RENORBIO
Programa de Pós-graduação em Biotecnologia

Vipericidinas e derivados: caracterização de alvo molecular em *Trypanosoma cruzi*, avaliação antiparasitária e antitumoral, visando o uso biotecnológico dos peptídeos terapêuticos

Juciane Vaz Rêgo

UFC-CE
2014

Juciane Vaz Rêgo

Vipericidinas e derivados: caracterização de alvo molecular em *Trypanosoma cruzi*, avaliação antiparasitária e antitumoral, visando o uso biotecnológico dos peptídeos terapêuticos

Tese apresentada ao Curso de Pós-graduação em Biotecnologia da Rede Nordeste em Biotecnologia (RENORBIO), com vistas à apreciação pela banca como requisito para a obtenção do título de Doutora em Biotecnologia, área de concentração Biotecnologia em Recursos Naturais.

Orientador: Gandhi Rádis Baptista

UFC-CE
2014

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

R267v Rêgo, Juciane Vaz.
Vipericidinas e derivados : caracterização de alvo molecular em Trypanosoma cruzi, avaliação antiparasitária e antitumoral, visando o uso biotecnológico dos peptídeos terapêuticos / Juciane Vaz Rêgo. – 2014.
153 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Pró-Reitoria de Pesquisa e Pós-Graduação, Programa de Pós-Graduação em Biotecnologia (Rede Nordeste de Biotecnologia), Fortaleza, 2014.
Orientação: Prof. Dr. Gandhi Rádis Baptista.

1. Peptídeos antimicrobianos. 2. Ciclofilina A (TcCyP19). 3. Trypanosoma cruzi. 4. Peixe zebra. 5. Células tumorais. I. Título.

CDD 660.6

JUCIANE VAZ REGO

Vipericidinas e derivados: caracterização de alvo molecular em *Trypanosoma cruzi,* avaliação antiparasitária e antitumoral, visando o uso biotecnológico dos peptídeos terapêuticos

Tese apresentada ao Curso de Pós-graduação em Biotecnologia da Rede Nordeste em Biotecnologia (RENORBIO), com vistas à apreciação pela banca como requisito para a obtenção do título de Doutora em Biotecnologia, área de concentração Biotecnologia em Recursos Naturais.

Aprovada em: 28/11/2014

BANCA EXAMINADORA

_____

Prof. Dr. Gandhi Radis Baptista
Orientador

_____

Prof. Dra Aparecida Tiemi Nagão Dias- Examinadora

_____

Profa Dra Alexsandra Fernandes Pereira- Examinadora

_____

Prof. Dr Daniel Barbosa Liarte - Examinador

_____

Profa Dra Luciana Magalhães Melo- Examinadora

Dedico este trabalho à Deus e a minha família pela bênção, força e apoio na finalização de mais uma etapa profissional.

AGRADECIMENTOS

À Deus, meu pai que me guia, protege e realiza todos os sonhos com muitas bênçãos.

Aos meus pais e irmãos que me deram força e incentivo na dedicação aos estudos.

Ao meu marido Elder pelo carinho, incentivo, apoio e companheirismo no desenvolvimento da tese.

As minhas sobrinhas Isa Gabrielle, Alícia e Isabelle que me fazem do dia um divertimento.

Ao Prof. Dr Gandhi Radis Baptista pela paciência, orientação e confiança no desenvolvimento do projeto de doutorado.

Á Profª Drª Silvane Maria pela colaboração, parceria e apoio durante esta etapa de formação profissional.

Ao amigo Daniel pela amizade, parceria, orientação e ajuda durante o desenvolvimento da pesquisa.

As minhas amigas Fernanda Barbosa e Lívia que me receberam em seu lar com boas conversas, carinho, apoio, amizade e ajuda para descontrair durante o desenvolvimento da pesquisa.

As queridas amigas Alexandra, Marcia, Carla, Aninha, Tais, Rute, Regiane, Janaína, Keylla e Carmen pelo apoio e carinho.

A aluna Francirlene pela amizade, conversas e desempenho em parte na pesquisa.

Aos colegas do (LABOMAR) Patrícia e Cláudio pela colaboração e apoio;

Aos colegas de trabalho e alunos da UFPI que me motivaram na realização desta jornada.

RESUMO

Os peptídeos antimicrobianos relacionados com as catelicidinas- as vipericidinas estão envolvidos na resposta imune inata, no sistema de defesa de muitos organismos, tendo um modo rápido de ação, podendo assim atuar em conjunto com esquemas terapêuticos existentes e/ou constituírem uma alternativa aos fármacos antibióticos convencionais. O presente estudo de tese tem como objetivos duas vertentes: a primeira caracterizar o gene alvo de *T cruzi*, que interage *in silico* com as vipericidinas: a Ciclofilina-A (TcCyP19) em populações sensíveis e resistentes do *T.cruzi* ao Benzonidazol; segunda avaliar *in vitro* a atividade antiparasitária e a antitumoral, bem como avaliar a citotoxicidade das vipericidinas e seus derivados, visando o uso biotecnológico desses peptídeos. Estudo *in silico* permitiu identificar uma ciclofilina que interage com o peptídeo antimicrobiano GA650- (lutzicidina) do veneno de jararacas. Esse achado é de interesse particular visto que nosso grupo de pesquisa havia realizado um estudo proteômico identificando a ciclofilina-A como uma proteína superexpressa (10 x) em populações resistentes do *T. cruzi* comparado com seu par sensível ao benzonidazol. Diante disso a Ciclofilina-A (*Tc*CyP19), uma peptidil prolil isomerase - molécula-chave com diversas funções biológicas, que incluem dobramento molecular, resposta ao stress, a modulação imunitária e transdução de sinal - compreende um potencial alvo de fármaco, e portanto foi selecionada para estudo. A análise filogenética revelou uma clara divergência entre as seqüências Ciclofilinas-A de tripanosomatídeos e mamíferos – o que a torna uma alvo diferencial. Uma combinação das análises de Southern blot e tempo real de RT-PCR (qPCR) mostraram que TcCyP19 é um gene de cópia única localizada em bandas cromossômicas que variam em tamanho de 0,68-2,2 Mb, dependendo da cepa de *T. cruzi*. Northern blot e qPCR indicaram que os níveis de mRNA TcCyP19 eram duas vezes maior em populações de *T. cruzi* resistentes a fármacos do que contrapartida de seu par sensível. Do mesmo modo , a expressão da proteína CyP19, conforme determinado por electroforese em gel bidimensional / western blot, foi aumentada na mesma ordem em populações resistentes do *T.cruzi* ao BZ. Essas cepas foram usadas para avaliação da atividade antiparasitária das vipericidinas. Além da atividade antiparasitária, nosso grupo determinou a ação inibitória das vipericidinas contra bactérias e fungos. Ensaio de atividade anti-*T.cruzi in vitro* de formas epimastigotas, tripomastigotas e amastigotas intracelulares, foi observado que a Vipericidina (crotalicidina – peptídeo C1AB) teve efeito citotóxico dose-dependente contra epimastigotas de *T.cruzi* (IC_{50} 24h = 4.72 µM; IC_{50} 48h = 4.37 µM). Para formas tripomastigotas e amastigotas intracelulares somente as vipericidinas -crotalicidina teve atividade seguindo de citotoxicidade celular e nas demais amostras foram inativas. Para avaliação a ação anticancerígena neste estudo, identificamos um oligopeptídeo de nove resíduos, KRFKKFFKK (EVP50) que é repetidamente codificado em conjunto dentro de seqüências da vipericidina. EVP50 exibiu ademais uma potente toxicidade letal para peixe-zebra (*Danio rerio*) (LD = 10 µM), somente quando o N-terminal do peptídeo foi quimicamente conjugado com rodamina B (RhoB). *In vitro*, a RhoB conjugado EVP50 exibiu um efeito antitumoral dependente da concentração, em relação às células MCF-7 de câncer da mama. Nas células MCF-7, o nonapeptídeo EVP50 RhoB conjugado se acumulou no citoplasma e núcleo dentro de minutos. No citoplasma, o RhoB-EVP50 induziu influxo de

cálcio extracelular, liberação de cálcio intracelular e lesões da membrana após a incubação com concentrações micromolares do EVP50 conjugado com o composto fluorescente. O peptídeo não conjugado foi desprovido de atividades tóxico e citotóxico em nossos modelos *in vivo* e *in vitro*. Assim, a interferência do conjugado com a homeostase do cálcio, sua função nuclear e sua disfunção membrana induzida (lesão e vacuolização) parecem agir em conjunto para romper o circuito da célula. Este mecanismo sinérgico da toxicidade foi restrita ao nonapeptídeo vipericidina estruturalmente modificado. Como um todo, o presente estudo vem contribuir para o entendimento do alvo molecular (Ciclofilina-A) que interage com peptídeo vipericidina e como potencial dessa classe de peptídeos em biotecnologia médica contra *T. cruzi* e atividade anticancerígena, quando o peptídeo é conjugado.

Palavras-chave: Peptídeos antimicrobianos- Vipericidinas- Ciclofilina A (TcCyP19)- *Trypanosoma cruzi*- Peixe zebra- células tumorais- citotoxicidade

ABSTRACT

Antimicrobial peptides related cathelicidins-vipericidins are involved in the innate immune response in the defense system of many organisms, and a mode of action rapid, and thus can operate in conjunction with existing and / or regimens constitute an alternative to conventional antibiotics drugs. The present study of thesis has as goals two aspects: first to characterize the target gene of *T.cruzi*, which interacts *in silico* with the vipericidin: Cyclophilin A (TcCyP19) in sensitive and resistant strains of *Trypanosoma cruzi* to benznidazole; second to evaluate *in vitro* antiparasitic and antitumor activity, as well as evaluate the cytotoxicity of vipericidinas and its derivatives, aiming at the use biotechnological of these peptides. In silico study allowed identifying a cyclophilin that interacts with the antimicrobial peptide GA650- (lutzicidina) from the venom of pit vipers. This finding is of particular interest given that our research group had conducted one study proteomic identifying the cyclophilin-A as an overexpressed protein (10 x) in resistant populations of *T. cruzi* compared with their pair-sensitive benznidazole. Therefore Cyclophilin A (TcCyP19), a peptidyl - prolyl isomerase - key molecule with diverse biological functions, including molecular folding, stress response, immune modulation and signal transduction - comprises a potential drug target, and thus was selected for study. Phylogenetic analysis revealed a clear divergence between the sequences of Cyclophilin-A trypanosomatids and mammals - which makes it a differential target. A combination of the Southern blot analysis and real-time RT-PCR (qPCR) showed that TcCyP19 is a single copy gene located in the chromosomal bands ranging in size from 0.68 to 2.2 Mb, depending on the strain *T. cruzi.* Northern blot and qPCR indicated that mRNA levels TcCyP19 were twice higher in populations of *T. cruzi* BZ resistant than its sensitive counterpart pair. Similarly, the expression of CyP19 protein, as determined by two-dimensional electrophoresis / western blot gel was increased in the order of resistant populations *T.cruzi* to BZ. Altogether, these data indicate that Cyclophilin A (TcCyP19) expression is up-regulated at transcriptional and translational levels in *T. cruzi* populations which were *in vitro*-induced and *in-vivo* selected for resistance to BZ. These strains were used to evaluate the antiparasitic activity of vipericidinas. Besides the antiparasitic activity, our group determined the inhibitory action of vipericidinas against bacteria and fungi. Activity assay *in vitro* anti-*Trypanosoma cruzi* epimastigotes, trypomastigote and intracellular amastigotes, it was observed that Vipericidin (crotalicidin – GA645 peptide) had dose-dependent cytotoxic effect against epimastigotes of *Trypanosoma cruzi* (24h IC_{50} = 4.72 uM; IC_{50} 48h = 4:37 mM). To trypomastigotes and intracellular amastigotes only had activity of crotalicidin vipericidin following cell cytotoxicity and the remaining samples were inactive. For trypomastigote and amastigote forms only vipericidin GA645-crotalicidin have activity followed by cell cytotoxicity and the other samples were inactive. For to evaluate anticancer action in this study, we identify a nine-residue cryptic oligopeptide, KRFKKFFKK (EVP50) that is repeatedly encoded in tandem within vipericidin sequences. EVP50 addition exhibited a potent lethal toxicity to zebrafish (*Danio rerio*) (DL = 10 mM), only when the N-terminal peptide was chemically conjugated to rhodamine B (RhoB). *In vitro*, RhoB-conjugated EVP50 exhibits a concentration-dependent antitumor effect toward MCF-7 breast cancer cells. In MCF-7 cells, the RhoB-conjugated EVP50 nonapeptide accumulated in the cytoplasm and the nucleus within minutes. In the cytoplasm, the EVP50 RhoB-induced influx of extracellular calcium, intracellular calcium release and membrane lesions after incubation with micromolar concentrations of EVP50 conjugated with the fluorescent compound. The naïve unconjugated peptide was devoid of toxic and cytotoxic activities in our models *in vivo* and *in vitro*. Thus, the conjugate's interference with calcium homeostasis, its nuclear function and its induced membrane dysfunction (lesion and vacuolization) seem to act in concert to break the cell circuitry. This synergistic mechanism of toxicity was restricted to structurally modified vipericidin

nonapeptide. As a whole, this study contributes to the understanding of the molecular target (cyclophilin-A) that interacts with vipericidin peptide and a potential of this class of peptides in medical biotechnology against *T. cruzi* and anticancer activity when the peptide is conjugated.

