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Post-translational import of protein into the endoplasmic reticulum of a trypanosome: an *in vitro* system for discovery of anti-trypanosomal chemical entities

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Abstract

HAT (human African trypanosomiasis), caused by the protozoan parasite *Trypanosoma brucei*, is an emerging disease for which new drugs are needed. Expression of plasma membrane proteins [e.g. VSG (variant surface glycoprotein)] is crucial for the establishment and maintenance of an infection by *T. brucei*. Transport of a majority of proteins to the plasma membrane involves their translocation into the ER (endoplasmic reticulum). Thus inhibition of protein import into the ER of *T. brucei* would be a logical target for discovery of lead compounds against trypanosomes. We have developed a TbRM (*T. brucei* microsome) system that imports VSG_117 post-translationally. Using this system, MAL3-101, equisetin and CJ-21,058 were discovered to be small molecule inhibitors of VSG_117 translocation into the ER. These agents also killed bloodstream *T. brucei in vitro*; the concentrations at which 50% of parasites were killed (IC_{50}) were 1.5 μM (MAL3-101), 3.3 μM (equisetin) and 7 μM (CJ-21,058). Thus VSG_117 import into TbRMs is a rapid and novel assay to identify 'new chemical entities' (e.g. MAL3-101, equisetin and CJ-21,058) for anti-trypanosome drug development.

Keywords

drug development; endoplasmic reticulum (ER); protein import; microsome; *Trypanosoma brucei*

INTRODUCTION

HAT (human African trypanosomiasis) threatens an estimated 60 million people with a debilitating disease. No vaccines are available to prevent infection by *Trypanosoma brucei*, a protozoan that causes HAT. The drugs currently in use are old, toxic and must be injected or

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delivered intravenuously (reviewed in [1]). As a result, new drugs are needed for the treatment of HAT.

In the host bloodstream *T. brucei* survives because a small fraction of parasites escape host immune responses by switching expression of VSGs (variant surface glycoproteins) during 'antigenic variation'. Many cell-surface proteins, including VSGs and cell-surface receptors, use the ER (endoplasmic reticulum) as a 'gateway' for transport to the plasma membrane. Most proteins destined for the lumen of the Golgi complex, lysosomes, endosomes and the inner nuclear membrane pass through the ER en route to their final destination.We propose that small molecules that interfere with the translocation of proteins into the ER of the parasite will provide new chemical entities that may be optimized into drugs to treat HAT, because the drugs might block the delivery of essential proteins to the plasma membrane and compromise the viability of the parasite.

In model eukaryotes, many mechanistic aspects of protein import into the ER have been described. In vertebrates, protein translocation into the ER is predominantly co-translational. Translocation requires an N-terminal signal peptide, a SRP (signal recognition particle), a SR (SRP receptor) and a Sec61 translocon complex (reviewed in [2]). In fungi, archaea and bacteria, proteins can be imported into the ER or, in bacteria, proteins can be secreted through the inner membrane post-translationally [i.e. after polypeptide (substrate) release from ribosomes] [3]. The SRP is not essential for post-translational import, which instead depends on chaperones in the cytoplasm and in the ER. In yeast, molecular chaperones in the cytoplasm, e.g. Ssa1p [Hsp (heat-shock protein) 70] and Ydj1p (Hsp40), and in the ER, e.g. BiP (immunoglobulin heavy-chain-binding protein) (Kar2p) and Sec63p, are important for ER protein import. In bacteria, SecA and SecB exhibit chaperone-like properties in mediating protein transport across the inner cell membrane.

Several aspects of the pathway for protein import into the trypanosome ER suggest that it might be a good target to discover new lead compounds for anti-trypanosome drug development. First, SRP54 is not essential for the import of protein into the ERof *T. brucei* [4]. Secondly, in general, signal peptides of *T. brucei* are not compatible with the canine microsomal protein import system [5,6] that is widely used to study ER protein translocation in eukaryotes [7]; even within vertebrates, non-equivalence of signal sequences is documented [8–10]. These ideas are consistent with reports that some small molecules inhibit a select group of signal peptides in vertebrates [9–11]. Since trypanosome and vertebrate signal sequences have intrinsic differences [5], one expects the ER protein translocation machinery in *T. brucei* to have coevolved to operate efficiently only with parasite signal peptides. Correspondingly, other components of the trypanosome protein translocation system may have diverged from the vertebrate system. Such mechanistic differences between host and parasite can be exploited for anti-trypanosome drug discovery.

In the present study, we describe a novel *in vitro* system using membranes from *T. brucei* [TbRMs (*T. brucei* microsomes)] that is able, unlike canine microsomes, to import a trypanosome VSG post-translationally. TbRMs were used in a 'focused screen' of small molecules predicted to inhibit the function of cytoplasmic chaperones involved in the post-translational import of proteins into the ER or in the secretion of polypeptides across the bacterial inner membranes. Compounds that prevented translocation of VSG into TbRMs were evaluated for inhibition of growth of bloodstream *T. brucei*. Using this method, we discovered two natural products, CJ-21,058 [12] and equisetin [13], that blocked import of VSG into TbRMs and also killed *T. brucei*. Similarly, MAL3-101, which was produced by combinatorial chemistry [14], blocked VSG translocation into TbRMs and was trypanocidal. Together, these previous studies established the ER protein import pathway as a target for the discovery of new chemical entities that can be developed into anti-trypanosome drugs.

MATERIALS AND METHODS

Reagents and chemicals

Plasmid pVSG_117 was provided by Dr J. Bangs (Department of Medical Microbiology, School of Medicine and Public Health, University of Wisconsin, Madison, WI,U.S.A.). The acyl tetramic acid (3-acyl-5-hydroxymethyl-2,4-dione) derivatives CJ-21,058 and equisetin were gifts from Pfizer (New York, NY, U.S.A.). Rabbit reticulocyte lysate and methionine-/cysteine-free amino acid mixture were purchased from Promega (Madison, WI, U.S.A.). [^{35}S]Redivue Promix™ was purchased from Amersham Biosciences (Piscataway, NJ, U.S.A.), etoposide from Sigma (St. Louis, MO, U.S.A.) and propidium iodide was from Invitrogen (Carlsbad, CA, U.S.A.). Ampliscribe™ T7 *in vitro* transcription kit was purchased from Epicentre Technologies (Madison, WI, U.S.A.).

DNA templates and RNA synthesis *in vitro*

DNA template (1 μg) was transcribed using the Ampliscribe™ T7 kit (Epicentre Technologies) following the manufacturer's protocol. A template for *in vitro* transcription was obtained by PCR using the forward primer 5′-CCC*TAATACGACTCACTATAGGG*AGGAGGGTTTTTACC ATGGACTGCCATACAAAGGAG-3′, which contains a T7 promoter (italicized) and a translation enhancer (underlined) [15]. The first 21 nucleotides of the VSG_117 coding sequence are in roman font. The forward primer for VSG_117$_{500}$ΔSP (VSG_117 with the signal peptide removed) was 5′-CCC*TAATACGACTCACTATAGGG*AGGAGGGTTTTTACC ATGGCCACACTGAGAAAGGTTGC-3′. It contains nucleotides 51–57 of the coding region (roman font) and the other features are the same as those mentioned above for the previous forward primer. The reverse primer forVSG_117$_{500}$ was 5′-*CATCATCAT*TTCCTAAAAAAGCAAGGC-3′. It contains nucleotides 516–525 of the coding region (roman font) and a T7 promoter (italicized). The substrate for synthesis of VSG_MVAT7 was plasmid SL/3 (provided by Dr John Donelson, Department of Biochemistry, University of Iowa, IA, U.S.A.). The forward primer for the reaction was 5′-CCC*TAATACGACTCACTATAGGG*AGGAGGGTTTTTACCATGTCAACAAGAGTCCA ACAA-3′. It contains a T7 promoter (italicized), a translational enhancer (underlined) and the first 21 nucleotides of the VSG_MVAT7 coding sequence (in roman font). RNase free DNase I {1 μl [1 MBU (molecular biology unit)]} was added to the reaction mixture that was incubated at 37°C for 15 min (1 MBU digests 1 μg of pUC19 DNA in 10 min). An equal volume of TE-saturated phenol/chloroform was then added to the mixture, which was agitated on a vortex mixer, and centrifuged at 14000 ***g*** for 15 min at 4 °C. The aqueous phase was withdrawn, combined with an equal volume of chloroform, agitated on a vortex mixer and centrifuged at 14000 ***g*** for 15 min at 4°C. To this aqueous phase, 0.3 M sodium acetate (final concentration) was added and precipitated with ethanol at−20°C overnight. The precipitate was recovered by centrifugation at 14000 ***g*** for 15 min at 4°C, rinsed with 70% (v/v) ethanol, air-dried and dissolved in 40 μl of nuclease-free water. The RNA concentration was determined by measuring the absorbance at 260 nm, and was confirmed by analysis with agarose-gel electrophoresis/ethidium bromide staining.

