Regulatory volume decrease in *Leishmania mexicana*: effect of anti-microtubule drugs

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The trypanosomatid cytoskeleton is responsible for the parasite’s shape and it is modulated throughout the different stages of the parasite’s life cycle. When parasites are exposed to media with reduced osmolarity, they initially swell, but subsequently undergo compensatory shrinking referred to as regulatory volume decrease (RVD). We studied the effects of anti-microtubule (Mt) drugs on the proliferation of *Leishmania mexicana* promastigotes and their capacity to undergo RVD. All of the drugs tested exerted antiproliferative effects of varying magnitudes [ansamitocin P3 (AP3) > trifluoperazine > taxol > rhizoxin > chlorpromazine]. No direct relationship was found between antiproliferative drug treatment and RVD. Similarly, Mt stability was not affected by drug treatment. Ansamitocin P3, which is effective at nanomolar concentrations, blocked amastigote-promastigote differentiation and was the only drug that impeded RVD, as measured by light dispersion. AP3 induced 2 kinetoplasts (Kt) 1 nucleus cells that had numerous flagella-associated Kts throughout the cell. These results suggest that the dramatic morphological changes induced by AP3 alter the spatial organisation and directionality of the Mt that are necessary for the parasite’s hypotonic stress-induced shape change, as well as its recovery.

Key words: microtubules - drugs - *Leishmania* - regulatory volume decrease

The cytoskeleton is a complex of fibrous elements that are essential for cellular form, cell division, motility, cytoplasmic structural organisation and the integrity of the cellular membrane. All flagellated trypanosomatid parasites are characterised by the presence of a cytoskeleton that is responsible for maintaining and modulating form throughout the cell cycle (Sherwin & Gull 1989). The microtubules (Mts) of the trypanosomatid subpellicular corset are connected to each other and to the cellular membrane and serve as a membrane cytoskeleton (Drenckhahn et al. 1985, Kohl & Gull 1998). The absence of transcellular cytoskeletal structures can result in decreased deformability, cell elasticity and cell modulation at different stages of the cell cycle (Sebeck et al. 1990). Mt-associated proteins, including actin, intermediate filaments and actin-associated proteins, are cytoskeletal components that are essential for cell functionality (Dagger et al. 1989, Hemphill et al. 1992, Hernandez et al. 1997, Sahasrabuddhe et al. 2004, Nayak et al. 2005). An interesting aspect of the biology of trypanosomatids is their capacity to survive in a wide range of environmental conditions; in particular, trypanosomatids are resistant to dramatic fluctuations in the external osmotic strength of the vector midgut, acidic phagolysosomes and the cytosol of vertebrate cells (Kollien et al. 2001). When exposed to media of reduced osmolarity, most animal cells, including trypanosomatids, initially swell but subsequently undergo rapid recovery to a normal cellular volume through a mechanism referred to as regulatory volume decrease (RVD). During this volume adjustment process, transport systems are activated and these systems can lead to a rapid extrusion of osmotically active inorganic solutes, including K\(^+\), Cl\(^-\) and water or the release of various organic osmolytes, such as amino acids, glyceryl phosphoryl choline, sorbitol and inositol (Hoffman & Simonsen 1989, Hoffman & Durham 1995, Vieira et al. 1996, Park et al. 1997, Rohloff et al. 2003). This mechanism is used by numerous protozoans, including *Giardia intestinalis*, *Crithidia luciliae* and multiple *Leishmania* and *Trypanosoma* species (Vieira 1998). Survival within different hydrodynamic environments requires a dynamic cell architecture that is highly elastic and tension-resistant. The characteristic trypanosomatid body form and the cytoskeletal structure that supports it may represent an environmental adaptation. The trypanosomatid cytoskeletal Mts are unusual in many aspects and behave differently than those in higher eukaryotic cells (Sherwin & Gull 1989); they are stable during extraction with a variety of buffers and detergents, are resistant to depolymerisation at low temperatures and are resistant to the action of many anti-Mt drugs that are effective in higher eukaryotic cells. Additionally, they persist during parasite division. The role of cytoskeletal elements and, specifically, Mts in RVD remains poorly defined. Nevertheless, the functional integrity of the cytoskeleton is required for RVD in all eukaryotic cells studied thus far (Haussing et al. 1994, Downey et al. 1995). Accordingly, we focus here on the role of *Leishmania mexicana* promastigote Mts in RVD by studying the cellular effects of known anti-Mt agents. The selected drugs include taxol, an ester complex that...
promotes Mts assembly and blocks cytokinesis in various cell types, including trypanosomatids (Baum et al. 1981, Hernandez 1996, Moulay et al. 1996); two potent and widely used tricyclic compounds, the phenothiazine drugs trifluoperazine (TFP) and chlorpromazine, which are potent antiproliferative agents at micromolar and nanomolar concentrations and exert highly effective antitumor activity in vivo (Tanida et al. 1979, Ootsu et al. 1980, Takahashi et al. 1989, 1990). In this paper, we report for the first time the application of anti-Mt drugs to Leishmania as a tool to understand the role of cytoskeletal components in the RVD process. Our results suggest that the spatial organisation of the subpellicular Mts provides the structural basis for the process of shape transition during RVD.