Keywords: Antimicrobial peptides- Vipericidins- Cyclophilin A (TcCyP19) *Trypanosoma cruzi*- Rhodamin- Zebrafish - tumor cells - cytotoxicity

LISTA DE SÍMBOLOS E ABREVIATURAS

17LER	Cepa de *T.cruzi* resistente ao Benzonidazol e derivada da cepa Tehuantepec cl2 , induzida *in vitro*
17WTS	Cepa de *T.cruzi* susceptível ao Benzonidazol e derivada da cepa Tehuantepec cl2
AcrAP1	Peptídeo antimicrobiano de veneno do escorpião árabe, *Androctonus crassicauda*
AcrAP2	Peptídeo antimicrobiano de veneno do escorpião árabe, *A. crassicauda*
APX	Ascorbato peroxidade
ATP	Adenosina trifosfato
B16	Células cancerígenas tumorais de melanoma
B16F10	Células do melanoma metastático
Bf_ PAMRCs	Peptideo antimicrobiano isolado do veneno de serpente *Bungarus fasciatus*
Bf_PAMRCs	*Bungarus fasciatus* - Peptídeos Antimicrobianos relacionados com catelicidinas
Bf-30	Peptídeo antimicrobiano de serpente *Bungarus fasciaus*
BMAP	Peptídeo antimicrobiano bovino mielóide
BZ	Benzonidazol
BZR	Cepa de *T.cruzi* resistente ao benzonidazol derivada da cepa Y selecionada *in vivo.*
BZS	Cepa de *T.cruzi* susceptível ao Benzonidazol e derivada da cepa Y.
Cbf-K 16	Derivado da catelicidina Bf-30
CsA	Ciclosporina A
CyPA	Ciclofilina A
D0870	Composto derivados do posaconazol
DTUs	“Discrete Typing Unit” - conjunto de isolados geneticamente semelhantes
GM	Gomesina
gp	Glicoproteína
GPI	Glicosilfosfatidilinositol
HBD-1	Peptídeo defesina natural humano
HGPRT	guanina fosforibosil transferase
LAFEPE	Laboratório Estadual de Pernambuco
LL-37	Catelicidina humana

LmCyP19	Ciclofilina A de *Leishmania major*
LR14	Peptídeo antimicrobiano isolado do *Lactobacillus plantarum cepa R14*
MCF-7	Células cancerígenas mamária
MDA-MB-231	Célula cancerígena da glândula mamária humana
Na_ PAMRC	*Naja atra*- Peptídeos Antimicrobianos relacionados com com catelicidinas
NF-AT	Fator nuclear de células T ativadas
NFX	Nifurtimox
NK-lisina	Peptídeo antimicrobiano derivado de NK-2
NTRI	Nitroredutases enzimas
Oh-PAMRs	Peptídeo antimicrobiano isolado de *Ophiophagus hannah*
OMS	Organização Mundial da Saúde
OPAS	Organização Panamericana da Saúde
PAMRCs ou CRAMPs	Peptídeos Antimicrobianos relacionados com catelicidinas or " *"Cathelicidin-related antimicrobial peptides"*
PAMs ou AMPs	Peptídeos Antimicrobianos ou "*Antimicrobial peptides*"
PC12	Células feocromocitoma
PFGE),	Técnica Eletroforese de campo pulsado ou "*pulse field gel electrophoresis"*
PGP	Fosfoglicoproteína de membrana
PPIases	Peptidil - prolil -isomerases enzimas
Pt_ PAMRC1	Peptídeo antimicrobiano isolado de *Pseudonaja textilis*
Rhob	Peptídeo antimicrobiano Rhodamine B
ROS	Espécies reativas de oxigênio
SCH 56592	Composto derivado do posaconazol
SH-SY5Y	Células neuroblastoma
SL	"splice leader" ou mini-exon,
TbCyP19	Ciclofilina A de *Trypanosoma brucei enzima*
TcCyP19	Ciclofilina A de *T.cruzi enzima*
TcFeSOD	Ferro-superóxido dismutase enzima
TcOYE	"*Old Yellow Enzyme*" ou Prostaglandina Sintetase enzima
TvCyP19	Ciclofilina A de *Trypanosoma vivax*
VEGF	Fator de crescimento vascular endotelial
VSG	Glicoproteína de superfície variante

LISTA DE FIGURAS

REVISÃO DE LITERATURA

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

LISTA DE TABELAS

REVISÃO DE LITERATURA

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

SUMÁRIO

INTRODUÇÃO

O impacto da crescente resistência aos fármacos disponíveis no mercado contra bactérias, fungos, parasitos e pelas células cancerígenas tem permitido o desenvolvimento de novos fármacos e formas terapêuticas que sejam capazes de lidar efetivamente com as estratégias de adaptação que esses organismos elaboram.

Os Peptídeos antimicrobianos (PAMs) representam alternativas desejáveis para produtos farmacêuticos convencionais, pois eles têm uma rápida ação e pode atuar em conjunto com esquemas terapêuticos existentes (Zasloff, M., 2002 e Adade et al., 2013). Os peptídeos antimicrobianos são parte do sistema imune inato de uma variedade de organismos eucariotos: plantas, vertebrados, invertebrados, bactérias, fungos e parasitos (Maróti et al., 2011; Torrent et a.; 2012; Meiyalaghan et al., 2014). Essas moléculas têm-se mostrado como potenciais componentes farmacológicos contra microorganismos e anti-tumorais.

Os peptídeos antimicrobianos têm sido identificados e caracterizados na família das serpentes. Esses peptídeos foram o foco desta pesquisa- Peptídeos antimicrobianos relacionados com catelicidinas (PAMRCs) oriundos da glândula de veneno dos viperídeos denominadas Vipericidinas (crotalicidina, batroxicidina, lutzicidina e lachesicidina) e da cobra Elapide compreendendo os peptídeos da *Bungarus fasciatus* (Bf- PAMRC), *Naja atra* (Na-PAMRC), e *Ophiophagus hannah* (Oh- PAMRC) (Falcao et al, 2014, Zhao et al, 2008 e Wang et al., 2008) .

Os mecanismos de atuação da maioria dos peptídeos antimicrobianos catiônicos promovem permeabilização ou perturbação na membrana. Revisões têm relatado sobre peptídeos que afetam a viabilidade microbiana em baixa concentração através da interação moderada com um ou mais alvos intracelulares tais como DNA, proteínas, chaperonas e outros (Nicolas, 2009).

Vários estudos demonstram que os peptídeos antimicrobianos têm ação contra uma variedade de bactérias e fungos pela sua entrada na membrana celular e desregulação da membrana. Peptídeo defensina isolado do fungo *Coprinopsis cinerea* - a Copsina foi

bactericida atuante contra uma diversidade de bactérias Gram-positivas, incluindo agentes patogénicos humanos, tais como *Enterococcus faecium* e *Listeria monocytogenes* (Essig et al., 2014). Além do mais, foi observado que a Cathelicidina-BF, peptídeo antimicrobiano isolado a partir de venenos de serpentes de *Bungarus fasciatus,* tem atividade contra *E. coli.* Os autores sugerem que devido sua conformação helicoidal anfipática podem interagir com as membranas (Wang et al., 2008).

Além disso, o mecanismo de ação em parasitos das catelicidinas foi observado em camundongos infectados com *Trypanosoma brucei* e envolve o rompimento da superfície da membrana plasmática, permitindo a diminuição da parasitemia e a sobrevida prolongada do organismo (McGwire et al, 2003). Por outro lado, estudo evidencia que a trialisina, um peptídeo antimicrobiano da saliva do *Triatoma infestans* ao se interagir com ciclofilina 19 (TcCyP19) bloquea a ação do PAM e protege os parasitos dos efeitos citolíticos. Os autores também observaram que modificando a estrutura da trialisina esta passa a ter atuação eliminando o *T.cruzi.* (Kulkarni et al., 2013).

Diante disso, com o propósito de buscar possíveis alvos que interagem com peptídeos antimicrobianos-vipericidinas, foi realizado um ensaio *in silico* que permitiu identificar alvos gênicos da base de dados do DrugBank. Desses alvos moleculares identificamos uma ciclofilina com função de dobramento protéico (função de chaperona) que se interagem com o peptídeo vipericidina GA650- lutzicidina conforme revisado por Nicolas et al (2009). A lista de alvos moleculares obtidos pela análise *in silico* das vipericidina GA650-lutzicidina e GA645-crotalicidina encontra-se no ANEXO E. Além disso, em outro estudo realizado por nosso grupo o gene ciclofilina (TcCyP19) foi encontrado como superexpresso em populações do *T. cruzi* resistentes ao BZ utilizando a metodologia de análise proteômica.

Então, selecionamos esse alvo molecular ciclofilina-A (TcCyP19) que pode se interagir com vipericidinas, pode estar associado com o fenótipo de resistência do *Trypanosoma cruzi* a fármacos e que participa de processos biológicos importantes para o parasito (enovelamento proteico). Nesta pesquisa foi estudado a análise filogenética do

TcCyP19 com outros organismos, e observado o nível de RNAm, organização gênica, número de cópias, localização cromossômica do TcCyP19 e expressão da proteína *Tc*CyP19 em cepas do *T. cruzi* sensíveis (S) e resistentes (R) ao BZ, com a finalidade de demonstrar a participação dessa proteína como alvo de fármaco anti-*T. cruzi e o* seu papel na resistência de agentes antiparasitários.

Além do mais, com intuito do uso biotecnológico das vipericidinas contra o *T. cruzi* e verificar sua atividade anti-parasitária*,* foi proposto avaliar a atividade *in vitro* dos peptídeos vipericidinas contra o *T. cruzi* e identificar o IC_{50} das vipericidinas contra as formas de *T. cruzi.*

Estudos também mostram que os efeitos citotóxicos dos peptídeos antimicrobianos podem ser inibidores para desenvolvimento das células cancerígenas. Wang e colaboradores (2013) monstrou que atuação da catelicidina de *Bungarus fasciatus* (Bf-30) destroi o melanoma de células B16F10 em dose dependente e inibe o crescimento do melanoma e metástase em camundongos. Com a mesma atividade foi observada pelo peptídeo mutante de BF-30 (BF30-K16) que exibiu citotoxidade seletiva *in vitro* contra a propagação de células cancerígenas (Tian et al., 2013).

Na busca das vipericidinas serem capazes de inibir o surgimento e a disseminação de células cancerígenas foi proposto sintetizar um pepitídeo conjugado para rhodamina-B e investigar a atividade biológica do peptídeo selecionado vipericidina criptografado e/or modificado na região hipervariável C-terminal das vipericidinas. As atividades biológicas e compartimentalização celular e toxicidade foram avaliadas *in vivo* utilizando peixe zebra e *in vitro* com células cancerígenas da mama humana- MCF-7.

A estrutura organizacional da Tese se apresenta da seguinte forma: **Introdução**, **Revisão de literatura** e **04 (quatro) capítulos**. O **CAPÍTULO I** - Ciclofilina A (*Tc*CyP19) e fenótipo de resistência*;* **CAPÍTULO II**- Peptídeos antimicrobianos, vipericidinas e sua ação contra *Trypanosoma cruzi;* **CAPÍTULO III** – Rhodamine B- nonapeptideo Vipericidina - atividade tóxica e citotóxica contra o modelo peixe-zebra e câncer de mama; **CAPÍTULO IV** -

Perspectiva Biotecnológicas da aplicação da crotalicidina e peptídeos terapêuticos análogos e **considerações finais**.

REVISÃO DE LITERATURA

1 Peptídeos antimicrobianos

Os peptídeos antimicrobianos (PAMs), também chamados de peptídeos de defesa, são componentes conservados da resposta imune inata de todos organismos, incluindo plantas, animais, e humanos (Kosciuczuk et al., 2012). PAMs exibem uma forte atividade inibidora contra microrganismos bactérias Gram-positivas e Gram-negativas, fungos, vírus, parasitas e células tumorais.