Preparation of TbRMs

Bloodstream trypanosomes were obtained from the blood of infected rats and purified by DE-52 chromatography. Cells (10^{10}) were resuspended in 5 ml of homogenization buffer [50 mM Hepes/KOH, 250 mM sucrose, 50 mM potassium acetate, 6 mM magnesium acetate, 1 mM EDTA, 1 mM DTT (dithiothreitol), 1 μg/ml Tos-Lys-CH_2Cl (tosyl-lysylchloromethane, 'TLCK'), 5 μg/ml leupeptin and 0.5 mM PMSF (final concentrations)]. Resuspended cells (2.5 ml) were added to a pre-chilled dounce homogenizer where they were lysed with 40 strokes

of a tight-fitting pestle on ice. After 1 min of rest, another 40 strokes were delivered with the pestle. The homogenized cells were kept on ice while the remaining 2.5 ml of resuspended cells were broken. Both sets of homogenized lysates were pooled and centrifuged at 2000 ***g*** for 10 min at 4°C in microfuge tubes. The supernatants were pooled, divided into new microfuge tubes and centrifuged at 14000 ***g*** for 20 min at 4°C, and the pellets were resuspended in 40 μl (total volume) of fresh RMB (rough microsome buffer) (50 mM Hepes/KOH, 250 mM sucrose, 50 mM potassium acetate, 6 mM magnesium acetate, 1 mM DTT, 0.5 μg/ml Tos-Lys-CH_2Cl and 2.5 μg/ml leupeptin). The absorbance of the resuspended pellet (TbRM) was determined at 260 nm, and the concentration of Tb-RMs was adjusted with RMB to an aborbance at 260 nm of 50. TbRM was divided into 20 μl aliquots, quick-frozen in liquid nitrogen and stored at −80 °C. One equivalent of TbRM has an absorbance at 260 nm of 50. The supernatant from the 14000 ***g*** centrifugation step (above) was also retained (see below).

Preparation of cytosol from *T. brucei*

The 14000 ***g*** supernatant obtained during the preparation of TbRMs (see above) was centrifuged at 65000 rev./min for 60 min at 4°C using a Beckman TLA 100.3 rotor. The resulting supernatant (2 ml)was concentrated 20-fold by ultrafiltration with a Centricon-10 filter (Amicon). The retentate was retrieved, an aliquot was diluted 50-fold with 0.1% SDS, and the absorbance at 280 nm was measured. One equivalent of cytosol has an absorbance at 280 nm of 50. Aliquots (50 μl) were quick-frozen in liquid nitrogen and stored at −80°C.

Protein import into TbRMs

RNA (2 μg) encoding VSG_117 (or VSG_MVAT7) was translated in a reaction mixture (30 μl) containing 15 μl of rabbit re-ticulocyte lysate, 60 μM amino acid mixture (without methionine and cysteine) and 2.4 μCi of [^{35}S]Promix. The reaction was incubated at 37°C for 15 min. Cycloheximide (50 μg/ml final concentration) was used to stop further translation, and the mixture was divided into two portions. TbRMs (1 equivalent) was added to one aliquot, and to the other portion an equal volume of RMB was added. The reactions were incubated at 37°C for 45 min. To measure the post-translational import of VSG_117 into TbRMs, each reaction mixture was divided into three portions (10 μl volume) and treated with one of the following on ice for 1 h: (i) RMB; (ii) 30 μg/ml proteinase K; (iii) NP-40 (Nonidet P40) [2% (v/v) final concentration] and proteinase K (30 μg/ml final concentration). PMSF (20 mM final concentration) was added to stop proteinase K digestion. Samples were precipitated with an equal volume of cold (4°C) ammonium sulfate (saturated) and total proteins were resolved by SDS/PAGE (14% total acrylamide and 3% cross-linker in Tricine/HCl system). The gels were dried and radioactive polypeptides were detected with a phosphorimager [Personal Molecular Imager FX (Bio-Rad, Hercules, CA, U.S.A.)]. Data were quantified with QuantityOne software (Bio-Rad) and graphs were plotted with DeltaGraph (Red Rock Software, Salt Lake City, UT, U.S.A.).

Assay of VSG binding to TbRMs

To a 20 μl translation mixture containing VSG_117, cycloheximide (1 mM) was added to terminate protein synthesis, and the mixture divided into four 5 μl aliquots. Two of the aliquots were left untreated, while the remaining two were supplemented with 1 μl (1 equivalent) of TbRMs, incubated at 37°C for 1.5 h and then incubated on ice to stop protein translocation. One pair of reactions was used for a flotation assay following the protocol described previously by Zhang et al. [16]. Each reaction mixture was combined with 300 μl of RMB (50 mM Hepes/KOH, 50 mM potassium acetate, 6 mM magnesium acetate, 1 mM DTT, 0.5 μg/ml Tos-Lys-CH_2Cl and 2.5 μg/ml leupeptin) containing 2.3 M sucrose (top layer). This solution was layered on to 200 μl of RMB containing 2.3 M sucrose (bottom layer) in an ultracentrifuge tube. Next, 400 μl of RMB supplemented with 1.5 M sucrose was layered on top of the 2.3 M sucrose top

layer, and a layer of 300 μl of RMB containing 0.25 M sucrose was layered on top of the 1.5 M sucrose layer. The sucrose gradients were centrifuged at 49 000 rev./min for 5 h at 4°C using a Beckman TLA 100.3 rotor. After centrifugation, the 0.25 M, 1.5 M and 2.3 M top sucrose layers were retrieved into new centrifuge tubes. All of the four sucrose layers were supplemented with saturated ammonium sulfate [60%(v/v) final concentration] and incubated on ice for 30 min. The samples were then centrifuged at 16060 ***g*** for 10 min to precipitate the protein pellets, and the supernatants were discarded. The pellets from the 16060 ***g*** centrifugation step were resuspended in 20 μl of 2.5 × SDS/PAGE buffer and resolved by SDS/PAGE. Radioactive polypeptides were visualized using a phosphorimager, and VSG protein bands were quantified using QuantityOne software (Bio-Rad). The percentage of VSG detected was obtained by dividing the amount of VSG in a lane by the sum of VSG in all of the lanes, and multiplying the ratio by 100. Gels were visually adjusted to detect weak phosphorimager bands: this visual optimization does not affect the quantification step mentioned above.

Cell culture

The bloodstream form of *T. brucei* CA427 was cultured in HMI-9 medium [17] to a maximum density of 10^6 cells/ml. For drug susceptibility studies, cells were seeded at 10^4 cells/ml and 100 μl of cells at this density was added to each well of a 96-well plate, with or without test compounds at the stated concentrations (see the relevant Figure legends for details). An equal volume of DMSO, the solvent in which the compounds were dissolved, was added to control cells. Cells were counted after 24 or 48 h using a haemocytometer, and the results were plotted. All experiments were performed in triplicate.

The HeLa cell line was maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 i.u./ml penicillin/streptomycin and 2 mM L-glutamine at 37°C in an atmosphere of 5% CO_2.

HeLa cell viability assays

Propidium iodide exclusion (reviewed in [18]) was used to determine HeLa cell viability after drug treatment. Cells were plated at a density of 10^4 cells/well (96-well plate) with or without the addition of test compounds for the stated time periods (see the respective Figure legends for details). An etoposide stock (2 mM in DMSO) was diluted in RPMI 1640 medium to a concentration of 100 μM immediately before use. Adherent cells were rinsed with PBS, trypsinized for 5 min at 37°C, pelleted by centrifugation at 2000 ***g*** for 2 min, resuspended in PBS (500 μl) containing propidium iodide (1 μg/ml) and DAPI (4′,6-diamidino-2-phenylindole) (5 μg/ml) and analysed by flow cytometry on a Dakocytomation CyAn (Dako) without gating. Single fluorophore controls were acquired prior to the collection of the experimental data. Other cell viability assays used (results not shown) were mitochondrial XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] reduction, total cell density determined with DNA fluorescence measurement and Trypan Blue uptake. Results from these assays were identical to the results obtained with protocols based on propidium iodide exclusion by viable cells.

RESULTS

TbRMs import trypanosome VSGs

Microsomes from canine pancreas are a model system used to study protein import into the ER of eukaryotes. The signal sequences of *T. brucei* are generally incompatible with the protein import machinery of canine microsomes [5,6]. Consequently, it was crucial to develop a trypanosomatid microsome system using ER membranes from *T. brucei* (TbRM) in order to study the ER import of a trypanosome protein. The substrate used for these studies was the VSG coat protein. Standard tests for successful microsomal ER protein import include: (i)

protease protection of the translocated protein and (ii) loss of protease protection after detergent permeabilization of the microsomes [7]. Native VSG is a protein which is retained on membranes by a GPI (glycosylphosphatidylinositol) anchor at its C-terminus. For the present study, all VSG constructs were engineered to produce 'soluble secreted proteins' by the addition of a stop codon upstream of the coding sequence for the GPI signal peptide. Following import into ER microsomes, a soluble secreted protein is protected by the ER membrane from digestion by exogenous proteases.

Full-length VSG_117 was tested as a substrate protein for TbRMs (see Figure 1A for an experimental scheme). In preliminary studies, truncated VSG_117 (an 86 amino-acid-long polypeptide whose mRNA contains a termination codon for release from the ribosome) was translocated in TbRMs (results not shown). However, full-length VSG_117 (500 amino acids long) was not imported into the microsomes (Figure 1B, lanes 1–3). Cytosolic chaperones that enable full-length proteins to maintain an 'import-competent status' are required for post-translational import of proteins into the ER of yeast [19,20]. Therefore we suspected that full-lengthVSG_117 was not imported into TbRMs because trypanosome chaperones were absent from the reaction mixture of reticulocyte lysate and TbRMs. In order to test this hypothesis, we prepared cytosol from *T. brucei* (see the Materials and methods section for details) and evaluated its effect on the translocation of full-length VSG_117 into TbRMs.

Addition of *T. brucei* cytosol to TbRMs led to protection VSG_117 from proteinase K digestion (Figure 1B, lanes 4 and 5). Detergent permeabilization of TbRMs allowed proteinase K to digest VSG_117 in the presence of cytosol (Figure 1A, lane 6), indicating that the protease-protected full-length VSG_117 was imported into TbRMs.We conclude that cytosol from *T. brucei* required for the import of full-length VSG_117 into TbRMs.

