Trypanosoma rangeli protein tyrosine phosphatase is associated with the parasite’s flagellum

Elisa Beatriz Prestes¹, Ethel Bayer-Santos¹, Patrícia Hermes Stoco¹, Thaís Cristine Marques Sincero¹, Glauber Wagner¹, Adriana Umaki², Stenio Perdigão Fragoso², Juliano Bordignon², Mário Steindel¹, Edmundo Carlos Grisard¹/²

¹Laboratórios de Protozoologia e de Bioinformática, Departamento de Microbiologia, Imunologia e Parasitologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brasil ²Instituto Carlos Chagas-Fiocruz, Curitiba, PR, Brasil

Protein tyrosine phosphatases (PTPs) play an essential role in the regulation of cell differentiation in pathogenic trypanosomatids. In this study, we describe a PTP expressed by the non-pathogenic protozoan Trypanosoma rangeli (TrPTP2). The gene for this PTP is orthologous to the T. brucei TbPTP1 and Trypanosoma cruzi (TcPTP2) genes. Cloning and expression of the TrPTP2 and TcPTP2 proteins allowed anti-PTP monoclonal antibodies to be generated in BALB/c mice. When expressed by T. rangeli epimastigotes and trypomastigotes, native TrPTP2 is detected as a ~65 kDa protein associated with the parasite’s flagellum. Given that the flagellum is an important structure for cell differentiation in trypanosomatids, the presence of a protein responsible for tyrosine dephosphorylation in the T. rangeli flagellum could represent an interesting mechanism of regulation in this structure.

Key words: Trypanosoma rangeli - protein tyrosine phosphatase - flagellum

Tyrosine phosphorylation is a key mechanism involved in cell regulation and differentiation. In kinetoplastid protozoans, such as trypanosomes, there is evidence that several proteins are phosphorylated on tyrosine residues (Andreeva & Kutuzov 2008). Determination of the TriTryp phosphatome (Brenchley et al. 2007) revealed that the Trypanosoma cruzi, Trypanosoma brucei and Leishmania major protein tyrosine phosphatases (PTPs) comprise 2.3%, 2.6% and 3.4% of the phosphatase complements of these organisms, respectively, in contrast to the 16% observed in humans. In addition, the significant difference between kinetoplast PTPs and their human homolog (33% amino acid identity) suggests a promising role for these enzymes as targets for therapeutic drugs (Brenchley et al. 2007).

Several phosphatases have been described in protozoans, suggesting their role in regulating the development of these organisms. T. brucei PTP (TbPTP1) is associated with the cytoskeleton and has been reported to be intrinsically involved in this parasite’s life cycle (Szoor et al. 2006), as its inactivation through chemical and genetic methods triggered the differentiation of epimastigotes to metacyclic forms. The downstream step in this developmental signalling pathway was determined to involve a DxDxT phosphatase found in T. brucei glycosomes (Szoor et al. 2010). Additionally, a T. cruzi PTP was recently purified and biochemically characterized (Gallo et al. 2011). Inhibition of this PTP using BZ3, a human PTP1b inhibitor, reduced the time required for the differentiation of epimastigotes to metacyclic forms and decreased the infectivity of trypomastigotes in L6 rat skeletal muscle cells by 50% (Gallo et al. 2011).

Considering the importance of PTPs in pathogenic trypanosomes, the present study focuses on the description of a PTP in T. rangeli (TrPTP2), a non-pathogenic Latin American protozoan closely related to T. cruzi. T. rangeli strains are divided into two major lineages that differ in their ability to infect certain Rhodnius species (Vallejo et al. 2002). These lineages are designated KP1(+) and KP1(-) based on the presence/absence of distinct types of kinetoplast DNA (kDNA) minicircles.

The life cycle of T. rangeli requires complex machinery to regulate cell differentiation, in which PTPs must play an important role. The first study on PTPs in T. rangeli showed that there are differences in tyrosine phosphatase activity between the long and short epimastigote forms (Gomes et al. 2006). The importance of this group of proteins for the T. rangeli life cycle was reaffirmed by the finding that inhibition of surface ecto-phosphatases impaired parasite proliferation (Fonseca-de-Souza et al. 2009). Additionally, ecto-phosphatase activity appears to be modulated by hydrogen peroxide (Cosentino-Gomes et al. 2009), which may be produced by the parasite, as well as by the concentration of inorganic phosphates in the medium (Dick et al. 2010). Although these studies have evaluated total ecto-phosphatase activity, the inhibitors used suggest the presence of PTPs among the surface phosphatases.