A maioria dos peptídeos antimicrobianos são pequenos (até 5-10 kDa) compostos por 12 a 50 resíduos de aminoácidos, são molécula catiônicas, devido ao excesso de resíduos de lisina, arginina e histidina (pH = 7), e em muitos casos são anfifílicos e hidrofóbicos, exibindo estruturas diversas (Hancock e Scott, 2000). Podem ser divididos em quatro grupos conforme a estrutura:

- **α hélices**: mais comum conformação que se estrutura em α-hélice quando interage com a membrana plasmástica. Exemplo: cecropinas P1, peptídeo isolado de nematoides do gênero *Ascaris* (Andersson et al., 2003),
- **Folhas β**: possuem resíduos de cisteínas que formam ligações dissulfeto, e se estruturam em folhas-β antiparalelas. Ocasionalmente podem ter pequenas porções de estrutura α -hélice; Exemplos: as defensinas apresentam seis resíduos de cisteína e três ligações dissulfeto com amplo espectro de atividade contra fungos, bactérias e vírus (Ganz, 2003).
- **Estruturas curvadas**: estruturas menos comuns formadas por ligações dissulfeto simples ou devido à presença de resíduos de prolinas na estrutura; Exemplo: Bactenecina, peptídeo de neutrófilos bovinos, tem dois resíduos de cisteína, que formam uma ligação dissulfureto, tornando-se uma molécula cíclica (Wu et al., 1999)

- **Estruturas estendidas**: são caracterizadas pelas altas repetições de um resíduo de aminoácidos na sequência primária; Exemplo: as indolicidinas, peptídeos ricos em resíduos de triptofano (Selsted et al., 1992); as histatinas, ricas em resíduos de histidina (Shamova et al., 1999); e as bactericinas, ricas em resíduos de prolina (Kavanagh and Dowd, 2004);

As principais classes de peptídeos antimicrobianos estruturalmente distintas são: α-defensinas, β-defensinas e as catelicidinas.

a) **Classes α- defensinas e β-defensinas** - são peptídeos catiônicos que exercem o seu efeito citolítico através permeabilização da membrana. As principais diferenças entre esses péptidos são aparentes nos seus perfis de expressão e estrutura. As defensinas são expressas em vários tecidos, tais como neutrófilos, células de Paneth e os revestimentos epiteliais do intestino, do pulmão e da pele;

b) **Catelicidinas** - são peptídeos catiônicos que exibem diversas estruturas linear, cíclico, α hélices e Folhas β.

1.1 Peptídeos antimicrobianos Catelicidinas

As catelicidinas são a segunda grande classe de peptídeos antimicrobianos encontrados nos eucariotos. Caracterizam-se por serem peptídeos antimicrobianos derivados por clivagem proteolíticas apartir de um pré-propeptídeo que possui domínio catelina em mamíferos e em outros eucariotos esse domínio é menos conservado.

Todas as catelicidinas possuem um domínio conservado N-terminal (catelina) de aproximadamente 100 aminoácidos. A porção de peptídeo antimicrobiano reside no C-terminal tornando ativo após clivagem proteolítica da catelina. Para todas as catelicidinas, os PAMs maduros são gerados por ativação proteolítica entre 12-100 resíduos. Apresentam-se ativos contra um amplo espectro de bactérias, e alguns fungos e vírus com invólucro, parasitas e canceres. Enquanto a parte catelina N-terminal é muito conservada entre espécies diferentes, os PAMs C-terminal são bastante diversificadas (Sørensen and

Borregaard, 2005). Os genes das catelicidinas são compostos por quatro exons e três introns conforme observado na Fig. 1 (Linde et al., 2013);

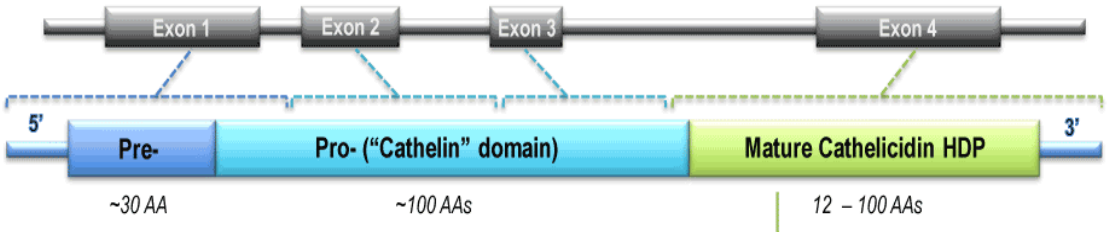


Fig. 1. Estrutura Geral das Catelicidinas
Fonte: (Linde et al., 2013)

Os peptídeos antimicrobianos relacionados com catelicidinas (PAMRCs) oriundos da glândula de veneno dos viperídeos denominadas Vipericidinas (crotalicidina, batroxicidina, lutzicidina e lachesicidina) e da cobra Elapide compreendem os peptídeos da *Bungarus fasciatus* (Bf- PAMRC), *Naja atra* (Na- PAMRC), e *Ophiophagus hannah* (Oh- PAMRC) tem sido descritas (Falcao et al, 2014, Zhao et al, 2008 e Wang et al., 2008) .

PAMRCs das Vipericidinas e Elapide são estruturalmente dispostas como pré-propetides com um peptídeo sinal de aproximadamente 20 resíduos, um domínio conservado catelina (inibidor da proteína catepsina L) e uma hipervariável carboxi-terminal trecho onde uma série de atividades antimicrobianas naturais é encontrada.

O PAMRC maduro compartilha um elevado grau de similaridade de aminoácidos, com mais de seus 34 resíduos idênticos ou substituições sendo estritamente conservados. Devido à elevada proporção de resíduos de lisina que alternam com resíduos hidrofóbicos, estes peptídeos têm uma rede carga positiva e um caráter anfipático. Além disso, uma característica estrutural distinta dos PAMRCs reptilianos foi observada quando comparado com outros membros familiares de catelicidinas é a presença de um fragmento adicional ácida na extremidade C-terminal da pró-sequência, que é rica em ácido aspártico e resíduo não carregado (Falcao et al., 2014).

Os PAMRCs Reptilianos (Vipericidinas e Elapide) possuem atividade de amplo espectro contra isolados clínicos e cepas padrões de bactéria e fungos (Wang et al.,2008. Essas moléculas tem mostrado atividade contra bactérias Gram-negativa e Gram-positiva (Falcao et al., 2014) (ver Tabela 1).

Pesquisas demonstram que as PAMRCs têm pouca atividade hemolítica e baixa citotoxicidade para células humanas o que torna interessante o uso dos peptídeos como potencial terapéutico. Foi observado que a Cathelicidina-BF, peptídeo antimicrobiano isolado a partir de venenos de serpentes de *Bungarus fasciatus,* além de sua atividade antibactericida e anticancerígeno apresenta pouca atividade hemolítica ou citotoxica em células sanguíneas humanas (Wang et al., 2008). Além do mais, Falcão e colaboradores (2014) observaram a atividade hemolítica de quatro peptídeos (Crotalicidina, Batroxicidina, Oh-PAMRC, Pt- PAMRC e LL-37) e mostraram que o peptídeo Oh-PAMRC apresentou menor citotoxicidade (10% de hemólise, a 100 µM) de eritrócitos (Fig 2). As vipericidinas crotalicidinas e batroxicidinas foram um pouco mais tóxico, que o Oh-PAMRC, mas ainda possuiu uma confortável diferencial de seletividade entre alvos bacterianos e eucarióticos.O Pt- PAMRC comparado com e Oh-PAMRC, teve uma forte atividade hemolítica tornando-o bastante não seletiva.

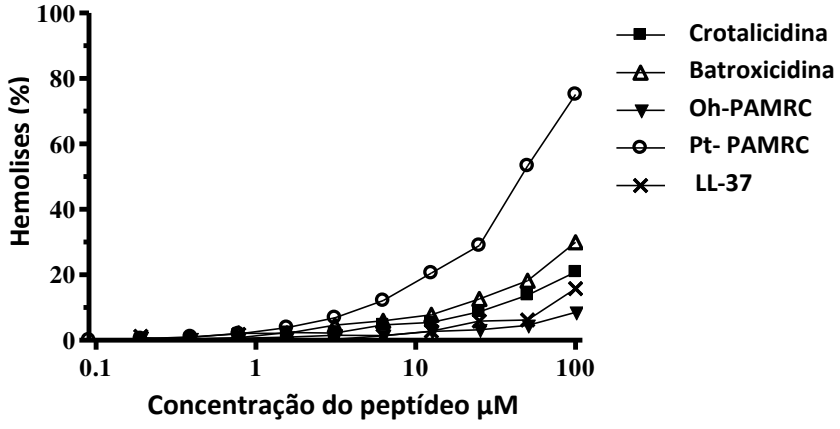


Fig. 2: Comparação da atividade hemolítica de vipericidinas (crotalicidina e batroxicidina), peptídeos relacionados com catelicidina de serpentes asiáticas, e catelicidina humano LL-37 *(Fonte: Falcao et al., 2014).*

Tabela 1: Peptídeos antimicrobianos catelicidinas encontrados em serpente com potencial terapêutico.[a]

Peptídeo	Origem	Atividade inibitória	Reference
Oh_PAMRC	*Ophiophagus Hannah*	Bactérias Gram positivas e negativas	Zhao et al.(2008); Li et al., (2012)
Derivado Oh_PAMRC 30	*O. Hannah*	Bactérias Gram positivas e negativas	Zhang et al.(2010) Zhang et al (2013)
Derivado Oh_CM6	*O. Hannah*	Bactérias Gram positivas e negativas	Zhao et al.(2008); Li et al., (2012)
Bf- PAMRC	*Bungarus fasciatus*	Bactérias Gram positivas e negativas Fungos Células cancerígenas	Wang et al.(2008)
Derivado Bf_PAMRC 30	*B. fasciatus*	Bactérias Gram positivas e negativas	Chen et al. (2011)
Derivado Bf_PAMRC15	*B. fasciatus*	Bactérias Gram positivas e negativas	Chen et al. (2011) Zhou et al., (2011)
Na_PAMRC	*Naja atra*	Bactérias Gram positivas e negativas	Zhao et al.(2008)
Pt_ PAMRC1	*Pseudonaja textilis*	Bactérias Gram positivas e negativas	Falcão et al.(2014)
Pt_ PAMRC2	*P. textilis*	Bactérias Gram positivas e negativas	Falcão et al.(2014)
Batroxicidina	*Crotalus durissus terrificus*	Bactérias Gram positivas e negativas	Falcão et al.(2014)
Crotalicidina	*Lachesis muta rhombeata*	Bactérias Gram positivas e negativas	Falcão et al.(2014)
Lachesicidina	*Bothrops atrox*	Bactérias Gram positivas e negativas	Falcão et al.(2014)
Lutzicidina	*Bothrops lutzi*	Bactérias Gram positivas e negativas	Falcão et al.(2014)

[a] *Fonte : Hoek et al.,2014 e Falcao et al., 2014*

1.2 Mecanismo de ação dos peptídeos antimicrobianos

O mecanismo de ação de peptídeos antimicrobianos (PAMs) ocorre pela combinação de efeitos hidrofóbicos e eletrostáticos, sendo que a maioria das suas atividades exerce seu efeito por uma única ação ou uma combinação de ações nas membranas lipídicas. A ações dos PAMs quando interagem na superfície de células provocam perturbações na estrutura da bicamada lipídica,e consequentemente a lise celular, formação de um poro, ruptura física, e translocação, seguido por interações subsequentes com alvos intracelulares como DNA, RNA e proteínas. Além disso, evidencia

que os PAMs são sinergicamente eficazes para matar as células, restringindo proliferação celular e recrutamento de componentes de imunidade adquirida (Zasloff, 2002; Sanderson, 2005; Nicolas et al., 2009).

Os PAMs podem eliminar os microorganismos através de mais de um mecanismo de ação, influenciado por fatores individuais como fase de crescimento, localização tecidual, e a presença ou ausência de outros mecanismos imunes ou agentes antimicrobianos exógenos (Yeaman & Yount, 2003), ou seja, podem ser multifuncionais e ter mecanismos de ação diferentes de acordo com o organismo alvo. O interessante que o peptídeo indolicidina elimina tanto bactérias, quanto fungos e vírus, porém cada um de maneiras diferentes. Em fungos o peptídeo permeabiliza a célula (Lee et al., 2003), em bactérias, a indolicidina inibe a síntese de DNA (Nan et al., 2009), em vírus, inibe a integrase do vírus HIV, mostrando assim a sua versatilidade de mecanismos frente a diferentes microorganismos.

Entretanto, o mecanismo pelo qual os peptídeos permeabilizam a membrana e como exercem sua atividade ainda não são completamente definidos, e vários mecanismos têm sido propostos para descrever a interação entre peptídeos e bicamadas lipídicas. (Shai, 2002; Bechinger e Lohner, 2012). Cruz e colaboradores (2014) em sua revisão expõem vários modelos propostos para explicar o mecanismo de ação dos péptidos antimicrobianos em membranas bacterianas e produzir permeabilização da membrana, os quais são dependendo da natureza (Ver Fig. 3). Estes modelos são os seguintes: Modelo de barril, modelo carpete, modelo de poro toroidal e modo de canal agregado. Estes diferem principalmente no modo de fixação de bactérias e inserção na membrana celular de péptidos antibacterianos.