To test whether our results with VSG_117 were applicable to other trypanosome proteins, we tested whether TbRMs could import another full-length trypanosome protein, VSG_MVAT7 (500 amino acids). We chose this substrate because the VSG_MVAT7 signal peptide primary structure is different from that of VSG_117 [5]. Nevertheless, in the absence of TbRMs, VSG_MVAT7 was degraded by proteinase K (Figure 1C, lane 2), but the addition of cytosol and TbRMs led to protection VSG_MVAT7 from proteinase K (Figure 1C, lanes 3 and 4). Without cytosol, the protein was not imported in TbRMs (results not shown). When detergent was introduced, proteinase degraded the protected VSG_MVAT7 (Figure 1C, lane 5). Based on these results, we conclude that VSG_MVAT7 is also imported into TbRMs.

N-terminal signal sequences mediate the import of proteins into the ER. Therefore the role of a signal sequence in VSG translocation into TbRM was evaluated using VSG_117_{500} ΔSP, which was obtained by deleting a 28-amino-acid signal peptide [5] from VSG_117. VSG_117_{500} ΔSP was not protected from proteinase K in the presence of both TbRMs and cytosol from *T. brucei* (Figure 1D, compare lanes 3 and 4). We conclude that an N-terminal signal peptide is essential for VSG_117 import into TbRMs.

Proteins imported into TbRMs were not N-glycosylated because the molecular mass of the protease-protected VSGs was identical to the size of the protein synthesized in the absence of TbRMs (Figures 1B and 1C). However, the absence of glycosylation does not mean that the TbRMs failed to import the proteins. Protein import into the ER and subsequent processing can be uncoupled, with OST (oligosaccharyltransferase) and signal peptidase acting after import of the substrate by the Sec61p complex [21,22]. We infer that TbRMs are deficient in OST activity, as has been observed in other *in vitro* ER systems [23]. Nevertheless, our TbRM import assay meets the 'gold standard' for demonstrating the movement of a polypeptide into a membrane vesicle (or organelle), i.e. (i) protease protection of the imported cargo, and (ii) digestion of the previously protected cargo by a protease in the presence of detergent that

permeabilizes the microsomal membrane [7,24]. Finally, we were unable to study co-translational protein import into TbRMs because the membranes inhibit polypeptide synthesis by the rabbit reticulocyte system for reasons that are not clear to us. Therefore our results alone cannot be used as proof that co-translational protein import into the ER does not occur in *T. brucei*.

Proteins imported into the ER must first bind to the membrane of the organelle before translocation across the lipid bilayer occurs. Furthermore, the imported proteins remain associated with intact microsomal vesicles after their import into the ER. We therefore sought evidence for VSG_117 association with TbRMs, an important step in the translocation pathway. In a 'membrane flotation' study without TbRMs, 90%of VSG_117 was detected in the 2.3 M sucrose layer, with only 5% in the 1.5 M sucrose layer where membranes sediment (Figure 2A, lanes 2 and 3). When TbRMs were added to the mixture, 4-fold more VSG (i.e. 20%) was membrane-bound and located in the 1.5 M sucrose cushion (Figure 2B). This result is consistent with our earlier conclusions: VSG_117 binds to TbRMs (Figure 2) and is imported into the microsomes (Figure 1).

MAL3-101 inhibits VSG translocation into TbRMs

As noted above, post-translational protein import into *Saccharomyces cerevisiae* ER and across the *Escherichia coli* inner membrane requires cytosolic chaperones or chaperone-like proteins respectively. Since the import of full-length VSG into *T. brucei* microsomes is post-translational and dependent on cytosol, we considered the possibility that the parasite cytosol contains Hsp70/Hsp40 that facilitate VSG_117 translocation into TbRMs. To explore this concept, we tested whether VSG import into TbRMs could be prevented by MAL3-101, a small molecule inhibitor of Hsp40-stimulated Hsp70 ATPase activity that blocks post-translational import of ppαMF(pre-pro-α-factor) into microsomes from *S. cerevisiae* [14]. The cytosol was pre-incubated with MAL3-101 (or an equal volume of DMSO) while VSG_117 mRNA was translated in a reticulocyte lysate. TbRMs were then added to reticulocyte lysate that had been supplemented with cytosol (MAL3-101 or DMSO-treated) containing VSG, and the mixture was incubated at 37°C to promote the import of VSG into the microsomes. Each mixture was treated with proteinase K (see Figure 3A for a flow chart of procedures) in order to detect the imported VSG.

TbRMs imported approx. 80% of VSG_117, as measured by protection from proteinase K digestion (Figure 3B, compare lanes 1 and 2) when only DMSO was added to the reaction mixture. In contrast, MAL3-101 inhibited the translocation of VSG_117 into TbRMs, as only 13% of the protein was protected from proteinase K when MAL3-101 was present (Figure 3B, lanes 3 and 4). MAL3-51 (Figure 4), which is structurally similar to MAL3-101 but has no effect on yeast translocation [14], had significantly lower impact on the import of VSG into TbRMs even when used at a 3-fold higher concentration (Figure 3B, lanes 5 and 6). We conclude that the import of VSG_117 into TbRM inhibited specifically by MAL3-101.

CJ-21,058 and equisetin inhibit protein translocation into TbRMs

Post-translational protein translocation in *E. coli* requires SecA [25,26] whose ATPase activity is inhibited by the small molecule CJ-21,058 (Figure 4C) [12]. In *T. brucei*, cytosolic chaperones involved in ER protein import have not been completely characterized. To test the possible involvement of a SecA-like domain in polypeptide import into TbRMs, we used CJ-21,058 to pharmacologically challenge TbRM protein import.

CJ-21,058 inhibited import of VSG_117 into TbRMs by 70% compared to a DMSO control (Figure 3B, compare lanes 1 and 2 to lanes 9 and 10). Similarly, equisetin [13], an analogue of CJ-21,058 (Figure 4D) inhibited VSG_117 translocation into TbRMs by 95% (Figure 3B,

compare lanes 7 and 8). Thus a protein with a SecA-like ATPase domain may contribute to the import of VSG_117 into TbRMs. Nevertheless, the target of equisetin (and CJ-21,058) in the trypanosome system is not likely to be a protein with extensive sequence similarity to the SecA protein, for two reasons. First, a *SECA* gene is not encoded in the *T. brucei* genome. Secondly, sodium azide, an inhibitor of SecA [26], does not inhibit the translocation of VSG_117 into TbRMs (results not shown). Interestingly, SecA contains a DEAD-like domain that is also found in some proteins in the *T. brucei* genome (http://www.genedb.org/genedb/tryp/). Therefore it is possible that equisetin inhibits the activity of a protein with this domain that is required for VSG import into the ER of *T. brucei*.

Inhibitors of VSG translocation into TbRMs are trypanocidal

Many essential plasma membrane proteins in *T. brucei* enter the secretory pathway at the ER. Therefore the inhibition of ER protein import is likely to deplete multiple proteins (e.g. receptors and nutrient transporters) from the plasma membrane, and as a result compromise the viability of the parasite. To test this concept, MAL3-101, CJ-21,058 and equisetin were examined for their ability to kill *T. brucei*.

As predicted, MAL3-101 killed *T. brucei* in a dose-dependent fashion (Figure 5A). Half of the parasites were killed by 1.5 μM MAL3-101 (IC_{50}), and 100% of parasites were killed in the presence of 3 μM of the inhibitor (results not shown). The related less active compound MAL3-51 had a minimal effect on VSG_117 translocation into TbRMs (Figure 3B, lanes 5 and 6) and was not trypanocidal at 50 μM (Figures 5A and 5B). Furthermore, both equisetin and CJ-21,058 killed *T. brucei*. The IC_{50} values for equisetin and CJ-21,058 were 3.3 μM (Figure 5D) and 7 μM (Figure 5C) respectively. We conclude that the inhibition of protein import into TbRMs correlates with the trypanocidal activity of the compounds. Thus VSG_117 import into TbRMs may be used to screen for compounds that can kill bloodstream *T. brucei*.

It is desirable that an anti-trypanosome ‘lead compound’ kill the parasite but have little or no effect on human cells. Therefore the new trypanocidal compounds were tested on human cells using concentrations of the inhibitors that killed 100% of *T. brucei*. In a cell-viability assay (Figure 6), no statistically significant differences were detected between drug-treated and control human HeLa cells (5% cell death) when MAL3-101 (10 μM), CJ-21,058 (12 μM) or equisetin (20 μM) were added to the cells. Recent work confirms that MAL3-101 is not generally toxic against mammalian cells [27]. In contrast, treatment with etoposide, an inhibitor of topoisomerase II, produced significant (40%) death of HeLa cells (Figure 6).

DISCUSSION

Cell-free protein import into trypanosome ER microsomes

We developed a cell-free system using TbRMs into which trypanosome VSG_117 could be imported post-translationally (Figures 1B and 1C) in a signal-sequence-dependent manner (Figure 1D). Cytosol from *T. brucei* is needed for the import of full-length VSGs into TbRMs (Figures 1B and 1C), reminiscent of the requirement for cytosol in the post-translational import of proteins into ER-derived microsomes from the fungi *S. cerevisiae* and *Candida maltosa* [23,28–33].

Factors involved in, or predicted to participate in, the import of proteins into the ER of *S. cerevisiae*, humans and *T. brucei* are summarized in Table 1. Some of the corresponding genes, for example *SEC62* and *SEC72*, which are found in *S. cerevisiae*, appear to be absent from the genomes of *T. brucei* and archaea. The human genome lacks *SEC71* and *SEC72*, whereas

archaea lack *HSC70*, a chaperone that is important for post-translational protein translocation in yeast [3]. However, absence of a protein homologue may not preclude activity of a 'functional analogue' [34,35] that may provide the biochemical function. In fact, for *T. brucei* there is precedent for this phenomenon; both the transferrin receptor and the nuclease Dicer lack sequence homology with their mammalian equivalents, although the biochemical functions of the proteins are retained [36,37]. In archaea, proteins can be secreted post-translationally [38,39], although the cells appear to lack cytoplasmic Hsp70 as well as Sec71p/Sec72p. Functional analogues of Hsp70 (or SecB) probably exist in archaea. Alternatively, it is possible that the protein secretion pathways evolved independently of these factors.

