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TOR induced resistance to toxic adenosine analogs in *Leishmania* brought about by the internalization and degradation of the adenosine permease

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Abstract

TOR is an atypical multidrug resistance protein present in the human protozoan parasite, *Leishmania*. Resistance to the toxic adenosine analog tubercidin was brought about by redirecting the adenosine permease from the plasma membrane to the multivesicular tubule lysosome. The cells became resistant to tubercidin because they were unable to take up and accumulate this toxic purine. The domain which was recognized by TOR in this internalization pathway was identified by expressing portions of this transporter in *Leishmania* and assessing whether they were capable of hindering the multidrug resistance capability of TOR. This approach identified the adenosine permease region spanning Met289 to Trp305. This region was also the epitope recognized by the internalization mechanism. An internal deletion mutant lacking Met289-Trp305 was functionally active but could no longer be internalized in cells with high TOR levels. The internalization and altered trafficking of the adenosine permease by TOR was observed in yeast and human embryonic kidney cells co-expressing these two *Leishmania* proteins indicating that the internalization process was conserved in evolutionary diverse organisms. The inability of *Saccharomyces* with a temperature sensitive ubiquitin ligase to internalize adenosine permease suggested that ubiquitination was involved in this altered trafficking.

Keywords

Leishmania; TOR; adenosine permease; protein trafficking; NT1

INTRODUCTION

Leishmania are flagellated protozoa belonging to the family Trypanosomatidae. Transmitted to humans by the bite of a sand fly, *Leishmania* are found in the tropical and subtropical regions on every continent except for Australia and Antarctica. It is estimated that 350 million people live in at risk areas, 12 million are infected and 1.5 to 2 million new cases occur each year [1,2].

Trypanosomatids are incapable of de novo purine synthesis and must rely entirely on exogenous purines for growth [3]. To satisfy this requirement, *Leishmania* possess multiple purine permeases and the necessary enzymes to convert these purines to nucleotides [3–11].

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Differences in the specificity of the parasite and human purine salvage/metabolizing enzymes could be exploited to the detriment of the parasite. The salvage enzyme hypoxanthine guanine phosphoribosyl transferase, for example, renders *Leishmania* more sensitive to allopurinol, which is converted to a toxic nucleotide by the parasite enzyme but not by the human enzyme [12,13].

For these toxic purines to be effective, however, they must first enter the cell. Resistance to toxic purine nucleosides occurs by the functional loss of the appropriate transporter by mutation or amplification of the TOR gene [9–11,14,15]. Whereas the functional loss of the adenosine or guanosine permease by mutation only renders the cell resistant to the toxic nucleosides transported by the mutated permease, both adenosine and guanosine permeases are adversely affected by TOR and the cell becomes resistant to the toxic nucleosides transported by both permeases. *Leishmania* TOR (Toxic nucleoside Resistance) is unrelated to the TOR (Target Of Rapamycin) kinase present in other eukaryotic cells.

TOR brings about resistance to toxic purine nucleosides by curtailing their entry into the cell. An analysis of the kinetics of purine uptake shows that TOR reduces the $V_{\max}$ for the substrates of the adenosine permease, guanosine permease and adenine/guanine permease(s) [14]. This communication will show that TOR brings about the reduction in $V_{\max}$ for the adenosine permease by reducing the amount of transporter per se and does so by rerouting the normal trafficking of this transporter from the plasma membrane to the multivesicular tubule lysosome. The *Leishmania* multivesicular tubule lysosome appears to be similar to the lysosome of higher eukaryotes [16,17]. This communication will also map the domain on the adenosine permease that is recognized by the internalization pathway and will show that this rerouting pathway is evolutionarily conserved.

MATERIALS and METHODS

Commercial antibodies and fluorescent compounds

Mouse anti chicken tubulin was purchased from Sigma Chemical Co. (St. Louis, MO), rabbit anti neomycin phosphotransferase II from Cortex Biochem (San Leandro, CA) and rabbit and mouse anti green fluorescent protein from Clontech (Palo Alto, CA). Goat anti rabbit and anti mouse IgG conjugated to horseradish peroxidase were obtained from Pierce Chemical Co. (Rockford, IL). FITC conjugated goat anti rabbit and Rhodamine RedTM-X-conjugated donkey anti mouse were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). FM4–64 and LysoTracker Red DND99 were obtained from Invitrogen (Carlsbad, CA).

Cell culture and transformation

Leishmania mexicana amazonensis (LV 78) was grown at 24°C in M199 medium supplemented with 5% (v/v) heat inactivated fetal calf serum and 25 mM HEPES (pH 7.4). *Leishmania* were transformed by electroporation [18]. The transformed cells were maintained under constant G418 and/or hygromycin selection to force the retention of the extrachromosomal plasmids.

Saccharomyces cerevisiae CDC25H (*Mat a ade2–101 his3–200 lys2–801 trp1–901 ura3–52 leu2–3, 112 cdc25-2 Gal+*), FW1810 (*Mat a, rsp5-1 his4–912 leu2 lys2–128 Gal2–1*) and BWG1–7a (*Mat a ade1–100 leu 2–2,2–112 his4–519, ura3–32*) were grown in YPD at 24 °C for CDC25H and 30 °C for FW1810 and BWG1–7a. Yeast were transformed with an extended PEG/LiCl protocol [19]. Transformed cells were selected on YNB medium supplemented with 1% (w/v) glucose and 50 µg/ml each of adenine, uracil and amino acids as required. Unless otherwise noted, transformed cells were grown at the same temperature as the parental wild type cells.

HEK293 were grown in DMEM (high glucose) supplemented with 10 % (v/v) heat inactivated calf serum and transformed by electroporation in DMEM without serum at 250 volts, 500 microfarad capacitance in a 4 mm cuvette.

Gene cloning

Unless otherwise noted, DNAs were amplified with Pfu (Stratagene, La Jolla, CA) or Phusion™ (MJ Research, Watertown, MA) and purified by size fractionation in low melting agarose gels. The region of the gel containing the amplified DNA was excised and the DNA therein phosphorylated with the Blunt End cloning kit (Novagen, Madison, WI) and cloned into the dephosphorylated Eco V site of pBC (Stratagene, LaJolla, CA). For some constructs, restriction sites in the multi cloning sequence of pBC were used for subsequent cloning into the final destination plasmid.

Sequences coding for small peptides were cloned with overlapping, complementary oligonucleotides. The oligonucleotides were first phosphorylated individually with T4 polynucleotide kinase. The oligonucleotides were then combined, denatured by boiling for five minutes and annealed by slow cooling at room temperature. The double stranded oligonucleotide pairs were then cloned into the destination plasmid.

To clone the *Leishmania mexicana amazonensis* adenosine permease NT1 gene, the open reading frame was amplified from *Leishmania mexicana amazonensis* genomic DNA with Pfu and primers #1 and #2 (Table 1). The insert was released with BamH I and Xba I and cloned into the BamH I/Xba I sites of pALT-NEO and 87366 (see Table 2 for plasmids used in this communication). Sub regions of the cloned NT1 ORF greater than 40 nucleotides were amplified with Pfu polymerase and primer pairs #3–10 and cloned into pBC before moving the inserts into the BamH I/Xba I sites in pALT-NEO. Smaller regions were cloned directly into pALT-NEO with complementary oligonucleotides (i.e., primers #11–20). Loop VII (Gln236-Lys331) was also cloned into the BamH I and Hind III sites of 87355 and 87357.