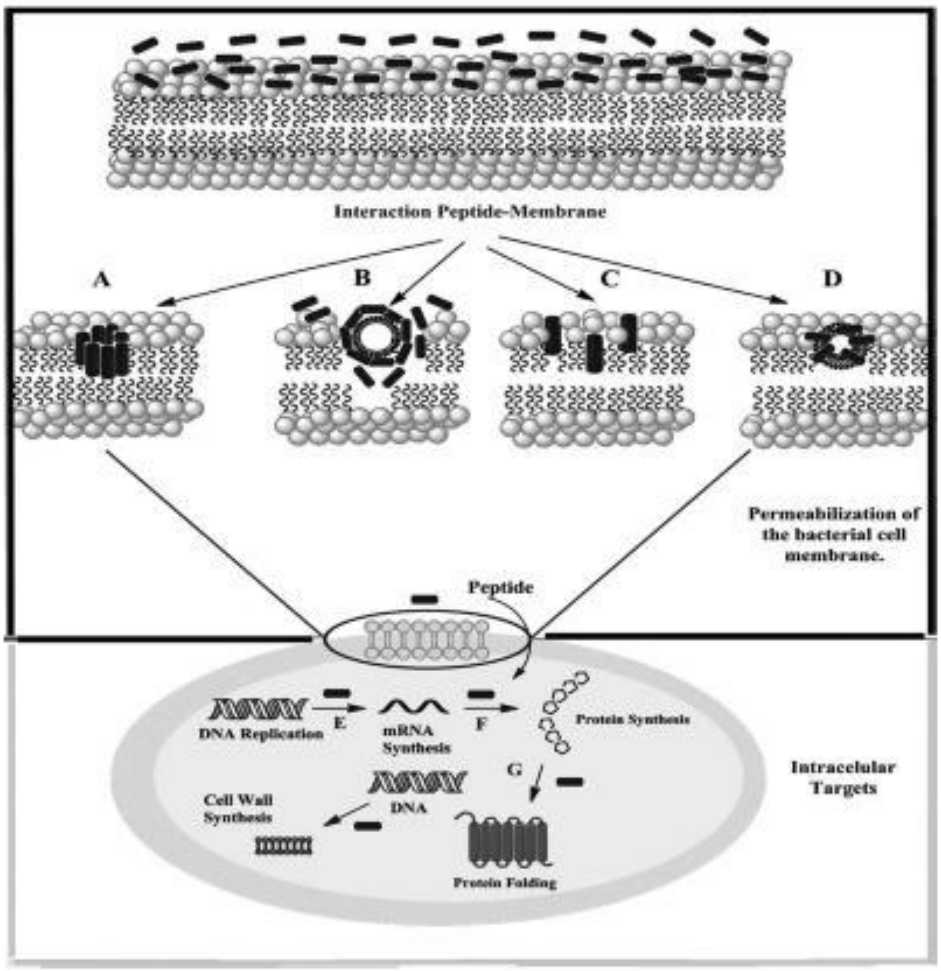


Fig. 3. Modo de ação dos PAMs. (A) Modelo Poro. (B) Modelo Carpete. (C) modelo formação de Barril. (D) Modelo canal de agregado. (E) PAMs inibindo a síntese de DNA. (F) PAMs inibindo a síntese de proteínas e (G) Interação dos PAMs com chaperonas auxiliadas por dobramento de proteínas.
(Fonte: Cruz et. al, 2014)

(A) Modelo de poro

Os peptídeos antimicrobianos, geralmente alfa hélice, são inseridos na membrana lipídica devido à curvatura contínua entre as monocamadas de lípidos e uma associação de superfícies de membrana. Neste caso, as regiões hidrofílicas dos peptídeos e a cabeça lipídica dos grupos interagem juntos formando um poro estrutural que geralmente é maior do que “aduelas barril” um tipo poro. Na formação do poro toroidal, as faces polares dos péptideos estão associados com os grupos de cabeça polares das fracções lipídicas. Os lípideos arranjados nos poros são, em seguida, inclinados a partir da estrutura lamelar

normal, e são ligados os dois folhetos da membrana, formando um contínuo dobrar a partir do topo para o fundo da forma de um buraco toroidal (Matsuzaki et al., 1996)

(B) Modelo Carpete

Inicialmente, os peptídeos ligam-se ao grupamento fosfato dos fosfolipídeos através de suas faces hidrofílicas. Em altas concentrações, os peptídeos são conectados e orientados paralelamente para a superfície da bicamada, o que se assemelha a uma forma ”carpete” Esta acumulação de moléculas peptídicas na membrana lipídica produz uma desintegração da membrana bacteriana por perturbar curvatura da bicamada e a formação posterior de micelas (Sanderson, 2005).

(C) Modelo formação de Barril

Envolve a formação de feixes anfipáticos de peptídeos alfa-hélice que oligomeriza e forma canais transmembranas ou poros com os resíduos hidrofílicos voltado para o lúmen do poro. A formação do poro transmembrana ocorre com agregação dos péptideos monómeros que se inserem perpendicular na bicamada lipídica. Em geral, o número de monómeros para a formação de poros é dependente da concentração de péptideos, que causa acumulação de monômeros e aumento do tamanho do poro (Pukala et al., 2006)

(D) Modelo canal de agregado.

O peptídeo aumenta somente a permeabilidade da membrana, mas este efeito não é severo suficientemente para causar a morte celular. A ação inicial do peptídeo envolve deslocamento competitivo de lipopolissacarídeo associado com cátions divalente ($Mg+^2$ e $Ca +^2$), onde os peptídeos destabilizam essa montagem supramolecular e tem acesso as membranas externas e internas. Esses peptídeos causam ruptura da membrana lipídica, formando domínios de péptideo específico, fase de segregação lateral dos fosoflipídeos aniônicos, e até mesmo a formação de fase lípido não-lamelar. A agregação dos péptideos forma canais que permite a difusão de íons através da membrana (Bond et al. 2008)

Modelos de Interação dos PAMs com Alvos intracelulares que inclui (E) inibição da síntese de DNA, (F) A inibição da síntese de proteínas, (G) Interação dos PAMs com chaperonas que desempenham dobramento de proteínas)

Uma vez dentro da célula, os PAMs podem se interagir com o alvo e se ligar eletrostaticamente ao DNA, RNA e proteinas (exemplos enzimas) causando inibição da parede celular, da síntese de DNA, proteínas, atividade da enzima (Hsu et al., 2005) e interferir *in vitro* ou *in vivo* a citocinese de células bacterianas. Consequentemente com a interferência desses processos metabólicos-chave necessários para sobrevivência da célula podem desenvolver mecanismos únicos, como translocar para o citoplasma e alterar a formação da membrana citoplasmática (Brown et al, 2006). Zanh et al., (2013), observou a capacidade da chaperona DnaK de se ligar em peptídeos antimicrobianos, bem como as propriedades específicas das sequências que determinam os modos de ligação de peptídeos.

1.3. Amplo espectro dos peptídeos antimicrobianos

1.3.1 Ação antibacteriana

Os peptídeos antibacterianos têm a capacidade de se ligar a estruturas lipídicas e grupos fosfolipídeos. Esses peptídeos têm como alvo principalmente as membranas celulares das bactérias e promovem a desintegração da estrutura da bicamada lipídica e podem inibir vias importantes da célula como síntese de proteínas, da parede celular, e replicação de DNA. Por outro lado podem eliminar as bactérias através de uma interação estereoespecífica com um receptor / molécula de acoplamento que pode ser um componente de um tipo permease do sistema transportador da membrana interna, seguido pela translocação do péptideo para dentro do interior da célula e se ligar ao DNA, RNA ou proteínas (Nicolas et al., 2009). Estudo evidencia que o peptídeo buforin II isolado entra na célula e se liga ao DNA e RNA sem causar danos à membrana celular (Park et al., 1998). Além disso, o “pyrrhocoricin”, um potente peptídeo, não tóxico, apresenta fortes relações

entre a ligação de um fragmento sintético da proteína alvo DnaK da bactéria e atividades antibacterianas deste peptídeo análogo modificado em posições estratégicas (Kragol et al., 2002).

Vários estudos mostram os peptídeos antimicrobianos Catelicidinas sendo encontrados em serpentes e envolvidos com atividade antibacteriana. O Peptídeo antimicrobiano relacionado com a Catelicidina-Bf (Bf_PAMRC), isolado a partir de venenos de serpentes de *Bungarus fasciatus,* Bf_PAMRC tem apresentado atividade contra diferentes bactérias (*E.coli*, *Staphylococcus aureus)* de forma eficiente e algumas espécies de fungos incluindo microorganismos isoladas de pacientes com fármaco-resistência (Wang et al.,2008). Outra pesquisa, O Bf_PAMRC foi encontrado exercendo forte atividade antibacteriana contra *Propionibacterium acnes e Staphylococcus epidermidis*. Os autores sugerem o potencial do Bf_PAMRC como uma nova opção terapêutica para acne vulgar (Wang et al., 2011). O peptídeo Oh_ PAMRC, isolado de serpente também exibiu atividade forte antibacteriana principalmente contra diferentes cepas de *E.coli* e de *Pseudomonas aeruginosa* (Zhao et al.,2008).

1.3.2 Ação antifúngica

Os mecanismos dos peptídeos antifúngicos têm ação na parede celular ou nos componente intracelulares, desregula a integridade da membrana, produz espécies reativas de oxigênio e induz a morte celular programada. A formação de espécies reativas de oxigênio tem sido um importante mecanismo fungincida de vários peptídeos (Narasimhan et al., 2001). Como se pode observar o peptídeo defensina da planta *Nicotiana alata* NaD1 que se interage com a superfície da célula fúngica de *Candida albicans* promoveu a permeabilização da membrana, a entrada no citoplasma, hiperprodução de espécies reativas de oxigénio, e morte celular induzida por danos oxidativos (Hayes et al., 2013).

Outro mecanismo que tem sido observado é a ligação dos peptídeos antifúngicos nos constituintes da parede celular. Por exemplo, a ligação de peptídeos antimicrobianos a quitina pertubam a integridade da membrana e aumenta a permeabilização da membrana plasmática ou formação de poro (Van der Weerden et al., 2010). Além do mais, foi

observado a eliminação *de Candida albicans* através da ligação de Histatina 5, um antimicrobiano com alta atividade fungicida, nas β-glicanas que constituem a parede celular do fungo. (Jang et al., 2010). Além disso, estudos mostram a importância do ergosterol da parede celular na seletividade dos peptídeos antimicrobianos. Os peptídeos Cecropina B e dermaseptina precisam se ligar ao ergosterol como parte do mecanismo que leva à morte de espécies do gênero *Aspergillus e Fusarium* (De Lucca et al., 1998).

1.3.3 Ação antiparasitária

A ação dos PAMS em vários estudos tem demonstrado uma atividade de amplo espectro contra parasitas. O alvo mais comum dos PAMs nos triponosomatideos é o dano na membrana citoplasmática e a interação com os alvos intracelulares dos parasitas (ver Fig. 4).

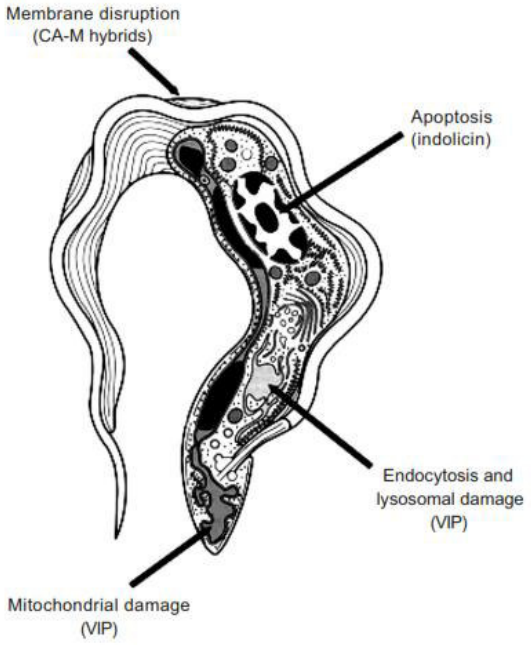


Fig. 4. Modos de ação e alvos dos peptídeos antimicrobianos em tripanosomatideos (Extraído Torrent et al., 2012)

Os danos na membrana ocorrem com rompimento da integridade da membrana, resultando em desequilíbrio osmótico, inchaço e lise celular. Este mecanismo envolve três mecanismos básicos visto anteriormente tais como: Formação de barril, modelo carpete e formação de poro. Inicialmente, o patógeno sofre um rápido colapso do potencial de

membrana e queda dos níveis intracelulares de ATP; aumento na permeabilidade da membrana, e alteração morfológica, como bolhas e rompimentos ou formação de domínios aniônicos ricos em fosfolipídios que causam defeitos na estruturação do fosfolípido e segue a permeabilidade da membrana (Torrent et al., 2012).