The aim of this study was to characterize the T. rangeli PTP gene by assessing both its intra and interspecific variability and to comparatively evaluate the levels and sites of TrPTP2 protein expression in different forms and strains of this parasite.
MATERIALS AND METHODS

Parasites - The T. cruzi Y strain and 17 T. rangeli strains from different hosts and geographical origins were used in this study (Table). The epimastigote forms were cultivated by weekly passages at 27°C in liver infusion tryptose medium supplemented with 10% foetal bovine serum after a cyclic mouse-triatomine-mouse passage. T. rangeli trypanomastigotes were obtained through in vitro differentiation (Koerich et al. 2002) and T. cruzi trypanomastigotes were obtained from the supernatant of infected Vero cells (Eger-Mangrich et al. 2001).

Nucleic acid extraction and gene amplification - Total DNA and RNA were extracted (Sambrook & Russell 2001) from the T. cruzi Y strain and 17 T. rangeli strains isolated from different hosts and geographical origins (Table). Different primer sets based on previously obtained T. rangeli cDNA sequences (Grisard et al. 2010) were designed for amplification and sequencing of the complete TrPTP2 coding region, as well as the 3' and 5' UTRs (Supplementary data).

Sequence assembly and phylogenetic analyses - The obtained amplification products were sequenced using the Megabace 1000® DNA Analysis System with the DYEnamic ET terminator kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Clusters were assembled with the Phred/Phrap/Consed® package and aligned using CLUSTALW software. Sequences of T. cruzi (GenBank XM_816368), T. brucei (GenBank XM_817818.1), Trypanosoma vivax (TriTrypDB TvY486_1006690) and Trypanosoma congoense (TriTrypDB TcIL3000.10.5750) PTP genes were retrieved from the GenBank or TriTrypDB database for comparative analysis. To construct a phylogenetic tree, the sequences were analysed with bootstrapped maximum parsimony and neighbour-joining methods using complete deletion and the Kimura-2 parameters in MEGA software version 4.0.2. The predicted TrPTP2 protein sequence was analysed using InterPro.

Southern and northern blotting - Southern and northern blot analyses were performed according to standard protocols (Sambrook & Russell 2001). A peroxidase-labelled probe was prepared using an 821 bp polymerase chain reaction fragment and the ECL Direct Nucleic Acid Labelling and Detection System (GE Healthcare).

Recombinant proteins and mass spectrometry (MS/MS) analysis - Recombinant His-tagged TrPTP2 and Trypanosoma cruzi TcPTP2 were produced in Escherichia coli BL21(DE3) cells transformed with the pET-14b vector (Novagen) containing the coding region from either TrPTP2 from the T. rangeli Choachi strain or TcPTP2 from the T. cruzi Y strain. Expression was induced by treatment with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C for 3 h and the recombinant proteins were purified through electroelution (4 h at 70 V) of the gel-excised proteins following dialysis in a buffer containing 25 mM Tris, 250 mM glycine and 0.1% sodium dodecyl sulfate (SDS) and assessment via SDS-polyacrylamide gel electrophoresis (PAGE). Mass spectrometry analysis (On-line Nano-LC MS-MS/MS) of gel-excised recombinant TrPTP2 was carried out with the NanoAcquity System (Waters Corporation) coupled to the LTQ Orbitrap Velos nanospray (Thermo-Scientific) using an established protocol (Williamson et al. 2010).