In *T. brucei,* three lines of evidence support the existence of post-translational protein translocation into the ER. First, SRP is dispensable for protein import into the ER [4], suggesting the existence of an alternative (i.e. chaperone-dependent) route for protein entry into the organelle. Secondly, the *T. brucei* genome lacks a gene for SRβ (*SRP102*) (Table 1), one of the two subunits of the SRP receptor that is required for co-translational protein import into the ER [40–42]. The caveat to this view is that a functional analogue of SRβ exists in *T. brucei*. Thirdly, we demonstrate post-translational import of VSG_117 into TbRM *in vitro*. Gene knockdown experiments *in vivo* [4,43] and *in vitro* microsomal studies of protein import into the ER of *T. brucei* (the present study) complement each other. Proposals for the existence of post-translational ER protein import *in vivo* are enhanced by supporting data from *in vitro* experiments (and vice versa), as SRP gene knockdowns do not prove the existence of a post-translational system (also see next paragraph). Furthermore, *in vivo* biochemical demonstration of post-translational ER protein import is not feasible for technical reasons. Cycloheximide, which is used to stop co-translational protein import in order for post-translational translocation to be demonstrated, also blocks synthesis of new proteins that need to be studied in the post-translational pathway [43a]. Thus only an *in vitro* system can provide unequivocal proof that ER microsomes from any biological system are capable of importing proteins post-translationally.

The presence of genes encoding SRP subunits does not imply that the ribonucleoprotein complex is involved in co-translational import into the ER of the trypanosome, since SRP has multiple functions in a cell. SRP can direct proteins that lack an N-terminal ER signal sequence to the ER post-translationally [44]. Therefore one cannot yet rule out a role for SRP in post-translational protein import into the ER of *T. brucei*.

The secretory pathway as a target for anti-parasite drug discovery

Cell-surface proteins, for example VSG, transferrin receptors, nucleobase transporters and GP63, are important for establishing trypanosomatid infections in humans. Movement of these proteins to the plasma membrane is dependent on entry into the parasite ER, following paradigms worked out in model eukaryotes (reviewed in [2]). The ER is a 'gateway' for the transport of proteins to the Golgi, endosomes, lysosomes and plasma membrane. Small molecules that interfere with the import of proteins into the ERof a trypanosomemay have value as anti-parasite agents. Moreover, mammals primarily translocate proteins into the ER by the co-translational route, whereas in *T. brucei* the most abundant cell-surface proteins use a post-translational route for entry into the ER [43]. Hence compounds that interfere with post-translational ER protein import in *T. brucei* may compromise the viability of the parasite without a significant effect on mammalian cells. Results supporting this premise are provided in Figure 6.

Molecular chaperones and chaperone-like proteins, such as cytoplasmic Hsp70/Hsp40, ER luminal Kar2p/BiP (in eukaryotes), SecB and SecA (in bacteria) are important for posttranslational protein import (reviewed in [45]). We predicted that small molecule modulators of Hsp70/Hsp40 might reduce post-translational import of VSG_117 into TbRMs.

MAL3-101 inhibits the co-chaperone-stimulated ATPase of Hsp70 and inhibits the translocation of ppαMF into yeast microsomes [14]. When added to the TbRM protein import system, MAL3-101 (Figure 4A) inhibited the translocation of VSG_117 into parasite microsomes (Figure 3B, lanes 3 and 4). Furthermore, MAL3-101 killed *T. brucei* (Figure 5A). The efficacy of MAL3-101 against *T. brucei* (IC_{50} of 1.5 μM) is comparable to that of suramin, a drug that is used to treat HAT (IC_{50} for suramin is 1.4–2.3 μM) [46]. These results indicate that MAL3-101 is a chemical entity that is worthy of further optimization for anti-trypanosome drug discovery. The synthesis of MAL3-101 is concise and readily amenable to analogue preparation [15a,48].

Equisetin (Figure 4D) and CJ-21,058 (Figure 4C), both fungal products [12,13], inhibited the import of VSG_117 into TbRMs (Figure 3), and these compounds were also trypanocidal (Figure 5). Fortunately, a total synthesis of equisetin has been described previously [49], an achievement that could guide future efforts, in the light of these anti-trypanosomal effects, to synthesize bioactive analogues for structure–activity studies on *T. brucei*. Finally, it will be interesting to test whether other equisetin-related compounds (e.g. altersetin, hexahydroaltersetin, dihydroaltersetin, tetrahydroequisetin and phomasetin) [50–52] are also trypanocidal.

The observations in the present study are consistent with a recent report from Michaeli and colleagues that the SRP-independent pathway for ER protein translocation is essential for viability of *T. brucei* [43]. Thus drugs that compromise the efficiency of the pathway should have anti-trypanosome activity. We have provided chemical validation of this concept. For genetic tests of the principle, future efforts will seek to knock down the SRP-independent post-translational pathway in *T. brucei* by targeting the cytosolic Hsp70 chaperone system.

Abbreviations used

BiP, immunoglobulin heavy-chain-binding protein
DTT, dithiothreitol
ER, endoplasmic reticulum
GPI, glycosyl-phosphatidylinositol
HAT, human African trypanosomiasis
Hsp, heat-shock protein
MBU, molecular biology unit
NP-40, Nonidet P40
OST, oligosaccharyl-transferase
ppαMF, pre-pro-α-factor
RMB, rough microsome buffer
SRP, signal recognition particle
TbRM, *Trypanosoma brucei* microsome
Tos-Lys-CH_2Cl, tosyl-lysylchloromethane
VSG, variant surface glycoprotein

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REFERENCES

1. Renslo AR, McKerrow JH. Drug discovery and development for neglected parasitic diseases. Nat. Chem. Biol 2006;2:701–710. [PubMed: 17108988]
2. Wickner W, Schekman R. Protein translocation across biological membranes. Science 2005;310:1452–1456. [PubMed: 16322447]
3. Albers SV, Szabo Z, Driessen AJ. Protein secretion in the Archaea: multiple paths towards a unique cell surface. Nat. Rev. Microbiol 2006;4:537–547. [PubMed: 16755286]
4. Liu L, Liang XH, Uliel S, Unger R, Ullu E, Michaeli S. RNA interference of signal peptide-binding protein SRP54 elicits deleterious effects and protein sorting defects in trypanosomes. J. Biol. Chem 2002;277:47348–47357. [PubMed: 12244113]
5. Al-Qahtani A, Teilhet M, Mensa-Wilmot K. Species-specificity in endoplasmic reticulum signal peptide utilization revealed by proteins from *Trypanosoma brucei* and *Leishmania*. Biochem. J 1998;331:521–529. [PubMed: 9531493]
6. Ramirez MI, Boscardin SB, Han SW, Paranhos-Baccala G, Yoshida N, Kelly JM, Mortara RA, Da Silveira JF. Heterologous expression of a *Trypanosoma cruzi* surface glycoprotein (gp82) in mammalian cells indicates the existence of different signal sequence requirements and processing. J. Eukaryot. Microbiol 1999;46:557–565. [PubMed: 10568029]
7. Connolly T, Collins P, Gilmore R. Access of proteinase K to partially translocated nascent polypeptides in intact and detergent-solubilized membranes. J. Cell Biol 1989;108:299–307. [PubMed: 2537313]
8. Hegde RS, Bernstein HD. The surprising complexity of signal sequences. Trends Biochem. Sci 2006;31:563–571. [PubMed: 16919958]
9. Harant H, Lettner N, Hofer L, Oberhauser B, de Vries JE, Lindley IJ. The translocation inhibitor CAM741 interferes with vascular cell adhesion molecule 1 signal peptide insertion at the translocon. J. Biol. Chem 2006;281:30492–30502. [PubMed: 16914554]
10. Besemer J, Harant H, Wang S, Oberhauser B, Marquardt K, Foster CA, Schreiner EP, de Vries JE, Dascher-Nadel C, Lindley IJ. Selective inhibition of cotranslational translocation of vascular cell adhesion molecule 1. Nature 2005;436:290–293. [PubMed: 16015337]
11. Garrison JL, Kunkel EJ, Hegde RS, Taunton J. A substrate-specific inhibitor of protein translocation into the endoplasmic reticulum. Nature 2005;436:285–289. [PubMed: 16015336]
12. Sugie Y, Inagaki S, Kato Y, Nishida H, Pang CH, Saito T, Sakemi S, Dib-Hajj F, Mueller JP, Sutcliffe J, Kojima Y. CJ-21,058, a new SecA inhibitor isolated from a fungus. J. Antibiot 2002;55:25–29. [PubMed: 11918061]
13. Vesonder RF, Tjarks LW, Rohwedder WK, Burmeister HR, Laugal JA. Equisetin, an antibiotic from *Fusarium equiseti* NRRL 5537, identified as a derivative of N-methyl-2,4-pyrollidone. J. Antibiot 1979;32:759–761. [PubMed: 541270]
14. Fewell SW, Smith CM, Lyon MA, Dumitrescu TP, Wipf P, Day BW, Brodsky JL. Small molecule modulators of endogenous and co-chaperone-stimulated Hsp70 ATPase activity. J. Biol. Chem 2004;279:51131–51140. [PubMed: 15448148]
15. Al-Qahtani A, Mensa-Wilmot K. A 5′ untranslated region which directs accurate and robust translation by prokaryotic and mammalian ribosomes. Nucleic Acids Res 1996;24:1173–1174. [PubMed: 8604355]
16. Zhang Y, Nijbroek G, Sullivan ML, McCracken AA, Watkins SC, Michaelis S, Brodsky JL. Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. Mol. Biol. Cell 2001;12:1303–1314. [PubMed: 11359923]
17. Hirumi H, Hirumi K. Axenic culture of African trypanosome bloodstream forms. Parasitol 1994;10:80–84.Today
18. Steff AM, Fortin M, Arguin C, Hugo P. Detection of a decrease in green fluorescent protein fluorescence for the monitoring of cell death: an assay amenable to high-throughput screening technologies. Cytometry 2001;45:237–243. [PubMed: 11746092]
19. McClellan AJ, Brodsky JL. Mutation of the ATP-binding pocket of SSA1 indicates that a functional interaction between ssa1p and ydj1p is required for posttranslational translocation into the yeast endoplasmic reticulum. Genetics 2000;156:501–512. [PubMed: 11014801]