Em *T. brucei*, a ação antiparasitária dos peptídeos pode desregular a integridade da membrana e conduzir a uma instabilidade osmótica causando inchaço e lise celular e morte do parasito. Pesquisadores sugerem que devido a presença de uma variedade de proteínas da superfície de membrana com ancoras glicosilfosfatidilinositol (GPI), presentes em trypanosomas podem permitir a afinidade e comunicação celular entre os peptídeos e essas moléculas (McGwire et al., 2003).

A morte dos Trypanosomas africanos mediada por endocitose também foi observada. Os peptídeos ligam-se as glicoproteinas aniônicas da superfíce da membrana tais com a glicoproteína de superfície variante-VSG e são rapidamente endocitados através da bolsa flagelar, acessando o lisossomo e perturbando sua membrana, que libera hidrolases que causam a morte do parasito. (Delgado et al., 2009; Torrent et al., 2012). Alternativamente, a apoptose pode mediar a ação dos peptídeos antimicrobianos contra parasitos promovendo morte celular. Este mecanismo ainda não é totalmente esclarecido, mas existem relatos que em *Leishmania* os PAMs promovem aumento na concentração de Ca^{2+} que são tóxicos para mitocôndria causando um colapso no potencial de membrana e perda de ATP ou liberação de Ca^{2+} para compartimentos intracelulares e interação com organelas ou proteínas intracelulares (Kulkarni et al., 2009).

A interação dos PAMs com alvos intracelulares eliminam o patógeno sem causar qualquer dano na membrana. A Histatin 5, um peptídeo salivar humano, promove alterações morfológicas na mitocôndria de *Leishmania* como: inchaço na matriz mitocondrial e alteração na definição das cristas mitocondriais, diminuição da taxa de respiração e consequente alteração bioenergética do parasito (Luque-Ortega et al 2008). Em *Leishmania major* foi observado que o peptídeo antimicrobiano bovino mielóide (BMAP) promove uma degradação de DNA nas formas promastigotas, evidenciando morte celular por apoptose

tardia, danos na membrana e inchaços de vacúolos citoplasmáticos, provavelmente devido a osmose e lise celular (Lynn et al., 2011). A ação dos peptídeos antimalária foi demosntrado no *Lactobacillus plantarum* e exibiu um peptideo antimicrobiano, AMPs LR14, não-tóxico, e com ação potente anti-plasmodial provocando a inibição do crescimento de *P. falciparum,* sem causar hemólise. Além disso, o Peptídeo de escorpião, Escorpine, exibiu atividade antibacteriana contra *Bacillus subtilis* e *Klebsiella pneumoniae* e inibiu o desenvolvimento de parasitas sendo 98% de mortalidade em estágios sexuais do *Plasmodium berghei* e 100% de redução do *Plasmodium falciparum* (Carballar- Lejarazu et al. 2008).

1.3.4 Ação Anticancerígena

O peptídeo antimicrobiano tem surgido como nova estratégia no tratamento do câncer devido apresentar baixa toxidade, não estimularem o aparecimento de resistência pelas células, boa penetrabilidade nos tecidos por tamanho reduzido, amplo espectro de atividade e serem capazes de eliminar tumores primários e metástase (Shadidi & Sioud, 2003; Bhutia & Maiti, 2008).

A ação dos PAMs contra as células cancerígenas pode estar relacionada com a carga positiva dessas moléculas. A interação eletrostática entre PAMs catiônicos e os componentes aniônicos da membrana pode ser uma característica de seletividade contra células cancerígenas. O NK-lisina, peptídeo derivado de NK-2 é um antibacteriano potente, mas não tóxica a uma linha celular de queratinócitos humanos e de baixa atividade hemolítica, tem interação com células cancerosas humanas baseando-se numa forte ligação preferencial às membranas contendo fosfolípidos aniônicos, que não são normalmente encontradas na superfície de células humanas (Schröder-Borm et al., 2005).

A indução de apoptose é o mecanismo mais importante de muitos agentes anti-cancerígeno. Esta ação foi observada pelo o peptídeo antimicrobiano, magainina, anfipático a-hélice, que induz à morte das células leucêmicas por apoptose e com relação às células normais tem baixa atividade (Cruz-Chamorro et al., 2006). Além disso, as Cecropinas, peptídeos antimicrobianos isolado da hemolinfa imune de *Hyalophora cecropia,* também exibem atividade citotóxica contra as células leucêmicas (HL60) (Ceron et al. 2010).

O envolvimento de canais iônico no mecanismo de ação dos PAMS foi relatado em estudo do peptídeo Gomesina (GM). PAM isolado da hemolinfa da aranha brasileira *Ancathoscurria gomesiana,* apresenta atividade citotóxica contra células cancerígenas tumorais de melanoma B16, neuroblastoma SH-SY5Y, e células feocromocitoma PC12. O mecanismo intracelular da GM ocorre através da indução da permeabilização da membrana precedida por eventos intracelulares específicos, tais como perturbação do retículo endoplasmático, aumento citosólico de Ca^{2+}, seguido por uma acumulação de Ca^{2+} em organelas, que induz a perda de potencial mitocondrial, levando ao colapso de mitocôndrias, que culmina no rompimento da membrana celular (Rodrigues et al., 2010; Soletti et al.,2010; Paredes-Gamero et al., 2013).

A interação do PAM de serpente em células cancerígenas com atuação na membrana plasmática e estruturas intracelulares tem sido estudada. Wang e colaboradores (2013) observaram a Catelicidina-BF (BF-30), um polipeptídeo antibacteriano extraída a partir do veneno de serpente *Bungarus fasciatus*, ao inibir o crescimento de células do melanoma metastático (B16F10) *in vitro e in vivo* promove pertubação na membrana citoplasmática e ligação no DNA genómico bloqueando transcrição e tradução do gene de VEGF (fator de crescimento vascular endotelial). Os autores destacam o potencial teraupêutico da BF-30 em pacientes com melanoma metastático. Outro peptídeo antimicrobiano Cbf-K 16 derivado da catelicidina (BF-30) inibe seletivamente a proliferação de células do carcinoma do pulmão por permeabilização da membrana citoplasmática e de ligação DNA, em vez de apoptose. Os autores consideram Cbf-K 16 um candidato para tratamento de câncer de pulmão (Tian et al., 2013).

Pesquisa revisada por Kerkis e colaboradores exibe que a crotamina, peptídeo antimicrobiano de veneno de serpente, inibe o crescimento de tumores e mata as células tumorais. Esta ação se processa através do aumento da concentração intracelular da crotamina nas células cancerígenas que levam a lise e morte celular. Além de representar uma ferramenta potencial para a identificação *in vivo* das células cancerosas (Kerkis et al., 2014)

1.4 Modificação dos PAMs

Vários estudos têm mostrado que as sequências de aminoácidos de catelicidinas podem ser modificados e sintetizados mais curtos e ativos, mas mantendo as características básicas de atividade antimicrobiana: carga catiônica e estrutura anfipática; e com o propósito de moderar as propriedades indesejáveis, tais como alta atividade hemolítica e citotóxica. Estas propriedades tornam catelicidinas modelos ideais em química combinatória para a concepção de novos péptidos antimicrobianos para utilização terapêutica (Midura-Nowaczek and Markowska, 2014).

Na tentativa de gerar uma molécula que combina a diversidade em termos de estrutura e atividade biológica, estudo sintetizou um peptídeo híbrido da combinação da região C-terminal de HBD-1, peptídeo defensina natural dos seres humanos com a θ-defensinas sintéticos humana, nonapétido sintético, e outra igual estrutura com diferença apenas com a presença de ponte dissulfeto. O peptídeo híbrido exibiu maior atividade antimicrobiana, com ou sem a ponte de dissulfeto contra ambas as bactérias Gram-negativas e Gram-positivas, contra fungos, incluindo isolados clínicos de infecções bacterianas oculares, *in vitro* não-hemolítico (Olli et al., 2014). Assim, o péptido híbrido foi gerado como um candidato terapêutico e tem um potencial como uma nova classe de antibióticos. Além do mais, os peptídeos, denominados AcrAP1 e AcrAP2, foram identificados no veneno do escorpião árabe, *Androctonus crassicauda*, exibindo atividade anticancerígena. Além disso, *a*nálogos desses péptidos foram sintetizados para maior cationicidade e obtiveram uma maior potência e espectro de actividade antimicrobiana a

linhas celulares de câncer humano comparado aos seus peptídeos nativos (Du et al.2014) Evidenciando que a modulação dos peptídeos naturais podem ser mais potenciais contra microorganismos e células cancerígenas.

Os peptídeos podem ser modificados desde que na sua natureza tenha sensibilidade a clivagem proteolítica. Os peptídeos sintéticos têm vantagens de ter sequencia curta e não sofrer com modificações pós-traducionais. E desde que o peptídeo mantenha alta potência, atividade de amplo espectro, baixa toxidade, alta seletividade e farmacocinética desejável comparado ao natural (Mok et al., 2014). Dessa forma, ampliam-se o arsenal de PAMs e permite criar peptídeos com maior eficácia sobre aqueles que ocorrem naturalmente, muitos grupos de pesquisa têm produzido peptídeo sintético imitando os peptídeos antimicrobianos naturais através do desenho racional. Outra abordagem utilizada é a combinação de peptídeos antimicrobianos com os convencionais antibióticos para o desenvolvimento de terapêuticos contra bactérias, fungos, parasitas e tumores (Cruz et. al., 2014).

A ferramenta de Base de Dados específica que armazena peptídeos antimicrobianos eucarióticos naturais pode ser aplicada na pesquisa para desenho de novos peptídeos. Com simulações dinâmicas moleculares *in silico*, pode-se modelar a conformação e movimento das mudanças virtuais ao longo do tempo, permitindo a previsão de seu mecanismo de ação (Mehta et al. 2014).

2. O *Trypanosoma cruzi* e a doença de Chagas

A Doença de Chagas ou Tripanosomíase Americana, causada pelo *Trypanosoma cruzi*, é um dos principais problemas de saúde pública na América Latina, sendo endêmica em grande parte do México, América Central e América do Sul, onde cerca de 8 milhões de pessoas estão infectadas (CDC, 2013). No entanto, nas últimas décadas, tem sido cada vez mais detectada nos Estados Unidos da América, Canadá, muitos países da Europa Ocidental e alguns do Pacífico (WHO, 2014). No Brasil, estima-se que de 2 a 3 milhões de

pessoas estão infectadas, com aproximadamente 6000 mortes anualmente (Ramos Jr. *et al.,* 2010; Martins-Melo *et al.,*2012).

O *T.cruzi* (*Protozoa, Sarcomastigophora, Kinetoplastida, Triponosomatidae*) é um protozoário primariamente, transmitido por insetos-vetores (*Triatominae, Hemíptera, Reduviidae*), transfusão sanguínea, transmissão congênita, acidentes de laboratório, via oral e transplantes de órgãos (Dias, 2011; Pereira *et al.,* 2013).

O ciclo de vida do *T. cruzi* apresenta basicamente três formas evolutivas: tripomastigota, amastigota e epimastigota. No hospedeiro vertebrado tem início quando as formas tripomastigotas metacíclicas eliminadas nas fezes dos triatomíneos durante o repasto sanguíneo, atingem a pele e invadem as células locais, onde se diferenciam em formas amastigotas, as quais se multiplicam por sucessivas divisões binárias no citoplasma. Posteriormente, as amastigotas se diferenciam em tripomastigotas, que rompem as células infectadas e ganham o meio extracelular. As formas tripomastigotas podem infectar as células vizinhas ou entram na circulação sanguínea, onde são, posteriormente, ingeridas pelo inseto vetor durante a alimentação, ou ainda infectarem células de outros tecidos do hospedeiro vertebrado (ver Fig. 5) (Tanowitz *et al.,* 1992).

A doença de Chagas tem duas, sucessivas fases: aguda e crônica. A fase aguda dura de 6 a 8 semanas, com alto grau de parasitismo no sangue e pode apresentar uma sintomatologia tão fugaz que passa inteiramente despercebida. Quando sintomática em torno de 8 a 10 dias após a infecção, há aparecimento de febre, astenia, mal estar geral, hiporexia e cefaléia. Além disso, podem aparecer alterações sistêmicas como edema subcutâneo, aumento de volume dos linfonodos, esplenomegalia, hepatomegalia, sinal de Romaña ou de outros tipos de chagoma de inoculação (Rassi et al*.,* 2012). Nas formas agudas graves, surgem quadros raros de miocardite e a maioria dos óbitos é devido à insuficiência cardíaca (Rassi *et al.,* 2012).

