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***Leishmania tarentolae*: Purification and characterization of tubulin and its suitability for antileishmanial drug screening**

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Abstract

Previously, tubulin has been purified from *Leishmania amazonensis* and used to identify novel molecules with selective antimitotic activity. However, *Leishmania amazonensis* is pathogenic and requires a relatively expensive medium for large-scale cultivation. Herein, the purification and characterization of tubulin from the non-pathogenic *Leishmania tarentolae* is reported, together with the sequence of α - and β -tubulin from this organism. This protein was purified by sonication, diethylaminoethyl-Sepharose chromatography, and one assembly-disassembly cycle in 1% overall recovery based on total cellular protein. *L. tarentolae* tubulin was indistinguishable from the corresponding *L. amazonensis* protein in terms of binding affinity for dinitroaniline sulfanilamides and sensitivity to assembly inhibition by these compounds. The amino acid sequences derived from the *L. tarentolae* α - and β -tubulin genes were 99.6% and 99.4% identical to the corresponding amino acid sequences from the *L. major* Friedlin strain. These results indicate that tubulin from *L. tarentolae* is suitable for use in drug screening.

Index Descriptors

Leishmania; tubulin; dinitroaniline; drug discovery

1. Introduction

Leishmaniasis, a spectrum of disease caused by parasites of the genus *Leishmania*, contributes to significant morbidity and mortality in developing countries (WHO, 2006). Traditional antileishmanial therapies include the use of pentavalent antimonial compounds, amphotericin B, and pentamidine. More recently, miltefosine (hexadecylphosphocholine) has demonstrated efficacy against visceral leishmaniasis when administered orally (Sundar et al., 2000). However, current treatment strategies all suffer from drawbacks which include toxicity, developing resistance, administration by injection, and expense (Ouellette et al., 2004; Singh and Sivakumar, 2004).

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Tubulin, a critical eukaryotic protein responsible for formation of the mitotic spindle, is the proposed target of clinically relevant anti-cancer and anthelmintic compounds. Dinitroaniline herbicides have shown activity versus both *Leishmania* and trypanosomes (Chan and Fong, 1990; Chan et al., 1991; Chan et al., 1993a; Chan et al., 1993b; Traub-Cseko et al., 2001), and tubulin has been implicated as the target of these compounds (Chan and Fong, 1990; Chan et al., 1991; Traub-Cseko et al., 2001). *N*¹-phenyl-3,5-dinitro-*N*⁴,*N*⁴-di-*n*-propylsulfanilamide (GB-II-5) (Werbovetz et al., 2003) and *N*¹-phenyl-3,5-dinitro-*N*⁴,*N*⁴-di-*n*-butylsulfanilamide (GB-II-150) (Bhattacharya et al., 2004) are derivatives of the dinitroaniline herbicide oryzalin that have displayed potent antimitotic effects on kinetoplastid parasites (see Figure 1 for structures). Initially, these oryzalin analogs were evaluated *in vitro* for their ability to inhibit the polymerization of tubulin purified from *L. amazonensis* (Werbovetz et al., 1999), and ongoing drug discovery efforts against this promising target will continue to require *in vitro* evaluation of novel compounds against purified leishmanial tubulin. Reports detailing the expression of tubulin from parasites have appeared (MacDonald et al., 2003; Fennell et al., 2006), some of which have demonstrated the assembly of recombinant $\alpha\beta$ tubulin (Oxberry et al., 2001; MacDonald et al., 2004). No such expression system currently exists for the leishmanial protein, however, and antileishmanial drug discovery work will continue to rely on the purification of tubulin directly from the parasite in the near future.

There are several drawbacks to purifying tubulin from *L. amazonensis*. This parasite is an etiological agent of cutaneous leishmaniasis and has been classified as a BSL-2 pathogen. To achieve significant yields of tubulin for compound screening, these parasites must be grown to high cell densities and large quantities of cells must be collected ($\sim 5 \times 10^{11}$ parasites), increasing the risk to laboratory personnel of accidental exposure. In contrast, *L. tarentolae*'s natural host is the Gecko lizard (*Tarentola mauritanica*) (Wenyon, 1921), and this parasite species does not infect humans. Furthermore, *L. tarentolae* can be cultured to higher cell densities than *L. amazonensis*; typical achievable cell densities are 1.8×10^8 parasites/ml (Sbicego et al., 2003) and 5×10^7 parasites/ml (Werbovetz et al., 1999), respectively. In addition, *L. amazonensis* requires supplemented Schneider's *Drosophila* medium for high density growth (Werbovetz et al., 1999), whereas *L. tarentolae* can be grown in Brain Heart Infusion supplemented with hemin (Sbicego et al., 2003), with the latter medium being considerably less expensive. Herein we report the purification and amino acid sequence of tubulin from *L. tarentolae*, along with a comparison of the effects of selected antimicrotubule agents on tubulin from *L. tarentolae* and *L. amazonensis*. Our studies demonstrate that *L. tarentolae* tubulin is a valid alternative to tubulin from pathogenic *Leishmania* species.

2. Materials and methods

2.1. Chemicals

Where not otherwise specified, materials were obtained from Sigma. Oryzalin analogues were synthesized as described in Bhattacharya *et al.* (Bhattacharya et al., 2004). All of the compounds assayed against tubulin were prepared in 100% DMSO stock solutions and stored in the dark at -4°C .