MATERIALS AND METHODS

Chemicals and solutions - Triton X-100, Schneider’s insect medium, poly-L-lysine, dimethylsulfoxide (DMSO), FITC-conjugated anti-alpha tubulin antibody (clone DM1A), taxol, chlorpromazine, ansamitocin P3 (AP3), TFP and rhizoxin were obtained from Sigma (St Louis, Mo). Stock solutions of each drug were prepared in DMSO and stored at 4°C or -20°C following the manufacturer’s instructions. Foetal bovine serum (FBS) was obtained from Electron Microscopy Sciences. All other reagents were analytical grade. Isotonic chloride buffer (137 mM NaCl, 4 mM KCl, 1.5 mM KH₂PO₄, 8.5 mM Na₂PO₄, 20 mM HEPES, 11 mM glucose, 1 mM CaCl₂, 0.8 mM MgSO₄) was adjusted to pH 7.4. The osmolarity of the buffer was 300 ± 5 mOsm (isosmotic) and 150 mOsm ± 7 mOsm as measured in an Advanced Digimatic Osmometer.

Parasite culture - L. mexicana promastigotes of strain NR (Ramirez & Guevara 1987) were isolated from infected mice and cultured at 26°C by serial passage every five days in Schneider’s insect medium supplemented with 10% inactivated FBS (pH 7.4). Axenic L. mexicana amastigote-like forms were obtained following incubation of promastigotes in Schneider’s insect medium supplemented with 20% FBS (pH 5.5) at 35°C in the presence of 5% CO₂ for 24 h.

Fluorescence microscopy - L. mexicana promastigotes were grown in the presence or absence of 100 nM AP3. Following three washes in phosphate-buffered saline (PBS) (150 mM NaCl, 20 mM phosphate buffer, pH 7.4), they were placed on glass cover slips coated with poly-L-lysine, fixed with 3% (v/v) freshly prepared formaldehyde solution in PBS at 26°C for 10 min, washed in PBS and permeabilised with 0.1% (v/v) Triton X-100 for 3 min. After 5 min of treatment with ammonium chloride followed by three washes in 0.2% gelatin in PBS, the cells were incubated with a 1:100 dilution of FITC-conjugated anti-tubulin antibody (clone DM1A, Sigma) for 40 min in a humidified chamber in the dark. The cover slips were washed with PBS, rinsed with water and finally mounted on glass slides with a drop of mounting medium (Moviol Hoechst). The slides were observed with a Nikon Eclipse E400 fluorescence microscope.

Transmission electron microscopy (TEM) - Parasite cells (1 x 10⁶) grown in the presence or absence of 100 nM AP3 for 48 h were washed in PBS and the pellets were fixed for 1 h with 2.5% glutaraldehyde in PBS at 4°C. The cells were washed again in PBS and post-fixed for 1 h in PBS containing 1% osmium tetroxide. The cells were then washed in the same buffer, dehydrated in a graded ethanol series followed by propylene oxide and finally embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEOL Transmission Electron Microscope.

Light scattering - RVD of control cells and 50% of inhibitory concentration (IC₅₀) drug-treated cells was monitored over 48 h by measuring light scattering of the cellular suspensions at 550 nm (A550) in 1 mL glass cuvettes at 26°C using a Beckman DU-7500 spectrophotometer (Park et al. 1997, Rohloff et al. 2003). The cells were washed once and resuspended in 300 mOsm chloride buffer to a final concentration of 1 - 2 x 10⁶ cells/mL. A550 readings were registered every 20 sec for 10 min. The cells remained evenly dispersed over this time interval. Hypoosmotic conditions were induced by diluting the isotonic cell suspension with an appropriate volume of deionised water to a final osmolarity of 150 mOsm. The blank sample contained PBS without cells. A decrease in the A550 reading indicated cell swelling (an increase in the cell volume).

