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Exploiting the Achilles' heel of membrane trafficking in trypanosomes

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Pathogenic protozoa are evolutionarily highly divergent from their metazoan hosts, reflected in many aspects of their biology. One particularly important parasite taxon is the trypanosomatids. Multiple transmission modes, distinct life cycles and exploitation of many host species attests to great prowess as parasites, and adaptability for efficient, chronic infection. Genome sequencing has begun uncovering how trypanosomatids are well suited to parasitism, and recent genetic screening and cell biology are revealing new aspects of how to control these organisms and prevent disease. Importantly, several lines of evidence suggest that membrane transport processes are central for the sensitivity towards several frontline drugs.

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African trypanosomes: novelty and conservation

Trypanosomatids cause a very broad range of diseases afflicting humans, animals, livestock, fish, plants and wild animals. Evidence has emerged for preadaptation to parasitism during evolution of the group and ongoing genetic modifications to suit distinct modes of infection and immune evasion [1••,2••]. Trypanosomes are classified as Excavata, and branched early from the eukaryotic lineage [3]. This fuelled considerable optimism that when the genomes of these organisms were characterized, a wealth of drug and potential therapeutic targets would emerge. Approximately forty percent of trypanosome protein-coding genes appear either lineage-specific or of such great divergence that they present a viable target, despite having an ortholog in higher eukaryotes [4,5]. This promise has, however, failed to emerge for several reasons, not least of which is translating initial hit compound activity against specific protein targets to leads with promising activity against whole cells, that is, trypanocidal or trypanostatic activity. Consequently, many efforts identifying new drugs remain focused on classical approaches such as phenotypic screens and do not, at least *a priori*, engage with either genetic divergence or those cell biological aspects unique to the kinetoplastids [6•]. Even compounds emerging from various screening efforts, with promising *in vitro* activity have experienced low rates of translation into viable (pre)-clinical candidates. However, serendipitously, it has emerged that many drugs presently used against these parasites, and specifically the African trypanosomes, do target rather well known unique aspects of trypanosome cell biology, and/or require these features, for their specificity and high potency.

The trypanosome cell is elongated, with a morphology supported by a sophisticated and elaborate subpellicular microtubule array [7,8]. This feature essentially precludes budding of conventional transport vesicles from the vast majority of the plasma membrane, and all membrane flow to and from the surface is restricted to the flagellar pocket. Thus, drugs that do not effectively diffuse across membranes, must reach their intracellular targets via the flagellar pocket or cross the membrane via an alternate mechanism, that is, a transporter or channel. The flagellar pocket crucially is devoid of the microtubule array, and while the membrane is continuous with the bulk plasma membrane, it has a distinct protein and lipid composition and is physically delineated by a complex collar surrounding the pocket neck, which likely also restricts fluid phase diffusion [8,9].

Trypanosomes possess a conventional endomembrane system, including a Golgi complex, early and recycling endosomes, late endosomes incorporating the ESCRT/multi-vesicular body system and a terminal endosome or lysosome, albeit somewhat streamlined, with several of these organelles probably present in interphase cells as single copy [10,11]. Several features are highly unique, for example, the mammalian-infective form of *T. brucei* has an extreme rate of endocytosis, capable of turning over the plasma membrane many times per hour, contributing

towards removal of surface-bound immune effectors, aiding immune evasion [12•]. The surface is dominated by the variant surface glycoprotein (VSG), a GPI-anchored homodimer comprising 90% of surface protein. Other surface proteins possess *trans*-membrane domains, but, importantly, are often highly divergent and trypanosome-specific [13•,14].

Trypanosome endocytosis is exclusively clathrin-dependent, setting it apart from higher eukaryotes where multiple modes of endocytosis operate [15]. Furthermore, the widely conserved heterotetrameric (AP)-2 adaptin complex is absent and is inversely correlated with the presence of VSG and thus antigenic variation, the principal mechanism of immune evasion [16,12•]. Additional clathrin adaptor proteins are present and include both ENTH and ANTH-domain phosphoinositide-binding proteins [17] and a cohort of trypanosomatid-specific proteins [18]. Sorting surface *trans*-membrane proteins requires ubiquitylation, likely performed by divergent ubiquitin ligases, although these remain unidentified [19••]. Whilst these details indicate a distinct endocytic system, the level of conservation with, for example, *Saccharomyces cerevisiae*, is considerable and the divergence certainly appears less extreme than in Apicomplexan parasites, where entire compartments and pathways have been repurposed [20,21•].