TABLE

Trypanosoma rangeli strains used in this study, their original geographical region and hosts, kDNA group classification according to presence (+) or absence (-) of KP1 minicircle and their respective GenBank accessions for TrPTP2 gene sequences

<table>
<thead>
<tr>
<th>Strain</th>
<th>Original host</th>
<th>Geographical origin</th>
<th>KP1 grouping</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC-58</td>
<td>Echimys dasythrix</td>
<td>Brazil</td>
<td>-</td>
<td>EU325665</td>
</tr>
<tr>
<td>SC-61</td>
<td>E. dasythrix</td>
<td>Brazil</td>
<td>-</td>
<td>EU325657</td>
</tr>
<tr>
<td>SC-76</td>
<td>Panstrongylus megistus</td>
<td>Brazil</td>
<td>-</td>
<td>EU325660</td>
</tr>
<tr>
<td>PII10</td>
<td>P. megistus</td>
<td>Brazil</td>
<td>-</td>
<td>EU325656</td>
</tr>
<tr>
<td>C23</td>
<td>Aotus sp.</td>
<td>Colombia</td>
<td>-</td>
<td>EU325667</td>
</tr>
<tr>
<td>S048</td>
<td>Homo sapiens</td>
<td>Colombia</td>
<td>-</td>
<td>EU325666</td>
</tr>
<tr>
<td>TRE</td>
<td>Not determined</td>
<td>Colombia</td>
<td>-</td>
<td>EU325668</td>
</tr>
<tr>
<td>B450</td>
<td>Rhodnius sp.</td>
<td>Brazil</td>
<td>+</td>
<td>EU325654</td>
</tr>
<tr>
<td>R1625</td>
<td>Homo sapiens</td>
<td>El Salvador</td>
<td>+</td>
<td>EU325664</td>
</tr>
<tr>
<td>H9</td>
<td>H. sapiens</td>
<td>Honduras</td>
<td>+</td>
<td>EU325662</td>
</tr>
<tr>
<td>H14</td>
<td>H. sapiens</td>
<td>Honduras</td>
<td>+</td>
<td>EU325658</td>
</tr>
<tr>
<td>Macias</td>
<td>H. sapiens</td>
<td>Venezuela</td>
<td>+</td>
<td>EU325659</td>
</tr>
<tr>
<td>Palma-2</td>
<td>Rhodnius prolirus</td>
<td>Venezuela</td>
<td>+</td>
<td>EU325663</td>
</tr>
<tr>
<td>Choachi</td>
<td>R. prolirus</td>
<td>Colombia</td>
<td>+</td>
<td>EU325653</td>
</tr>
<tr>
<td>D3493</td>
<td>R. prolirus</td>
<td>Colombia</td>
<td>+</td>
<td>EU325655</td>
</tr>
<tr>
<td>San Agustín</td>
<td>H. sapiens</td>
<td>Colombia</td>
<td>+</td>
<td>EU325661</td>
</tr>
<tr>
<td>1545</td>
<td>R. prolirus</td>
<td>Colombia</td>
<td>+</td>
<td>EU325652</td>
</tr>
</tbody>
</table>
with slight modifications. The m/z spectrum was processed using Mascot Distiller and Mascot Software (Matrix Science) was employed to perform database searches among T. rangeli expressed sequence tags (Grisard et al. 2010) and predicted open reading frames (ORFs).

Monoclonal antibody (mAb) production - mAbs were obtained through immunization of BALB/c mice with TrPTP2 (50 μg) according to an established protocol (Mazzarotto et al. 2009). Clones were selected based on exclusive recognition of recombinant and/or native TrPTP2.

Western blotting - Parasite protein extracts were obtained by lysis in an appropriate buffer (50 mM NaCl, 200 mM Tris-HCl pH 8.0, 1% Triton X-100) at 80°C. NP-40 fractionation was carried out according to a previously described protocol (Woods et al. 1989), with ethylene glycolbis(beta-aminoethyl ether)-N,N,N',N'-tetra acetic acid substituted for ethylene-diamine tetraacetic acid to enable concentration of proteins from the cytoskeletal fraction. After resolution in SDS-PAGE gels, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) in an appropriate buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) using a blotting transfer unit (Bio-Rad). After blocking with non-fat milk, the membranes were incubated for 90 min with anti-TrPTP2 or anti-TcPTP2 polyclonal antisera (1:250), anti-PTP2 mAbs (1:2), an anti-His-Tag mAb (Sigma-Al drich, 1:12,000) or an anti-a-tubulin mAb (1:10,000), the last of which was used as a loading control.