20. Caplan AJ, Cyr DM, Douglas MG. YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. Cell 1992;71:1143–1155. [PubMed: 1473150]
21. Nothwehr SF, Folz RJ, Gordon JI. Uncoupling of co-translational translocation from signal peptidase processing in a mutant rat preapolipoprotein-A-IV with a deletion that includes the COOH-terminal region of its signal peptide. J. Biol. Chem 1989;264:4642–4647. [PubMed: 2647742]
22. Ruiz-Canada C, Kelleher DJ, Gilmore R. Cotranslational and posttranslational N-glycosylation of polypeptides by distinct mammalian OST isoforms. Cell 2009;136:272–283. [PubMed: 19167329]
23. Brodsky JL, Hamamoto S, Feldheim D, Schekman R. Reconstitution of protein translocation from solubilized yeast membranes reveals topologically distinct roles for BiP and cytosolic Hsc70. J. Cell Biol 1993;120:95–102. [PubMed: 8416998]
24. Walter P, Blobel G. Preparation of microsomal membranes for cotranslational protein translocation. Methods Enzymol 1983;96:84–93. [PubMed: 6656655]
25. Watanabe M, Blobel G. SecA protein is required for translocation of a model precursor protein into inverted vesicles of *Escherichia coli* plasma membrane. Proc. Natl. Acad. Sci. U.S.A 1993;90:9011–9015. [PubMed: 8415645]
26. Knott TG, Robinson C. The secA inhibitor, azide, reversibly blocks the translocation of a subset of proteins across the chloroplast thylakoid membrane. J. Biol. Chem 1994;269:7843–7846. [PubMed: 8132499]
27. Rodina A, Vilenchik M, Moulick K, Aguirre J, Kim J, Chiang A, Litz J, Clement CC, Kang Y, She Y, et al. Selective compounds define Hsp90 as a major inhibitor of apoptosis in small-cell lung cancer. Nat. Chem. Biol 2007;3:498–507. [PubMed: 17603540]
28. Hansen W, Garcia PD, Walter P. *In vitro* protein translocation across the yeast endoplasmic reticulum: ATP-dependent posttranslational translocation of the prepro-alpha-factor. Cell 1986;45:397–406. [PubMed: 3009026]
29. Brodsky JL, Goeckeler J, Schekman R. BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum. Proc. Natl. Acad. Sci. U.S.A 1995;92:9643–9646. [PubMed: 7568189]
30. Wiedmann M, Wiedmann B, Voigt S, Wachter E, Muller HG, Rapoport TA. Post-translational transport of proteins into microsomal membranes of *Candida maltosa*. EMBO J 1988;7:1763–1768. [PubMed: 3169003]
31. Chirico WJ, Waters MG, Blobel G. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature 1988;332:805–810. [PubMed: 3282179]
32. Ngosuwan J, Wang NM, Fung KL, Chirico WJ. Roles of cytosolic Hsp70 and Hsp40 molecular chaperones in post-translational translocation of presecretory proteins into the endoplasmic reticulum. J. Biol. Chem 2003;278:7034–7042. [PubMed: 12493732]
33. Waters MG, Chirico WJ, Blobel G. Protein translocation across the yeast microsomal membrane is stimulated by a soluble factor. J. Cell Biol 1986;103:2629–2636. [PubMed: 3025220]
34. Zaltash S, Palmblad M, Curstedt T, Johansson J, Persson B. Pulmonary surfactant protein B: a structural model and a functional analogue. Biochim. Biophys. Acta 2000;1466:179–186. [PubMed: 10825441]
35. Miller RV, Kokjohn TA. Cloning and characterization of the c1 repressor of Pseudomonas aeruginosa bacteriophage D3: a functional analog of phage lambda cI protein. J. Bacteriol 1987;169:1847–1852. [PubMed: 3106321]
36. Best A, Handoko L, Schluter E, Goringer HU. *In vitro* synthesized small interfering RNAs elicit RNA interference in african trypanosomes: an *in vitro* and *in vivo* analysis. J. Biol. Chem 2005;280:20573–20579. [PubMed: 15781462]
37. Steverding D. The transferrin receptor of *Trypanosoma brucei*. Parasitol. Int 2000;48:191–198. [PubMed: 11227758]
38. Irihimovitch V, Eichler J. Post-translational secretion of fusion proteins in the halophilic archaea *Haloferax volcanii*. J. Biol. Chem 2003;278:12881–12887. [PubMed: 12566448]
39. Osborne AR, Rapoport TA, van den Berg B. Protein translocation by the Sec61/SecY channel. Annu. Rev. Cell. Dev. Biol 2005;21:529–550. [PubMed: 16212506]

40. Ogg SC, Barz WP, Walter P. A functional GTPase domain, but not its transmembrane domain, is required for function of the SRP receptor beta-subunit. J. Cell Biol 1998;142:341–354. [PubMed: 9679135]

41. Fulga TA, Sinning I, Dobberstein B, Pool MR. SRbeta coordinates signal sequence release from SRP with ribosome binding to the translocon. EMBO J 2001;20:2338–2347. [PubMed: 11331598]

42. Tajima S, Lauffer L, Rath VL, Walter P. The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. J. Cell Biol 1986;103:1167–1178. [PubMed: 3021779]

43. Goldshmidt H, Sheiner L, Butikofer P, Roditi I, Uliel S, Gunzel M, Engstler M, Michaeli S. Role of protein translocation pathways across the ER in *Trypanosoma brucei*. J. Biol. Chem 2008;283:32085–32098. [PubMed: 18768469]

43a. Duszenko M, Kang X, Böhme U, Hömke R, Lehner M. *In vitro* translation in a cell-free system from *Trypanosoma brucei* yields glycosylated and glycosylphosphatidylinositol-anchored proteins. Eur. J. Biochem 1999;266:789–797. [PubMed: 10583372]

44. Abell BM, Pool MR, Schlenker O, Sinning I, High S. Signal recognition particle mediates post-translational targeting in eukaryotes. EMBO J 2004;23:2755–2764. [PubMed: 15229647]

45. Mitra K, Frank J, Driessen A. Co- and post-translational translocation through the protein-conducting channel: analogous mechanisms at work? Nat. Struct. Mol. Biol 2006;13:957–964. [PubMed: 17082791]

46. del Rayo Camacho M, Phillipson JD, Croft SL, Rock P, Marshall SJ, Schiff PL Jr. *In vitro* activity of *Triclisia patens* and some bisbenzylisoquinoline alkaloids against *Leishmania donovani* and *Trypanosoma brucei brucei*. Phytother. Res 2002;16:432–436. [PubMed: 12203262]

47. Wright CM, Chovatiya RJ, Jameson NE, Turner DM, Zhu G, Werner S, Huryn DM, Pipas JM, Day BW, Wipf P, Brodsky JL. Pyrimidinone-peptoid hybrid molecules with distinct effects on molecular chaperone function and cell proliferation. Bioorg. Med. Chem 2008;16:3291–3301. [PubMed: 18164205]

48. Werner S, Turner DM, Lyon MA, Huryn DM, Wipf P. A focused library of tetrahydropyrimidinone amides via a tandem Biginelli-Ugi multicomponent process. Synlett 2006:2334–2338.

49. Burke LT, Dixon DJ, Ley SV, Rodriguez F. A short stereoselective total synthesis of the fusarium toxin equisetin. Org. Lett 2000;2:3611–3613. [PubMed: 11073657]

50. Hellwig V, Grothe T, Mayer-Bartschmid A, Endermann R, Geschke FU, Henkel T, Stadler M. Altersetin, a new antibiotic from cultures of endophytic *Alternaria* spp. Taxonomy, fermentation, isolation, structure elucidation and biological activities. J. Antibiot 2002;55:881–892. [PubMed: 12523821]

51. De Clercq E. Current lead natural products for the chemotherapy of human immunodeficiency virus (HIV) infection. Med. Res. Rev 2000;20:323–349. [PubMed: 10934347]

52. Tziveleka LA, Vagias C, Roussis V. Natural products with anti-HIV activity from marine organisms. Curr. Top. Med. Chem 2003;3:1512–1535. [PubMed: 14529524]