To construct the GFP-NT1 chimera for expression in *Leishmania*, *Saccharomyces* and mammalian cells, the PCR insert in the pBC/NT1 plasmid described above was released with BamH I and Hind III and inserted into the BamH I/Hind III sites in pEGFP-c1 (Clontech Palo Alto, CA). The GFP-NT1 open reading frame was amplified from pEGFPc1-NT1 with Pfu and primers #21 and #2 and cloned into pBC. The GFP-NT1 insert was then released from pBC/EGFP-NT1 with BamH I and Xba I and inserted into the Bam HI/Xba I sites of pALT-NEO for expression in *Leishmania*. For expression in *Saccharomyces*, the GFP-NT1 insert was released from pBC/EGFP-NT1 with BamH I and Hind III and cloned into the BamH I/Hind III sites of 87366. For expression in mammalian cells, the GFP-NT1 insert from a second pBC clone was released with BamH I and cloned into the BamH I site of pcDNA3.1 (Invitrogen, Carlsbad, CA).

To add a histidine tag to the C terminus of adenosine permease, the open reading frame was amplified with Phusion and primers #22 and #23. The insert was removed from pBC with Eco RI and Hind III and cloned into the Eco RI/Hind III I sites in pTEX.

To add a FLAG tag to the N terminus of loop VII (Gln236-Lys331), the complementary oligos #24 and #25 were inserted into the BamH I site of pALT-NEO/loop VII described above.

The TOR open reading frame was amplified from the 2.3 kb Xho I/Pst I fragment from the amplified DNA in tubercidin resistant cells with primers #26 and #27 [9,14]. The insert was released from pBC with Bgl II and Xba I and moved into the BamH I/Xba I sites of pHdX (hereafter called pHdX/TOR). The Xba I site between the PARP and hygromycin phosphotransferase sequences of pB-HyB- $\alpha\beta$ was deleted by partial Xba I digestion coupled

with fill in by polymerase I to yield pHdX [20]. For expression in *Saccharomyces*, TOR was released from pBC with Bgl II and Xho I and cloned into the BamH I/Xho I sites of 87333. For expression in mammalian cells, the TOR DNA was released with Xba I and cloned into the Nhe I site of pCEP (see next paragraph for construction of pCEP).

To construct pCEP, the RSV promoter-multicloning sequence-terminator region of pREP10 (Invitrogen, Carlsbad, CA) was removed with Sal I and replaced with the CMV promoter-multicloning sequence-terminator region from pcDNA3.1 that had been amplified with Phusion™ (MJ Research, Watertown, MA) and primers #30 and #31.

To construct the hSOS-TOR chimera, the DNA coding for TOR Met(31)-Ala(250) was amplified with Pfu and primers #28 and #29. The insert was released from pBC with BamH I and Nco I and cloned into the BamH I/Nco I sites of pSOS.

To express the *Candida albicans* nucleoside transporter in *Leishmania*, the *Candida* permease DNA was cut from pBluescript with Bgl II and Hind III and cloned into the BamH I/Xba I sites in pALT-NEO [21]. The Hind III and Xba I sites had been made blunt ended by fill in with Klenow fragment prior to the ligation.

To clone the *Leishmania* GT1glucose transporter, GT1 DNA was amplified from *Leishmania mexicana amazonensis* genomic DNA using Phusion™ and primers #32 and #33. The insert was released from pBC with BamH I and Xba I and cloned into the BamH I/Xba I sites of pALT-NEO. To tag the glucose transporter with green fluorescent protein, the GT1 open reading frame minus stop signal was amplified using Phusion™ and primers #32 and #34. The insert was released from pBC with BamH I and Bgl II and cloned into the BamH I site of pEGFPN3. The GT1-GFP DNA was cut from the pEGFPN3 plasmid with BamH I and Xba I and cloned into the BamH I/Xba I site of pALT-NEO.

The internalization domain on the adenosine permease DNA was eliminated from the pBC/adenosine permease open reading clone described above using the Quickchange® Site Directed Mutagenesis kit (Stratagene, LaJolla, CA) and primers #35 and #36.

Western blots

To prepare whole cell lysates, log phase *Leishmania* were harvested at 2000 xg, washed once with 150 mM NaCl, resuspended in a small volume of 2% (w/v) SDS and boiled for two minutes to inactivate proteases. After protein levels were determined with the Micro BCA™ Protein Assay reagent (Pierce Chemical Co., Rockford, IL), the proteins were lyophilized and dissolved in sample buffer (60 mM Tris (pH 6.8), 5 % (v/v) 2-mercaptoethanol, 2 % (w/v) SDS) at 4 µg/ml. The proteins were separated on a 4–20% PAGEr® polyacrylamide gel (Cambrex BioScience Inc., Walkersville, MD) under denaturing conditions and electroblotted onto PVDF membrane (Millipore, Billerica, MA) [22,23]. The membranes were blocked for 1 hour with 5% (w/v) nonfat dry milk in TBS (20 mM Tris (pH 7.5), 500 mM NaCl) and then incubated overnight at 4 °C with the primary antibody and for 1 hour at room temperature with the secondary goat anti rabbit or goat anti mouse antibody conjugated to horseradish peroxidase. The blots were washed three times for five minutes per wash with TBS-0.2% (v/v) Tween 20 after each antibody. The antibodies were diluted in the above wash buffer plus 1 % (w/v) nonfat dry milk. Horseradish peroxidase activity was detected with the chemiluminescent substrate, SupraSignal West Dura (Pierce Chemical Co., Rockford, IL) and the emitted light captured with a LumiImager cooled CCD camera (Boehringer Mannheim, Indianapolis, IN).

Fluorescence microscopy

Unfixed cells were photographed under epifluorescence microscopy (Nikon TE300 attached to a Hamamatsu digital camera) through an enhanced GFP filter set. Images were acquired with MetaMorph (Universal Imaging Corp., Downingtown, PA).

Confocal microscopy was conducted with the Olympus IX70 Fluoview 300 laser scanning biological microscope using a 100X oil immersion objective. Green fluorescent fluorochromes were excited with an argon laser at 488 nm with a detection range of 510–530 nm. Red fluorochromes were excited with a HeNe laser at 543 nm with a detection range of 543 nm and above. Images were captured with the Fluoview software packaged with the Fluoview 300. Figures were composed with Adobe PhotoShop 7.0.

TOR activity assay

In *Leishmania*, TOR activity was assessed through its ability to alter the sensitivity of the cells to tubercidin, formycin B, sinefungin and puromycin. *Leishmania* from late log or early stationary phase were diluted to $3\text{--}5 \times 10^5/\text{ml}$ with fresh medium and grown for two to three days under a range of concentrations of the toxic compounds. Cells were counted under phase contrast microscopy at the end of this period and cell numbers were compared to cultures grown in the absence of toxin [9,14].

In *Saccharomyces cerevisiae*, TOR activity was assessed by an adaptation of the Cytotrap™ two hybrid system (Stratagene, LaJolla, CA). Recombinant TOR-hSOS activity was detected through the interaction of TOR with yeast plasma membrane protein(s) allowing the hSOS component of the chimeric protein to activate the Ras signaling pathway thereby enabling the growth of *S. cerevisiae* CDC25H at the nonpermissive 35°C.

Flow cytometry

Log *Leishmania* phase were washed twice with 150 mM NaCl and resuspended with a volume equal to the original culture volume of 150 mM, NaCl, 1 % (v/v) formaldehyde. The cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson).

Glucose accumulation

Glucose accumulation was assessed as described previously for purine and arginine accumulation except that the RPMI was replaced with 150 mM NaCl, 10 mM HEPES (pH 7.4) and [^{14}C]glucose (New England Nuclear, 5 mCi/mmol) [9].

Antibody production

The DNA for the adenosine permease loop VII (Gln236-Lys331) was released from the pBC clone described in the cloning section with BamH I and Hind III and cloned into the BamH I and Hind III sites of pQE9 (QIAGEN, Valencia CA). Recombinant protein production was induced with IPTG and the HIS tagged loop VII protein was purified by Ni chromatography and preparative polyacrylamide gel electrophoresis. Antibodies against the purified recombinant protein were raised in rabbits by ProSci, Inc. (Poway, CA).

Rabbit polyclonal antibodies against *Leishmania* ubiquitin was raised against the peptide, CKTIALEVEPSDTIE, by ProSci, Inc. (Poway, CA).

