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Short communication

Evidence for a non-LDL-mediated entry route for the trypanocidal drug suramin in *Trypanosoma brucei*

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The polyanionic aromatic suramin is a classical trypanocidal drug, and whilst first introduced in 1922, remains an important first line therapeutic for African sleeping sickness. Suramin contains two symmetrical polysulphonated napthylamine groups giving rise to six negative charges at physiological pH, preventing passive diffusion across biological membranes. Hence, specific uptake mechanisms into the parasite must exist and a complex mode of action such as interference with the import of newly synthesised glycolytic enzymes into glycosomes has been proposed to underpin the antiparasitic action of suramin [1–4]. Suramin may also bind to nascent cytosolic glycolytic enzymes and prevent glycosomal import [5]. However, an unequivocal description of how suramin enters the trypanosome and exhibits its toxicity remains to be presented. Interest in suramin has grown recently with its new use as an antitumour agent.

Coppens et al. described evidence for receptor-mediated endocytosis of host low density lipoprotein (LDL) in both bloodstream (BSF) and procyclic (PCF) stages [6–8] and proposed an LDL-receptor cycle similar to that in metazoans [7]. Vansterkenburg et al. [9] suggested entry of suramin via the LDL-receptor based on several observations. Firstly, suramin binds with considerable affinity to both human LDL and serum albumin in vitro and suramin uptake into trypanosomes requires pre-binding to LDL, suggesting a carrier function for the lipoprotein. Secondly, addition of more LDL to the incubation medium increases suramin accumulation, but conversely, addition of serum albumin decreases suramin uptake, suggesting partitioning between these plasma components. Thirdly, preincubation of suramin with albumin alone abrogates drug uptake, consistent with accumulation of the drug via a trypanosomal LDL-receptor and not fluid phase endocytosis, the route of entry for serum albumin [6]. Finally, suramin interferes with LDL-binding and uptake into live *Trypanosoma brucei* in a concentration-dependent manner, suggesting competition for the LDL-binding site on the LDL-receptor [9]. As the trypanosomal LDL-receptor gene has not been cloned, direct genetic evidence characterising the role of this receptor in suramin uptake is not available.

Several important proteins involved in endocytosis have been described recently, including members of the trypanosome Rab family [10,11]. Rab proteins are small GTPases, which control a number of central aspects of vesicle transport; in endocytosis Rab5 and Rab4 are responsible for the transport of cargo through early endosomes and recycling endosomes, respectively. These two processes are clearly closely coupled, but distinct as unique protein effectors interact with Rab4 and Rab5, and mutations in these proteins have specific effects on endocytosis [12]. Most importantly, the membranous compartments to which trypanosomal Rab4 and Rab5 (TbRAB4 and TbRAB5) localise are overlapping, but nonidentical [10]. Recently, we have demonstrated that the functions of the two TbRAB5 isoforms (termed A and B) are distinct, with TbRAB5A having a clear role in transport of GPI-anchored proteins and LDL, and TbRAB5B being involved in transmembrane protein endocytosis [11]. Creation of point mutations in Rab proteins to prevent GTP hydrolysis (Q→L substitution) locks the protein in the GTP-bound state (effectively active); such a strategy, pioneered in higher eukaryotes,

Abbreviations: BSA, bovine serum albumin; BSF, bloodstream form; LDL, low density lipoprotein; PCF, procyclic culture form.

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is applicable to trypanosomes and has the advantage that the mutant proteins are *trans*-dominant [11]. Here we have used two mutant cell lines, with alterations in the TbRAB4 and TbRAB5A pathways to investigate the relationship between LDL and suramin endocytosis. We have performed these experiments in the procyclic form as all evidence indicates that the LDL-receptor system is essentially identical in procyclic and bloodstream forms (BSF) in terms of receptor number and affinity. Hence, a lack of correlation between LDL and suramin-uptake in procyclics would invalidate the concept that suramin enters the cell via the LDL-receptor. In addition, in previous work with TbRAB5, we were unable to manipulate endocytosis levels in the BSF despite a marked effect on the system in procyclics (see [11] for details).