2.2. Parasites

Leishmania tarentolae (UC strain) were a generous gift from Dr. Larry Simpson (Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, CA). The parasites were maintained in Brain Heart Infusion medium (Becton Dickinson) at 37 g/l which was sterilized by autoclave and supplemented with 10 mg/L filter sterilized hemin (added from a 2 mg/ml stock dissolved in 0.05N NaOH). Parasites were maintained by serial passage in T-25 flasks (Corning) at 25 $^{\circ}\text{C}$. Large scale cultures were grown in a 1 liter flask in an orbiting shaker incubator set at 125 rpm and 25 $^{\circ}\text{C}$. Cells were harvested in late log phase

($\sim 1\text{--}2 \times 10^8$ parasites/ml) by centrifugation at $1200 \times g$ at 4°C . Cell pellets were then washed twice with phosphate buffered saline (PBS) solution and stored at -80°C until use.

2.3. Purification of tubulin

A modification of the earlier protocol for purification of tubulin from *L. amazonensis* was employed for the isolation of *L. tarentolae* tubulin (Werbovetz et al., 1999). PBS washed parasites (7.3×10^{11} cells) were thawed and resuspended in three 40 mL portions in PME + P buffer (PME plus protease inhibitors, consisting of 0.1M PIPES (pH 6.9), 1 mM MgCl_2 , 1 mM EGTA, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 25 $\mu\text{g/ml}$ leupeptin) on ice. These cell suspensions were extensively sonicated on ice using a Misonix (Microson XL) probe sonicator (Farmingdale, NY) using ten 30 s bursts at full power ($\sim 25\text{ W}$) with two minute cooling intervals between bursts. The resulting sonicated suspensions were allowed to cool on ice for 30 min, then were centrifuged in an Optima L-90K Ultracentrifuge (Beckman, Fullerton, CA) using a Ti-70 rotor at $40,000 \times g$ for 40 min at 4°C . The resulting supernatant was filtered through glass wool, then loaded via peristaltic pump at a rate of 2.5 ml/min on a column containing 13 ml DEAE-Sepharose Fast Flow matrix (Amersham Biosciences) that was previously equilibrated with 2 column volumes PME + P. The column was then washed with two column volumes PME + P, followed by 4 column volumes PME + P containing 0.1 M KCl and 0.25 M glutamate (pH 6.9). Tubulin was eluted with two column volumes PME + P containing 0.3 M KCl and 0.75 M glutamate (pH 6.9). Approximately 3 ml fractions were collected after beginning the 0.3 M KCl/0.75 M glutamate elution. Fractions were assayed for the presence of tubulin by performing assembly reactions. Briefly, 20 μl of each fraction were added to the 96-well half-area microplates (Costar) on ice, along with distilled H_2O and $5 \times$ PME (to restore the final PME concentration to $1 \times$), assembly was initiated by the addition of 10 μl of 5 mM GTP in 50% DMSO; the final assay volume was 50 μl . Assembly was assessed by measuring the turbidity at 351 nm over a twenty minute period in a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA) with thermostat control set at 30°C . Assembly competent fractions were pooled and diluted in PME buffer supplemented with GTP, dimethylsulfoxide and additional MgCl_2 (final concentrations $1 \times$ PME, 10 mM MgCl_2 , 8% DMSO (v/v), 2 mM GTP). The solution was then incubated at 37°C for 30 min to promote assembly, then spun at $50,000 \times g$ at 30°C for 30 min. Following centrifugation, the supernatant was removed, the remaining pellet consisting of microtubules was rinsed once with warm PME ($\sim 37^\circ\text{C}$), and resuspended in ~ 1.5 ml cold PME. The remaining pellet was further solubilized via probe sonication (three ~ 5 s bursts at 10 W) using the Misonix sonicator. The tubulin rich solution was incubated on ice for an additional 30 min, then spun at $50,000 \times g$ at 4°C for 30 min. The supernatant containing heterodimeric tubulin was stored at -80°C in 50 μl aliquots. Protein purity was assessed by SDS-PAGE using 12% polyacrylamide Ready Gels (BioRad). Gels were stained with Coomassie Brilliant Blue R250 (0.25 g dissolved in 20% MeOH, 8% acetic acid in distilled H_2O) for 30 min, then destained with 20% methanol and 8% acetic acid.

2.4. Tubulin/drug Assembly Assays

Tubulin/drug assembly assays were performed as outlined previously (Werbovetz et al., 2003). Assembly reactions were carried out in 96-well half-area microplates (Costar) in a final volume of 50 μL and contained final concentrations of 1.5 mg/ml (15 μM) tubulin, 0.1 mM PIPES (pH 6.9), 1 mM EGTA, 1 mM MgCl_2 , 10% (v/v) DMSO, and 1 mM GTP with and without compounds of interest. Solutions containing PIPES, EGTA, MgCl_2 , and compounds were added to the microplate on ice. The compounds were added to reaction mixtures in 5 μl volumes containing 50% DMSO, then assembly was initiated by adding 10 μL of 5 mM GTP in 25% DMSO. Absorbance values were recorded in a SpectraMax Plus microplate reader at 351 nm with the thermostat control set at 30°C . IC_{50} values are reported as the mean $\pm$ range of two duplicate experiments.