RESULTS AND DISCUSSION

Toxic nucleoside sensitivity in *Leishmania* with high levels of adenosine permease

TOR exhibited the greatest homology with Oct 6, a mammalian transcription factor of the Pou family [14,24]. Although transcription of most genes does not appear to be controlled in *Leishmania* [25,26], this homology suggested that TOR may act as a repressor of the purine transport genes, a hypothesis consistent with the decreased purine transport capability of cells with high levels of TOR. However, GFP tagged TOR was found in multiple locations in *Leishmania* (i.e., mitochondria and Golgi/trans Golgi regions) but not in the nucleus [27 and manuscript submitted]. It appears instead that TOR acts at the protein level and affects the activity and/or amount of a class of transporters and other proteins that we refer to as TOTs (i.e., Target Of TOR). Operationally, TOTs are defined as permeases or enzymes whose activities and/or location are affected by TOR. The emphasis shifted to the levels of the purine transporters as a means to determine how TOR exerted its effect.

Previous publications dealt with cells with high levels of TOR [9,14]. Our model suggested that if TOR adversely interacted with a handful of TOTs, then cells expressing a large amount of a single TOT should exhibit greater activity of the other TOTs. The rationale is that an excess abundance of a single recombinant TOT would saturate the limited amount of TOR normally present in cells thereby preventing TOR from adversely affecting the other TOTs. To test this possibility, additional adenosine permease was expressed from the extrachromosomal pALT-NEO plasmid and the sensitivity of these transformed cells to tubercidin, formycin B, sinefungin and puromycin was examined (Table 3). The toxicity of these purine based compounds has been found to be reduced in cells with high levels of TOR [9, 14 and data not shown]. Tubercidin is transported by the adenosine permease, formycin B by the guanosine permease and sinefungin by the S-adenosylmethionine transporter [4,7,9,14,28]. It is not known how puromycin enters *Leishmania*.

As expected, *Leishmania* expressing elevated levels of adenosine permease exhibited an increased sensitivity to tubercidin, a toxic adenosine analog. The additional adenosine permease would enable cells to accumulate tubercidin faster than cells transformed with the empty pALT-NEO plasmid resulting in a decrease in the observed ED₅₀ relative to the empty pALT-NEO transformed *Leishmania*. In these toxicity assays, changes in the ED₅₀ for tubercidin arose from changes in the transport capacity of the cell (personal observation). In addition to the increased sensitivity to tubercidin, these cells also became more sensitive to formycin B, sinefungin and puromycin, toxic compounds not transported by the adenosine permease. The differences between cells with normal and elevated levels of adenosine permease were significant ($p < 0.01$) as determined by Student's T test. These results are consistent with the "squenching" of TOR activity by a large amount of a single TOT.

The ability to merely accumulate adenosine/tubercidin at a greater rate is not sufficient to elicit these squenching effects. *Leishmania* expressing the *Candida albicans* nucleoside transporter also became more sensitive to tubercidin and formycin B. In contrast to *Leishmania* which possess two nucleoside permeases each specific for a single family of structurally related nucleosides, the *Candida* transporter transports both adenosine and guanosine so these cells should also become more sensitive to formycin B [21]. The *Candida* nucleoside transporter, however, did not render the cells more sensitive to sinefungin or puromycin.

During the initial characterization of impaired purine transport in TUB cells (the original toxic nucleoside resistant cells which had amplified the TOR gene), we showed that arginine transport was unaffected by high levels of TOR [9]. Glucose accumulation was also found not to be impaired in these cells. In a ten minute incubation, wild type *Leishmania* accumulated 341 ± 1 nmole glucose/ 10^9 cells whereas TUB cells accumulated 455 ± 55 nmole glucose/

10⁹ cells (mean $\pm$ standard deviation of two experiments). This difference was not statistically significant as determined by Student's T test ($p > 0.05$). Since LmGT1 transports glucose poorly relative to the other two *Leishmania* glucose transporters, the absence of any significant decrease in the ability to accumulate glucose in cells with high TOR levels suggest that the LmGT2 and LmGT3 glucose transporters which are found in the plasma membrane as is NT1 are also not TOTs [29]. To pursue LmGT1 further, additional LmGT1 was expressed in *Leishmania*. There was no difference in the sensitivity to tubercidin or sinefungin indicating that this glucose transporter was not a TOT (Table 3). I will show later in this communication that TOR did not affect the normal trafficking of this protein.

TOR and altered trafficking of the adenosine permease in *Leishmania*

An analysis of the kinetic parameters of the purine transporters in cells expressing high levels of TOR indicated that TOR affected the $V_{\max}$ of the adenosine permease, guanosine permease and guanine/adenine permease(s) but not the K_m for the substrates for each of these purine transporters [14]. As $V_{\max}$ is a function of the turnover number as well as the amount of transporter, TOR could have negatively affected either or both of these components resulting in decreased activity. To determine which parameter was affected by TOR, the fate of the adenosine permease in cells expressing high levels of TOR was first assessed. To examine adenosine permease levels in cells with different levels of TOR and to follow the trafficking of this transporter, adenosine permease was tagged with the green fluorescent protein. Epifluorescence microscopy showed that a large fraction of the GFP-NT1 was peripherally located in *Leishmania* co-transformed with the empty pHdX plasmid and most likely reflects the expected plasma membrane localization (Figure 1C). A small amount was located in the vicinity of the flagellar pocket and an internal site which will be show later to be the multivesicular tubule lysosome. On the other hand, in cells co-expressing additional TOR from a second independent plasmid (i.e., pHdX/TOR), the peripheral fluorescence and the fluorescence near the flagellar pocket were absent (Figure 1D). The internal multivesicular tubule lysosome fluorescence remained. Quantitation of the fluorescence by flow cytometry indicated that the amount of GFP-NT1 in the GFP-NT1/elevated TOR transformed cells was greatly reduced (Figure 2).

In contrast to the altered trafficking of the GFP-NT1 chimera in the presence of high TOR levels, GFP was distributed throughout the cell regardless of the level of TOR (Figures 1A and B). Furthermore, flow cytometry showed that the amount of fluorescence from GFP was similar in cells with normal TOR levels (i.e., pHdX transformed cells) as in cells with elevated levels of TOR (i.e., pHdX/TOR transformed cells) (Figure 2).

As a negative control, I also examined the effect of elevated TOR levels on the trafficking of the LmGT1 glucose transporter. I had shown earlier in this communication that TOR did not affect glucose transport nor did additional recombinant LmGT1 affect the sensitivity to tubercidin or sinefungin as did the adenosine permease. There was no evidence that TOR affected the trafficking of this transporter. This conclusion was supported by the observation that the LmGT1 glucose transporter-GFP chimera was primarily located on the flagellum whether the cells expressed normal or high levels of TOR (Figures 1E and F). Because of the apparent absence of any decrease in glucose transport in cells with high levels of TOR, the trafficking of the LmGT2 and LmGT3 glucose transporters was not pursued.

Identification of the internal location of the GFP-NT1 chimera in cells with elevated TOR levels

To identify the organelle to which the GFP-NT1 was redirected, *Leishmania* co-expressing the GFP-NT1 chimera and recombinant TOR was incubated with FM4-64. This fluorescent lipid is rapidly incorporated into the plasma membrane and flagellar pocket followed by what appears to be the *Leishmania* equivalent of early endosomes [17]. With longer periods of

exposure, the bar like multivesicular tubule lysosome (MVT) becomes labeled. Cells photographed simultaneously under phase contrast and epifluorescence microscopy showed that the fluorescence resided internally and frequently at higher amount at the end of a tubular structure, properties consistent with a multivesicular tubule lysosome location (Figure 3, top panel). When these cells were co-labeled with FM4–64, the merged image showed that the green fluorescence from GFP-NT1 coincided with the bar like red fluorescent signal of the MVT body (Figure 3, bottom panel).