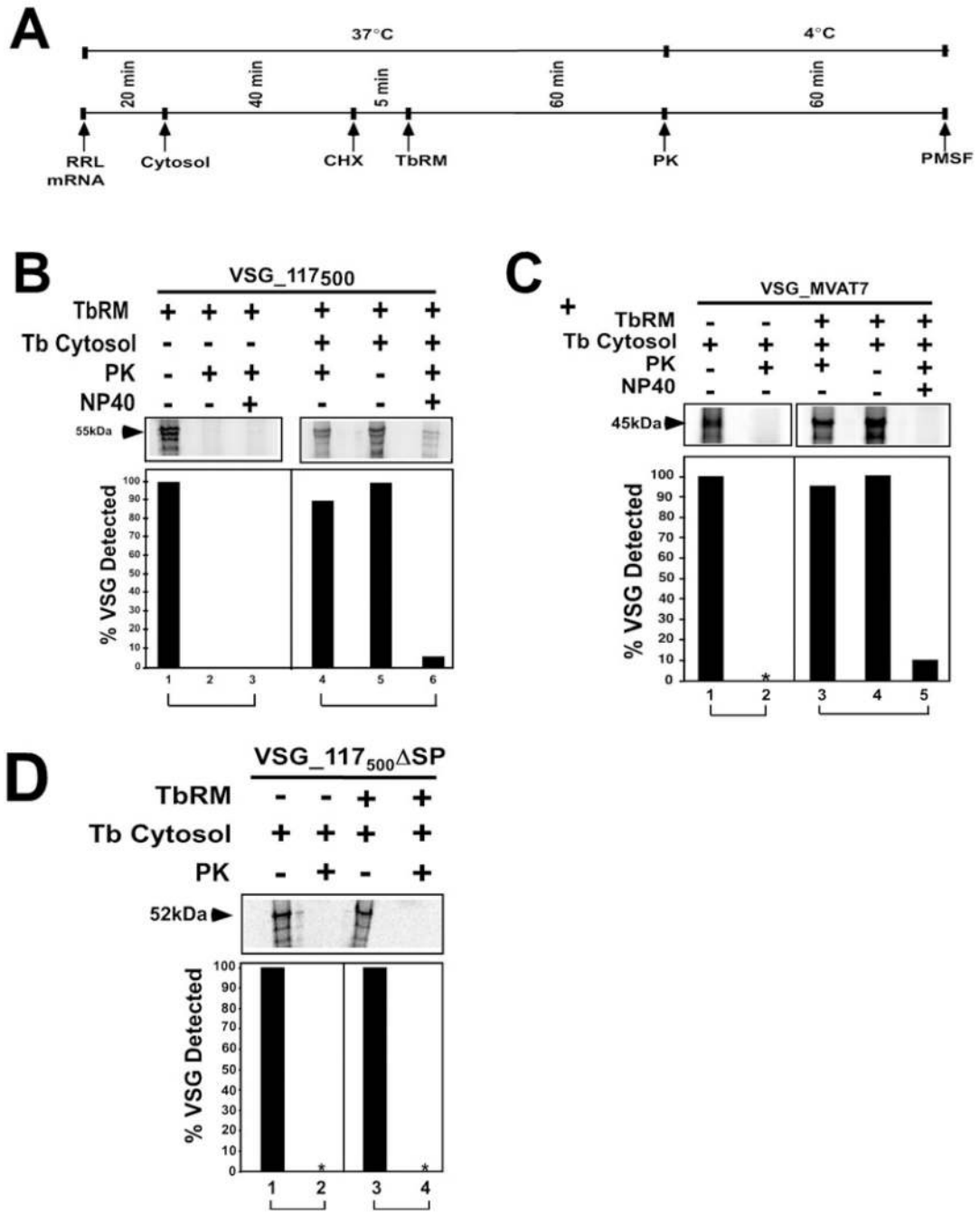

Figure 1. Cell-free protein import into *T. brucei* ER membranes

(**A**) Protocol for post-translational import of VSG. Depicted are the various steps, duration and temperatures at which the reactions took place. CHX, cycloheximide; PK, proteinase K; RRL, rabbit reticulocyte lysate. (**B**) Import of VSG_117 into TbRMs. VSG_117 mRNA was translated in rabbit reticulocyte lysate for 15 min and then cycloheximide (50 μg/ml final concentration) was added. Cytosol from *T. brucei* (Tb) was also added to the reactions analysed in lanes 4, 5 and 6. Reaction mixtures were incubated with TbRMs (one equivalent) for 45 min at 37°C, followed by proteinase K digestion (300 μg/ml final concentration) on ice for 60 min. Proteins were resolved by SDS/PAGE and detected by phosphorimaging. Lane 1, VSG_117 translated in reticulocyte lysate; lane 2, VSG_117 digested with proteinase K; lane 3, VSG_117

digested with proteinase K in the presence of NP-40 (NP40) (2 %); lane 4, VSG_117 incubated with TbRMs; lane 5, VSG_117 incubated with TbRMs and digested with proteinase K (PK); lane 6, VSG_117 was translocated into TbRMs and then permeabilized with 2% NP-40 during proteinase K digestion. (**C**) Translocation of VSG_MVAT7 into TbRMs. Lane 1, VSG_MVAT7 translated in reticulocyte lysate; lane 2, VSG_MVAT7 produced in reticulocyte lysate and digested with proteinase K; lane 3, VSG_MVAT7 incubated with TbRMs; lane 4, VSG_MVAT7 incubated with TbRMs and then challenged with proteinase K; lane 5, VSG_MVAT7 incubated with TbRMs, permeabilized with NP-40 (2%) and digested with proteinase K. (**D**) A signal peptide is required for VSG import into TbRMs: VSG_117_{500} ΔSP translocation. VSG_117_{500} ΔSP mRNA was translated in a rabbit reticulocyte lysate with *T. brucei* cytosol (1.5 equivalents) for 60 min and treated with cycloheximide (50 μg/ml final concentration). VSG_117_{500} ΔSP was incubated with TbRMs (one equivalent) for 60 min at 37°C and digested with proteinase K (20 μg/ml final concentration) for 60 min on ice. Proteins were separated by SDS/PAGE and radiolabelled polypeptides were detected by phosphorimaging. Lane 1, untreated VSG_117_{500} ΔSP; lane 2, VSG_117_{500} ΔSP treated with proteinase K; lane 3, VSG_117_{500} ΔSP incubated with TbRM; lane 4, VSG_117_{500} ΔSP incubated with TbRM and treated with proteinase K. The rectangular brackets underneath the sets of bars denote those data points that were directly compared for quantification, and the asterisks denote instances where no signal was detected. Results are representative of four experiments that produced similar results.

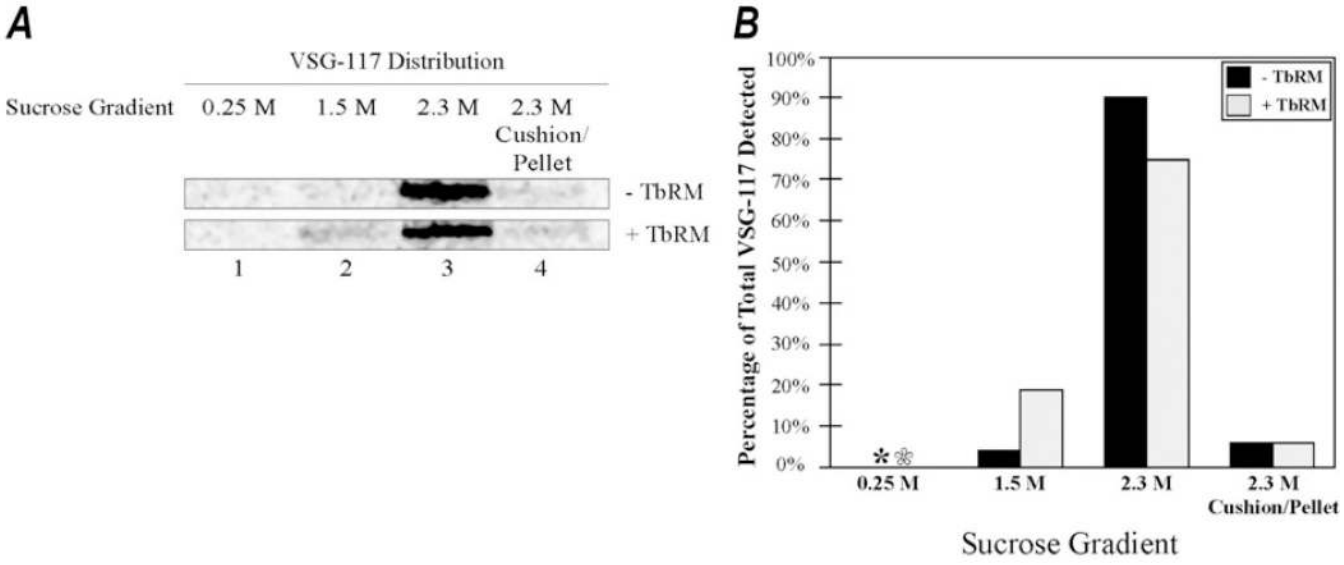

Figure 2. Sucrose flotation of [^{35}S]methionine-labelled VSG_117 after incubation with TbRMs

(**A**) Floatation of VSG_117 in 2.3 M sucrose in the absence (−TbRM, top panel) or presence (+TbRM, bottom panel) of TbRMs. [^{35}S]Methionine-labelled VSG_117 was translated in a rabbit reticulocyte lysate and incubated with (+) or without (−) TbRMs. Reaction mixtures were loaded on to a 2.3 M sucrose cushion with layers of 1.5 M and 0.25 M sucrose above (see the Materials and methods section for details). After centrifugation, each sucrose layer was retrieved, proteins were precipitated, separated by SDS/PAGE, detected with a phosphorimager and quantified. Lane 1, proteins from the 0.25 M sucrose layer; lane 2, proteins obtained from 1.5 M sucrose layer; lane 3, proteins obtained from the 2.3 M sucrose layer; and lane 4, proteins from the 2.3 M sucrose cushion and those that pelleted through the cushion. (**B**) Quantification of the results in (**A**). Asterisks (*) represent 0% VSG_117 detected. The images of the bands from lanes 1 and 2 and 3 and 4 were adjusted separately to detect relatively weak bands. Raw data (i.e. not visually adjusted bands) were used for quantification using QuantityOne software. Results are representative of four experiments that produced similar results.