2.5. Determination of Dissociation Constants by Fluorescence Quenching Assays

The strong 300–350 nm absorbance of the dinitroaniline compounds makes them useful acceptors of resonance energy transfer from photoexcited tryptophan residues within tubulin. Quenching of intrinsic tubulin fluorescence by dinitroaniline compounds was assessed based on a previous method (Werbovetz et al., 2003). Samples were studied using sub-micro 50 μ L quartz spectrophotometer cells (Starna Cells). Tubulin samples (1 μ M) with varying concentrations of oryzalin analogue were excited at 290 nm and tryptophan fluorescence was measured between 310 and 340 nm. Spectra were recorded using a Perkin Elmer LS 50B luminescence spectrometer with excitation and emission slits of 2.5 nm and 15 nm, respectively. Emission intensity at 325 nm was taken for calculation of quenching. In contrast to the previous study (Werbovetz et al., 2003), multiple tubulin samples with varying concentrations of compound in a fixed volume of solvent (DMSO, 9.7% v/v) were prepared. Since the DMSO concentrations were fixed and the fluorescence was only measured once per sample, it was unnecessary to correct for solvent effects and photobleaching. Inner filter effects were corrected by measuring the quenching of N-acetyl-L-tryptophanamide (8 μ M) fluorescence in the presence of the compounds. K_d values were calculated by plotting decrements in fluorescence as a function of oryzalin analogue concentration. These values were fit to a single-site binding model using the nonlinear fitting routines of Prism software (GraphPad Software, San Diego, CA) and are reported as the fitted $K_d \pm$ standard error of the fit.

2.6. Genomic Cloning and Sequencing

To determine the sequence of *Leishmania tarentolae* α -tubulin, genomic DNA from *L. tarentolae* was amplified by the polymerase chain reaction (PCR) using 40 pmol forward (5'-ACCCCTCTTTCTTCTTTTCAGCCATG-3') and 40 pmol reverse (5'-TTAGTACTCCTCGACGTCCTCCTCACC-3') oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) designed to match the 5'-untranslated region and 3'-terminal region of the *L. donovani* α -tubulin gene (Joshi et al., 1995), respectively. β -tubulin was PCR amplified using 40 pmol forward (5'-ATGCGTGAGATCGTTTCCTGCCAGGC-3') and 40 pmol reverse (5'-CTAGTAGGCTTCCTCCTCGTCG-3') oligonucleotide primers matching the 5'-terminal and 3'-terminal regions of the β -tubulin gene (c β T_{2.2} isotype) from *L. major* (Coulson et al., 1996), respectively. PCR reactions were performed using PCR SuperMix (Invitrogen) and incubated in a thermal cycler using a program consisting of a 94 °C denaturation step, 40 °C and 50 °C annealing steps for 40 s, and an elongation step of 72 °C repeated for a total of 25 cycles, following the manufacturer's instructions (Perkin Elmer). PCR products were gel purified and cloned into pCR2.1-TOPO (Invitrogen). Insert containing clones were isolated after transformation of *E. coli* DH5 α and sequenced at the Plant-Microbe Genomics Facility at The Ohio State University using a 3730 DNA analyzer (Applied Biosystems, Inc.).

Results and discussion

3.1. Purification of *L. tarentolae* tubulin

L. tarentolae tubulin was purified based on a previous protocol for the isolation of *L. amazonensis* tubulin (Werbovetz et al., 1999). The initial step in the earlier protocol involved lysis of cells by sonication, followed by centrifugation to generate a cytoplasmic fraction and a pellet rich in tubulin (most likely from subpellicular microtubules and flagella). Since there was little tubulin in the cytoplasmic fraction, it was discarded and the pellet was resuspended and sonicated to solubilize tubulin. In our current protocol, the initial fractionation was eliminated in favor of extensive sonication, thus shortening the purification protocol. Figure 2 indicates that the DEAE-Sepharose column was nonetheless capable of providing nearly homogeneous tubulin despite the presence of cytoplasmic proteins. Further purification and

concentration of the protein using one cycle of assembly-disassembly provided tubulin with approximately 1% overall recovery based on total cellular protein. Fong and Chang determined that tubulin comprised 11.4% of the total cellular protein in *L. mexicana* promastigotes (Fong and Chang, 1981). Assuming this to be a reasonable approximation for *L. tarentolae* tubulin, the overall yield of the present purification is roughly 9 – 10%. Protein recoveries obtained at different stages of the protocol are summarized in Table 1; a typical procedure can be completed in ~11 hours.