Plasma membrane-cytoskeleton complex preparation - Leishmania promastigotes grown in the absence of drugs were separated into two batches. One batch was subjected to the procedure described in this section for immunofluorescence visualisation of Mts (control cells). The other batch, together with Leishmania promastigotes, grown in the presence of 100 nM AP3 over 48 h, was washed three times in PBS and resuspended in buffer A (20 mM HEPES - 20 mM NaCl pH 7.4) for 5 min. The cells were centrifuged at 1500 g for 10 min and the pellet was resuspended in buffer B [20 mM HEPES - 100 mM NaCl - 0.5% (v/v) Triton X-100, pH 7.4]. The cells were sedimented again under the same conditions and were washed and resuspended in buffer C (100 mM HEPES - 100 mM NaCl, pH 7.4) following a modification of the procedure previously reported for preparing the Leishmania plasma membrane cytoskeleton complex (“ghost”) (Bordier et al. 1982). The entire procedure was carried out at 4°C. Control cells and Triton X-100 “ghosts” of control and drug-treated cells were used for immunofluorescence.

Antiproliferative activity of drugs on Leishmania promastigotes - The effects of the five distinct anti-Mt drugs on promastigote proliferation were assessed by measuring the optical density of the cell cultures at 560 nm every 24 h for five days. The drugs were added to the log-phase promastigote cultures when they reached a final density of 2 x 10⁶ parasites/mL. Each drug was added
to the cultures in a range of concentrations determined previously by measuring the antiproliferative activities of the drugs. Viability was assessed by measuring the mobility of the cells. The drug stocks were dissolved in DMSO and the final concentration of DMSO in the assays was always less than 1%. At this low concentration, DMSO does not affect cell growth. The results presented were obtained in three independent experiments performed with duplicate samples. The IC\textsubscript{50} values for each drug were determined after 48 h by performing a sigmoidal regression on the concentration response curves.

**Cellular differentiation** - AP3 (100 nM) was added to the *L. mexicana* promastigote cultures in Schneider’s insect medium (pH 7.4) at 26ºC. The cultures were then transferred to a growth chamber and cultured at 34ºC in the presence of 5% CO\textsubscript{2}. Inactivated FBS (20%) was added to the Schneider’s insect medium (pH 5.5) to allow differentiation of the promastigotes to the amastigote form. After 24 h of incubation, the cultures were returned to the original temperature and pH conditions. The cell cultures that were exposed to the same experimental conditions without drugs were used as controls. The stage changes were observed under an inverted light microscope (results not shown).

**RESULTS**

**Effect of anti-Mt agents on *L. mexicana* promastigote proliferation** - The antiproliferative activities of AP3, chlorpromazine, taxol, TFP and rhizoxin were tested in vitro. Drug treatment resulted in a dose-dependent inhibition of parasite growth. The IC\textsubscript{50} values of these drugs were determined after 48 h of treatment (Table). Of the agents studied, AP3 was the most effective, strongly inhibiting the proliferation of *L. mexicana* promastigotes at nanomolar concentrations. As observed by phase contrast microscopy, control cells were slender and spindle-shaped, each with a single flagellum (Fig. 1A). The taxol-treated cells had a single flagellum and were pear-shaped or had shortened spindles (Fig. 1B). The TFP-treated cells had a single motile flagellum and had spindle-shaped bodies with an occasional enlarged brilliant area at the anterior end (Fig. 1C). The chlorpromazine-treated cells (photographs not taken) maintained a slender form with several cells presenting a brilliant spot at the anterior part of the cell body. A single flagellum was present in all of the cell populations. The cells that were treated with rhizoxin presented a morphologically mixed population of pear-shaped, round or spindle-shaped cells with slightly shortened lengths; however, all rhizoxin-treated cells displayed a single flagellum (Fig. 1D). In contrast,

| Drugs                | IC\textsubscript{50} (µM) |
|----------------------|--------------------------|
| Taxol                | 15                       |
| Chlorpromazine       | 50                       |
| Trifluoperazine      | 5                        |
| Rhizoxin             | 22                       |
| Ansamitosin P3       | 0.092                    |

IC\textsubscript{50}: 50% of inhibitory concentration.

