The toxic effect of *Vu-Defr*, a defensin from *Vigna unguiculata* seeds, on *Leishmania amazonensis* is associated with reactive oxygen species production, mitochondrial dysfunction and plasma membrane perturbation

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Abstract: Plant defensins are plant antimicrobial peptides that present diverse biological activities *in vitro*, including the elimination of *Leishmania amazonensis*. Plant defensins are considered promising candidates for the development of new drugs. This protozoan genus has great epidemiological importance and the mechanism behind the protozoan death by defensins is unknown, thus, we chose *L. amazonensis* for this study. The aim of the work was to analyze the possible toxic mechanisms of *Vu*-Defr against *L. amazonensis*. For analyses, the antimicrobial assay was repeated as previously described, and after 24 h, an aliquot of the culture was tested for viability, membrane perturbation, mitochondrial membrane potential, reactive oxygen species and nitric oxide inductions. The results of these analyses indicated that after interaction with *L. amazonensis*, the *Vu*-Defr causes elimination of promastigotes from culture, membrane perturbation, mitochondrial membrane collapse and reactive oxygen species induction. Our analysis demonstrated that NO is not produced after *Vu*-Defr and *L. amazonensis* interaction. In conclusion, our work strives to help to fill the gap relating to effects caused by plant defensins on protozoan and thus better understand the mechanism of action of this peptide against *L. amazonensis*.

Keywords: antimicrobial peptide; mechanism of action; cowpea; protozoan

Introduction

All existing organisms depend on an ancient defense strategy, in which the front line is the production of antimicrobial peptides (AMPs) (Ageitos et al. 2017). These molecules have been abundantly described as participating in plant defence against microbial attack (Tam et al. 2015). Among the plant AMPs, the family of plant defensins stands out. Plant defensins are peptides that have a primary structure encompassing from 45 to 54 amino acid residues and are rich in basic amino acid residues, which become positively charged at physiological pH (Carvalho and Gomes 2011). This plant peptide family is also rich in cysteine residues, which are extremely well conserved in the primary structure and bound to each other forming disulfide bridges (Carvalho and Gomes 2011). Plant defensins commonly present eight cysteine residues and four disulfide bridges (Carvalho and Gomes 2011), with the exception of *PhD₁* (*Petunia hybrida* defensin 1), which contains five disulfide bridges (Bonds et al. 2003). These bridges stabilize the tri-dimensional structure composed of one α -helix and three antiparallel β -sheets (Carvalho and Gomes 2011), with some exceptions such as *VrD₁* (*Vigna radiata* defensin 1), which had a 3_{10} helix between the first β -strand and the α -helix (Liu et al. 2006), and *Sd₅* (sugarcane defensin 5), which possesses an unstructured C-terminal extension (Paula et al. 2011). The disulfide bridges of plant defensins ensure them high stability, as demonstrated by their exposure to harsh conditions such as high temperatures (Osborn et al. 1995), extreme pH values, proteases and mammal serum (Wong and Ng 2005, Beer and Vivier 2008, Tavares et al. 2008b), after which their biological activity remains.

Plant defensins present diverse biological activities such as the antimicrobial activity *in vitro* (Carvalho and Gomes 2011). In addition, other compelling evidence such as the expression of plant defensin genes in response to microbial infection (Terras et al. 1995, Penninckx et al. 1996), positive correlation of the *in vivo* concentration of plant defensins and the antimicrobial activity observed *in vitro* (Terras et al. 1995) and the production of transgenic plants resistant to fungal pathogens (Abdallah et al. 2010, Portieles et al. 2010) and insects (Choi et al. 2009) clearly relate the function of plant defensins with plant defense *in vivo*.

A few plant defensins have been tested against protozoan as follows: *PTH₁*, a defensin from *Solanum tuberosum*, was tested against *Leishmania donovani* (Berrocal-Lobo et al. 2009), *Vu-Def*, a defensin from *Vigna unguiculata*, was tested against *Leishmania amazonensis* (Souza et al. 2013) and *PvD1*, a defensin from *Phaseolus vulgaris*, was tested against *L. amazonensis* (Nascimento et al. 2015).