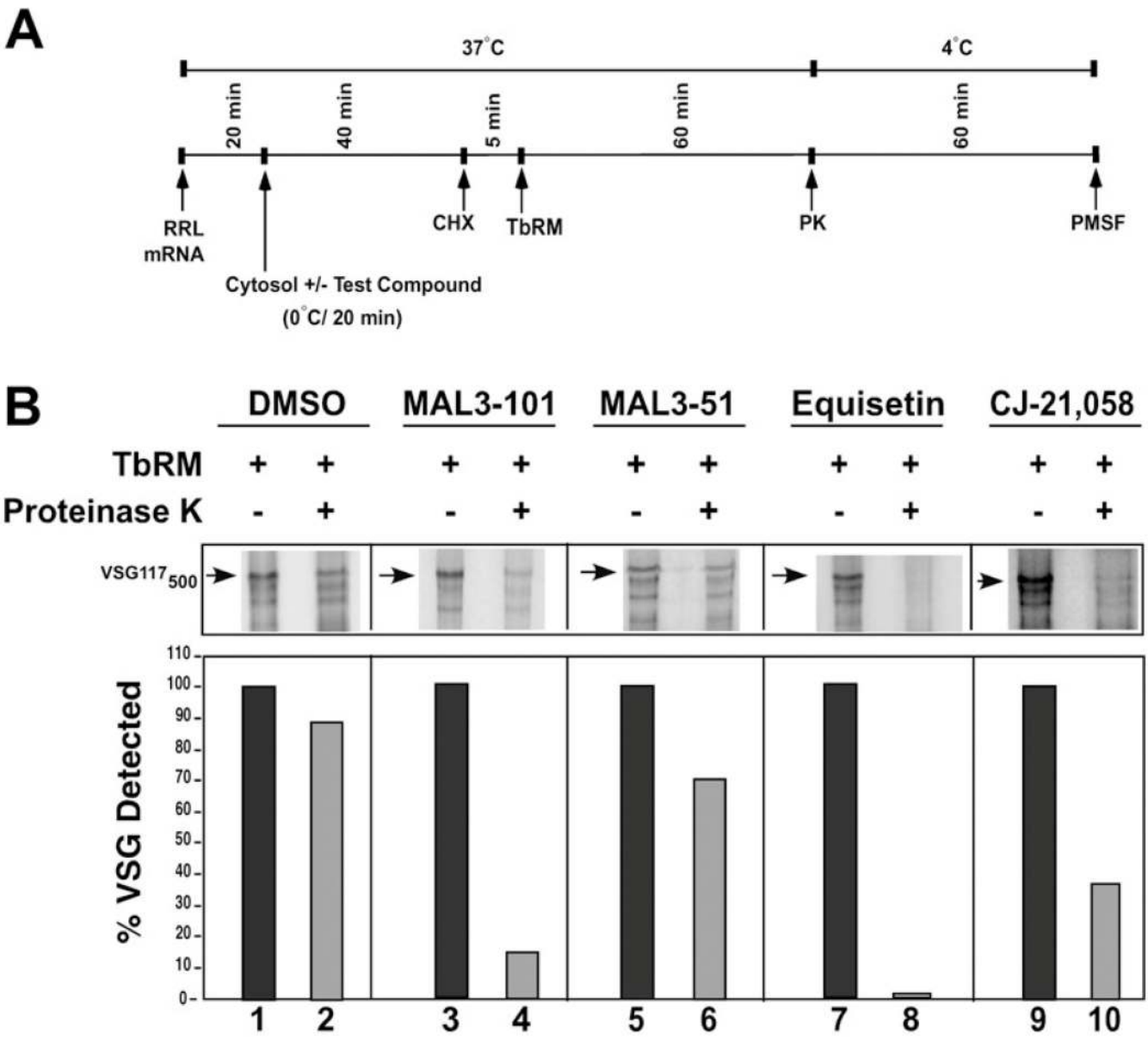

Figure 3. MAL3-101, equisetin and CJ-21,058 inhibit VSG translocation into TbRM

(**A**) Schematic diagram of the protocol used for import of full-length VSG_117. CHX, cycloheximide; PK, proteinase K; RRL, rabbit reticulocyte lysate. (**B**) VSG_117 mRNA was translated in rabbit reticulocyte lysate with 1.5 equivalents of *T. brucei* cytosol pre-treated with MAL3-101 (3 μM), MAL3-51 (10 μM), CJ-21,058 (20 μM) or equisetin (50 μM) for 60 min. The reaction mixtures were treated with cycloheximide (50 μg/ml final concentration) and were incubated with TbRMs (one equivalent). After incubation at 37°C for 60 min, reaction mixtures were transferred to an ice-water bath and treated with proteinase K (30 μg/ml final concentration) for 60 min. Proteins were resolved by SDS/PAGE and detected by phosphorimaging. Lane 1, VSG_117 incubated with TbRMs; lane 2, VSG_117 digested with proteinase K after incubation with TbRMs; lane 3, VSG_117 incubated with MAL3-101 in the presence of TbRMs; lane 4, VSG_117 and TbRMs incubated with MAL3-101 and then digested with proteinase K; lane 5, VSG_117 incubated with MAL3-51 and TbRMs; lane 6, VSG_117 incubated with TbRMs in the presence of MAL3-51 and digested with proteinase

K; lane 7, VSG_117 incubated with TbRMs; lane 8, VSG_117 incubated with equisetin and digested with proteinase K; lane 9, VSG_117 incubated with CJ-21,058 and TbRMs; lane 10, VSG_117 incubated with TbRMs and CJ-21,058 and digested with proteinase K. For quantification, only the upper band corresponding to the full-length VSG_117 (indicated by an arrow) was taken into consideration. Furthermore, each proteinase-digested sample was compared to a control experiment containing the drug but without proteinase addition. Thus lane 2 is compared to lane 1, while lane 4 is compared to lane 3 etc. Results are representative of four experiments that produced similar results.

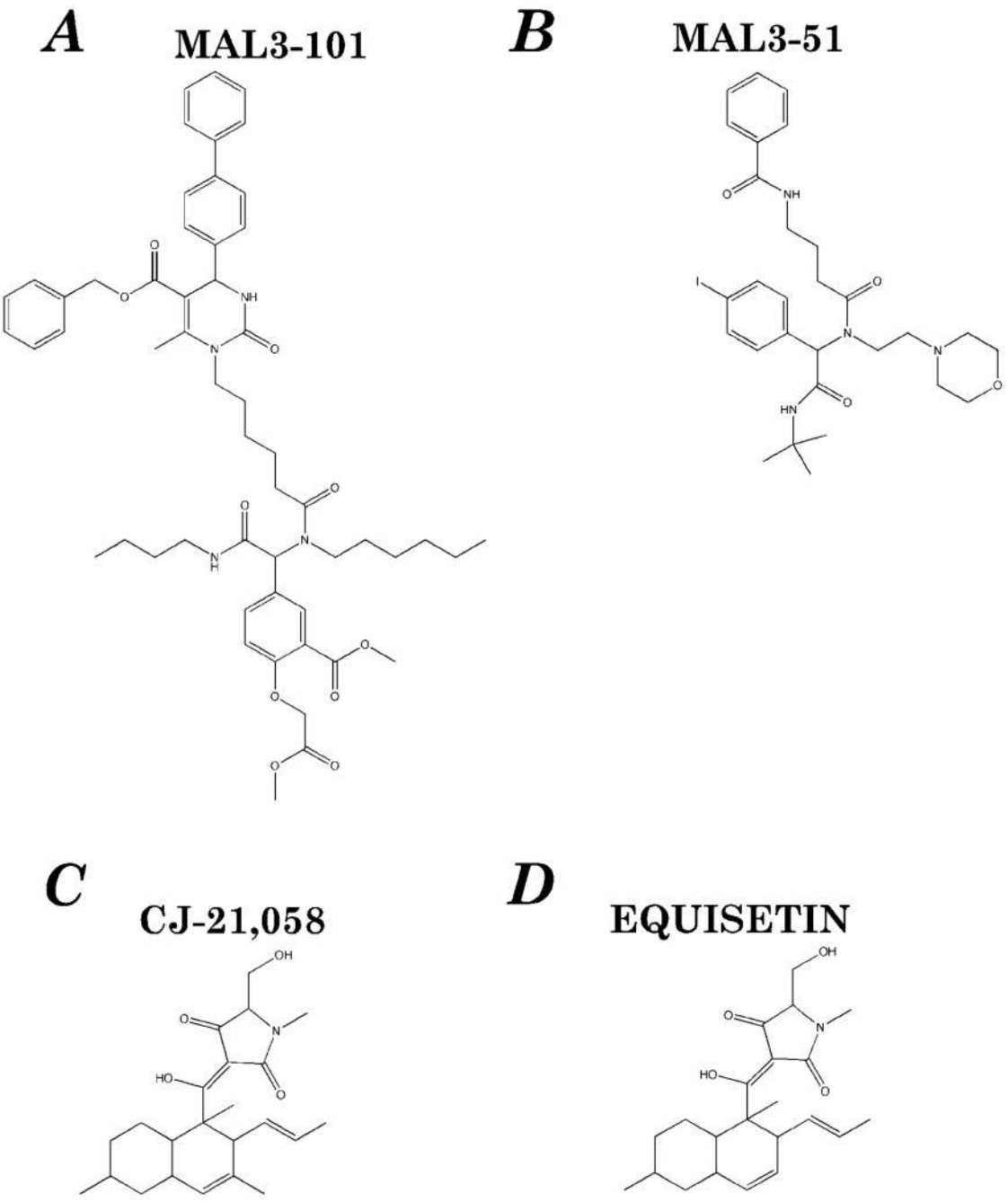

Figure 4. Structures of MAL3-101, MAL3-51, CJ-21,058 and equisetin

The structures of these compounds have been published previously [12–14].