3.2. Sequences of *L. tarentolae* α - and β -tubulin genes and comparison with tubulins from other *Leishmania* species and other organisms

To investigate the similarity between *L. tarentolae* tubulin and tubulin from other *Leishmania* species and other organisms, both α - and β -tubulin genes of *L. tarentolae* were sequenced. The *L. tarentolae* α -tubulin gene (accession number [DQ309032](#)) and β -tubulin gene (accession number [DQ309033](#)) share 97.3% and 97.5% nucleotide sequence identity with the corresponding genes from the *L. major* Friedlin strain, respectively (Ivens et al., 2005). As expected, the tubulin amino acid sequences from *L. tarentolae* are highly similar to the tubulin sequences from other *Leishmania* species and with other organisms. *Leishmania* species possess a single mRNA for α -tubulin (Fong et al., 1984; Burchmore and Landfear, 1998), while multiple β -tubulin mRNAs have been observed in this parasite. Three distinct β -tubulin mRNAs have been detected in *L. amazonensis* (Fong et al., 1984) and *L. major* (Coulson et al., 1996), while multiple β -tubulin mRNAs were also found in *L. mexicana* (Burchmore and Landfear, 1998). The identity between amino acid sequences for *L. tarentolae* α -tubulin and α -tubulins from *L. donovani*, *L. major* and *L. infantum* present in available databases is 98% or greater, while the identity between the *L. tarentolae* β -tubulin sequence reported here and the various β -tubulin sequences (from *L. infantum*, *L. braziliensis*, *L. major*, *L. mexicana*, and *L. amazonensis*) is at least 96%. For *L. tarentolae* β -tubulin, this includes 98% identity with *L. major* β -tubulin c β T_{2.8} and over 99% identity with *L. mexicana* β -tubulin A850, the predominant β -tubulin isotypes from amastigotes of these species (Coulson et al., 1996; Bellatin et al., 2002). Based on the high amino acid identity observed between *L. tarentolae* tubulin and tubulins from other *Leishmania* species, the former protein would be expected to be suitable for antimitotic drug screening against this parasite.

Alignments of the *L. tarentolae* α - and β -tubulin amino acid sequences with the corresponding sequences from *L. major* (Ivens et al., 2005), *Trypanosoma brucei* (Berriman et al., 2005), *Trypanosoma cruzi* (El-Sayed et al., 2005), *Plasmodium falciparum* (Holloway et al., 1989), and *Sus scrofa* (pig) (Krauh et al., 1981; Ponstingl et al., 1981) are shown in Figures 3 and 4, respectively. A comparison of these sequences provides some insight into similarities and differences in antimitotic drug susceptibility among these organisms. Compounds that are effective antimitotic agents against *Leishmania* would also be expected to be effective against African trypanosomes, as has been observed for GB-II-5 and GB-II-150 (Werbovetz et al., 2003; Bhattacharya et al., 2004) based on the 94% amino acid identity between the α - and β -tubulin sequences from *T. brucei* and *L. tarentolae*. The lower amino acid sequence identity between *L. tarentolae* and mammalian tubulin (81% and 84% for α - and β -tubulin, respectively) is reflected by differences in susceptibility to antimitotic ligands. Although vinca domain agents and the microtubule stabilizer taxol affect microtubules in mammalian cells and in *Leishmania* (Werbovetz et al., 1999; Havens et al., 2000), mammalian colchicine-site agents have little effect on *Leishmania* (Werbovetz et al., 1999) while kinetoplastid tubulin is much more sensitive to dinitroaniline sulfanilamides than porcine tubulin (Werbovetz et al., 2003; Bhattacharya et al., 2004). Interestingly, kinetoplastid tubulin appears to be nearly as distant from *Plasmodium* tubulin as it is from porcine tubulin. This may help to explain differences in susceptibility to dinitroaniline sulfanilamides between the kinetoplastids and malaria parasites. Oryzalin has been shown to possess greater potency against *Plasmodium*

than against kinetoplastids (compare refs. (Dow et al., 2002; Werbovetz et al., 2003)), while GB-II-5 is approximately 5-fold more active against *L. donovani* axenic amastigotes and about 60-fold more active against *T. brucei* bloodstream forms than against intraerythrocytic *P. falciparum* (K. Werbovetz, unpublished data). Experimental evidence indicates that the binding site for oryzalin is on α -tubulin (Anthony and Hussey, 1999; Morrisette et al., 2004). In the apicomplexan parasite *Toxoplasma gondii*, molecular simulations suggest that the oryzalin binding site consists of α -tubulin residues Arg2, Glu3, Val4, Trp21, Phe24, His28, Ile42, Asp47, Arg64, Cys65, Thr239, Arg243, and Phe244 (Morrisette et al., 2004). Only one of these residues, where Val4 is replaced with Ala, differs between *Toxoplasma* and *Leishmania* α -tubulin. This does not necessarily account for the susceptibility differences between kinetoplastid and apicomplexan tubulin to dinitroanilines, as the amino acid at position 42 differs between *Plasmodium* α -tubulin and all the other tubulins listed in Figure 3 and the amino acid at position 65 differs between the *Leishmania* and *Trypanosoma* α -tubulin. Morrisette *et al.* have suggested that amino acid differences at positions other than the binding site causing allosteric effects are responsible for the selectivity of dinitroanilines for plant and protozoal tubulin (Morrisette et al., 2004). Further structure-activity studies and molecular modeling data are required to develop a binding site model for the dinitroaniline sulfanilamides on kinetoplastid tubulin that will facilitate the design of improved ligands for this important drug target.