Therefore, *L. amazonensis* was chosen for this study because this protozoan genus has great epidemiological importance (Pace 2014), the disease caused by this protozoan genus requires new drugs (Akbari et al. 2017) and the mechanism behind the protozoan death and elimination by this peptide is unknown. Additionally, plant defensins are considered promising candidates for the development of new drugs (Thevissen et al. 2007) as indicated by the injection of the plant defensins *Rs*-AFP₂ (from *Raphanus sativus* seeds) in mouse model of candidiasis which did not cause toxicity to the mice and had a prophylactic effect (Tavares et al. 2008a). In this sense we had used the defensin from *V. unguiculata* in this study. In a previous work, we have demonstrated the inhibitory activity of *Vu*-Def against *L. amazonensis* (Souza et al. 2013). In this work, we continued that study, analyzing possible toxic mechanisms imposed by the interaction of *Vu*-Def with *L. amazonensis*.

Materials and methods

Sequence of the recombinant *Vu*-Defr

The sequence of the recombinant *Vu*-Defr was obtained in Carvalho et al. (2006). To the amino acid sequence of the recombinant *Vu*-Defr was added a methionine in the first position because this amino acid residue is added to the sequence of the recombinant *Vu*-Defr as a cloning requirement, as described by Santos et al. (2010). The biochemical of the recombinant *Vu*-Defr we calculated by the ProtParam features (isoelectric point, charge at pH 7.0 and molecular weight) at ExPASy and Peptide Property Calculator (hydrophobicity at pH 6.8) at <http://www.biosyn.com>.

Escherichia coli strain

Escherichia coli strain Rosetta-gami₂ (DE₃) pLysS [genotype: $\Delta ara-leu7697 \Delta lacX74 \Delta phoA PvuII phoR araD139 ahpC galE galK rpsL$ (DE₃) F'⁺[*lac*⁺ *lacI*^f *pro*] *gor522::Tn10 trxB* pLysSRARE2 (Cam^R, Str^R, Tet^R)] containing the construct pET-Def (pET-32 EK/LIC vector fused with the coding sequence of the defensin from *Vigna unguiculata* L. Walp.) (Santos et al. 2010) was used for the recombinant production of *Vu*-Defr (recombinant defensin from *V. unguiculata*) (Souza et al. 2013). Luria-Bertani (LB, 0.5% yeast extract, 1% tryptone, 1% NaCl) and Terrific Broth (TB, 2.4% yeast extract, 1.2% tryptone, 0.45% glycerol, 1.3% K₂HPO₄, 0.38% KH₂PO₄) were used as culture and

expression media, respectively. Both were supplemented with ampicillin ($50 \mu\text{g}\cdot\text{mL}^{-1}$) (Sigma-Aldrich) and chloramphenicol ($34 \mu\text{g}\cdot\text{mL}^{-1}$) (Sigma-Aldrich).

***Leishmania amazonensis* strain**

Leishmania amazonensis, LV 79 strain, at the promastigote stage, was supplied by Laboratório de Biologia Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil. The protozoan was cultivated in 5 mL of Warren's medium (90% brain heart broth (Fluka) containing 10% heat-inactivated foetal bovine serum (Nutricell)), enriched with 0.01% folic acid (Sigma-Aldrich) and 0.4% hemin (Sigma-Aldrich) at 28 °C with the transfer of 1 mL to new medium every three days.

Expression and purification of recombinant *Vu*-Defr

The production and purification of the *Vu*-Defr was performed as described by Souza et al. (2013).

Growth inhibition assay on *L. amazonensis* promastigotes treated with *Vu*-Defr

The growth inhibition assay on *L. amazonensis* was performed as described by Souza et al. (2013). At the time of 24 h, samples were used for fluorescence analyses and after 48 h samples were tested for viability.

Viability assay of *L. amazonensis* promastigotes treated with *Vu*-Defr

At the end of the growth inhibition time of 48 h the control and treated samples were centrifuged for 10 min, 1.700 rpm and 21 °C. The harvested *L. amazonensis* cells were washed in Warren's medium and incubated with new Warren's medium at 28 °C for 24 h without the addition of *Vu*-Defr. The number of parasite was determined as described by Souza et al. (2013).