As part of a study to understand the role of TbRAB4, we developed a procyclic clone, TbRAB4^{WT}, overexpressing the wild type version of TbRAB4. The open reading frame for TbRAB4 was subcloned by PCR into the expression vector pXS219, and the plasmid transfected into procyclic cells [11]. Following selection and cloning by serial dilution, several transformants were obtained with marked overexpression of the transgene as verified by Western analysis (Fig. 1). This transgenic cell line exhibts severe disruption of surface protein expression and endocytic processes (to be described in detail elsewhere) and we wished to assess the impact these alterations may have on suramin interaction.

Initially, we considered suramin sensitivity as a crude indicator of interaction with the compound. Unexpectedly, we observed increased resistance to suramin in the TbRAB4-overexpressor (4^{WT}) compared to the parental Lister 427 line (Par). To quantify drug sensitivity, growth curves for parental and TbRAB4^{WT}-overexpressor cells were recorded in flask cultures in SDM79 at various suramin concentrations (Fig. 1). At the growth-inhibitory concentration of suramin (100 μM, [9]), replication of the parental cells was inhibited, but growth of the TbRAB4^{WT} clone was essentially unaffected, even at 150 μM. For the parental cells, the ID_{50} for growth was ~70 μM. This observation prompted us to determine in more detail the effect of ectopic TbRAB4 expression on suramin interaction by the parasite. Initially we asked if surface binding of the drug had been altered. We assessed the ability to bind suramin in parental and TbRAB4^{WT} cells by incubation at 4 °C for 1 h in medium that had been preequilibrated with ^{3}H-suramin for 16 h, and following washing, determined the level of suramin bound by scintillation counting. The nonspecific background levels were determined by an identical incubation, but in the presence of excess unlabelled suramin. Typically we obtained 140 pmol suramin bound/10^{7} parental cells, in agreement with previous observations [9], whilst the TbRAB4^{WT} cells were clearly decreased by ~50% in their ability to bind suramin (Fig. 1).

The decrease in suramin interaction was even more dramatic when we assessed cellular uptake of suramin, i.e. endocytosis. Here we assayed uptake following a 2 h incubation at 27 °C in media preequilibrated with radiolabelled suramin. Nonspecific uptake was assessed by incubation of identical cultures at 4 °C. We observed that the TbRAB4^{WT} cells had an almost negligible ability to take up suramin (Fig. 1) suggesting that reduced interaction with the drug most probably is responsible for the resistance of the TbRAB4^{WT} cells. These data suggest that the TbRAB4^{WT} cells have an altered endocytic system that decreases surface expression and endocytosis of a putative suramin receptor. Remarkably, when we determined the levels of ^{125}I-LDL endocytosed by the TbRAB4^{WT} cells by incubation with radioiodinated LDL for 1 h at 27 °C (experimental details in [11]), there was no detectable change between the mutant and the parental lines. These observations strongly suggest that LDL and suramin uptake are uncoupled processes, and hence unlikely to share the same molecular mechanism/receptor.

Previous work in our laboratory had generated a mutant procyclic trypanosome with greatly increased LDL-binding and endocytosis activity [11]. This parasite expresses an activated version of TbRAB5A (TbRAB5A^{QL}), a further Rab protein which is implicated as having a role in endocytosis of a number of cell surface proteins, incuding the variant surface glycoprotein [10,11]. ^{125}I-LDL internalisation is strongly increased in procyclics expressing TbRAB5A^{QL} (Fig. 2). The QL mutation ablates the majority of GTP hydrolytic activity and retains TbRAB5A^{QL} in the active GTP-bound form. Hence we could assess the sensitivity of a cell line with a well documented increase in LDL accumulation, and which would be predicted to have increased suramin sensitivity/uptake should suramin enter trypanosomes via an LDL-receptor system.