3.3. Tubulin-ligand interactions

The amino acid sequences of *L. tarentolae* α - and β -tubulin suggest that this heterodimeric protein would be a suitable alternative to leishmanial tubulin isolated from a pathogenic species for compound screening. This was verified by comparing data generated in assembly assays and fluorescence quenching assays performed with tubulin from *L. amazonensis* and *L. tarentolae*. The IC₅₀ values for GB-II-5, GB-II-150, and oryzalin in the assembly assay with tubulin purified from *L. tarentolae* were $6.8 \pm 1.4 \mu\text{M}$, $5.8 \pm 0.6 \mu\text{M}$, and $>40 \mu\text{M}$, respectively. Previously, the IC₅₀ values for these same compounds were shown to be $6.7 \pm 0.7 \mu\text{M}$, $6.9 \pm 0.0 \mu\text{M}$, and $>40 \mu\text{M}$ against *L. amazonensis* tubulin assembly when assayed under identical assay conditions (Werbovetz et al., 2003; Bhattacharya et al., 2004). Thus, the activities of these compounds against both tubulins are comparable in this assay.

3.4. Fluorescence quenching results

To further investigate the similarity of ligand binding properties between tubulin from *L. tarentolae* and from *L. amazonensis*, we examined the binding affinities of GB-II-5 and oryzalin for the two proteins by employing fluorescence quenching to determine dissociation constants (K_d). The K_d s determined for GB-II-5 and oryzalin against *L. tarentolae* tubulin were $2.4 \pm 0.9 \mu\text{M}$ and $17 \pm 8 \mu\text{M}$, respectively, which were similar to the K_d values of $1.7 \pm 0.4 \mu\text{M}$ and $19 \pm 3 \mu\text{M}$ reported for these compounds against tubulin purified from the pathogenic *L. amazonensis* (Werbovetz et al., 2003).

3.5. Conclusions

The modified purification of leishmanial tubulin described here proved advantageous in that the isolation procedure can be shortened by ~ 1.5 hours without sacrificing yield or purity. Furthermore, by utilizing *L. tarentolae* the overall process is shortened in that the parasites can be grown to high cell densities, reducing the time required to harvest adequate numbers of parasites for a large-scale purification of tubulin. The *L. tarentolae* α - and β -tubulin genes were 97.3% and 97.5% identical to *L. major* respectively. A majority of the nucleotide sequence substitutions are silent in that the α - and β -tubulin protein sequences are 99.6% and 99.4% identical to the corresponding genes from *L. major*, respectively. Fluorescence quenching and assembly inhibition assays further illustrated the interchangeability of *L. tarentolae* tubulin

with the corresponding protein from *L. amazonensis*, as differences in the results between *L. tarentolae* and *L. amazonensis* tubulin for the dinitroaniline sulfanilamides were negligible. Tubulin from *L. tarentolae* is thus suitable for tubulin-ligand assays. Given that the *L. tarentolae* protein can be obtained more cheaply and quickly than tubulin from pathogenic species of *Leishmania*, these findings should be a great benefit to drug discovery efforts targeting this essential parasite protein.

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Abbreviations

DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
EGTA	ethyleneglycol-bis(β -aminoethyl ether)
GB-II-5	<i>N</i> ¹ -phenyl-3,5-dinitro- <i>N</i> ⁴ , <i>N</i> ⁴ -di- <i>n</i> -propylsulfanilamide
GB-II- 150	<i>N</i> ¹ -phenyl-3,5-dinitro- <i>N</i> ⁴ , <i>N</i> ⁴ -di- <i>n</i> -butylsulfanilamide
PBS	phosphate-buffered saline

PME

buffer consisting of 0.1M PIPES (pH 6.9), 1 mM MgCl₂, and 1 mM EGTA

Figure 1.

Structure of oryzalin and its derivatives N^1 -phenyl-3,5-dinitro- N^4,N^4 -di-*n*-propylsulfanilamide (GB-II-5) and N^1 -phenyl-3,5-dinitro- N^4,N^4 -di-*n*-butylsulfanilamide (GB-II-150).

Figure 2.

Assessment of tubulin purity by SDS-PAGE: Lane 1, crude whole cell lysate obtained by sonication (30 μ g); lane 2, cell particulate free supernatant obtained by ultracentrifugation (30 μ g); lane 3, pooled fractions from DEAE chromatography (30 μ g); lane 4, cycled leishmanial tubulin (10 μ g); lane 5, purified porcine brain tubulin (10 μ g); lane 6, cycled leishmanial tubulin (30 μ g).

