto, G., Fogg, C., Balasegaram, M., Erphas, O., Louga, A., Checchi, F., Ghabri, S. and Piola, P. (2006) Three drug combinations for late-stage *Trypanosoma brucei* gambiense sleeping sickness: a randomized clinical trial in Uganda. PLoS Clin. Trials. **1**, (8), e39. doi:10.1371/journal.pctr.0010039

5 Picozzi, K., Fèvre, E. M., Odiit, M., Carrington, M., Eisler, M. C., Maudlin, I. and Welburn, S. C. (2005) Sleeping sickness in Uganda: a thin line between two fatal diseases. BMJ **331**, 1238–1241

6 Thuita, J. K., Karanja, S. M., Wenzler, T., Mdachi, R. E., Ngotho, J. M., Kagira, J. M., Tidwell, R. and Brun, R. (2008) Efficacy of the diamidine DB75 and its prodrug DB289, against murine models of human African trypanosomiasis. Acta Trop. **108**, 6–10

7 Stockdale, C., Swiderski, M. R., Barry, J. D. and McCulloch, R. (2008) Antigenic variation in Trypanosoma brucei: joining the DOTs. PLoS Biol. **6**, (7), e185. doi:10.1371/journal.pbio.0060185

8 Sheader, K., Vaughan, S., Minchin, J., Hughes, K., Gull, K. and Rudenko, G. (2005) Variant surface glycoprotein RNA interference triggers a pre-cytokinesis cell cycle arrest in African trypanosomes. Proc. Natl. Acad. Sci. U.S.A. **102**, 8716–8721

9 Mikolajczak, S. A., Aly, A. S. and Kappe, S. H. (2007) Pre-erythrocytic malaria vaccine development. Curr. Opin. Infect. Dis. **20**, 461–466

10 Freymann, D. M., Metcalf, P., Turner, M. and Wiley, D. C. (1984) 6A-resolution X-ray structure of a variable surface glycoprotein from *Trypanosoma brucei*. Nature **311**, 167–169

11 Ferguson, M. A., Homans, S. W., Dwek, R. A. and Rademacher, T. W. (1988) Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. Science **239**, 753–759

12 Bangs, J. D., Andrews, N. W., Hart, G. W. and Englund, P. T. (1986) Post-translational modification and intracellular transport of a trypanosome variant surface glycoprotein. J. Cell Biol. **103**, 255–263

13 Patham, B., Duffy, J., Lane, A., Davis, R. C., Wipf, P., Fewell, S. W., Brodsky, J. L. and Mensa-Wilmot, K. (2009) Post-translational import of protein into the endoplasmic reticulum of a trypanosome: an *in vitro* system for discovery of anti-trypanosomal chemical entities. Biochem. J. **419**, 507–517

14 Mayor, S., Menon, A. K. and Cross, G. A. (1991) Transfer of glycosyl-phosphatidylinositol membrane anchors to polypeptide acceptors in a cell-free system. J. Cell Biol. **114**, 61–71

15 Liu, L., Liang, X. H., Uliel, S., Unger, R., Ullu, E. and Michaeli, S. (2002) RNA interference of signal peptide-binding protein SRP54 elicits deleterious effects and protein sorting defects in trypanosomes. J. Biol. Chem. **277**, 47348–47357

16 Lustig, Y., Goldshmidt, H., Uliel, S. and Michaeli, S. (2005) The *Trypanosoma brucei* signal recognition particle lacks the Alu-domain-binding proteins: purification and functional analysis of its binding proteins by RNAi. J. Cell Sci. **118**, 4551–4562

17 Zhang, X., Kung, S. and Shan, S. O. (2008) Demonstration of a multistep mechanism for assembly of the SRP x SRP receptor complex: implications for the catalytic role of SRP RNA. J. Mol. Biol. **381**, 581–593

18 Subramaniam, C., Veazey, P., Redmond, S., Hayes-Sinclair, J., Chambers, E., Carrington, M., Gull, K., Matthews, K., Horn, D. and Field, M. C. (2006) Chromosome-wide analysis of gene function by RNA interference in the African trypanosome. Eukaryot. Cell. **5**, 1539–1549

Received 16 February 2009/9 March 2009; accepted 9 March 2009
Published on the Internet 27 March 2009, doi:10.1042/BJ20090283