GFP-NT1 levels as assessed by western blotting

The previous micrographs showed that elevated levels of TOR redirected the NT1 from its normal plasma membrane destination to the multivesicular tubule lysosome. Furthermore, the micrographs and flow cytometry data indicated that the amount of NT1 was greatly reduced in an environment characterized by high TOR levels. To verify these results and examine the physical state of the GFP-NT1 TOT, the level of TOR and GFP-NT1 in these cells was also determined from western blots (Figure 4). The level of tubulin in each lane was similar for all four transformed cells indicating that an equal amount of protein was in fact analyzed in each lane. The amount of TOR expressed from pHdX in addition to the TOR normally found in these cells greatly exceeded that levels normally present in *Leishmania* transformed with the empty pHdX plasmid. The TOR signal in the empty pHdX plasmid transformed cells was similar to that of *Leishmania* transformed with an empty pALT-NEO plasmid or that of wild type *Leishmania* (personal observation).

GFP was readily detected in these co-transformed cells regardless of the amount of TOR. Densitometry showed that the amount of GFP was equal in the cells co-transformed with the empty pHdX plasmid as compared to the cells co-transformed with pHdX/TOR. In contrast, GFP-NT1 was only easily detected in cells co-transformed with the empty pHdX plasmid and barely seen in the cells co-transformed with pHdX/TOR and only with a much longer exposure than that shown in Figure 4. Faintly visible in this figure is a small amount of what appears to be GFP from the original GFP-NT1 chimera. The low level of GFP-NT1 in these westerns is in agreement with the fluorescence microscopy and flow cytometry discussed above.

To verify that the cells did carry pALT-NEO and that the apparent absence of GFP-NT1 in cells with high levels of TOR was not due to exceedingly large differences in copy number of this plasmid or its level of expression, the amount of neomycin phosphotransferase, the G418 resistance marker encoded on pALT-NEO, was also quantified by western blotting. Densitometry indicated that the level of neomycin phosphotransferase (NPT) expressed from the pALT-NEO/GFP-NT1 transformed cells was approximately 25% of the level expressed from the pALT-NEO/GFP transformed cells (Figure 4). The reason for this difference is not clear. All four cultures were propagated in the presence of 10 µg/ml G418 and 50 µg/ml hygromycin so it was expected that they should have a similar number of copies of the pALT-NEO and pHdX plasmids respectively. Figure 4 also shows that the amount of NPT expressed from pALT-NEO/GFP-NT1 transformed cells is approximately the same regardless of the amount of TOR. These data indicate that TOR does not affect the amount of protein expressed from the pALT-NEO vector and that apparent absence of the GFP-NT1 was not due to gross differences in the amount of product expressed from pALT-NEO. The absence of the GFP-NT1 signal in cells with high TOR levels in these western blots is consistent with our conclusion that TOR redirected this protein to the multivesicular tubule lysosome where it was degraded.

Figure 4 shows a small amount of what appears to be GFP based on its size in GFP-NT1 transformed *Leishmania* expressing high levels of TOR (lane 4). It is possible that the altered trafficking/degradation observed for the GFP-NT1 chimera by TOR may simply be due to cleavage between the adenosine permease and GFP domains releasing the fluorescence tag from the adenosine permease. The observable effect would be the apparent loss of the adenosine

permease. To test this possibility, the GFP tag was replaced with a small six amino acid histidine tag. The commercial anti HIS antibodies that were tested were too low in titer to detect the recombinant HIS tagged adenosine permease in whole cell lysates of *Leishmania* so antibodies against loop VII of the adenosine permease were used.

When the samples were prepared at 100 °C as described in the methods section, a prominent, broad, diffuse band was seen in the upper third of the gel on western blots of *Leishmania* transformed with pALT-NEO/HIS-NT1. A few minor, fainter bands were observed lower in the gel but they were not of the correct size to be NT1. The intensity of this broad band was much reduced in cells co-transformed with pHdX/TOR as compared to cells co-transformed with the empty pHdX plasmid supporting our previous conclusion that elevated levels of TOR result in a diminution of adenosine permease levels (data not shown). On the other hand, when the samples were only heated to 50 °C to denature and solubilize the proteins, multiple sharp bands were seen (Figure 5, panel B). The band indicated by the single arrow migrated with a mass expected for completely denatured NT1. The other major bands may also represent NT1 even though they have an apparent size inappropriate for NT1. These bands were not present in samples denatured by boiling. Furthermore, they were recognized by the anti NT1 antibody whereas pre-immune serum did not yield any detectable bands (Panel A) and most of these bands disappeared with antibody pre-incubated with purified recombinant NT1 loop VII peptide (Panel C). The high hydrophobicity and/or incomplete denaturation of NT1 at this lower temperature may be responsible for the multiple bands and their abnormal migration in these denaturing gels. Quantification of the fluorescence intensity of the bands in panel B that were absent in panel C showed that there was a 80% decrease in this HIS tagged NT1 in cells with high TOR levels (Panel G). The difference was statistically significant as determined by Student's T test ($p < 0.01$). The band indicated by the single arrow in panel B (i.e., the putative completely denatured NT1) was also reduced by a comparable amount in cells with high levels of TOR.

This decrease is consistent with the decrease of GFP tagged NT1 observed in cells with high TOR levels. Panel F showed that there was similar amount of tubulin in each lane indicating that the difference in NT1 was not due to differences in the amount of total protein electrophoresed. Furthermore, panel D showed the cells had a similar level of neomycin phosphotransferase. Neomycin phosphotransferase is co-expressed from pALT-NEO with NT1 and the similar level of this marker protein between these cells indicated that they had approximately an equal number of copies of the pALT-NEO plasmid. The difference in NT1 level could, therefore, not have been due to differences in plasmid copy number. The level of NT1 was too low in wild type *Leishmania* to unequivocally detect with this antibody (data not shown).

Endocytosis and protease inhibitors

The preceding data indicated that TOR brought about resistance to toxic adenosine analogs by redirecting adenosine permease from its plasma membrane destination to the multivesicular tubule lysosome. To determine if this altered trafficking could be blocked and to attempt to increase the amount of GFP-NT1 in cells with high TOR levels, cells were treated overnight with 40 nM Bafilomycin A1 or 50 μ M ALLN. Bafilomycin A1 is an inhibitor of vacuolar-type H^+ -ATPase and interferes with endosomal and lysosomal function through acidification whereas ALLN inhibits cysteine neutral proteases and proteasomes [30,31]. There were no obvious differences by confocal microscopy in either the intensity or distribution of fluorescence (data not shown). Flow cytometry, on the other hand, yielded an approximately two fold increase in the fluorescent signal intensity for both Bafilomycin A1 and ALLN. For Bafilomycin A1 treated cells the mean fluorescence channel value was 25.0 ± 16.6 as compared to 13.6 ± 12.6 for the untreated cells whereas the mean fluorescence channel value for ALLN

treated cells was 35.2 ± 22.5 as compared to 17.8 ± 12.7 for the untreated cells ($N = 10,000$ cells). The differences are statistically significant as determined by Student's T test ($p < 0.01$). The cysteine protease inhibitor K11777 was also tested at 1 and 5 μM for sixteen hours. The cells fluoresced much more intensely but the distribution of the fluorescence did not change significantly (Figure 6). Western blots showed that the increase in fluorescence was due to an increase in the amount of the residual GFP component. There was no apparent increase in the amount of the precursor GFP-NT1 chimera.