<i>L. tarentolae</i>	MREAICIHIGAGCQVGNACWELFCLEHGIQPDGSMPSDKICIGVEDDAFNTFFSETGAGK	60
<i>L. major</i>	MREAICIHIGAGCQVGNACWELFCLEHGIQPDGSMPSDKICIGVEDDAFNTFFSETGAGK	60
<i>T. brucei</i>	MREAICIHIGAGCQVGNACWELFCLEHGIQPDGSMPSDKICIGVEDDAFNTFFSETGAGK	60
<i>T. cruzi</i>	MREAICIHIGAGCQVGNACWELFCLEHGIQPDGSMPSDKICIGVEDDAFNTFFSETGAGK	60
<i>P. falciparum</i>	MREVIHSHVGGAGTQVGNACWELFCLEHGIQPDGSMPSDKASRANDDAFNTFFSETGAGK	60
<i>S. scrofia</i>	MRECIHSHVGGAGTQVGNACWELFCLEHGIQPDGSMPSDKICIGGGDSFNTFFSETGAGK	60
<i>L. tarentolae</i>	HVPRCLFLDLEPTVVDEVRTGTGYROLFNPEQLVSGKEDAANNYARGHYTIGKEIVDLALD	120
<i>L. major</i>	HVPRCLFLDLEPTVVDEVRTGTGYROLFNPEQLVSGKEDAANNYARGHYTIGKEIVDLALD	120
<i>T. brucei</i>	HVPRAVFLDLEPTVVDEVRTGTGYROLFNPEQLVSGKEDAANNYARGHYTIGKEIVDLALD	120
<i>T. cruzi</i>	HVPRAVFLDLEPTVVDEVRTGTGYROLFNPEQLVSGKEDAANNYARGHYTIGKEIVDLALD	120
<i>P. falciparum</i>	HVPRCVFVLDLEPTVVDEVRTGTGYROLFNPEQLVSGKEDAANNYARGHYTIGKEIVDLALD	120
<i>S. scrofia</i>	HVPRAVFVLDLEPTVVDEVRTGTGYROLFNPEQLVSGKEDAANNYARGHYTIGKEIVDLALD	120
<i>L. tarentolae</i>	RIRKLDNCTGLOGFMVFAVGGGTGSGLGALLERLSVDYGGKSKLGYTVYPSPOVSTA	180
<i>L. major</i>	RIRKLDNCTGLOGFMVFAVGGGTGSGLGALLERLSVDYGGKSKLGYTVYPSPOVSTA	180
<i>T. brucei</i>	RIRKLDNCTGLOGFMVFAVGGGTGSGLGALLERLSVDYGGKSKLGYTVYPSPOVSTA	180
<i>T. cruzi</i>	RIRKLDNCTGLOGFMVFAVGGGTGSGLGALLERLSVDYGGKSKLGYTVYPSPOVSTA	180
<i>P. falciparum</i>	RIRKLDNCTGLOGFMVFAVGGGTGSGFECTMLERLSVDYGGKSKLNFCCWPSPOVSTA	180
<i>S. scrofia</i>	RIRKLDNCTGLOGFMVFAVGGGTGSGFECTMLERLSVDYGGKSKLNFESHVPSPOVSTA	180
<i>L. tarentolae</i>	VVEPYNVLSLTHSLLEHTDVAITMLDNEAIYDLTRRLDIERPSYTNVNRLLIGOVVSSLTA	240
<i>L. major</i>	VVEPYNVLSLTHSLLEHTDVAITMLDNEAIYDLTRRLDIERPSYTNVNRLLIGOVVSSLTA	240
<i>T. brucei</i>	VVEPYNVLSLTHSLLEHTDVAITMLDNEAIYDLTRRLDIERPSYTNVNRLLIGOVVSSLTA	240
<i>T. cruzi</i>	VVEPYNVLSLTHSLLEHTDVAITMLDNEAIYDLTRRLDIERPSYTNVNRLLIGOVVSSLTA	240
<i>P. falciparum</i>	VVEPYNVLSLTHSLLEHTDVAITMLDNEAIYDLTRRLDIERPSYTNVNRLLIGOVVSSLTA	240
<i>S. scrofia</i>	VVEPYNVLSLTHSLLEHTDVAITMLDNEAIYDLTRRLDIERPSYTNVNRLLIGOVVSSLTA	240
<i>L. tarentolae</i>	SLRFDGALNVDLTFOTNLVYPRIHFLVTSYAPVVSAAEKAYHEQLSVSDITNSVFPFAP	300
<i>L. major</i>	SLRFDGALNVDLTFOTNLVYPRIHFLVTSYAPVVSAAEKAYHEQLSVSDITNSVFPFAP	300
<i>T. brucei</i>	SLRFDGALNVDLTFOTNLVYPRIHFLVTSYAPVVSAAEKAYHEQLSVSDITNSVFPFAP	300
<i>T. cruzi</i>	SLRFDGALNVDLTFOTNLVYPRIHFLVTSYAPVVSAAEKAYHEQLSVSDITNSVFPFAP	300
<i>P. falciparum</i>	SLRFDGALNVDLTFOTNLVYPRIHFLVTSYAPVVSAAEKAYHEQLSVSDITNSVFPFAP	300
<i>S. scrofia</i>	SLRFDGALNVDLTFOTNLVYPRIHFLVTSYAPVVSAAEKAYHEQLSVSDITNSVFPFAP	300
<i>L. tarentolae</i>	MLTKCDPRHGKYMCCCLMYRGDVVPKDVNAATIKTKRTIQFVDMCPTGFKCGINIQPP	360
<i>L. major</i>	MLTKCDPRHGKYMCCCLMYRGDVVPKDVNAATIKTKRTIQFVDMCPTGFKCGINIQPP	360
<i>T. brucei</i>	MLTKCDPRHGKYMCCCLMYRGDVVPKDVNAATIKTKRTIQFVDMCPTGFKCGINIQPP	360
<i>T. cruzi</i>	MLTKCDPRHGKYMCCCLMYRGDVVPKDVNAATIKTKRTIQFVDMCPTGFKCGINIQPP	360
<i>P. falciparum</i>	MMAKCDPRHGKYMCCCLMYRGDVVPKDVNAATIKTKRTIQFVDMCPTGFKCGINIQPP	360
<i>S. scrofia</i>	QMVTKCDPRHGKYMCCCLMYRGDVVPKDVNAATIKTKRTIQFVDMCPTGFKCGINIQPP	360
<i>L. tarentolae</i>	TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEEMEEGFSE	420
<i>L. major</i>	TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEEMEEGFSE	420
<i>T. brucei</i>	TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEEMEEGFSE	420
<i>T. cruzi</i>	TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEEMEEGFSE	420
<i>P. falciparum</i>	TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEEMEEGFSE	420
<i>S. scrofia</i>	TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEEMEEGFSE	420
<i>L. tarentolae</i>	AREDLAALKDYEEVGAEASDDMGEEED--VEEY	451
<i>L. major</i>	AREDLAALKDYEEVGAEASDDMGEEED--VEEY	451
<i>T. brucei</i>	AREDLAALKDYEEVGAEASDDMGEEED--VEEY	451
<i>T. cruzi</i>	AREDLAALKDYEEVGAEASDDMGEEED--VEEY	451
<i>P. falciparum</i>	AREDLAALKDYEEVGAEASDDMGEEED--VEEY	453
<i>S. scrofia</i>	AREDLAALKDYEEVGAEASDDMGEEED--VEEY	451