No significant difference was found in suramin sensitivity (Fig. 2). At 100 μM suramin, both cell lines died after ~48 h. As suramin has a slow inhibitory action on the parasite, unaltered suramin sensitivity in a background of increased LDL uptake cannot rule out suramin endocytosis via the LDL-receptor [13]. Thus, TbRAB5A^{QL} trypanosomes may more rapidly accumulate suramin, but a downstream slow killing step could make this refractory to detection by growth curve analysis. To measure suramin endocytosis directly in these cells, we again used radiolabelled suramin [6] as described above. Cells were washed in serum-free medium and resuspended in SDM–79 supplemented with either 20% foetal bovine serum (FBS) or 1% bovine serum albumin (BSA), using identical conditions as those for the TbRAB4^{WT} analysis. In this case, an identical experiment was also performed with 427

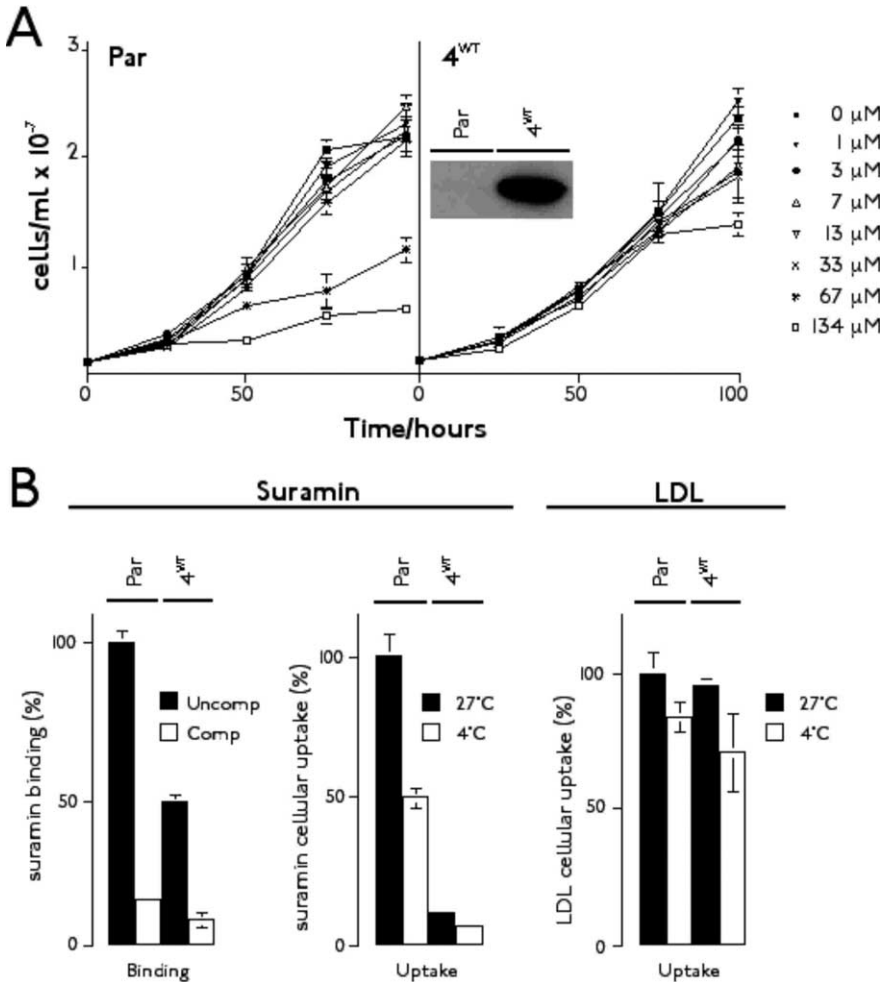

Fig. 1. Overexpression of TbRAB4^{WT} reduces suramin sensitivity. *Panel A:* Growth curves of procyclic parental Lister 427 (Par) and TbRAB4^{WT} (4^{WT}) in the presence of suramin. Cells in exponential growth were inoculated at 10^6 cells ml^{-1} into SDM79 supplemented with 10% FBS. Cultures with various suramin concentrations were set up in triplicate and cell densities determined using a Coulter Z1 Cell Counter. Suramin concentrations and the corresponding plot symbols are shown at right. All data were accumulated from triplicate cultures; error bars are the standard deviation. ID_{50} values were calculated by regression analysis [11]. In some cases, the error bars are obscured by the plot symbols. Inset; Western blot analysis of Lister 427 parental cells (Par) and TbRAB4^{WT} (4^{WT}) overexpressors, demonstrating overexpression of TbRAB4. Lysates corresponding to 10^7 cells were loaded in each slot, and equivalence of protein and transfer assayed by Ponceau S staining of the blot membrane prior to blocking. Data verifying overexpression of TbRAB5A can be found in [11]. *Panel B:* Binding and uptake of radiolabelled suramin to parental Lister 427 (Par) and TbRAB4^{WT} (4^{WT})-expressing 427 procyclic cells. Cells were harvested at