Plasma membrane integrity assay of *L. amazonensis* cells treated with *Vu*-Defr by Sytox green stain

Sytox green dye is a green-fluorescent stain that has high-affinity for nucleic acids and does not cross intact membranes but penetrates cells with compromised plasma membranes (Sytox green nucleic

149 acid stain instruction manual, Thermo Fisher), making this stain an indicator of compromised plasma
150 membranes.

151 For this analysis, after 24 h of the growth inhibition assay on *L. amazonensis* promastigotes
152 treated with *Vu*-Defr, an aliquot of 120 µL of each control and treated sample was removed. Each sample
153 was separately centrifuged for 10 min at 500 x g at room temperature, and then, 80 µL of supernatant was
154 discarded. The remaining 40 µL was used to gently suspend the protozoan cells, and then, they were
155 incubated with 7 µL of Sytox green dye (diluted in DMSO to a final concentration of 0.2 µM) for 30 min
156 at room temperature, protected from light and with slight shaking as described by Nascimento et al.
157 (2015).

158 The cells were observed with an Axioplan optical microscope (Zeiss) coupled with a DP72
159 digital camera (Olympus), which was equipped with a set of fluorescence detection filters (excitation:
160 450-490 nm; emission: 500 nm). The *Soft Imaging System Cell* program was used to visualize and acquire
161 the images. All fluorescence images were captured at the same exposure time.

162 The glass slides used in this assay were treated with 0.1% poly-L-lysine and dried in an oven at
163 37 °C for 24 h.

165 **Mitochondrial membrane potential assay of *L. amazonensis* cells treated with *Vu*-Defr by** 166 **rhodamine 123 dye**

167 Rhodamine 123 is a cell diffusible fluorescent dye that is sequestered by active mitochondria
168 without cytotoxic effects (rhodamine 123 instruction manual, Thermo Fisher). Therefore, using this dye,
169 it is possible to evaluate the mitochondrial membrane potential. This assay was performed as described in
170 section **Plasma membrane integrity assay of *L. amazonensis* cells treated with *Vu*-Defr by Sytox**
171 **green stain** with the following modifications: the final probe concentration was 20 µM and detection
172 filters were for excitation at 488 nm and emission at 550 nm.

173

174 **ROS and NO induction assays in *L. amazonensis* cells treated with *Vu*-Defr**

175 The detection of the endogenous production of reactive oxygen species (ROS) in cells
176 of *L. amazonensis* treated with *Vu*-Defr was performed using 2,7-dichlorofluorescein diacetate
177 fluorescent dye (H₂DCFDA, Thermo Fisher) as described by Aerts et al. (2007) with

178 modifications. H₂DCFDA is a reduced nonfluorescent form of fluorescein, which after cleavage
179 of the acetate group by intracellular esterases and oxidation by ROS, becomes fluorescent. This
180 assay was performed as described in section **Plasma membrane integrity assay of *L. amazonensis***
181 **cells treated with *Vu*-Defr by Sytox green stain** with the following modifications: the final
182 concentration of the probe was 20 µM and the incubation time was 2 h.

183 The same procedure of ROS was performed for the detection of nitric oxide (NO) using 3-
184 amino-4-aminomethyl-2,7-dichlorofluorescein diacetate (DAF-FM-DA, Thermo Fisher) at 20 µM. This
185 dye is cell permeant and freely diffuses across cell membranes, and upon reaching the intracellular milieu,
186 it is deacetylated by intracellular esterases becoming DAF-FM. DAF-FM is essentially nonfluorescent
187 and becomes fluorescent when it reacts with NO.

188

189 **Statistical analysis**

190 All assays were performed in triplicate and repeated three times. Statistical analysis were
191 performed by Tukey's test using Prism software (version 5.0).