Immunofluorescence assays - Indirect immunofluorescence assays were carried out as described previously (Stoco et al. 2012) in T. rangeli epimastigotes and trypomastigotes. Anti-PTP2 mAbs (no dilution) and a mAb directed against the flagellar calcium-binding protein (FCaBP) (1:50) (Schenkman et al. 1991) were used as primary antibodies, with anti-mouse IgG antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes; diluted 1:1,000) being used as secondary antibodies and 4',6-diamidino-2-phenylindole was added to a final concentration of 1 μg/mL. The slides were examined using a Leica TCS SP5 confocal microscope (Leica Microsystems).

Ethics - All procedures involving animals were previously approved by the Ethical Committee on Animal Use of Federal University of Santa Catarina (reference 23080.025618/2009-81).

RESULTS

The TrPTP2 gene has a 987 bp ORF, with a predicted polypeptide product of ~36 kDa, which is approximately the same as the predicted size of TcPTP2. The TrPTP2 sequences from all strains were of high quality (Phred ≥ 60) and are available in the GenBank database under the accessions listed in Table.

The same tree topology was obtained from the phylogenetic analyses using nucleotide sequences regardless of the methodology employed, illustrating that TrPTP2 is useful as a marker for interspecific characterization, but lacks the appropriate bootstrap support for intraspecific characterization (Fig. 1). Interestingly, the KPI(-) strains from Brazil were separated from the Colombian KPI(-) strains, which were grouped with KPI(+), indicating a reduced, but clear intraspecific variability between the KPI(-) lineages.

The complete TrPTP2 gene sequence was obtained for the 17 T. rangeli strains and nucleotide alignment revealed several substitutions within the coding region, as well as in the 5’ and 3’ UTRs (data not shown). A comparison of the PTP nucleotide sequences from T. rangeli KPI(+) with KPI(-) showed 23 substitutions. The Colombian KPI(-) strains differed from the KPI(+) strains and from the Brazilian KPI(-) strains in three exclusive nucleotide substitutions. However, the identity between the nucleotide sequences from all strains was still close to 99%.

The predicted TrPTP2 protein sequence showed a single PTP domain between amino acids 238 and 247, similar to what is observed for the TriTryps PTPs. Comparisons with orthologous sequences from other trypanosomatid species showed high intra and interspecific conservation (Supplementary data). The amino acid sequence identities between TrPTP2 and its orthologs are as follows: TcPTP2, 73%, T. brucei TrPTP1, 59%, T. congolense, 58%, T. vivax, 57%, and L. major, 37%.

Southern blot analysis revealed that TrPTP2 is present as a single copy gene in the genome of the T. rangeli Choachí strain (Fig. 2A, B), which is in agreement with the number of copies of orthologous PTP genes observed in other kinetoplastid species. Northern blot assays showed that TrPTP2 encodes a ~1.5 kb mRNA transcript in both the epimastigote and trypomastigote forms of the T. rangeli Choachí strain (Fig. 2C).

Heterologous expression of both TrPTP2 and TcPTP2 using the pET-14b vector produced a protein of approximately 36 kDa in the insoluble fraction of the lysate, corresponding to the theoretically expected size for these

Fig. 1: Dendogram analysis based on the Trypanosoma rangeli protein tyrosine phosphatase (TrPTP2) gene. A neighbour-joining (1,000 replicates) based-dendogram derived from the analysis of the TrPTP2 gene and its orthologous sequences from distinct trypanosome species. Branches with bootstrap values < 50 are not shown.
proteins (data not shown). Mass spectrometry analysis of the recombinant TrPTP2 covered more than 50% of its peptides and confirmed its identity (data not shown).

Immunization of BALB/c mice with the purified recombinant proteins resulted in the production of anti-TrPTP2 and anti-TcPTP2 polyclonal antisera. The anti-TrPTP2 antiserum recognized a ~65 kDa protein in T. rangeli extracts, but not in T. cruzi extracts, whereas the anti-TcPTP2 antiserum recognized both TcPTP2 in T. cruzi extracts and TrPTP2 in the T. rangeli extracts (data not shown). Based on the cross-reactivity obtained using the polyclonal antisera, as well as the identification of a protein that was larger (~65 kDa) than predicted, anti-PTP2 mAbs were produced and selected based on the recognition of both recombinant and native TrPTP2 in T. rangeli extracts.