Figure 3.

Amino acid sequence alignment of *L. tarentolae* α -tubulin (**DQ309032**) with the corresponding sequences from *L. major* (**CAJ02503**), *T. brucei* (**XP_846749**), *T. cruzi* (**XP_802499**), *P. falciparum* (**CAD51722**, tubulin α -I isotype, expressed in both asexual and sexual blood stages), and *S. scrofia* (pig, **P02550**, 99% identical to the human major neural isotype, **ba1**). Identical and conserved amino acids are shaded in black and gray, respectively. Alignments were performed with the aid of the program ClustalW (Chenna et al., 2003).

L. tarentolae MREIVSCQAGCCGQIGSKFWEVISDEHGVDPGTGYQGSDDLQLERINVYFDESSGGRYV 60
L. major MREIVSCQAGCCGQIGSKFWEISDEHGVDPGTGYQGSDDLQLERINVYFDESSAGGRYV 60
T. brucei MREIVCQAGCCGQIGSKFWEISDEHGVDPGTGYQGSDDLQLERINVYFDEATGGRYV 60
T. cruzi MREIVCQAGCCGQIGSKFWEISDEHGVDPGTGYQGSDDLQLERINVYFDEATGGRYV 60
P. falciparum MREIVHIOAGCCGQIGAKFWEISDEHGTDPSCGYQGSDDLQLERVDVYINBATGGRYV 60
S. scrofia MREIVHIOAGCCGQIGAKFWEISDEHGTDPSCGYQGSDDLQLERINVYINBAAGKNRYV 60

L. tarentolae PRAVLMDLEPGTMDSVRAGPYGOLFPRDNFI FGQSGAGNNWAKGHYTEGAELIDSVLLDVC 120
L. major PRAVLMDLEPGTMDSVRAGPYGOLFPRDNFI FGQSGAGNNWAKGHYTEGAELIDSVLLDVC 120
T. brucei PRSVLMDLEPGTMDSVRAGPYGOLFPRDNFI FGQSGAGNNWAKGHYTEGAELIDSVLLDVC 120
T. cruzi PRAVLMDLEPGTMDSVRAGPYGOLFPRDNFI FGQSGAGNNWAKGHYTEGAELIDSVLLDVC 120
P. falciparum PRAVLMDLEPGTMDSVRAGPYGOLFPRDNFI FGQSGAGNNWAKGHYTEGAELIDSVLLDVC 120
S. scrofia PRAVLMDLEPGTMDSVRAGPYGOLFPRDNFI FGQSGAGNNWAKGHYTEGAELIDSVLLDVC 120

L. tarentolae RKEAESCDCLQGFPOLSHSLGGGTGSGMGTLLISKLRREYDPDRIMMTFSVIPSPRVS DTVV 180
L. major RKEAESCDCLQGFPOLSHSLGGGTGSGMGTLLISKLRREYDPDRIMMTFSVIPSPRVS DTVV 180
T. brucei RKEAESCDCLQGFPOLSHSLGGGTGSGMGTLLISKLRREYDPDRIMMTFSVIPSPRVS DTVV 180
T. cruzi RKEAESCDCLQGFPOLSHSLGGGTGSGMGTLLISKLRREYDPDRIMMTFSVIPSPRVS DTVV 180
P. falciparum RKEAESCDCLQGFPOLSHSLGGGTGSGMGTLLISKLRREYDPDRIMMTFSVIPSPRVS DTVV 180
S. scrofia RKEAESCDCLQGFPOLSHSLGGGTGSGMGTLLISKLRREYDPDRIMMTFSVIPSPRVS DTVV 180