192

193 **Results**

194

195 ***Vu*-Defr amino acid sequence**

196 The *Vu*-Defr presents 48 amino acid residues (Figure 1A), being the first amino acid residue a
197 methionine which was added to the sequence of *Vu*-Defr was a requirement of cloning in the pET-32
198 EK/LIC vector (Santos et al., 2010). The 48 amino acid residues of *Vu*-Defr confer a molecular mass of
199 5544.26 Da, a charge at pH 7.0 of +1 and an isoelectric point of 7.54 (Fig. 1B).

200

201 **Growth inhibition and viability**

202 In order to we had *L. amazonensis* cells to do the viability assay, a growth inhibition assay was
203 repeated as described in Souza et al. (2013) and showed similar results, *i.e.* at 48 h of incubation, *L.*
204 *amazonensis* culture was inhibited at 56% (Fig. 2A) and in the previous work the inhibition was 46.9%.
205 At this time, *L. amazonensis* cells of control and *Vu*-Defr treated samples were harvested and seeded in a
206 new medium without *Vu*-Defr and allow to growth for 24 h. The result indicated that *Vu*-Defr treated *L.*

207 *amazonensis* cells were able to regrown (Fig. 2B). Therefore, the concentration 18.5 μ M of *Vu*-Defr is
208 lethal to the majority of *L. amazonensis* cells from culture and the remaining live cells (3.5%) were able to
209 growth, causing a 96.4% of inhibition.

210

211 **Plasma membrane integrity assay**

212 *L. amazonensis* cells treated with 18.5 μ M of *Vu*-Defr for 24 h were observed using an optical
213 microscope in the presence of the dye Sytox green and showed fluorescent parasites, indicating that
214 protozoan plasma membranes were compromised (Fig. 3). Protozoan cells treated with *Vu*-Defr were also
215 rounded (Fig. 3, 4 and 5, white boxes indicate the inserts).

216

217 **ROS and NO induction assays in *L. amazonensis* cells treated with *Vu*-Defr**

218 *L. amazonensis* cells treated with 18.5 μ M *Vu*-Defr for 24 h were observed using an optical
219 microscope in the presence of the fluorescent probe H₂DCFDA, which is an indicator for ROS in cells. *L.*
220 *amazonensis* cells in contact with *Vu*-Defr for 24 h present a green fluorescence stain, indicating that ROS
221 were induced in the treated cells (Fig. 4). No fluorescent cells were observed among *L. amazonensis* cells
222 treated with *Vu*-Defr in the presence of the fluorescent probe DAF-FM-DA, indicating that NO is not
223 involved in the toxic effect of *Vu*-Defr on *L. amazonensis* (Results not shown).

224

225 **Mitochondrial membrane potential assay of *L. amazonensis* cells treated with *Vu*-Defr by** 226 **rhodamine 123 dye**

227 *L. amazonensis* cells treated with 18.5 μ M *Vu*-Defr for 24 h were observed using an optical microscope
228 in the presence of the dye rhodamine 123 and showed weakly fluorescent parasites, indicating that, in the
229 presence of *Vu*-Defr, a decrease of energized mitochondria occurs in *L. amazonensis* cells (Fig. 5).

230

231 **Discussion**

232 Plant defensins are considerate promising candidates for the development of new drugs due to
233 their broad and strong antimicrobial activity as well as selective toxicity to microbial cells (Vriens et al.
234 2014). However, to be used as a drug, the mechanism of inhibition of the target microorganism has to be
235 understood. In our previous work, we produced the recombinant defensin from *V. unguiculata* seeds (*Vu*-
236 Defr) in *E. coli* and tested its activity against *L. amazonensis* (Souza et al. 2013). In this work, we

investigated part of the toxic effect of *Vu*-Defr on *L. amazonensis*. For this, viability assay, membrane perturbation, mitochondrial membrane potential, ROS induction and NO induction tests were evaluated.