mid-log phase and resuspended at 2×10^7 cells ml^{-1} in radiolabelled suramin (30 μM) preincubated for 16 h in complete SDM79 medium. Cells were incubated for 1 h at 4 °C in the presence (open bars) and absence (filled bars) of 100-fold excess competing unlabelled suramin then washed and solubilised in 1% sodium deoxycholate and counted in scintillant. Data are presented as percentage of the basal level of binding in parental cells. Each point represents the mean of triplicate samples ±SD. For uptake measurements, cells were incubated at 2×10^7 cells ml^{-1} in suramin (30 μM) preincubated as described above for 2 h at 4 °C (open bars) and 27 °C (filled bars). Results are presented as a percentage of the basal level of uptake in parental cells. Each point represents the mean of triplicate samples ± SD. *Panel B:* Uptake of ^{125}I-LDL by parental Lister 427 (Par) and TbRAB4^{WT} (4^{WT})-expressing 427 procyclic cells (method described in [11]). Results are presented as a percentage of the basal level of uptake in parental 427 cells. Each point represents the mean of triplicate samples ±SD.

bloodstream form cells at 37 °C to measure BSF uptake. After incubation, cells were washed and cell-associated radioactivity was determined by scintillation counting. Our data showed indistinguishable suramin accumulation for the wild type and TbRAB5A^{QL} cells (Fig. 2). Preincubation of the radiolabel with BSA rather than total serum abolished uptake in both stages consistent with previous data. Cell-associated radioactivity was grossly decreased in both BSFs and PCFs after incubation at 4 °C or in the presence of excess cold suramin confirming a specific receptor-mediated process for accumulation of radioactivity. BSFs displayed ~10-fold greater uptake of the drug compared to the insect stage indicating that should there have been an increase in suramin endocytosis of similar magnitude to LDL uptake in the TbRAB5A^{QL} expressors, our assay should have detected it. As LDL endocytosis in TbRAB5A^{QL} and BSF are equivalent but uptake of suramin is greater in the latter (Fig. 2), this is further evidence against an LDL-receptor-mediated mechanism in the BSF. These