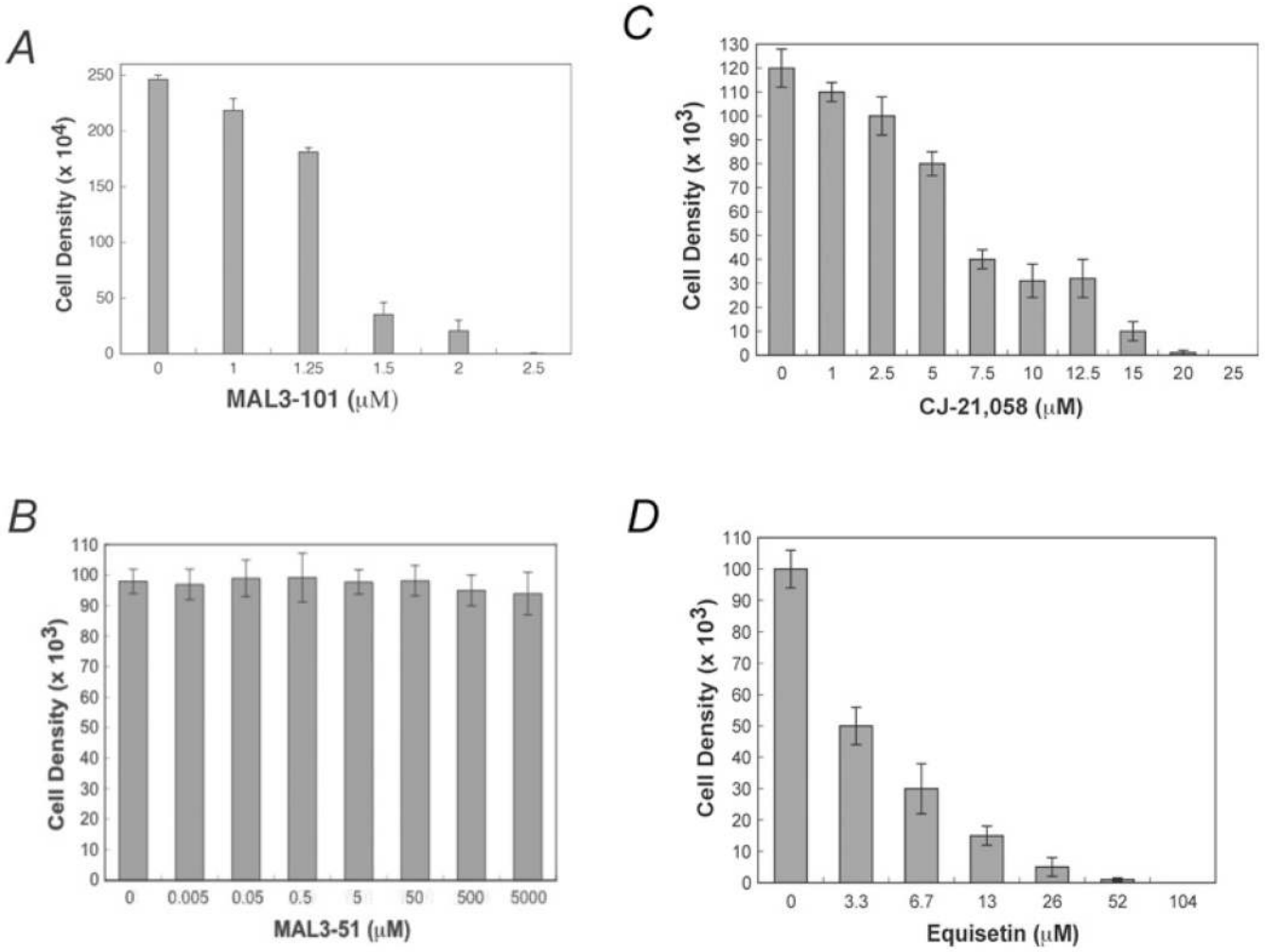

Figure 5. Trypanocidal effect of translocation inhibitors

Cultured bloodstream *T. brucei* CA427 was seeded in medium at a density of 10^4 cells/ml in 96-well plates. The indicated concentrations of MAL3-101, MAL3-51, CJ-21,058 and equisetin were added to the cells (duplicate cultures) and were incubated for 24 h. In control studies (i.e. no drug), equal volumes of DMSO were added to the cultures. The cell density was determined after 24 h, and the results were plotted. Results are means ± S.D. (n = 4).

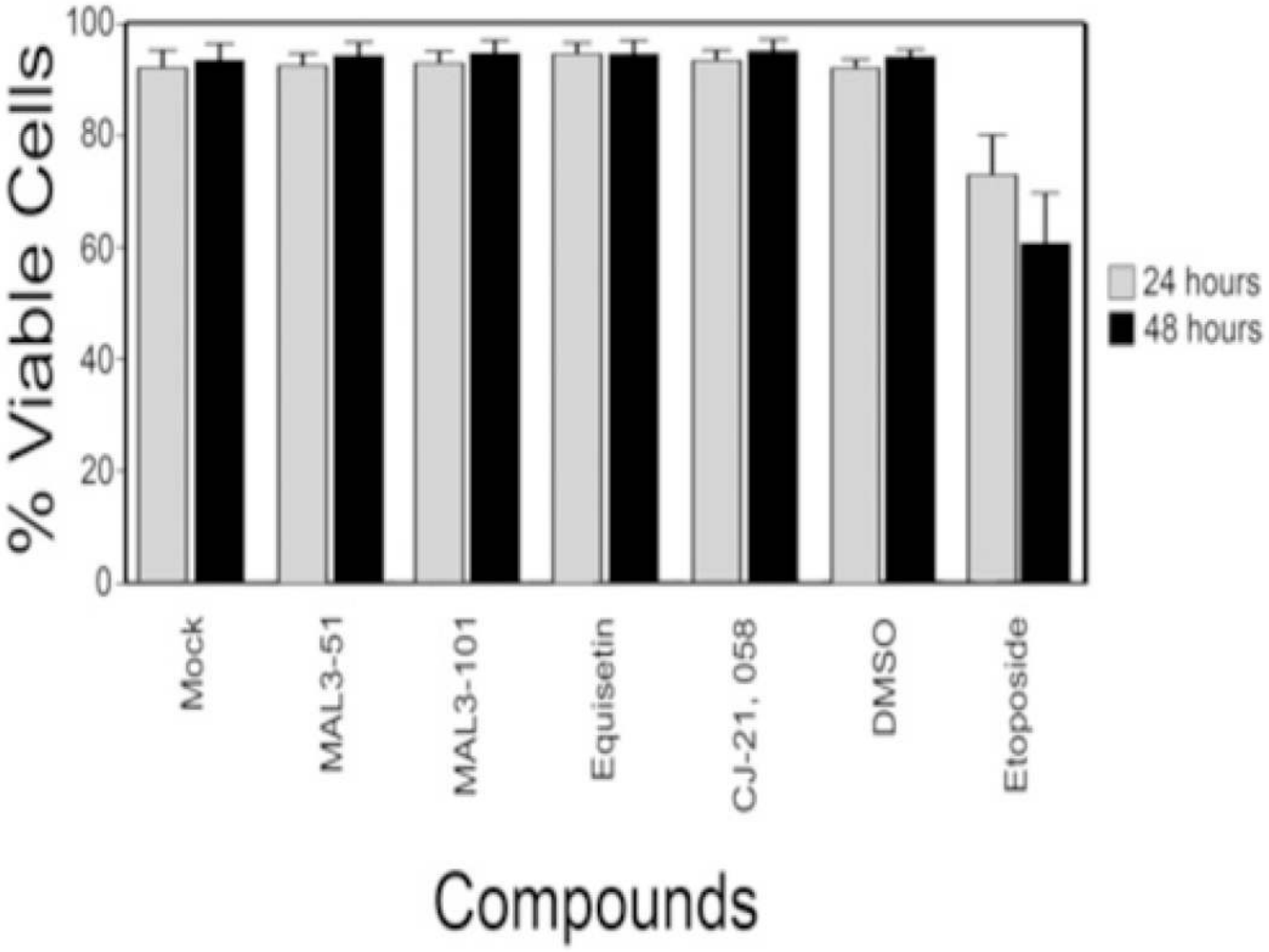

Figure 6. Effect of MAL3-101, equisetin and CJ-21,058 on a human cell line

HeLa cells (10^4 cells/well in a 96-well plate) were treated with one of the following agents: MAL3-101 (10 μM), MAL3-51 (10 μM), CJ-21,058 (12 μM), equisetin (20 μM), etoposide (20 μM), DMSO (5 μM) or mock-treated for 24 or 48 h. Cell viability was determined with a propidium iodide exclusion assay (see the Materials and methods section for details). Results are means ± S.D. (n = 4)

Table 1

Prediction of *T. brucei* polypeptides required for ER protein translocation

Gene	Required for ER protein translocation		
	T. brucei	***S. cerevisiae***	***Homo sapiens***
KAR2 (BIP)	Yes	Yes	Yes
HSC70 (SSA1)	Yes	Yes	Yes
HSP40 (DJ1)	Yes	Yes	Yes
SEC61α (SEC61)	Yes	Yes	Yes
SEC61β	Not detected	Yes	Yes
SEC61γ	Yes	Yes	Yes
SEC62	Not detected	Yes	Yes
SEC63	Yes	Yes	Yes
SEC71	Yes	Yes	Not detected
SEC72	Not detected	Yes	Not detected
SRP54	Yes	Yes	Yes
SRP101 (SRα)	Yes	Yes	Yes
SRP102 (SRβ)	Not detected	Yes	Yes