In our previous work, we established that a *Vu*-Defr concentration of 18.5 μ M (100 μ g/mL) with an incubation time of 24 h caused the elimination of 54.3% of *L. amazonensis* from culture (Souza et al. 2013). Therefore, we chose these concentration and time parameters for the toxicity assays. The *L. amazonensis* growth inhibition assay was repeated in the conditions previously established, and afterwards, we evaluated the viability of *L. amazonensis* cells. The growth inhibition assay at 48 h indicated an inhibition of 56% in regard to the control (Fig. 2A). This result is in accordance with the previously obtained by Souza et al. (2013). After the time of 48 h of growth inhibition assay, protozoan cells were harvested and seeded in a new medium without *Vu*-Defr. The result indicated that after the treatment of *Vu*-Defr the *L. amazonensis* 3.6% of the cells were able to regrown (inhibition of 96.4%) (Fig. 2B). Therefore, this result indicates that *Vu*-Defr at 18.5 μ M had lethal effect that eliminates the great majority of the *L. amazonensis* cells from culture, and the remaining living cells were responsible for the regrown observed. Antimicrobial substances that causes a lethal effect on microorganisms are preferable taking into consideration drug development because the lower probability of rise of resistance (Levy and Marshall 2004, Thevissen et al. 2007, Vieira et al. 2015).

Following the determination that *Vu*-Defr at 18.5 μ M has a 96.4% lethal effect, protozoan cells treated with this concentration were observed using an optical microscope. Initially, we determined the *L. amazonensis* membrane integrity by the stain Sytox green and observed fluorescent parasites in treated samples, indicating that protozoan plasma membranes were compromised (Fig. 3). In our assay conditions, a concentration of 18.5 μ M and an incubation time of 24 h caused 96.4% of *L. amazonensis* elimination (Fig. 2B) (Souza et al. 2013), indicating that *Vu*-Defr killed *L. amazonensis* in a process involving membrane damage. The interaction of plant defensins with fungal membrane components is well documented, e.g., *Rs*-AFP₂ and *Ms*Def₁ (from *Mendicago sativa*) bind glycosylceramide (GlcCer) (Ramamoorthy et al. 2007), *Dm*AMP₁ (defensin from *Dahlia merckii*) binds mannosyldiinositolphosphorylceramide (M(PI)₂C) (Thevissen et al. 2003), *Mt*Def₄ (defensin from *Mendicago truncatula*) binds phosphatidic acid (Sagaram et al. 2013), *Ps*D₁ (from *Pisum sativum*) binds phosphatidylcholine (Neves et al. 2010) and TPP3 (from *Solanum lycopersicon*) as well as *Na*D₁ (from *Nicotiana glauca*) bind phosphatidylinositol (4,5)-bisphosphate (Poon et al. 2014, Baxter et al. 2015). The membrane of *Leishmania* contains lipid components that may be the target of *Vu*-Def (Zhang and

267 Beverley 2010), which upon interaction, induces the observed toxic effect on fungi, *i.e.*, membrane
268 damage. The initial interaction of positive charged plant defensins (with negative component of
269 microorganism membranes are abrogated in the presence of cations (Terras et al. 1993, Segura et al.
270 1998, van der Weerden et al. 2008), therefore, the *Vu*-Defr positive charge in neutral pH (Fig. 1A and B),
271 may exert an important attraction for the initial interaction.

272 The membrane damage *per se* can cause membrane depolarization, as observed for Bac8c (peptide
273 variant derived from batenecin from bovine neutrophils) when it interacts with the opportunistic yeast
274 *Candida albicans* (Lee and Lee 2015), which seriously impairs cell homeostasis. This mechanism may be
275 responsible for the observation of rounded *Leishmania* cells in the samples treated with *Vu*-Defr (Fig. 3, 4
276 and 5, white boxes represent the inserts). Two synthetic peptide derivatives from the platelet kinocidins
277 RP-1 and AA-RP-1 caused this rounding effect on promastigotes of *L. infantum chagasi*, and the
278 protozoan had its membrane permeabilized (Erfe et al. 2012), as we observed for the interaction of *Vu*-
279 Defr and *L. amazonensis*. However, some authors have suggested that the membrane permeabilization is
280 an indirect action of the fungal-defensin interaction (Aerts et al. 2007, Soares et al. 2017).