Na fase crônica o *T. cruzi* multiplica-se lentamente e apresenta, em geral, um parasitismo tecidual baixo. Nesta fase a maioria dos pacientes permanece assintomática por um tempo ou por toda a vida, evidenciando a forma indeterminada da doença. Enquanto

acerca de 20 a 50% de casos analisados de áreas endêmicas, desenvolveram características sintomáticas desta fase como: comprometimento do sistema cardíaco (forma cardíaca), digestivo (forma digestiva) ou de ambos (forma cardio-digestiva) (Brener *et al.,* 1987).

.

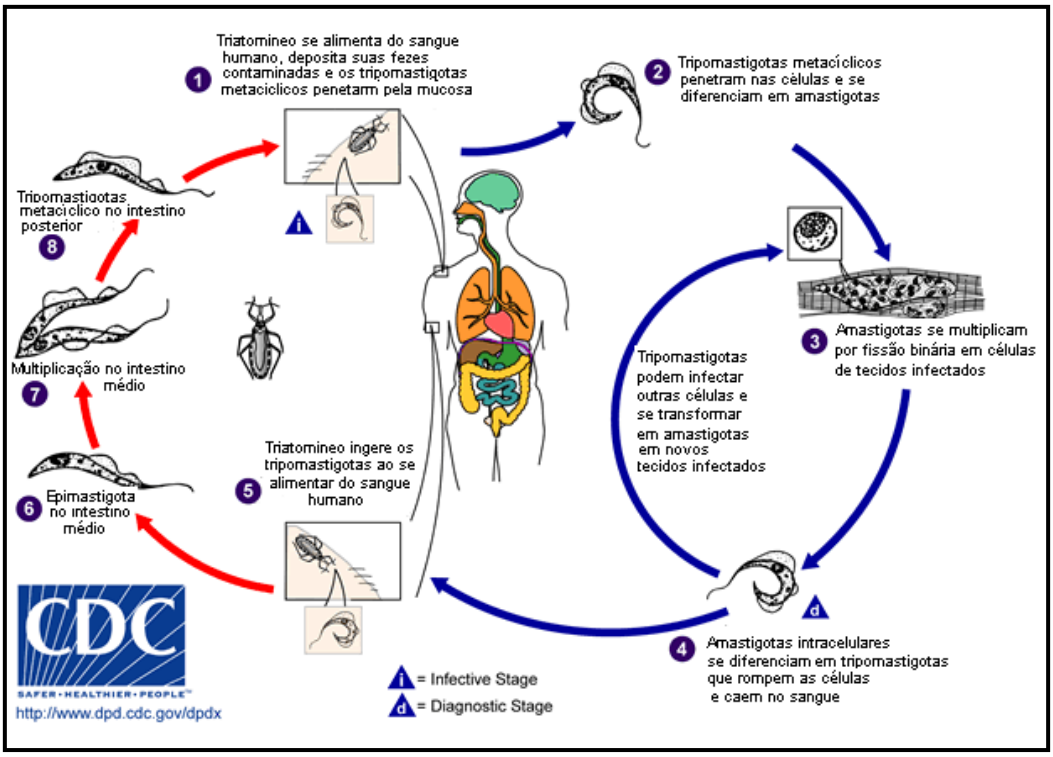


Fig. 5: Ciclo de vida do *Trypanosoma cruzi*.
Fonte: CDC,2000.

2.1 Diversidade genética do *T.cruzi*

O *T. cruzi* é uma população heterogênea e complexa que diferem entre si quanto à sua morfologia, conteúdo de DNA, virulência, patogenicidade e suscetibilidade a fármacos. Estudos de caracterização biológica e molecular utilizando populações clonadas de *T. cruzi*, reforçam esta heterogeneidade do parasita e demonstram que as cepas são compostas de uma variedade de subpopulações com características distintas (Finley & Dvorak, 1987, Machado *et al.,* 2006).

Inicialmente a diversidade genética foi demonstrada por Miles *e col.* (1977, 1978 e 1980) apresentando a existência de três diferentes grupos de cepas de *T.cruzi*: zimodemas 1 (Z1), 2 (Z2) e 3 (Z3). Os zimodemas 1 e 3 estão associados ao ciclo silvestre de transmissão. O zimodema 2 caracteriza o ciclo doméstico (Miles *et al.,* 1977, 1978, 1980 e 1981; Devera *et al.,* 2003). Posteriormente, estudo de marcadores moleculares como RAPD, microssatélites e genes que codificam o mini-exon e RNA ribossomal 24Sα, permitiram a divisão das cepas de *T. cruzi* em duas linhagens filogenéticas distintas denominadas grupo I e grupo II (Souto *et al.,* 1996, Devera *et al.,* 2003). O *T. cruzi* I (zimodema 1) é geralmente observado em mamíferos e triatomíneos silvestres, enquanto que *T. cruzi* II (zimodema 2) é normalmente encontrado em humanos (Devera *et al.,* 2003). Pedroso e col. (2003) observaram distâncias filogenéticas entre os grupos *T. cruzi* I, *T. cruzi* II e híbridos baseados no tamanho dos cromossomas. Esses dados reforçam a hipótese de que cada grupo pode ter evoluído independentemente.

Em 1999, uma nova classificação de *T. cruzi* foi proposta com intuito de padronização, durante uma reunião satélite, na FIOCRUZ, foi definida a separação do parasito em duas grandes linhagens evolutivas, denominadas *T. cruzi* I e *T.cruzi* II (Anonymous 1999). Posteriormente, com o avanço da comunidade científica na estrutura da classificação da população de *T. cruzi,* na segunda reunião satélite, precedendo o XIII Congresso Internacional de Protistologia, a XXV Reunião Anual da Sociedade Brasileira de Protozoologia e a XXXVI Reunião Anual de Pesquisa Básica em Doença de Chagas, foi estabelecido o conceito de DTUs "Discrete Typing Unit" um conjunto de isolados geneticamente semelhantes podendo ser identificados através de marcadores genéticos, moleculares ou imunológicos comuns" (Tibayrenc, 2003). Dessa forma, por consenso científico ficou compreendido que a população de *T. cruzi* se divide em seis DTU's (*T. cruzi* I a VI) (Zingales et al., 2009).

2.2 Organização genômica do *T. cruzi*

O tamanho do genoma do *T. cruzi* (100-200 x 10^6 pb) é relativamente superior ao tamanho dos genomas de outros protozoários parasitas, como por exemplo, *Leishmania* (45-65 x 10^6 pb) ou *T. brucei* (25 x 10^6 pb). O sequenciamento do genoma de *T.cruzi* revelou que o genoma diploide contem cerca de 22.570 proteínas preditas, em 12.570 representam pares alélicos. Em torno de 50% do genoma consiste de sequencias repetidas, tais como retrotransposons e genes de famílias de moléculas de superfície como mucinas, trans-sialidases, gp63s e nova família de mucinas associadas a proteínas de superficie (El-Sayed *et al.,* 2005).

A organização dos genes dos tripanosomatídeos difere daquela conhecida em outros eucariotos. O DNA satélite do *T.cruzi* apresenta repetições de 195 nucleotídeos presente aproximadamente em 10^5 cópias do genoma do parasito, correspondendo a 10% do total de DNA (Elias *et al.,* 2003). Muitas sequencias repetitivas - e a maioria dos genes do *T. cruzi* que codificam proteínas - estão presentes em múltiplas cópias na célula. A presença de múltiplas cópias gênicas pode ser uma maneira encontrada pelo parasita de compensar eventuais perdas de genes essenciais durante essas constantes mudanças do genoma devido à amplificação e translocação das sequencias gênicas. Essa organização em "tandem" também pode estar relacionada com a transcrição policistrônica existente nos tripanossomas, o que facilitaria a síntese e manutenção dos níveis de mRNAs na célula (Silveira, 2000). Além disso, os genes de cópia única são encontrados em *T. cruzi* e estão presentes em um único lócus. Não são muitos os genes em cópia únicos descritos no *T. cruzi* até a presente data. Como exemplo, de tais casos, tem-se: os genes da DNA polimerase II, guanina fosforibosil transferase - HGPRT e glicoproteína (gp) 72 (Silveira, 2000).

O estudo da expressão gênica dos tripanosomatídeos tem revelado a existência de mecanismos genéticos peculiares, como, por exemplo, a organização dos genes de proteínas em unidades transcricionais policistrônicas, o processamento dos transcritos através de trans-splicing e a edição de RNAs polimerases.

O processamento dos mRNAs nos tripanosomatídeos difere dos demais organismos eucariotos, pois transcrevem longos pré-mRNAs policistrônicos, que devem ser processados em mRNAs individuais antes de serem traduzidos (Nilsen, 1992). Durante este processo, uma sequencia nucleotídica líder, conhecida como "splice leader" (SL) ou mini-exon, pelo fato de não ser codificada de uma maneira contínua com os genes estruturais, é transferida para a extremidade 5' de cada mRNA individual. Esta transferência ocorre através de uma reação denominada "trans-splicing". A SL é derivada de um RNA de 110 nucleotídeos codificado por genes localizados em um ou dois cromossomas, dependendo da cepa de *T. cruzi* (Wagner, 1990). A SL está teoricamente presente em todos os mRNAs dos tripanosomatídeos. Pouco se conhece sobre a origem evolutiva do trans-*splicing* e sua função biológica na célula. No entanto, este mecanismo de transcrição parece ter evoluído para acomodar a necessidade dos tripanosomas em processar seus pré-RNAs policistrônicos, servindo como um meio de individualizar os mRNAs e ao mesmo tempo adicionar a eles uma estrutura de quepe (cap), tornando-os maduros e competentes para tradução. Além da adição da SL na extremidade 5' dos mRNAs individuais, ocorre também nos tripanosomatídeos a adição da cauda poli-A na extremidade 3' dos mRNAs (Vanhame, 1995).

Os cromossomas do *T. cruzi* não se condensam durante a divisão celular, dificultado a sua análise pelos métodos convencionais de citogenética (Solari, 1980). No entanto, com a utilização da técnica de "*pulse field gel electrophoresis"* (PFGE), foi possível verificar que o genoma do *T. cruzi* está organizado em pelo menos 20-25 bandas cromossômicas, variando de 0,45 a 1,6 Mb (Gibson & Miles, 1986; Henriksson *et al.,* 1990, 1995 e 1996). Outra peculiaridade marcante deste parasita é a grande variação no conteúdo de DNA entre as diferentes cepas (Dvorak *et al.,* 1982) e até mesmo entre clones de uma mesma cepa (McDaniel & Dvorak, 1993).

2.3 Quimioterapia da Doença de Chagas

Os primeiros compostos analisados experimentalmente para o tratamento da Doença de Chagas foram logo depois da descoberta por Carlos Chagas em 1909. Entre eles citamos atoxil, fucsina, antimônio pentavalente e cloreto de mercúrio (revisto por Coura & Castro, 2002). Em 1957, o uso de Nitrofurazona apresentou resultados promissores, mas depois foi substituído por outro nitrofurano, o Nifurtimox (Brener,1984).

Desde 1960, dois fármacos nitroheterociclicos têm ação para o tratamento da Doença de Chagas: um nitrofurano, nifurtimox (NFX) e um nitroimidazol, benzonidazol (BZ). Estes compostos são da mesma família química (dos nitroheterocíclicos), mas possuem diferentes grupos químicos e diferentes mecanismos de ação contra *T. cruzi*. O mecanismo de ação do NFX age sobre a via de redução do grupo nitro por nitroredutases, gerando radicais nitroânion, que na presença do oxigênio causa a produção de compostos altamente tóxicos, tais como: superóxido, peróxido de hidrogênio e radicais de hidroxila.

Atualmente, o BZ é o único fármaco disponível no Brasil para o tratamento de Doença de Chagas. Segundo a Organização Panamericana da Saúde (OPAS) o Brasil é reponsável pela demanda regional e único fornecedor mundial desse produto, sendo produzido pelo Laboratório Estadual de Pernambuco (LAFEPE). O modo de ação do BZ ainda não está claro. Pesquisas relatam que o mecanismo de ação do BZ induz a formação de radicais livres e de metabolitos electrofílicos que são gerados quando o grupo nitro ($N0_2$) é reduzido ao grupo amino (NH_2) através das enzimas nitroredutases (Maya *et al.,*2003; Wilkinson et al.,2008). Estudo evidencia que o BZ está envolvido com modificação de macromoléculas através da ligação de seus intermediários nitroreduzidos (Maya et al., 2003). Pedrosa e coloaboradores (2001) indicam que, em doses terapêuticas, o BZ induz o estress oxidativo em hepatócitos de rato. Além disso, foi observado que o BZ age no DNA promovendo uma descompactação da heterocromatina e catalisa a formação de quebras na dupla fita do DNA genômico do *T. cruzi*. Também demonstrou a superexpressão de proteínas de reparo do DNA mitocondrial indicando que o BZ promove lesões no DNA mitocondrial (Rajão et al., 2014).