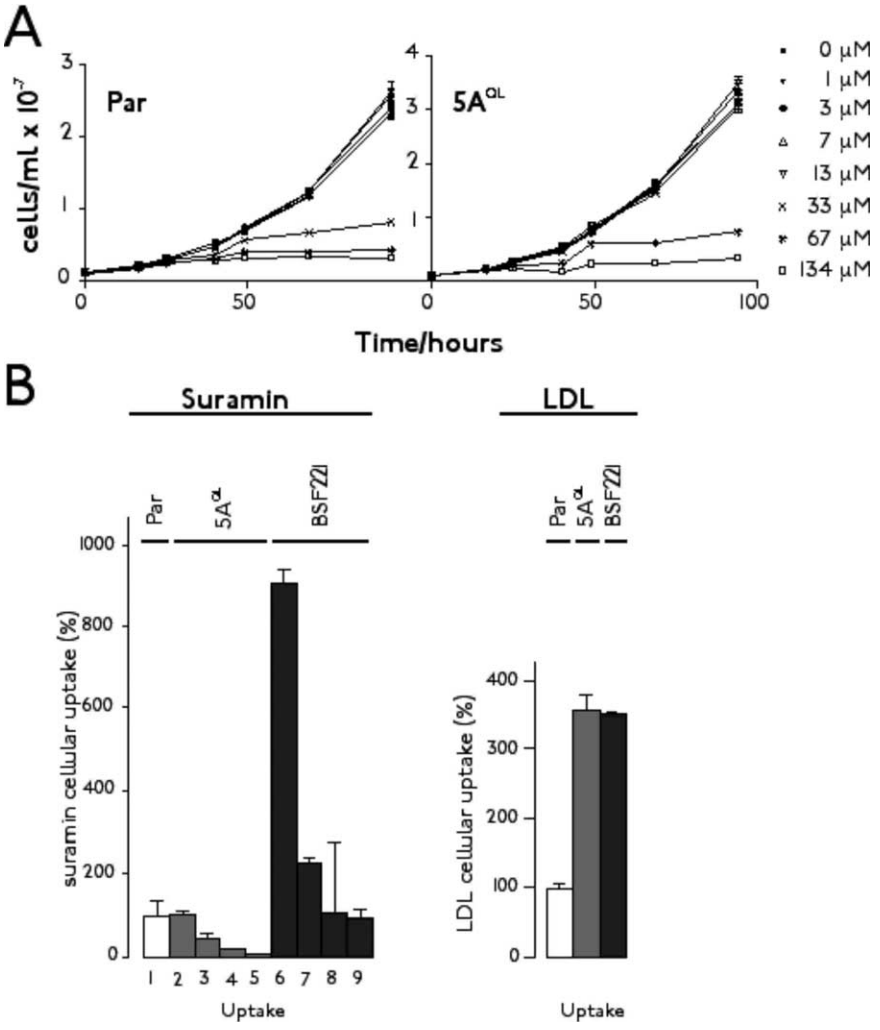

Fig. 2. Overexpression of TbRAB5A^{QL} in procyclic form *T. brucei* alters LDL endocytosis but not suramin uptake. *Panel A:* Growth curves of procyclic parental Lister 427 (Par) and TbRAB5A^{ql} (5A^{WT}) in the presence of suramin. Cells in exponential growth were inoculated at 10^6 cells ml^{-1} into SDM79 supplemented with 10% FBS. Cultures with various suramin concentrations were set up in triplicate and cell densities determined using a Coulter Z1 Cell Counter. Suramin concentrations and the corresponding plot symbols are shown at right. All data were accumulated from triplicate cultures; error bars are the standard deviation. ID_{50} values were calculated by regression analysis [11]. In some cases, the error bars are obscured by the plot symbols. *Panel B:* Uptake of suramin is unaltered in TbRAB5A^{QL} expressors, but LDL uptake is increased. Cells in exponential growth were harvested from flask cultures, washed and resuspended in incubation media pre-incubated with radiolabelled suramin, supplemented with either FBS or BSA as indicated. Accumulation of labelled suramin was allowed with or without excess cold suramin. Cells were washed and cell-associated radioactivity determined by scintillation. *Lane 1:* Procyclic parental Lister 427 wild type (Par) in FBS at 27 °C. *Lanes 2–5:* Procyclic TbRAB5A^{QL} overexpressor (5^{QL}) in FBS at 27 °C (lane 2); BSA at 27 °C (lane 3); excess cold suramin and FBS at 27 °C (lane 4) and FBS at 4 °C (lane 5). *Lanes 6–9:* 427 bloodstream form (BSF221) in FBS at 37 °C (lane 6); BSA at 37 °C (lane 7); excess cold suramin in FBS (lane 8) and FBS at 4 °C (lane 9). Error bars represent the standard deviation of assays performed in triplicate. For LDL uptake, procyclic parental Lister 427 (Par), TbRAB5A^{QL} overexpressors (5^{QL}) and bloodstream form wild type strain 427 (BSF221) exponential growth cultures were harvested and ^{125}I-LDL uptake assayed as described [11]. Error bars represent the standard error of triplicate determinations.

data also confirm that suramin uptake relies on prior binding to one or several serum compounds and is by an energy-dependent mechanism.

Suramin accumulation by trypanosomes fulfils the criteria for specific, receptor-mediated endocytosis, but unaltered suramin uptake in the presence of raised LDL internalisation, and decreased suramin uptake in cells with normal LDL endocytosis both clearly argue that suramin and LDL uptake are only indirectly related. The data presented here suggest that suramin entry into cells is not mediated via an LDL-specific receptor and furthermore also suggest that the suramin receptor is a distinct entity that has yet to be identified at the molecular level. Interestingly, in a mutant trypanosome selected for suramin resistance in vitro culture, STIB 386, we could not detect altered TbRAB levels (data not shown); this may indicate a different resistance mechanism is operating in these cells [14]. Finally, it remains unclear how suramin kills trypanosomes or other cells; recently this compound has been demonstrated to

influence intracellular transport of the cellular prion protein, PrP^C [15], raising the possibility that trafficking of GPI-linked molecules may be involved in the trypanocidal activity of this classic drug.

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