281 One common effect induced by several AMPs on fungal cells is the production of ROS (Aerts et
282 al. 2007, 2011, Choi et al. 2014). With the aim of assessing whether *Vu*-Defr is also capable of inducing
283 the production of ROS in *L. amazonensis* cells, we used the fluorescent probe H₂DCFDA. This probe is
284 the chemically reduced form of fluorescein and is used as an indicator for ROS in cells, and after cleavage
285 of the acetate groups by intracellular esterase and oxidation, the nonfluorescent H₂DCFDA is converted to
286 the highly fluorescent 2',7'-dichlorofluorescein, thus serving as an indicator of ROS induction in cells
287 (Invitrogen-Molecular Probes 2006). Observing the image of the ROS induction assay (Fig. 4), it is
288 possible to note that in contact with *Vu*-Defr, the *L. amazonensis* cells present green fluorescence
289 staining, indicating that ROS were induced in the treated cells. Aerts et al. (2007) also observed this effect
290 while studying the interaction of *Rs*-AFP₂ with *C. albicans* cells. In that work, they showed that ROS
291 production is dose-dependent, and they also demonstrated that antifungal activity is ROS-dependent.
292 Other works also correlate the involvement of ROS production with the death pathway trigger by plant
293 defensins on fungi. For example, the plant defensins *PvD*₁, *NaD*₁ and *ApDef*₁ (from *Adenanthera*
294 *pavonina* seeds) are also capable of inducing ROS production in fungi (Aerts et al. 2007, Weerden et al.
295 2008, Mello et al. 2011, Soares et al. 2017).

296 Due to the induction of ROS, we speculated that mitochondria could be involved in this process.
297 We based our assumption on a piece of information that the main site of ROS production in eukaryotic
298 cells is the mitochondria as a by-product of the respiration process (Batandier et al. 2002, Venditti et al.
299 2013). In fact, Soares et al. (2017) had demonstrated that *S. cerevisiae* cells died after *ApDef₁* interaction
300 by ROS production and also presented mitochondrial damage, as indicated by transmission electronic
301 microscopy. The work of Fardin et al. (2016), in which proteins of *Bertholletia excelsa* (Brazil nut) seeds
302 were tested against *L. amazonensis*, also corroborates this hypothesis in the sense that the authors showed
303 that those proteins are capable of the induction of ROS production and cause functional mitochondria
304 collapse. Therefore, we investigated if *Vu-Defr* was also capable of causing some damage to the
305 mitochondria. For this, rhodamine 123 dye that marks mitochondrial membrane potential was used,
306 serving as an indicator of energized mitochondria. Therefore, the loss of mitochondrial membrane
307 potential is observed as a decrease in the fluorescent signal. In Fig. 5, it is possible to observe that the
308 treatment of *L. amazonensis* cells with 18.5 μ M *Vu-Defr* impairs the accumulation of the fluorescent
309 probe by the mitochondria, indicating that in the presence of *Vu-Defr*, a decrease of energized
310 mitochondria occurs in *L. amazonensis* cells. Histatin-5, a human salivary antimicrobial peptide, is also
311 capable of leading to *Leishmania* death, and the toxic effects caused by that peptide include
312 morphological alterations in mitochondria and severe decay of the respiration rate as well as
313 mitochondrial membrane potential, in addition to limiting and mostly reversing the depolarization of the
314 plasma membrane. With this data, those authors concluded that the main target for histatin-5 in
315 *Leishmania* is the mitochondria through interaction with F_1F_0 -ATP_{ase} (Luque-Ortega et al. 2008).
316 Quercetin, a polyphenolic flavonoid from plants, is another antileishmanicidal substance that also causes
317 the induction of ROS and loss of mitochondrial membrane potential; however, the mitochondrial damage
318 is attributed to occur through a large quantity of ROS (Fonseca-silva et al. 2011). Additionally, work from
319 Silva et al. (2012) indicated that one of the targets of quercetin is the arginase, which is metallohydrolase
320 and is involved in polyamine biosynthesis, which is involved in ROS detoxification.