Location of the adenosine permease domain responsible for TOT activity

To identify the domain in the adenosine permease that affected TOR activity, fragments of the adenosine permease were cloned into pALT-NEO and the constructs were assessed for their ability to affect the sensitivity of the cells to tubercidin. I did not examine transmembrane domains theorizing that a sequence embedded in the membrane would most likely not be visible to TOR. Loop VII (Gln236-Lys331) when expressed from pALT-NEO was able to increase the sensitivity of the cells to tubercidin as efficiently as the whole permease (Figure 7, peptide labeled B). To further define the domain affecting TOR activity, loop VII was subcloned into progressively smaller fragments and each fragment was assessed for its ability to affect TOR activity. If a peptide did not start with methionine an ATG codon was engineered into the 5' primer immediately upstream of the peptide sequence and all DNAs coding for the peptide had a translation stop signal at the end of the last amino acid. The middle two quarters (i.e., Val258-Glu308, peptide D) and the C terminal half of Loop VII (i.e., Leu284-Lys331, peptide E) were capable of altering the sensitivity of the cells to tubercidin as effectively as the whole transporter. The region that was shared by these two fragments was then subdivided into five smaller nine through twelve amino acid long, overlapping fragments. The two fragments spanning the region from Met289 to Trp305 (i.e., peptides G and H) were each independently able to render *Leishmania* as sensitive to tubercidin as was the entire adenosine permease.

It is extremely unlikely that these smaller fragments possess adenosine transport capability. Their ability to affect tubercidin sensitivity most likely stems from their ability to be recognized by TOR or components associated with TOR. By a mass-action effect these recombinant fragments would saturate the limited amount of TOR in cells to the extent that less TOR would be available to adversely affect the other proteins (i.e., TOTs) that would be recognized by TOR. If this model is correct then the fragments which affect the sensitivity to tubercidin should also affect the sensitivity to toxic purine based compounds transported by other permeases that are adversely affected by TOR. Table 4 shows that the fragments which were capable of increasing the sensitivity of the cell to tubercidin were also capable of affecting the sensitivity to formycin B, a toxic guanosine analog transported by the guanosine permease, and sinefungin, a toxic S-adenosylmethionine analog transported by the S-adenosylmethionine transporter.

In addition to competing with the larger TOTs for TOR's attention, recombinant loop VII also appeared to be targeted for degradation in cells with high TOR levels. When cloned into pALT-NEO and expressed in cells with high levels of TOR (i.e., *Leishmania* co-transformed with the TOR gene cloned into pHdX plasmid), loop VII was present in lower amounts than in cells with normal TOR levels (i.e., *Leishmania* co-transformed with the empty pHdX plasmid, Figure 8). Both pHdX and pHdX/TOR co-transformed *Leishmania* have similar levels of neomycin phosphotransferase (the selection gene product encoded in the pALT-NEO plasmid) indicating that they have a similar number of copies of the pALT-NEO plasmid. The pHdX/TOR co-transformed cells, on the other hand, only have half of the amount of recombinant loop VII. The difference is statistically significant by Student's T test ($p < 0.01$). We conclude that the high levels of TOR were responsible for the decrease in the levels of recombinant loop VII.

The preceding data showed that the region between Met289 and Glu310 to be able to affect TOR's ability to impair purine transport. The data suggested that this also was the region that was recognized by TOR or components associated with TOR when TOR altered the normal trafficking of the adenosine permease and redirected it to the multivesicular tubule. Deletion of this region could result in an adenosine permease that would no longer affect TOR's activity or be redirected to the multivesicular tubule. It was not possible to test this hypothesis in *Leishmania*. Deletion of the 22 amino acid peptide from Met289 to Glu310 altered the normal trafficking of this transporter. The permease remained internally in what I hypothesize to be the endoplasmic reticulum based on the similarity of its distribution to that observed for GFP tagged endoplasmic reticulum resident protein, Ca²⁺-transporting ATPase (Imaa1) in a separate experiment [data not shown and reference 32]. Furthermore, the fluorescence intensity of this mutated GFP-NT1 was greatly diminished as compared to fluorescence from wild type NT1 tagged with GFP rendering the mutated GFP-NT1 very difficult to visualize by confocal microscopy. The absence of any observable effect on the sensitivity to tubercidin of *Leishmania* transformed with the gene for this mutated adenosine permease could have been due to too little protein being present in *Leishmania* and/or its placement in an inappropriate location masking it from TOR's attention (data not shown). Later in this communication, I will show that this region was required for the altered trafficking of the adenosine permease when expressed in yeast co-expressing *Leishmania* TOR.

Conservation of the internalization process of GFP-NT1 in *Saccharomyces* co-expressing high levels of *Leishmania* TOR

Previous experiments had shown that the yeast *Saccharomyces* could properly target the *Leishmania* adenosine permease to the plasma membrane and that *Saccharomyces* could use this transporter to transport adenosine (data not shown). To determine if the retargeting system elicited by TOR was also present in yeast, *Saccharomyces* BWG1-7a were co-transformed with the *Leishmania* GFP-NT1 chimera in the constitutive plasmid 87366 and *Leishmania* TOR in the galactose inducible plasmid 87333. Confocal microscopy showed that the GFP-NT1 was targeted primarily to the plasma membrane in the absence of high levels of TOR (Figure 9, panel C). There was a faint punctate fluorescence throughout the cell but the primary distribution was along the periphery. When *Leishmania* TOR was induced from the Gal1 promoter by growing the yeast on media containing galactose as a carbon source (panel I) instead of glucose (panel D), the peripheral distribution greatly decreased or disappeared and the fluorescence was scattered throughout the interior of the yeast in a punctate fashion. Yeast co-transformed with the empty 87333 plasmid showed the strong peripheral signal for GFP-NT1 when grown on media with galactose as the carbon source (panel H). This peripheral location indicated that the different carbon source per se was not directly responsible for the altered distribution.

Western blots were not effective in determining if GFP-NT1 was degraded in yeast with high TOR levels as it was in *Leishmania*. The 87366 plasmid has a relatively weak TEF promoter and a CEN/ARS origin of replication effectively rendering this a low copy number plasmid in yeast [33]. The GFP-NT1 chimera was present in too low an abundance to be unequivocally detected above the background on western blots with anti GFP antibodies even with long exposures. A more efficient plasmid vis-à-vis promoter strength and plasmid copy number would not be feasible as the increased levels of GFP-NT1 would have completely saturated the available TOR and the altered trafficking of the GFP tagged adenosine permease would have been masked by the excess free GFP-NT1. The fluorescence intensity observed during confocal microscopy appeared to be reduced in cells with high TOR levels but the difference could not be accurately quantified.

Altered trafficking of NT1 lacking the Met289-Glu310 region in *Saccharomyces* co-expressing high levels of TOR

To revisit the role of the Met289-Glu310 region in NT1 trafficking, the fate of adenosine permease lacking this domain was examined in *Saccharomyces*. GFP-NT1 with the Met289-Glu310 deletion (i.e., NT1 Δ IAD) was properly targeted to the periphery of the cell in yeast (Figure 9, panel E). Furthermore, NT1 Δ IAD appeared to be functional. *Saccharomyces* BWG1-7a (*ade1-100*) transformed with the NT1 Δ IAD gene could grow on defined media with adenosine as the purine source whereas yeast transformed with the empty 87366 vector could not. *Saccharomyces* lack a nucleoside transporter so yeast lacking the ability to synthesize purines *de novo* (i.e., *ade* mutants) can only grow on exogenous adenosine if they express a functional recombinant nucleoside transporter [21]. The absence of this domain also rendered the adenosine permease insensitive to the internalization process elicited by TOR. This permease exhibited a very strong peripheral signal even when high levels of TOR were induced by growing the cells on galactose as a carbon source (panel J) instead of glucose (panel E). These data indicate, that this region was a critical epitope in the internalization process mediated by TOR.

Altered TOR activity in *Saccharomyces* expressing high levels of loop IV of the adenosine permease

We had originally intended to use Cytotrap (Stratagene, LaJolla, CA) to investigate the putative interaction between TOR and the *Leishmania* purine permeases. Cytotrap is a variation of the classical yeast two hybrid protocol and investigates protein-protein interactions taking place on the plasma membrane. For interacting pairs, the bait fused to the hSOS gene would interact with the target fused to the Myr transmembrane domain. The hSOS would thereby be brought to the plasma membrane where it would activate Ras thereby initiating cell division. The hSOS complements a temperature sensitive yeast SOS at the nonpermissive temperature only when the bait interacts with the target.