O NFX e BZ são compostos efetivos em reduzir a duração e a severidade clínica da doença de Chagas aguda e congênita, levando aproximadamente a 50% dos pacientes tratados à cura parasitológica (Kirchhoff, 1993 e Urbina et.al., 2003). Em uma avaliação do tratamento da doença de Chagas com o BZ foi observado cura em 76% dos pacientes tratados na fase aguda e apenas 8% dos pacientes tratados na fase crônica (Cançado, 2002). Além da baixa eficácia de cura, os mecanismos de ação de ambos compostos podem estar relacionados com reações indesejáveis relatadas por pacientes e médicos tais como: perda de peso, náuseas e vômitos, excitação nervosa, insônia, depressão psíquica, adinamia, paraestesias, convulsões, vertigem e esquecimento (Castro *et al.,* 2006).

Os alvos bioquímicos da quimioterapia incluem, enzimas como a tripanotiona redutase, superóxido dismutase, cisteína protease, hipoxantina-guanina fosforibosiltrnasferase, gliceraldeído-3-fosfato dehidrogenase, DNA topoisomerases, dihidrofolato redutase e farnesilpirofosfato sintase (Coura & de Castro, 2002). Alguns inibidores da biossíntese de esterol pode ser útil para o tratamento da Doença de Chagas. Além disso, derivados de ázois como D0870 e SCH 56592 (posaconazol) foram capazes de induzir cura parasitológica em modelos murinos de ambas as fases (aguda e crônica) da doença de Chagas (Liendo et al 1998 e Urbina 2001).

2.4 Resistência do *T. cruzi* à Fármacos

Tem sido sugerido que essa resistência natural à fármacos é um fator importante para explicar as baixas percentagens de cura detectadas em pacientes chagásicos tratados. Filardi & Brener (1987) descreveram a presença de cepas do *T. cruzi* com resistência natural ao benzonidazol e nifurtimox. Outros autores demonstraram diferenças na susceptibilidade ao fármaco por cepas de *T. cruzi* (Hauschka, 1949; Neal & Van Bueren, 1988). Murta e Romanha (1998) selecionaram *in vivo* uma população e clones de *T. cruzi* com resistência ao benzonidazol. Posteriormente esses autores mostraram que ao contrário do que ocorre em outros protozoários (Ullman, 1995), a superexpressão dos genes que

codificam a fosfoglicoproteína de membrana (PGP) não está associada com o fenótipo de resistência do *T. cruzi* à fármacos (Murta *et al.,* 2001).

A resistência a fármacos é um complicador para o tratamento de doenças parasitárias, inclusive na doença de Chagas. As alterações bioquímicas e os mecanismos moleculares pelos quais os parasitas tornam-se resistentes são ainda pouco conhecidos. Diante disso, o estudo do mecanismo de resistência a fármacos é de fundamental importância para o desenvolvimento de estratégias terapêuticas mais eficazes para cura. Estudos que visam o conhecimento dos alvos intracelulares do BZ e os potenciais mecanismos de defesa celular ou do parasita contribuem decisivamente para se atingir essa finalidade (de obter fármacos seletivos e eficazes).

O estudo da resistência a fármacos não é simples e pode envolver vários mecanismos distintos. Os principais mecanismos podem ser relacionados (Borst & Ouellette, 1995), como: a diminuição da entrada do fármaco na célula, eliminação do fármaco pela fosfoglicoproteína de membrana (PGP) ou outras proteínas transportadoras dependentes de ATP, diminuição da ativação do fármaco, inativação do fármaco, alteração da formação do complexo alvo-fármaco e eficiência no sistema de reparo.

Uma questão importante foi o comportamento de resistência cruzada entre NFX e BZ. Ambos os compostos apesar de pertencerem a uma mesma família, possuem diferentes grupos químicos e diferentes mecanismos de ação contra o parasita (Pontes & Andrade, 1984; Filardi & Brener, 1987;Neal & Van Bueren, 1988).

2.5 Cepas de *T.cruzi* selecionadas *in vivo* (BZR e BZS) e induzida *in vitro* (17LER e 17 WTS) no estudo do fenótipo de resistência

A grande heterogeneidade do *T.cruzi* tanto genômica como fenotípica, entre as cepas e também clones da mesma cepa, são obstáculos para os estudos de fenótipo de resistência a fármacos. Então, com o intuito de elucidar as bases moleculares desse mecanismo de resistências do *T.cruzi* a fármacos que vem sendo utilizando modelos experimentais através de cepas com resistência selecionada *in vivo* e *in vitro* aos

nitroderivados (Nirdé et al, 1995; Murta & Romanha, 1998; Wilkinson et al, 2008) e também as utilizam em estudos de expressão gênica diferencial. Abdo (1991), conseguiu selecionar *in vitro* uma população de *T. cruzi* 8 vezes mais resistente ao BZ e outra 3 vezes mais resistente ao NFX, do que a cepa selvagem. Murta & Romanha (1998) *em seu* estudo obtiveram pares de cepas de *T.cruzi* resistentes (BZR) selecionadas ao Benzonidazol e sensíveis (BZS), oriundas da mesma cepa Y (resistência mediana e alto pico de parasitemia, no 7° dia após a infecção). Nirdé et al,(1995) induziram a cepa Tehuantepec cl2 a resistência ao BZ e conseguiram uma cepa 23 vezes mais resistente (17LER) e seu par sensível (17 WTS). Várias pesquisas têm utilizados esses modelos em ensaios de expressão gênica diferencial (Villarreal et al., 2005; Murta et al.2008 e Meija et al., 2012).

2.6 Expressão diferencial de proteínas

A análise proteômica de diferentes estágios do ciclo de vida de organismos como *Plasmodium falciparum* (Lasonder *et al.,* 2002 e Florens *et al.,* 2002), *Leishmania donovani* (Thiel & Bruchhauss 2001), *Leishmania infantum* (El Fakhry *et al.,* 2002), *Leishmania (Viannia) guyanensis* e *L. (V.) panamensis* (Gongora *et al.,* 2003) e *T. cruzi* (Atwood *et al.,* 2005) tem sido descritos. O uso da ferramenta proteômica para identificar alvos terapêuticos e para o entendimento do mecanismo de resistência a fármacos de alguns organismos tem sido relatado em *Helicobacter pylori*, *Leishmania major* e *Staphylococcus aureus* (McAtee *et al.,* 2001; Drummelsmith *et al.,* 2003; Scherl *et al.,* 2006).

Genes promissores associados com fenótipos de resistência a fármacos de *T. cruzi* tem sido estudados. Murta *et al.,* (2006) identificou a deleção de cópias do gene que codifica uma nitroredutase tipo I TcOYE (Old Yellow Enzyme, também chamada Prostaglandina Sintetase) está associada com o fenótipo de resistência do *T. cruzi* ao BZ. Estas nitroredutases podem ser responsáveis pela ativação do BZ. As enzimas de defesa antioxidantes Triparedoxina peroxidase, Ascorbato peroxidade (APX) e Ferro-superóxido dismutase (TcFeSOD) foram observadas superexpressas em populações do *T. cruzi* resistentes a benzonidazol (Nogueira et al 2006, 2009 , 2012); Wilkinson *et al.,* (2008)

mostrou que uma nitroredutase dependente de NADH (NTRI) apresenta um papel na ativação do fármaco. Os autores sugerem que o mecanismo de resistência do *T. cruzi* ao BZ e NFX pode ocorrer pela redução nos níveis de nitroredutases (NTRI) nos parasitos resistentes, enquanto que a superexpressão desta nitroredutase pode resultar na excessiva sensibilidade do parasito.

Em muitos casos, os genes expressos diferencialmente ou mutados são desconhecidas ou a sua participação na resistência à fármacos não está bem definido. A identificação de genes que são expressos diferencialmente nas populações *T. cruzi* resistentes e suscetíveis ao BZ podem ajudar a aumentar nossa compreensão das bases moleculares de resistência do parasita a fármacos e conduzir à descoberta de novos alvos para quimioterapia. Neste estudo, o Ciclofilina A (TcCYP19) foi caracterizada em cepas de *T. cruzi* suscetíveis e resistentes a BZ .

2.7 Ciclofilina- A

Ciclofilina A (CyPA) são proteínas conservadas e tem sido isolada de procariotos e eucariotos, incluindo protozoários (Bell et al, 2006). A Ciclofilina -A pertencem ao grupo de peptidil - prolil -isomerases enzimas (PPIases) que catalisam a isomerização cis/trans . Esta classe de enzima é envolvida em muitos processos biológicos, incluindo o dobramento de proteínas, transdução do sinal, a interacção proteína - proteína, resposta ao stress, imunossupressão, sinalização celular, a divisão celular, regulação da transcrição (Wang et al, 2005; Nigro et al. 2013). A localização da CyPA é citosólica, no entanto pesquisas monstram que podem ser secretada em resposta a estímulos inflamatórios, tais como infecção, hipoxia e estresse oxidativo (Jin et al., 2000; Suzuki et al,2006).

O Mecanismo pelo qual as ciclofilinas participam de proteção sob estresses não está bem claro. Estudo sugere que a Ciclofilina pode atuar como proteínas chaperonas facilitando o dobramento de proteínas envolvidas com estresses ou protege essas proteínas da degradação proteolítica ou ocorre agregação sob condições de estresses (Kumari et al., 2013). Andreeva et al. (1999) evidencia o seu envolvimento com a resposta de choque de

calor. Pesquisadores observaram que em baixas concentrações, o H_2O_2 pode atuar como um segundo mensageiro, transdução do sinal oxidativo em respostas biológicas por meio da modificação de proteínas pós-tradução. Estado redox intracelular está intimamente regulado pelo equilíbrio entre oxidantes e sistemas antioxidantes e seu desequilíbrio pode causar estresse oxidativo, levando a danos celulares e desregulação (Satoh et al.,2013).

Além disso, foi observado o papel potencial da CyPA relacionada com várias doenças humanas: Em doenças cardiovasculares ocorre aumento de espécies reativas de oxigênio (ROS) e conseqüente a CyPA se supreexpressa promovendo envolvimento com recrutamento de células inflamatórias; Em diabetes, a CyPA secretada a partir de monócitos poderia ser um importante estímulo pró-inflamatório para inflamação vascular em pacientes com diabetes. CyPA pode funcionar como um sensor de célula-intrínseca capazes de reconhecer as proteínas de superfice do vírus HIV a fim de promover a infecção e replicação viral . A CyPA da célula hospedeira de mamífero pode estar envolvida no ciclo de replicação intracelular de *Leishmania, Plasmodium falciparum e Toxoplasma goodi* (revisado por Nigro et al., 2013). Estudo monstra que a CyPA é modulada e superexpressa em cancer sugerindo que esta proteína pode ser um alvo promissor para a terapia do câncer (Lee et al., 2010).

A família de Ciclofilinas do *T. cruzi* compreende várias isoformas, incluindo Ciclofilina A (TcCyP19), que variam em tamanho de 19-110 kDa (Potenza et al., 2006). Análise de organização molecular da família de genes de ciclofilina de *T. cruzi* mostrou que o TcCyP19 , TcCyP22 , TcCyP28 TcCyP40 e ligam-se a ciclosporina A (CsA) (Bua et al. 2001). A Ciclofilina A do *T. cruzi* (TcCyP19) é expressa em todos os estágios evolutivos do parasito, entretanto é bem mais abundantemente na fase de epimastigota do *T. cruzi.* Esta isoforma é uma ciclofilina citosólica clássica com massa teórica de 18,782 Da (19 kDa). Búa e colaboradores (2001) observaram que TcCyP19 é homóloga à ciclofilina A a partir de *T. brucei* (TbCyP19), *L. major* (LmCyP19) e *T. vivax* (TvCyP19). Foi relatado que TcCyP19 e outros membros da ciclofilina de *T. cruzi* (por exemplo, TcCyP22, TcCyP28 e TcCyP40)

também são capazes de se ligar ao compostoimunossupressor ciclosporina A (CsA), e evidencia o seu papel na mediação de respostas imunossupressores (Potenza et al., 2006).

Além disso, pesquisa demonstra a interação de um pepítideo antimicrobiano salivar α-helicoidal do Triatoma infestans- a trialisina, com a TcCyP19. Foi observado que a TcCyP19 secretada pelo parasita, se liga e inativa a trialisina através de uma ação no seu resíduo de prolina para induzir ativação bio-energética do parasita protegendo-o contra os efeitos citolíticos e favorecendo um aumento de infectividade. Támbem os autores perceberam que modificando os resíduos da trialisina torna-se com efeito ativo contra o *T.cruzi* (Kulkarni et al., 2013).

Apesar de várias funções, o envolvimento de TcCyP19 no fenótipo de resistência à BZ de *T. cruzi* não tenha sido ainda demonstrada – o que se deu a partir do presente estudo.

3 Câncer

A Organização Mundial da Saúde (OMS) estimou que, para o ano 2030, podem-se esperar 27 milhões de novos casos de câncer, 17 milhões de mortes por câncer e 75 milhões de pessoas vivas, anualmente, com câncer. (OMS, 2012).