321 The probe DAF-FM-DA is susceptible to NO, and it penetrates the cell and emits fluorescence in
322 the presence of NO. In this assay, neither untreated nor *Vu-Defr*-treated protozoan cells emit fluorescence,
323 thus indicating that the production of NO is not involved in the toxic effect of *Vu-Defr* on *L. amazonensis*
324 (Results not shown).

Although part of the mechanism of action and the toxic effects of antimicrobial agents have been described, among them the plant AMPs, the knowledge mainly regarding plant defensin and its action against *Leishmania* is lacking. In this work we showed that the lethal effect of Vu-Defr on *L. amazonensis* is the result of membrane perturbation, ROS production and mitochondria membrane potential loss. In this context, our work helps to fill the gap about effects caused by the plant defensin and thus aids a better understanding of the mechanism of action of this peptide against *L. amazonensis*.

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528 **List of legends**

529

530 **Fig 1.** A. Amino acid sequence of the recombinant *Vu*-Defr in one letter code. Numbers above the531 sequence indicate the size of the *Vu*-Defr in amino acid residues. M (in green) indicates the methionine532 residue that was added to the sequence of the *Vu*-Defr as a requirement of cloning in to the pET-32

533 EK/LIC vector as described by Santos et al. (2010). Negatively charged amino acid residues are indicated

534 in red. Positively charged amino acid residues are indicated in blue. B. The biochemical features of the
535 *Vu*-Defr.

536

537 **Fig 2.** A. Growth inhibition assay of *Leishmania amazonensis* in the absence (control) and presence of the
538 18.5 μ M of *Vu*-Defr. B. Viability assay of *L. amazonensis* after growth inhibition assay in the presence of
539 *Vu*-Defr. The percent of protozoan elimination in regard to their respective controls is shown above the
540 bars. Different uppercase letters indicate significance by the Tukey test ($P < 0.05$) among the experiments
541 and their respective controls. The graphics are shown as mean with standard deviations of one
542 independent assay.

543

544 **Fig. 3.** Images of *Leishmania amazonensis* cells treated with *Vu*-Defr (18.5 μ M) for 24 h and incubated
545 with *Sytox green* fluorescent dye. Note the green fluorescence in cells treated with *Vu*-Defr, which
546 indicates compromised plasma membranes. Bars = 50 μ m. Images shown are representative of one
547 independent experiment out of three ($n = 3$). Large white boxes indicate inserts to show protozoan cell
548 details of the small white boxes.

549

550 **Fig. 4.** Images of *Leishmania amazonensis* cells treated with *Vu*-Defr (18.5 μ M) for 24 h and incubated
551 with 3-amino-4-aminomethyl-2,7-dichlorofluorescein diacetate dye. Note the green fluorescence in
552 protozoan cells treated with *Vu*-Defr, indicating the induction of ROS in these cells. Bars = 50 μ m.
553 Images shown are representative of one independent experiment out of three ($n = 3$). Large white boxes
554 indicate inserts to show protozoan cell details of the small white boxes.

555

556 **Fig. 5.** Images of *Leishmania amazonensis* cells treated with *Vu*-Defr (18.5 μ M) for 24 h and incubated
557 with Rhodamine 123 dye. Note the decrease of the green fluorescence signal in cells treated with *Vu*-Defr
558 (D), which indicates compromised mitochondrial membrane potential. Bars = 50 μ m. Images shown are
559 representative of one independent experiment out of three ($n = 3$). Large white boxes indicate inserts to
560 show protozoan cell details of the small white boxes.

561

A

Vu-Defr **M** **K** **T** **C** **E** **N** **L** **A** **D** **T** **Y** **R** **G** **P** **C** **F** **T** **T** **G** **S** **C** **D** **D** **H** **C** **K** **N** **K** **E** **H** **L** **L** **S** **G** **R** **C** **R** **D** **D** **V** **R** **C** **W** **C** **T** **R** **N** **C**

10 20 30 40

B

Peptide name	Size in amino acids	Isoelectric point	Charge (pH 7.0)	Net charge	Hydrophobicity at pH 6.8	Molecular weight (Da)
<i>Vu</i> -Defr	48	7.54	+ 1	+ 3	11.79	5544.26