Pilot experiments, however, showed that the hSOS-TOR chimeric protein enabled cellular multiplication at the nonpermissive temperature even if no recombinant target-Myr chimeric protein was present. hSOS without TOR attached did not. I concluded from these observations that TOR interacted with component(s) of the yeast plasma membrane.

This interaction with component(s) of the yeast plasma membrane was used to investigate the ability of loop VII (i.e., Gln236-Lys331) to affect TOR activity (i.e., binding of TOR to the yeast plasma membrane) in *Saccharomyces* as it did in *Leishmania*. High levels of Loop VII should compete with the yeast plasma membrane components for TOR's attention and thereby reduce the amount of the TOR-hSOS chimera that would be free to interact with the plasma membrane. When grown at the nonpermissive temperature, these yeast would be expected to yield smaller and/or fewer colonies. To test this hypothesis, the hSOS-TOR chimera was cloned into the constitutive 87366 and 87374 plasmids whereas the DNA for loop VII was cloned into the constitutive 87355 and 87357 plasmids. This combination was chosen to take advantage of the different promoters and origins of replication to obtain a wide range in the level of recombinant proteins synthesized. *Saccharomyces* CDC25H were co-transformed with the combinations of these plasmids as described in Figure 10 and cloning efficiency was assessed at the permissive and nonpermissive temperatures. In every combination tested, *Saccharomyces* co-expressing Loop VII and TOR-hSOS exhibited a lower cloning efficiency at the nonpermissive temperature as compared to *Saccharomyces* co-expressing hSOS-TOR and the empty 87355 and 87357 vectors (Figure 10). The data show that the expression of loop VII was able to affect the interaction of TOR with component(s) of the yeast plasma membrane. I conclude from these data that Loop VII was able to affect TOR activity in *Saccharomyces* as in *Leishmania*.

Conservation of the internalization process of GFP-NT1 in human cells co-expressing high levels of *Leishmania* TOR

To probe further the evolutionary conservation of the internalization process of the adenosine permease in a high TOR environment, the effect of *Leishmania* TOR on *Leishmania* adenosine permease in human embryonic kidney cells (i.e., HEK293) was also investigated. The *Leishmania* GFP-NT1 chimera in these human cells was targeted to the plasma membrane as it was in *Leishmania* and yeast (Figure 11). There was a small amount of internal fluorescence in a punctate pattern. If these cells were co-transformed with the *Leishmania* TOR gene, the internal punctate fluorescence increased greatly. Staining these cells with LysoTracker™ showed that the red fluorescence from the LysoTracker™ coincided with many of the internalized GFP-NT1 containing vesicles indicating that this fraction of GFP-NT1 had been redirected into lysosomes. These data indicated that the mechanism by which TOR redirected NT1 from the plasma membrane to the lysosome was conserved from mammals to yeast to *Leishmania*.

Ubiquitination in the altered trafficking of the GFP-NT1 in *Saccharomyces*

Internalization and retargeting of membrane proteins is frequently initiated by ubiquitination [34–37]. Rabbit polyclonal antibodies were raised against *Leishmania* ubiquitin but they did not yield any signal on western blots that coincided with the position of GFP-NT1 in whole cell lysates from *Leishmania* co-expressing high levels of TOR. The amount of ubiquitinated GFP-NT1 in these cells may have been too low to detect or the ubiquitination may have been too fleeting of a modification. The anti ubiquitin antibodies did recognize several proteins which may be the polyubiquitin precursor or ubiquitin fusion proteins present in this trypanosomatid [data not shown and references 38,39].

To pursue further the possible role of ubiquitination, the internalization of GFP-NT1 in *Saccharomyces* FW1810 which possess a temperature sensitive ubiquitin ligase (*rsp5-1*) was examined. FW1810 were co-transformed with GFP-NT1 in the constitutive 87366 plasmid and TOR in the galactose inducible 87333 plasmid. At the permissive 24 °C, FW1810 were able to alter the trafficking of the NT1 to the plasma membrane when TOR expression was induced with galactose (Figure 12, panel F). At 37 °C, however, the GFP-NT1 fluorescence remains concentrated around the periphery of the cell (panel D). The peripheral fluorescence is similar to yeast transformed with the empty 87333 plasmid as well as in 87333/TOR transformed yeast grown on glucose to silence the Gal1 promoter (panels B and E respectively). These data suggest that ubiquitination of the GFP-NT1 chimera is a prerequisite step in the TOR mediated altered trafficking of this chimera.

Experiments to detect the putative adenosine permease TOR interaction

I have not been able to demonstrate a TOR-adenosine permease interaction by chemical cross linking in *Leishmania* or with the yeast and bacterial two hybrid systems (i.e., Cytotrap™ and BacterioMatch™ (Stratagene, La Jolla, CA)) or with the yeast split ubiquitin complementation protocol [data not show, see reference 40 for split ubiquitin]. There are several strong possibilities to account for this failure. There may be an undetected intermediary protein(s) involved in this interaction that would not be present in bacteria. TOR exists as tetramer in *Leishmania* and chemical crosslinking suggests the possibility of additional components in this complex (personal observation). This explanation does not suffice for yeast as TOR is capable of causing the internalization of the adenosine permease in this organism indicating that the yeast homolog of this putative accessory protein(s) exists. Alternatively, the interaction could have been so fleeting/labile that these techniques were insufficiently sensitive to detect this putative interaction.

It is also possible that the protein-protein interaction experiments were just not sufficiently sensitive because there was an insufficient amount of TOR as bait in these protocols. As described earlier in this communication, TOR interacts with component(s) of the yeast plasma membrane. TOR also interacts with component(s) of bacteria. A phage display library of *Leishmania* DNA was constructed to search for TOTs by screening with purified, recombinant TOR. Every plaque lit up with the TOR probe showing that TOR was binding to components released by the lysed bacteria. The endogenous proteins in bacteria and yeast may simply have absorbed too much recombinant TOR leaving too little for these protein-protein interaction protocols to work.

For *Leishmania*, there is just too little adenosine permease present in the cells with high TOR levels. The amount of adenosine permease in these cells is so low that it is very difficult to routinely detect the residual protein. Chemical crosslinking would yield an even weaker signal as the proteins are not quantitatively crosslinked.

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Abbreviations

GFP	green fluorescent protein
MVT	multivesicular tubule
NPT	neomycin phosphotransferase
ORF	open reading frame
TOR	toxic nucleoside resistance
TOT	target of TOR

Figure 1. Altered trafficking of adenosine permease in cells with elevated TOR levels

Live, log phase *Leishmania* were photographed under phase contrast and epifluorescence microscopy. GFP, GFP-NT1 adenosine permease and LmGT1 glucose transporter-GFP were cloned in pALT-NEO whereas TOR was cloned in pHdX. The cells were grown under constant G418 and hygromycin selection in order to retain the extrachromosomal pALT-NEO and pHdX plasmids.

Figure 2. Changes in the amount of GFP-NT1 as a function TOR levels as evaluated by flow cytometry

The fluorescence of *Leishmania* transformed with the plasmids shown in each panel was quantified by flow cytometry. Ten thousand *Leishmania* of each culture were analyzed. *Leishmania* transformed with the empty pALT-NEO plasmid were used as a negative control.

Figure 3. Localization of GFP-NT1 in *Leishmania* expressing elevated amounts of TOR
 Promastigotes were labeled with FM4–64 for two hours at 4 °C to label the plasma membrane and multivesicular tubule lysosome [17]. The location of the green (GFP tagged adenosine permease) and red fluorescence (FM4–64) was determined by epifluorescence microscopy. In the top panel, the merged image is cells photographed simultaneously under phase contrast and epifluorescence microscopy.