Membrane transport activity is rather different between life stages and species of parasite, which may in part explain the differential sensitivity to some front-line drugs. Specifically, the bloodstream forms of *T. brucei* have much greater endocytic transport rates compared with the insect forms, and this correlates with sensitivity to suramin and pentamidine for example, with procyclic cells being much less sensitive to either drug, although other changes to the surface composition are considerable and may also contribute to the differential sensitivity. Although pentamidine is almost exclusively used against *T. brucei gambiense*, the West African form of the disease, and suramin usually for the East African *T. brucei rhodesiense*, both subspecies are fully sensitive to either drug. Both drugs are used exclusively to treat early stage (haemolymphatic) trypanosomiasis, because neither drug penetrates sufficiently into the central nervous system; it is not known how trypanosomes adapt after crossing the blood–brain barrier, and whether this might impact drug sensitivity.

In addition to endocytic mechanisms for entry into the cell, trypanosomes also possess a considerable array of surface nucleoside and nucleobase transporters, together with hexose and amino acid permeases, plus a small family of aquaporins [22]. These systems obviously also present a potential mechanism for accumulation of drugs as well as natural metabolites, whilst themselves also being proteins that are subject to turnover by the endocytic system. By contrast to many surface proteins, the transporters appear to be more broadly conserved with higher eukaryotes. Significantly, traffic focused at the flagellar pocket, high rates of endocytosis, novel surface protein composition and the presence of conserved transporters all directly contribute towards sensitivity of African trypanosomes to drugs that have been in clinical use since the 1920s.

A grandfather therapeutic: suramin

Suramin emerged from early synthetic chemistry and development of aniline dyes [23]. The trypanocidal diazo dyes, trypan red and trypan blue, were developed by Bayer in 1916 and led to suramin, still a frontline treatment against some forms of trypanosomiasis [24]. High molecular weight and negative charge prevent passive membrane diffusion, suggesting specific uptake. A thousand-fold reduced potency against insect stage parasites suggested involvement of endocytosis, as endocytic trafficking is much decreased in this life stage [25]. However, extensive surface proteome remodelling between life stages also suggests the possibility of bloodstream stage-specific expression of a 'suramin-receptor' [14]. Genome-wide loss-of-function screens identified multiple genes that sensitize trypanosomes to suramin [26••] (Figure 2), many of which have roles and/or locations at the endocytic pathway, for example, invariant surface glycoprotein 75 (ISG75), two deubiquitylating enzymes (DUBs) Usp7 and Vdu1, the AP-1 adaptin complex and the lysosomal protein p67 [27]. An ISG75-dependent pathway is required for lysosomal delivery of suramin while an AP-1-dependent path is likely connected to lysosomal composition and a requirement for transport of p67 and other factors (Figure 1) [19••]. ISG75 stability is regulated by ubiquitylation [28] and evidence that trypanosome Usp7 and Vdu1 modulate ISG75 turnover is consistent with this model [19••]. Significantly, the suramin-uptake pathway is highly specific and does not involve the closely related invariant surface glycoprotein 65 (ISG65) family.

Together these observations are consistent with a hypothesis that ISG75 is the suramin receptor, but failure to demonstrate binding *in vitro* to recombinant ISG75 (unpublished data, MZ, MCF) suggests that additional factors may be involved. Suramin binds various serum proteins with high affinity, including Low Density Lipoprotein (LDL), and the influence of LDL on suramin uptake has led to a proposed model of an LDL-dependent receptor-mediated pathway for suramin internalization [29]. However, this was overturned by the mutation of trafficking pathway components [25]. Formal proof of receptor identity and precise mechanisms for suramin uptake remain elusive, but what is clear is the essential role for endocytosis and a protein with an itinerary that includes transport through the endosomal system. Significantly, the lysosomal proteases CatL and CBP1 are also implicated for suramin-sensitivity, and potentially these