The anti-PTP2 mAbs showed a strong signal when tested against the recombinant TrPTP2 and specifically recognized the ~65 kDa protein in both epimastigotes and trypomastigotes from the T. rangeli Choachi strain (Fig. 3A). Similar to TbPTP1 in T. brucei, TrPTP2 is detected in the cytoskeletal fraction in T. rangeli (Fig. 3B). Remarkably, mAbs preincubated twice with recombinant TrPTP2 embedded in PVDF membranes could no longer recognize the ~65 kDa protein in T. rangeli protein extracts (data not shown). Although TcPTP2 is predicted to be 73% identical to TrPTP2, native TcPTP2 was not detected in T. cruzi by the mAbs generated in this study. This absence of cross-reactivity could be due to the few differences in the amino acid compositions of TrPTP2 and TcPTP2, as shown in the supplementary data.

The observed ~65 kDa protein is almost 30 kDa heavier than the predicted mass of 36 kDa for both TrPTP2 and TcPTP2. This unexpected increase in mass was investigated in several different ways. Although glycosylation sites have been predicted within the amino acid sequence of TrPTP2, analyses using staining with Schiff’s reagent did not detect aldehydes in the region corresponding to the ~65 kDa protein in the T. rangeli extracts (data not shown), suggesting a lack of significant glycosylation sites within this region. Because dimer formation could also explain the ~30 kDa increase in mass, T. rangeli extracts were resolved via PAGE using 8 M urea as a denaturing agent. This process produced the same ~65 kDa protein (Fig. 3C). Thus, if TrPTP2 forms dimers, they are very stable and resistant to strong denaturing conditions.

Staining with anti-PTP2 mAbs revealed a strong signal in the T. rangeli flagellum in both epimastigotes and trypomastigotes that overlaps with the signal from anti-FCaBP mAb, suggesting that TrPTP2 is concentrated in
this structure and co-localizes with a flagellar protein (Fig. 4). In epimastigotes, there was also a weak signal detected in the cell body that was not observed in trypomastigotes. In T. cruzi, the staining with the mAbs was observed to be subtle and diffuse.

DISCUSSION

Given that PTPs from pathogenic trypanosomatids, such as T. brucei and T. cruzi, have been reported to play a role in the parasites’ in vitro differentiation into infective forms (Szoor et al. 2006) and mammalian host cell infectivity (Gallo et al. 2011), finding a highly similar PTP in a non-virulent species like T. rangeli is intriguing. The TrPTP2 gene is present in a single copy in this parasite’s genome and encodes a ~1.5 kb transcript, which is in accordance with a former transcriptome analysis (Grisard et al. 2010). The TrPTP2 gene proved to be a good phylogenetic marker to differentiate T. rangeli from other trypanosomes, but did not exhibit high intraspecificity, despite the variability between the KP1(+) and KP1(-) strains, as well as among the KP1(-) strains.

The TrPTP2 transcript is close in size to the T. brucei transcript (~1.8 kb) (Szoor et al. 2006), predicting a polypeptide product with a size of approximately 36 kDa. This predicted molecular mass is almost identical for both TrPTP2 and TcPTP2 and is close to the mass reported for the homologous TbPTP1 protein (~34 kDa) (Szoor et al. 2006). In contrast to the recombinant forms of TpPTP2 and TcPTP2 produced in this study, which were detected in the insoluble fraction of the lysate, Gallo et al. (2011) expressed a recombinant, active 36 kDa T. cruzi PTP in the soluble fraction, which is consistent with the similar sizes of T. rangeli and T. cruzi PTP2 obtained herein.