L. tarentolae EPNYNTLSVHQLVENSDESMCIDNEALYDIFRFTLKLTPPTFGDLNHLVAAMSGVTCCCL 240
L. major EPNYNTLSVHQLVENSDESMCIDNEALYDIFRFTLKLTPPTFGDLNHLVAAMSGVTCCCL 240
T. brucei EPNYNTLSVHQLVENSDESMCIDNEALYDIFRFTLKLTPPTFGDLNHLVAAMSGVTCCCL 240
T. cruzi EPNYNTLSVHQLVENSDESMCIDNEALYDIFRFTLKLTPPTFGDLNHLVAAMSGVTCCCL 240
P. falciparum EPNYNTLSVHQLVENSDESMCIDNEALYDIFRFTLKLTPPTFGDLNHLVAAMSGVTCCCL 240
S. scrofia EPNYNTLSVHQLVENSDESMCIDNEALYDIFRFTLKLTPPTFGDLNHLVAAMSGVTCCCL 240

L. tarentolae RFPQQLNSDLRLKAVNLVFPRLHFFMMGFAPLTSRGSQOYRGLSVAELTQOMFADAKNMM 300
L. major RFPQQLNSDLRLKAVNLVFPRLHFFMMGFAPLTSRGSQOYRGLSVAELTQOMFADAKNMM 300
T. brucei RFPQQLNSDLRLKAVNLVFPRLHFFMMGFAPLTSRGSQOYRGLSVAELTQOMFADAKNMM 300
T. cruzi RFPQQLNSDLRLKAVNLVFPRLHFFMMGFAPLTSRGSQOYRGLSVAELTQOMFADAKNMM 300
P. falciparum RFPQQLNSDLRLKAVNLVFPRLHFFMMGFAPLTSRGSQOYRGLSVAELTQOMFADAKNMM 300
S. scrofia RFPQQLNSDLRLKAVNLVFPRLHFFMMGFAPLTSRGSQOYRGLSVAELTQOMFADAKNMM 300

L. tarentolae QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVONKNSSYFIEWIPNNIKSSICDIPPKG 360
L. major QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVONKNSSYFIEWIPNNIKSSICDIPPKG 360
T. brucei QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVONKNSSYFIEWIPNNIKSSICDIPPKG 360
T. cruzi QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVONKNSSYFIEWIPNNIKSSICDIPPKG 360
P. falciparum QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVONKNSSYFIEWIPNNIKSSICDIPPKG 360
S. scrofia QAADPRHGRYLTASALFRGRMSTKEVDEQMLNVONKNSSYFIEWIPNNIKSSICDIPPKG 360

L. tarentolae LKMSVTFIGNNTCQEMFRRVGEQFTCMFRKAPLHWYTGEQMDMEFTTEAESNMNDLVS 420
L. major LKMSVTFIGNNTCQEMFRRVGEQFTCMFRKAPLHWYTGEQMDMEFTTEAESNMNDLVS 420
T. brucei LKMSVTFIGNNTCQEMFRRVGEQFTCMFRKAPLHWYTGEQMDMEFTTEAESNMNDLVS 420
T. cruzi LKMSVTFIGNNTCQEMFRRVGEQFTCMFRKAPLHWYTGEQMDMEFTTEAESNMNDLVS 420
P. falciparum LKMSVTFIGNNTCQEMFRRVGEQFTCMFRKAPLHWYTGEQMDMEFTTEAESNMNDLVS 420
S. scrofia LKMSVTFIGNNTCQEMFRRVGEQFTCMFRKAPLHWYTGEQMDMEFTTEAESNMNDLVS 420

L. tarentolae EYQQYQDATVEEAGEYDEEEAY-- 443
L. major EYQQYQDATVEEAGEYDEEEAY-- 443
T. brucei EYQQYQDATVEEAGEYDEEEAY-- 442
T. cruzi EYQQYQDATVEEAGEYDEEEAY-- 442
P. falciparum EYQQYQDATVEEAGEYDEEEAY-- 445
S. scrofia EYQQYQDATVEEAGEYDEEEAY-- 445

Figure 4.

Amino acid sequence alignment of *L. tarentolae* β -tubulin (**DQ309033**) with the corresponding sequences from *L. major* (**CAJ06134**, tubulin β T_{2.2} isotype, the predominant β -tubulin isotype in promastigotes), *T. brucei* (**XP_846748**), *T. cruzi* (**XP_816690**), *P. falciparum* (**NP_700558**), and *S. scrofia* (**P02554**, a class II β -tubulin, the major β -tubulin isotype expressed in neural tissues). Identical and conserved amino acids are denoted as in Figure 3, and alignments were again performed with the aid of ClustalW.

Table 1

Purification of tubulin from *Leishmania tarentolae* promastigotes as described in Section 2.3.

[[Fraction]]	[[Volume (ml)]]	[[Protein conc. (mg/ml)]]	[[Total protein (mg)]]	[[Recovery (%)]]
[[Cell lysate]]	[[135]]	[[11.7]]	[[1580]]	[[100]]
[[Centrifuged supernatant]]	[[85]]	[[14.1]]	[[1200]]	[[75.6]]
[[Pooled DEAE fractions]]	[[9.0]]	[[2.75]]	[[24.8]]	[[1.56]]
[[Cycled tubulin]]	[[1.55]]	[[10.7]]	[[16.5]]	[[1.04]]