Figure 4. Western blot analysis of GFP tagged adenosine permease levels in cells with different amounts of TOR

Whole cell lysates (20 μ g protein) were resolved by SDS-PAGE and transferred to PVDF membrane as described in the methods section. The blots were probed individually with the antibodies indicated adjacent to each panel. The single arrow points to the position of the GFP-NT1 chimera whereas the double arrow points to the position of GFP. The four cell lines shown were co-transformed with the plasmid combination listed under the last panel. NPT, neomycin phosphotransferase.

Figure 5. Western blot analysis of HIS tagged adenosine permease levels in cells with different amounts of TOR

Whole cell lysates (20 µg protein) were resolved by SDS-PAGE and transferred to PVDF membrane as described in the methods section except that the samples were prepared at 50 °C instead of 100 °C. MagicMark™ molecular weight standards from Invitrogen were used to determine the size of proteins. Lane 1, *Leishmania* co-transformed with pALT-NEO/HIS-NT1 and pHdX; lane 2, *Leishmania* co-transformed with pALT-NEO/HIS-NT1 and pHdX/TOR. Panel A, preimmune serum; panel B, anti NT1 loop VII antibodies; panel C, anti NT1 loop VII antibodies pre-incubated for one hour with 10 µg purified recombinant NT1 loop VII peptide; panel D, anti neomycin phosphotransferase; panel E, anti TOR; panel F, anti tubulin. Panel G, quantification of the luminescence of panel B. The luminescence was summed for all bands which were present in greater amount in lane 1 as compared to lane 2. BLU, Boehringer luminescence units. Statistical significance as assessed by Student's T test: *, $p < 0.01$. The arrow adjacent to panel B indicates the position of a completely denatured NT1 migrating normally as based on its calculated mass.

Figure 6. Partial inhibition of the degradation of the GFP-NT1 chimera in the multivesicular tubule lysosome by cysteine protease inhibition

Leishmania co-transformed with GFP-NT1 in pALT-NEO and either TOR in pHdX or the empty pHdX plasmid were grown overnight in the presence of the cysteine protease inhibitor K11777 at the concentration shown in the figures. Left panel, phase contrast and epifluorescence microscopy of live, unfixed cells. Right panel, western blots probed with anti GFP or tubulin antibodies. For these westerns, whole cell lysates (20 μ g protein) were resolved by SDS-PAGE and transferred to PVDF membrane as described in the methods section. The blots were probed individually with the antibodies indicated underneath each panel.

Figure 7. Location of the domain on loop VII of the *Leishmania* adenosine permease affecting TOR activity

Leishmania transformed with the intact adenosine permease or different regions of loop VII cloned in pALT-NEO were assessed for their sensitivity to the toxic adenosine analog, tubercidin. The ED₅₀ values for tubercidin are the average $\pm$ standard deviation of at least three determinations. The upper portion of the figure shows the position of the peptides B–J relative to the intact adenosine permease labeled A in this figure. EV is *Leishmania* transformed with the empty pALT-NEO plasmid and serves as the reference.

Figure 8. Western blot quantification of FLAG tagged Loop VII levels in cells with different amounts of TOR

Whole cell lysates (20 μ g protein) of cells co-transformed with pALT-NEO/Loop VII-FLAG and pHdX or with pALT-NEO/Loop VII-FLAG and pHdX/TOR were resolved by SDS-PAGE and transferred to PVDF membrane as described in the methods section. The blots were probed individually with anti neomycin phosphotransferase (NPT) and anti FLAG. The relative amount is the chemiluminescence signal from cells co-transformed with pALT-NEO/Loop VII-FLAG and pHdX/TOR divided by chemiluminescence from cells co-transformed with pALT-NEO/Loop VII-FLAG and pHdX. Statistical significance by Student's T test: *, $p < 0.01$.

Figure 9. Conservation of the altered trafficking of *Leishmania* adenosine permease in yeast co-expressing high levels of *Leishmania* TOR

Yeast were co-transformed with the *Leishmania* GFP-NT1 chimera in the constitutive 87366 plasmid and *Leishmania* TOR in the galactose inducible 87333 plasmid and cloned on 1x YNB supplemented with adenine, histidine and glucose. The cells from one clone from each transformation were grown overnight in 1x YNB supplemented with adenine, histidine and either 0.5% glucose or galactose. The DIC (rows 1 and 3) and fluorescence images (rows 2 and 4) were obtained by confocal microscopy. The combination of plasmids is shown above each lane of micrographs. 87366 and 87333 are the empty vectors. NT1ΔIAD is GFP tagged adenosine permease without the internalization domain cloned in 87366. The internalization domain was deleted with the Quickchange site specific mutagenesis kit as described in the methods section.

Figure 10. Squelching of hSOS-TOR complementation in *Saccharomyces CDC25H* by co-expression with *Leishmania* loop VII

Saccharomyces CDC25H were co-transformed with either 87366/TOR-hSOS or 87374/TOR-hSOS and 87355 with (+) and without (-) loop VII insert or 87357 with (+) and without (-) loop VII insert. The cells were grown for five days at 24 or 35 °C. The relative cloning efficiency is the number of clones at 35 °C/number of clones at 24 °C. The data is the average $\pm$ standard deviation, N = 3. Significance as determined by Student's T test: *, $p < 0.01$; **, $p < 0.02$.

Figure 11. Conservation of the altered trafficking of *Leishmania* adenosine permease in human embryonic kidney cells co-expressing high levels of *Leishmania* TOR

HEK293 were transformed with *Leishmania* GFP-NT1 cloned in pcDNA3.1 (top panel) or co-transformed with *Leishmania* GFP-NT1 cloned in pcDNA3.1 and *Leishmania* TOR cloned in pCEP (bottom panel). The cells were grown for one hour at 37 °C with 100 nM LysoTracker in complete medium to label the lysosomes. DIC, Differential Interference Microscopy.

Figure 12. Identification of an ubiquitination involvement in the altered trafficking of *Leishmania* adenosine permease in yeast co-expressing high levels of *Leishmania* TOR
Saccharomyces FW1810 with a temperature sensitive ubiquitin ligase (*rsp5-1*) were co-transformed with *Leishmania* GFP-NT1 chimera in the constitutive 87366 plasmid and *Leishmania* TOR in the galactose inducible 87333 plasmid or with the empty 87333 plasmid. The cells were grown overnight at a permissive 24 °C or nonpermissive 37 °C temperature in 1x YNB supplemented with adenine, histidine and either 0.5% glucose or galactose. The DIC (columns 1 and 3) and fluorescence images (columns 2 and 4) were obtained by confocal microscopy.