While the molecular mass of ~65 kDa detected for PTP2 in T. rangeli is similar to a 55-60 kDa L. major PTP (Aguirre-Garcia et al. 2006), the increase in mass compared to the predicted 36 kDa protein was unexpected and was investigated in several different ways. Post-translational modifications, such as glycosylations, have been predicted for TrPTP2 and may dramatically increase protein mass (Sakurai et al. 2008). Formation of dimers could also account for the detection of a protein that was heavier than expected; however, no specific antibody signal near 36 kDa (the expected mass for TrPTP2) was detectable under strong denaturing conditions. We cannot rule out the possibility of covalent oligomerization within the TrPTP2 product itself or with another protein, but the results obtained from urea denaturation suggest that if TrPTP2 forms dimers, they are very stable. A previous study in T. cruzi (D’Orso & Frasch 2001) detected a difference of 20 kDa between the deduced and obtained mass of the TcUBP-1 protein and parasite transfection was employed to confirm that this increase in mass occurred naturally, possibly due to oligomerization or post-translational modifications. A similar situation might involve TrPTP2, which could be post-translationally modified through phosphorylation or methylation, both of which are predicted to occur within the TrPTP2 sequence. Additionally, the anomalous electrophoretic migration observed might be due to the estimated high pI of TrPTP2 (8.95) (Chiou & Wu 1999).

The detection of TrPTP2 in the T. rangeli flagellum was a surprising and interesting result obtained in this study. Given that the flagellum in eukaryotic cells is constantly modulated by phosphorylation, it is very likely that dephosphorylation and thus phosphatases play an

Fig. 4: Trypanosoma rangeli protein tyrosine phosphatase (TrPTP2) is associated with the T. rangeli flagellum. TrPTP2 concentrates in the flagellum and co-localizes with the flagellum calcium-binding protein (FCaBP) in T. rangeli epimastigotes and trypomastigotes as revealed by immunofluorescence assays using anti-PTP2 monoclonal antibodies and anti-FCaBP monoclonal antibodies. Parasites were counter-stained with 4',6-diamidino-2-phenylindole (DAPI). White bars represent 5 µm. mAb: monoclonal antibody.
important role in this structure. It has been shown that phosphorylation of axonemal proteins is crucial for regulating flagellar motility, independent of cyclic AMP, in response to an increase in pH (Nakajima et al. 2005). Interestingly, the in vitro differentiation of \textit{T. rangeli} is successful when the culture pH is 8.0, but is drastically impaired when it is 6.0 or 7.0 (Koerich et al. 2002).

Although \textit{TbPTP1} is mainly found in the \textit{T. brucei} cytoskeletal fraction, a specific association with the flagellum was not detected (Szoor et al. 2006). However, a mAb directed against phosphotyrosine allowed the identification and cytolocalization of tyrosine-phosphorylated proteins in \textit{T. brucei} (Nett et al. 2009), which were found to be concentrated in the nucleolus region, the flagellum basal body and the flagellum itself. The recognition signal detected throughout the parasite's flagellum disappears in the distal extremity, suggesting that tyrosine-phosphorylated proteins could be directly associated with the axoneme, either adjacent to it or connected to membranous structures near the flagellum (Nett et al. 2009). With the exception of the basal body, the recognition signal for \textit{TbPTP2} observed in \textit{T. rangeli} epimastigotes and trypomastigotes precisely follows the same distribution pattern throughout the flagellum.

Given that the \textit{T. brucei} flagellum contains proteins modulated by specific phosphorylation on tyrosine residues, it is likely that these proteins may also be found in phylogenetically related organisms, such as \textit{T. rangeli}, \textit{T. cruzi} and \textit{Leishmania} species. Therefore, the presence of \textit{TbPTP2} in the flagellum is quite intriguing, as concentration of proteins regulated by tyrosine phosphorylation most likely requires tyrosine kinases as well as tyrosine phosphatase enzyme activity.

The mechanisms involved in adhesion to a substrate in \textit{T. brucei} (Ersfeld & Gull 2001) \textit{T. cruzi} (Ferreira et al. 2008) and \textit{T. rangeli} (Meirelles et al. 2005) require the crucial and active participation of the flagella of these parasites. During metacyclogenesis, the flagellar membrane remains connected to the epithelial surface (Ersfeld & Gull 2001) representing an important interface between the parasite and its host that is essential for the fulfilment of its biological cycle. The presence of proteins such as PTPs in the flagellum reinforces the role of this structure in both cell differentiation and cell cycle regulation.

ACKNOWLEDGEMENTS

To Dr. Sergio Schenkman, for the anti-FCaBP antibody, to Dr Claudia Nunes Duarte dos Santos, for providing facilities for mAbs production, and to Dr Hercules Moura, for the MS/MS analysis.

REFERENCES