![Fig. 1: morphology changes observed in *Leishmania mexicana* promastigotes after antimicrotubule drug treatment. Parasites were cultured in the absence (control) (A) or in the presence of taxol at 15 µM 50% of inhibitory concentration (IC\textsubscript{50}) (B) or trifluoperazine at 5 µM (IC\textsubscript{50}) (C) or rhizoxin at 22 µM (IC\textsubscript{50}) (D) or ansamitocin P3 at 92.2 nM (IC\textsubscript{50}) (E-F) during 48 h. Light photographs of non fixed cells were taken using an electronic flash.](image-url)
the AP3-treated cells displayed irregular or mostly round forms and were larger than the control cells. Furthermore, they had multiple actively beating flagella emerging from different parts of the cell body (Fig. 1E, F). In summary, except for the AP3-treated cells, the drug-treated cells presented normal or slightly altered morphology with a single flagellum. Light microscopy images of unfixed cells were taken after 48 h of growth in the presence of each drug at a concentration equivalent to the IC_{50} value.

Effect of anti-Mt drugs on RVD in L. mexicana promastigotes under hypotonic stress - The drugs used in this study have shown strong antiproliferative effects on Leishmania promastigotes. Based on these results, we next investigated the capacity of these drug-treated promastigotes to carry out RVD under hypotonic stress. When control cells were subjected to a reduction in osmolarity from 300-150 mOsm, they immediately swelled until they became quite rounded, but within a few minutes they began to shrink, reaching the same degree of motility and morphology observed at 300 mOsm. These interesting changes lasted nearly 10 min (results not shown). Light-scattering experiments allowed us to follow the process of volume recovery by monitoring the A550 of the cell suspensions, as cell swelling leads to a decrease in the absorbance reading (Park et al. 1997, Rohloff et al. 2003). All of the experiments were carried out under the same conditions. The promastigotes were cultured for 48 h and the drugs were added at concentrations near the IC_{50} values. The control cells and the drug-treated cells were centrifuged and resuspended in 300 mOsm buffer. Following a two-fold dilution of the isosmotic cell suspensions with distilled water, the absorbances of the suspensions were monitored for at least 10 min. No differences in the lag time or rate of volume recovery were observed among control, chlorpromazine, taxol or TFP-treated cells (Fig. 2A) (and results not shown). The cells completed RVD in less than 5 min. Our findings were quite different for the AP3 and rhizoxin-treated cells. A decrease in absorbance was also observed in these cells 1 min post-treatment, but this decrease in absorbance was much more dramatic than that in the control cells. Volume recovery was not observed for the AP3-treated cells, whereas the rhizoxin-treated cells slowly returned to their original volume (Fig. 2B).

Effect of AP3 on the ultrastructure of L. mexicana promastigotes - Light microscopy showed that AP3 dramatically affected the growth and morphology of L. mexicana promastigotes at nanomolar concentrations. The unusual forms observed and the loss of the cells’ capacity to accomplish RVD led us to investigate the impact of AP3 treatment on the promastigote ultrastructure. Thin sections of cells were processed after 48 h of culture with 100 nM AP3 and were viewed by TEM. In contrast with the slender shape of the control cells (Fig. 3A), the treated cells were round or amorphous with flagella-associated kinetoplasts (Kts) in various cell positions. Interestingly, only one nucleus per cell could be visualised. The untreated cells displayed a rounded nucleus with a central prominent nucleolus and dense chromatin patches adhered to the internal nuclear membrane. In contrast, the treated cells displayed anisotropic nuclei that appeared to sequester part of the cytoplasm; moreover, the heterochromatin was arranged in dense reticular patches and the nucleolus was no longer visible. The treated cells often displayed fragmented, condensed mitochondria accompanied by an increasing number of electron-transparent vesicles that occupied a considerable portion of the cytoplasm. Furthermore, many treated cells displayed multiple flagella emerging from one flagellar pocket (Fig. 3B-D). Abnormally elongated and duplicated Kts were frequently observed both in association with and independent of flagella (Fig. 3E1-4). Despite the dramatic changes in cell morphology in the presence of the drug, no changes in the corset Mts or the distance between them were observed (Fig. 3F1-2).