TABLE 1

Primers for DNA amplification and cloning^a

Primer	Region	Sequence
Primers for NT1 adenosine permease		
1. Met(1)-His(491) ^b	5'	ggatccATGGACACTGCACCCGATCATCGCGAGCCC
2.	3'	gaattcaagcttctagaTCACTAGTGGCGCTCGCGGATGGTCTGCGTCATGAT
3. Gln(236)-Lys(331) ^b	5'	ggatccatgCAGAACTACTTCGGTGACCTCGGTGCTGTG
4.	3'	aagctttctagactaTCACTTGACACGGCGCAGCGTGCTGAAGATGGC
5. Gln(236)-Ala(283) ^b	5'	agatctaATGCAGAACTACTTCGGTGAC
6.	3'	aagctttctagactaGGCCGGCACCTCCTTGCCCGA
7. Val(258)-Glu(308) ^b	5'	agatctaagGCGCTCTGCCACACCGACGAG
8.	3'	aagctttctagactaCTCGTGCGGCCATGABCTCTC
9. Leu(284)-Lys(331) ^b	5'	agatctaagCTGGGTGAGGTGCAGACGGCC
10.	3'	aagctttctagactaCTTGACACGGCGCAGCGTGCT
11. Leu(284)-Ser(294) ^c	5'	gatcatgTTGGGTGAGGTGCAGATGGTCACCGCCAAGTCGtag
12.	3'	ctagctaCGACTTGGCGGTGACCATCTGCACCTCACCCAA
13. Met(289)-Ala(300) ^c	5'	gatcATGGTCACCGCCAAGTCGGAGAGCCCGGATGCGGCGtag
14.	3'	ctagctaCGCCGCATCCGGGCTCTCCGACTTGGCGGTGACCAT
15. Glu(295)-Trp(305) ^c	5'	gatcatgGAGAGCCCGGATGCGGCGGAAGAGGCCTCGTGtag
16.	3'	ctagctaCCACGAGGCCTCTTCCGCCGCATCCGGGCTCTC
17. Ala(300)-Glu(308) ^c	5'	gatcatgGCGGAAGAGGCCTCGTGGCCGCAGGAGGTGGAGtaggaattc
18.	3'	ctaggaattcctaCTCCACCTCCTGCGGCCACGAGGCCTCTTCCGCcat
19. Trp(305)-Asn(315) ^c	5'	gatcatgTGGCCGCAGGAGGTGGAGGGCCCGACGAGTAACtaggaattc
20.	3'	ctaggattctaGTTACTCGTCGGGCCCTCCACCTCCTGCGGCCAcat
Primers for green fluorescent protein -NT1 chimera^d		
21.	5'	ggatccATGGTGAGCAAGGGCGAGGAGCTG
Primers for NT1-HIS tagged chimera		
22. Met(1)-His(491) ^b	5'	gaattcATGGACACTGCACCCGATCATCGCGAGCCC
23.	3'	ggatcctcactagtagtgatggtgatggtgGCGCTGCGGATGGTCTGCGTCATGAT
Primers for FLAG-Loop VII chimera		
24. FLAG peptide	5'	gatcgctgcagATGGACTACAAGGACGATGACGATAAAc
25.	3'	gatcgTTTATCGTCATCGTCCTTGTAGTCCA Tctgcagc
Primers for TOR^e		
26. Met(1)-Glu(458)	5'	agatctATGCATCTCTCTGCC
27.	3'	tctagactgcagTCATGGGAAATCGGC
28. Met(31)-Ala(250)	5'	ggatcccaATGTACTTCGGCTGCCTCAAG
29.	3'	ccatggctaCGCATATGCCCACTGGTCCAA
Primers for CMV promoter-multicloning sequence		
30.		gtcgacGATGTACGGGCCAGATATACG
31.		gtcgacATCCCCAGCATGCCTGCTATT
Primers for <i>Leishmania mexicana</i> GT1 glucose transporter^f		
32. Met(1)-Glu(610)	5'	ggatccATGCTGCCACCGCCTCAGATTCT
33.	3'	aagctttctagaCCGCAAAGCGCCAAGTCCTGACAT ^g
34.	3'	agatctCTACGCCCTGTTGCCGCTGAGCGA
Primers for site specific mutagenesis		
35. NT1 Δ Met(289)-Glu(310)		TTGGGTGAGGTGCAGGGCCCCGACGAGTAAC
36.		GTTACTCGTCGGGCCCTGCACCTCACCCAA

^aUpper case letters are targeting nucleotides. Lower case letters are nucleotides for cloning and expression purposes.^bTargeting sequence is based on the *L. donovani* NT1 nucleoside transporter 1.2 sequence, AF041473 [11].^cTargeting sequence is based on the *Leishmania mexicana amazonensis* NT1 adenosine permease sequence, AF452412.^dTargeting sequence is based on the GFP in pEGFP-C1 (Clontech, Palo Alto, CA).^eTargeting sequence is based on the *Leishmania mexicana amazonensis* TOR sequence, AF016581[14].^fTargeting sequence is based on the *Leishmania mexicana* GT1 sequence AF071217.^gPrimer recognition site resides in the GT1 downstream intergenic region.

TABLE 2

Plasmids used in this research.

Plasmid	Host	Source
pBC	<i>E. coli</i>	Stratagene
pEGFPC1	<i>E. coli</i>	Clontech
pEGFPN3	<i>E. coli</i>	Clontech
pALT-NEO	<i>Leishmania</i>	[41]
pB-HyB- $\alpha\beta$	<i>Leishmania</i>	[20]
pHdX	<i>Leishmania</i>	This communication
pTEX	<i>Leishmania</i>	[42]
pCDNA3.1	Mammalian cells	Invitrogen
pCEP	Mammalian cells	This communication
pSOS	<i>Saccharomyces</i>	Stratagene
87333 ^a	<i>Saccharomyces</i>	a.k.a., p426 GAL1, Gal1 promoter, 2 μ , <i>ura3</i> [33]
87355 ^a	<i>Saccharomyces</i>	a.k.a., p423 GPD, GPD promoter, 2 μ , <i>his3</i> [33]
87357 ^a	<i>Saccharomyces</i>	a.k.a., p424 GPD, GPD promoter, 2 μ , <i>trp1</i> [33]
87366 ^a	<i>Saccharomyces</i>	a.k.a., p415 TEF, TEF promoter, CEN/ARS, <i>leu2</i> [33]
87374 ^a	<i>Saccharomyces</i>	a.k.a., p415 ADH, ADH promoter, CEN/ARS, <i>leu2</i> [33]

^aYeast plasmids were purchased from the American Type Culture Collection and are referenced in this communication by their catalog number. The source column lists the original name, the promoter, origin of replication and yeast selection marker gene.

TABLE 3
Sensitivity of *Leishmania* transformed with potential TOTs

Plasmid	Tubercidin	Formycin B	ED ₅₀ ^a	Sinefungin	Puromycin
<i>Leishmania mexicana</i> NT1 adenosine permease ^b	7.3 ± 0.1 *	2.4 ± 0.2 *		0.33 ± 0.02 *	150 ± 12 *
<i>Candida albicans</i> nucleoside permease ^{b,c}	6.4 ± 0.8 *	1.7 ± 0.2 *		1.9 ± 0.1	530 ± 66
<i>Leishmania mexicana</i> GT1 glucose transporter ^{b,d}	40	ND ^e		2.1	ND
pALT-NEO vector	34 ± 3.0	17 ± 1.0		1.9 ± 0.2	540 ± 52

^a ng/ml, data is the average ± standard deviation, N = 3 except for the glucose transporter where N = 1. Statistical significance by Student's T test:

*, p < 0.001.

^b Cloned in pALT-NEO.

^c GenBank accession number AF016246 [21].

^d GenBank accession number AF071217.

^e ND, not determined.

TABLE 4Comparison of selected regions of loop VII of the NT1 permease for their ability to affect TOR activity.^{a, b}

NT1 fragment	ED ₅₀			AMINO ACID
	Tubercidin	Sinefungin	Formycin B	
B) Gln236-Lys331	6.4 ± 0.8	0.32 ± 0.07	1.5 ± 0.2	←→
C) Gln236-Ala283	32 ± 0.6	1.3 ± 0.3	17 ± 0.6	←→
D) Val258-Glu308	5.8 ± 0.2	0.32 ± 0.02	2.4 ± 0.1	←→
E) Leu284-Lys331	6.9 ± 0.6	0.43 ± 0.03	2.6 ± 0.4	←→
F) Leu284-Ser294	26 ± 1.5	1.7 ± 0.1	12 ± 1.0	←→
G) Met289-Ala300	5.9 ± 0.3	0.37 ± 0.01	1.3 ± 0.2	←→
H) Glu295-Trp305	5.3 ± 0.71	0.71 ± 0.03	2.3 ± 0.2	←→
I) Ala300-Glu310	14.2 ± 0.3	ND	ND	←→
J) Trp305-Asn315	23 ± 2.1	ND	ND	←→
pALT-NEO	29 ± 4.6	1.6 ± 0.3	15 ± 2.1	

^{a)} *Leishmania* were transformed with the DNA coding for the NT1 peptides cloned in pALT-NEO. The sensitivity of the transformed cells to the three toxins used in table was assessed as described in the methods section. The ED₅₀ is in ng/ml and is the average ± standard deviation, N ≥ 3

^{b)} The figure at the top of the table shows the position of loop VII relative to the whole NT1 permease. The dark rectangles show the position of the transmembrane domains.