Fig. 2: regulatory volume decrease in Leishmania mexicana promastigotes after anti-microtubule (Mt) drug treatment. Relative changes in cell volume of parasites cultured in the absence or presence of the drugs were followed by monitoring absorbance at 550 nm. Traces followed by control, trifluoperazine (TFP) and taxol treated cells (A) and cells treated with rhizoxin and ansamitocin P3 (AP3) (B). Results are representatives of those obtained from five independent experiments and are expressed in arbitrary absorbance units. OD: optical density.
Direct immunofluorescence of AP3-treated promastigotes and membrane-cytoskeleton complex - Based on these findings, we used fluorescence microscopy to test whether *Leishmania* Mts remain unaltered following 48 h of promastigote culture with 100 nM AP3, as has been observed by TEM. Direct immunofluorescence of cells stained with FITC-conjugated anti-alpha tubulin showed a similar number and distribution of labelled Mts in the slender, elongated control cells and the rounded treated parasites (Fig. 4A, B). The fluorescence distribution was uniform within the parasite body and within the detergent-extracted cytoskeletons of the control (Fig. 4C) and drug-treated cells, and Mts were abundant in both preparations (Fig. 4D, E).

Effect of AP3 on Leishmania differentiation - In addition to its effect on promastigote cell division, AP3 also suppressed *Leishmania* differentiation. AP3 (100 nM) was added to promastigotes cultured at 26ºC in Schneider’s insect medium (pH 7.4) and the cells were transferred to a growth chamber and cultured in medium with pH 5.5 at 35ºC in the presence of 5% CO₂. Twenty-four hours later, the control cells had been transformed to the amastigote form, were oval shaped, had free flagella and were immobile. The AP3-treated cells were of different shapes and sizes, had short flagella, formed cell aggregates and had a 40% cell death rate. After 24 h, the cells were returned to 26ºC and cultured in Schneider’s insect medium (pH 7.4) to allow differentiation from the amastigote to the promastigote form. After 36 h, the untreated cells had completely reverted to the motile, slender promastigote form, whereas AP3 treatment completely inhibited differentiation to the promastigote form, resulting in predominantly large, non-motile cell aggregates.

Fig. 4: tubulin fluorescent labelling of promastigotes and membrane cytoskeleton complex after ansamitocin P3 (AP3) treatment. In slender control cells (A), fluorescence is distributed in all the cell body and flagellum. After AP3 treatment for 48 h, the labelling is observed in the rounded cell bodies and flagella. Tubulin labelling (B) of the membrane cytoskeleton complex prepared from normal (C) and treated cells (D, E).

Fig. 3: ultrastructural changes observed in *Leishmania mexicana* after ansamitocin P3 (AP3) treatment. Promastigotes after 48 h culture in Schneider’s insect medium in the absence (A) or in the presence of 100 nM AP3 (B-F). Rounded cells (B-C) with two distinct replicating kinetoplasts (Kts) were observed in different parts of the cell associated to flagella. Anisotropic nucleous (n) enclosing part of the cytoplasm was commonly visualized with chromatin arranged as a dense blocks. Cell with amorphous shape (D) presented branched condensed mitochondria (m) and an enlarged flagellar pocket containing five emerging flagella (f). Multiple elongated dividing Kts associated or not to basal bodies were frequently observed (E1-4). Subpellicular microtubules (Mts) remain unaltered, as well as the distance between them (F1-2).
DISCUSSION

The traditional approach to understanding the role of cytoskeletal components in RVD has been to use drugs that interfere with the cellular function of these components. Here, we selected drugs that were previously reported to affect the proliferation of various *Leishmania* species, suggesting that these drugs may act on the nuclear Mts, the kts, the basal body or the assembly of new Mts, which is necessary for cytokinesis. Except for the two ansamitocin antibiotics, treatment with these drugs resulted only in slight changes in cell morphology. For the most part, the cell populations remained slender or oval-shaped with one motile flagellum at the anterior end of the cell body. This finding indicates that the antiproliferative effects of these drugs involved the inhibition of basal body replication, one of the earliest events of the cell cycle in *Leishmania* and trypanosomatids (Robinson & Gull 1991, Harmanton et al. 2003). The drugs taxol, TFP and chlorpromazine (results not shown) did not alter promastigote RVD. Our findings were quite different for the drug rhizoxin: the antiproliferative action of this drug was associated with mixed morphological forms characterised by pear-shaped bodies or shortened spindles and a single flagellum. These shape changes could account for the differences observed in the extent of cell swelling and in the rate of volume recovery under hypotonic conditions compared to the control cells. The dose-dependent antiproliferative effect of AP3 observed at nanomolar concentrations, which was associated with profound changes in cell morphology and the inhibition of RVD, led us to focus on understanding the basis of this cell behaviour in response to AP3. Our observation of large, rounded cells with numerous beating flagella throughout the cell body by light microscopy was supported by previous reports that electron micrographs showed several replicated flagellum-associated kts displaced throughout the cell. In addition, the decreased proliferation of these cells and the electron microscopy data showing a single nucleus per cell with altered heterochromatin seemed to indicate an impairment in the assembly of the mitotic spindle and subpellicular Mts following AP3 treatment. These results are in agreement with those obtained in *Leishmania donovani* promastigotes, in which AP3 induced the accumulation of cells in the G2 and M phases of the cell cycle. This result is consistent with an anti-tubulin mechanism of action that generates 2 Kt 1 nucleus cells and multi-Kts cells (Havens et al. 2000). Previous results also support our finding that drug treatment interferes with the polymerisation or assembly of new Mts, which is necessary for body enlarge-ment and the emergence of free flagella. These functions enable the differentiation from the amastigote to the promastigote form through a mechanism similar to that involved in cilia regeneration in *Tetrahymena* sp. (Takahashi et al. 1989). We also observed by electron microscopy that the integrity of the corset Mts of AP3-treated promastigotes was preserved. A similar observation has been reported for *L. donovani* promastigotes subjected to AP3 treatment under the same conditions (Havens et al. 2000). This finding was confirmed by labelling the Mts of control and AP3-treated cells and their detergent-extracted cytoskeletons (“ghosts”) with an anti-tubulin an-tibody. No differences were found in the distribution or fluorescence intensity of tubulin between the control and AP3-treated cells. These data support the hypothesis that AP3 does not have a depolymerising effect on the cytoplasmic Mts of *L. mexicana* promastigotes. Therefore, its ability to impair RVD, particularly during the recovery phase, cannot be attributed to the instability of the subpellicular Mt corset. Together, the results reported here suggest that drugs can induce strong antiproliferative effects without affecting volume regulation under hypotonic stress. Despite their antiproliferative effects, none of the drugs used in this study impeded RVD, except AP3. The amorphous or round shape of the AP3-treated cells differed from those of the cells treated with the other drugs, which presented only slight morphological changes; they maintained elongated shapes and a single flagellum, indicating that they had not undergone basal body replication and separation. It has been proposed that the RVD process in trypanosomatids involves a shape transition from an elongated to an almost spherical form. This process depends on the medium osmolarity and is accompanied by the efflux of water, amino acids and osmolytes (Cosgrove & Kessel 1958, Van Rossum et al. 1987). It has also been suggested that the linear subpellicular Mts of elongated promastigotes of *Herpetomonas megaseliae* undergo a spiralisation process upon phagocytosis in vitro, which results in the helicoidal arrangement that is found in round intracellular forms (Janovy et al. 1974). Identical findings have also been reported for *L. donovani* (Van Rossum et al. 1987), indicating that the architectural rearrangement of Mts might be a general process underlying shape trans-ition in trypanosomatids and some euglenoids (Hoffman & Bouck 1976). The forms assumed by the cells following an increase in volume are limited by ultrastructural constraints, as a constant distance must be maintained between the Mts (Meyer & de Souza 1976). Taking these findings into consideration, a model has been proposed to account for the relationship between Mt rearrangement, changes in cell volume and the transition from an elongated (promastigote) form to a spherical form. Whereas some Mts become linear, others assume a compensatory over-spiralised shape, allowing for a change in the cellular volume with little change in the cell surface area (de Andrade & de Almeida 1980). As we have shown in AP3-treated cells, various Kts are displaced to different parts of the cell bodies and are connected to flagella. Mts may then anchor themselves at locations that correspond to Kts migration. Consequently, Mts are arranged parallel to various axes throughout the cells, resulting in Mts with different directionals. This hypothesis suggests that during AP3 treatment, the process of Mt spiralisation, followed by cell body torsion during hypotonic condition-induced shape change, becomes disorganised, asynchronous and ineffective. Therefore, the AP3-treated cells are not able to carry out the process of cell transformation that underlies RVD.

The successful search for new drugs for the treatment of leishmaniasis requires prior knowledge of the cellular biology and biochemistry of the parasites. Our results support the hypothesis that Mts are viable therapeutic targets by demonstrating their important role in RVD in trypano-
somatids. The effect of AP3 is remarkable, particularly because it is effective at concentrations in the nanomolar range. Therefore, future studies should explore the potential therapeutic use of AP3 in leishmaniasis.

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