O Brasil vem sofrendo mudanças em seu perfil demográfico, conseqüência, entre outros fatores, do processo de urbanização populacional, da industrialização e dos avanços da ciência e da tecnologia. A essas novas características da sociedade brasileira, unem-se os novos estilos de vida e a exposição, ainda mais intensa, a fatores de risco próprios do mundo contemporâneo. Esse processo de mudança demográfica, denominado de “envelhecimento” da população, associado à transformação nas relações entre as pessoas e seu ambiente, trouxe uma alteração importante no perfil de morbimortalidade, diminuindo a ocorrência das doenças infectocontagiosas e colocando as doenças crônico-degenerativas como o câncer, novo centro de atenção dos problemas de doença e morte da população brasileira (INCA, 2014).

O câncer é um termo genérico para um amplo grupo de doenças que podem afetar qualquer lugar do corpo. Outros termos utilizados são tumores e neoplasias malignas. Uma característica marcante do câncer é a formação rápida de células anormais que podem invadir tecidos próximos, bem como se disseminar para outros tecidos e órgãos (OMS, 2012). As neoplasias podem diferir de acordo com o tipo de célula, tecido ou órgão em que se desenvolvem. Geralmente as células neoplásicas levam a formação de uma massa tumoral, podendo esta ser benigna ou maligna (INCA, 2008).

Seletividade molecular e mecanismo molecular do cancer

3.1 Câncer de Mama

O Câncer de Mama (CM) é o segundo tipo mais frequente no mundo e mais comum entre as mulheres, respondendo por 22% dos casos novos a cada ano. Na população mundial, a sobrevida média após cinco anos é de 61%. No Brasil, a estimativa deste ano 2014 aponta para a ocorrência de aproximadamente 57 mil casos novos de câncer de mama e as taxas de mortalidade continuam elevadas, muito provavelmente porque a doença ainda é diagnosticada em estádios avançados. (INCA, 2014). Segundo a Organização Mundial da Saúde (OMS), nas décadas de 60 e 70 registrou-se um aumento de 10 vezes nas taxas de incidência ajustadas por idade nos Registros de Câncer de Base Populacional de diversos continentes. Estatísticas indicam aumento de sua incidência tanto nos países desenvolvidos quanto nos em desenvolvimento.

A etiologia do câncer de mama é variada podendo ser influenciada por agentes físicos e químicos do meio ambiente ou por produtos tóxicos da própria célula, como os radicais livres, ou fator genético de cada indivíduo. Na maioria das vezes o gene do câncer inicia por mutações em células epiteliais constituintes dos túbulos e lóbulos mamário (Ellsworth et al., 2010). Esses genes mutados- são oncogenes, e acarretam a desregulação de vias importantes como perda do controle da proliferação e da divisão celular, imortalização celular, devido ativação da enzima telomerase, alterações cromossômicas, perda de adesão da membrana plasmática, perda de função e da capacidade de diferenciação celular e capacidade para invadir o tecido vizinho promovendo a

carcinogênese. Enquanto os genes supressores mantêm essa proliferação sob controle, restringindo assim o crescimento celular. Os genes reparadores de danos do DNA têm por função refazer as moléculas que sofrem mutação. Quando ocorre o mau funcionamento dos mecanismos de regulação do ciclo celular, permitindo a passagem das células mutadas pelo ciclo, o acúmulo dessas mutações, acaba por contribuir no surgimento das características do tumor maligno (Belizário, 2002; Vermeulen, 2003).

As estratégias para controle do CM podem ser dirigidas a indivíduos assintomáticos ou sintomáticos. As ações de saúde voltadas para indivíduos assintomáticos objetivam evitar o câncer mediante o controle da exposição aos fatores de risco (prevenção primária), bem como detectar a doença e/ou lesões precursoras em fase inicial, chamadas de rastreamento – é a aplicação de um exame em uma população presumivelmente assintomática (WHO, 2007). Por outro lado, as ações que identificam indivíduos sintomáticos são chamadas de diagnóstico precoce – reconhecimento pelo paciente ou pelo profissional de saúde de sinais e sintomas precoces da doença tais como: o surgimento de alterações na pele que recobre a mama, como abaulamentos ou retrações, inclusive no mamilo, ou aspecto semelhante a casca de laranja. Além disso, a liberação de secreção no mamilo também é um sinal de alerta. O sintoma do câncer palpável é o nódulo (caroço) no seio, acompanhado ou não de dor mamária. Podem também surgir nódulos palpáveis na axila (WHO, 2007; INCA, 2014,).

O câncer de mama quando detectado em sua fase inicial tem enormes chances de cura, no entanto, o diagnóstico tardio da doença no Brasil deve-se à dificuldade de acesso da população aos serviços públicos de saúde, baixa capacitação dos profissionais envolvidos na atenção oncológica, incapacidade do sistema público para atender à demanda ou baixa capacidade dos gestores municipais e estaduais em definir o fluxo de casos suspeitos em diferentes níveis de atenção, e a própria demora do paciente em procurar atendimento, seja por medo ou negação da doença (INCA, 2012; Janime et al.,2011).

3.2 Quimioterapia do câncer de mama

O tratamento de câncer de mama avançado é bastante heterogêneo na prática clínica, podendo envolver quimioterapia, fármacos alvo-específicas, hormonioterapia e, e alguns casos, cirurgia e radioterapia paliativa. Além disso, diversos fármacos, isolados ou em combinação, têm mostrado atividade importante para esses pacientes, compondo um arsenal terapêutico bastante diversificado (Schutz et al., 2007)

Os quimioterápicos estão divididos em várias classes, com base no mecanismo pelo qual interferem na divisão celular. Quimioterápicos de diferentes classes podem ser combinados para ter maior eficácia contra o câncer, mas com consequente maior toxicidade.. Quando a quimioterapia é administrada em pacientes com doença metastática e incurável, exceto em situações onde é urgente a resposta rápida ao tratamento, é dado preferência à utilização de quimioterápicos isoladamente, para evitar interferir em demasia na qualidade de vida das pacientes. Assim, administrando um quimioterápico, só muda-se para um segundo, somente quando a doença não responde mais ao primeiro, frequentemente podemos proporcionar sequencialmente o benefício de várias linhas de quimioterapia a estas pacientes, prolongando ao máximo a sobrevida (INCA, 2012).

3.3 Peixe-zebra (*Danio rerio*) e células MCF-7 em estudos de cancer

O peixe-zebra (*Danio rerio*), também conhecido popularmente como “paulistinha”, é um peixe teleósteo com tamanho de 3 a 4 cm e pertencente a família Cyprinidae. As vantagens do peixe-zebra como modelo de estudo do desenvolvimento dos vertebrados deve-se a produção de grandes posturas, que podem atingir 200 embriões a cada sete dias, e ao fato dos embriões serem pequenos, transparentes e com um rápido desenvolvimento externo. Além do Peixe-zebra ter uma taxa muito baixa de neoplasia espontânea, apenas aproximadamente 10% dos peixes zebras tem possibilidade de desenvolver um tumor (Tavares et al,2013).

Outro aspecto importante é que o genoma do peixe-zebra está completamente sequenciado, apresentando 26.206 genes, mais do que em qualquer outro vertebrado. A comparação com o genoma humano de referência mostra que aproximadamente 70% dos genes humanos possuem pelo menos um ortólogo do peixe-zebra (Howe et. al.,2013.)

A primeira publicação na pesquisa do cancer utilizando o peixe-zebra foi realizado por Stanton e colaboradores (1981) para testar os efeitos de substâncias cancerígenas. Atualmente, o peixe-zebra tem sido um interessante modelo para estudo de cânceres. Vários estudos mostram que os melanomas, leucemia linfóide e hepatocarcinoma do peixe-zebra se assemelham a doença humana morfologicamente, geneticamente e epigeneticamente, demonstrando adequação para esse fim terapeutico (Liu et al., 2011; Tavares et al., 2013). Outras pesquisas têm usado painéis de compostos farmacêuticos ou pequenas moléculas, onde os embriões do peixe-zebra são expostos a produtos químicos para testar os níveis de dose-resposta de fármacos, potencial de toxicidade, e potencial terapêutico de compostos conhecidos ou novos (Phillips & Westerfield, 2014)

A célula “Michigan Foundation-7 Câncer” (MCF-7) deve-se esta denominação referindo-se ao instituto onde foi criada, são células de câncer de mama que apresentam um fenótipo tumoral (Soule et al., 1973). Representam bons modelos para estudo de cancer de mama, seja pela rápidez na duplicação, maior resistência aos quimioterápicos e mantêm características diferenciadas específicas de tumores mamários em cultura celular. Estas células têm sido utilizadas em diversos ensaios que envolvem teste de fármacos e radioterapia. (Shenvi et al., 2014) O fármaco doxorrubicina induz apoptose das células MCF-7 e MDA-MB-231 cancerígenas mamárias (Wang et al., 2014). A atividade do composto lupeol atua como um agente anticancerígeno contra células MCF-7 (Pitchai et al., 2014).

CAPÍTULO I

Ciclofilina (TcCyP19) e fenótipo de resistência do *Trypanosoma cruzi*.

Artigo aceito para publicação no periódico ***Experimental Parasitology****. As normas de publicação do periódico foram respeitadas (ANEXO A). O comprovante de submissão segue no (ANEXO C)*

Molecular characterization of Cyclophilin (TcCyP19) in *Trypanosoma cruzi* populations susceptible and resistant to benznidazole

Juciane Vaz Rêgo[a,b,e/+], Ana Paula Duarte[b], Daniel Barbosa Liarte[c], Francirlene de Carvalho Sousa[a], Humberto Medeiros Barreto[c], Jacqueline Búa[d], Alvaro José Romanha[b], Gandhi Rádis-Baptista[e], Silvane Maria Fonseca Murta[b*]

[a]Campus Amilcar Ferreira Sobral,Universidade Federal do Piauí-UFPI, Floriano, PI, Brazil

[b]Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisa René Rachou-CPqRR/FIOCRUZ, Belo Horizonte-MG, Brazil

[c] Campus Petrônio Portela, Universidade Federal do Piauí-UFPI,Teresina-PI, Brazil

[d]Instituto Nacional de Parasitología “Dr. M. Fatala Chabén”, A.N.L.I.S. Buenos Aires, Argentina

[e]Instituto de Ciências do Mar, Universidade Federal do Ceará-Labomar/UFC, Fortaleza-CE, Brazil

* Corresponding author. Tel.: +55-31-33497780, Fax: +55-31-32953115.

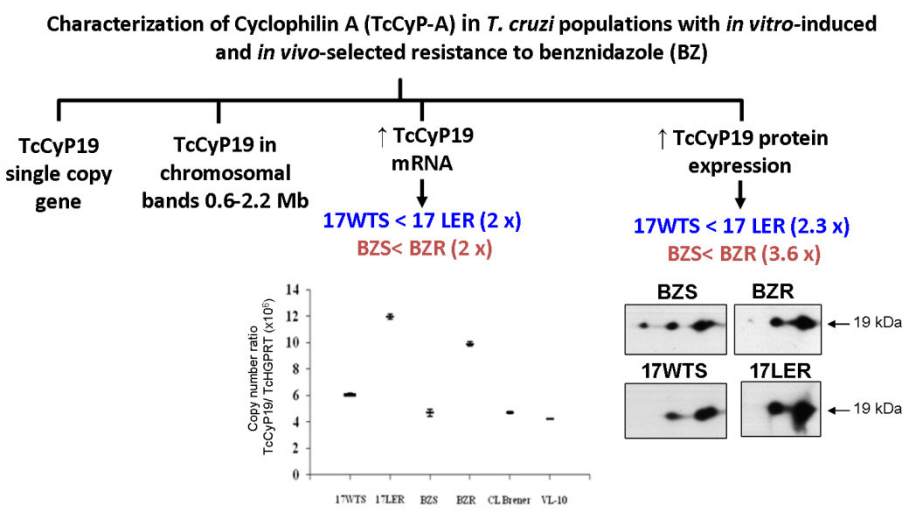
E-mail address: silvane@cpqrr.fiocruz.br (Silvane.M. F. Murta), juciane@ufpi.edu.br (J.V. Rego)

Abbreviations: CyP-A, cyclophilin-A; TcCyp19, *T. cruzi* 19 kDa cyclophilin; BZ, Benznidazole; HGPRT, Hypoxanthine-guanine phosphoribosyltransferase; LIT, Liver-infusion tryptose; NFX, Nifurtimox; PCR, Polymerase chain reaction; qRT-PCR, real time PCR; PFGE, Pulsed-field gel electrophoresis.

HIGHLIGHTS

> Cyclophilin A (CyP19) gene was characterized in 11 strains and clones of *T. cruzi*
> TcCyP19 is a single copy gene located in chromosomal bands varying from 0.6 to 2.2 Mb
> There is a clear divergence between CyP-A of trypanosomatids and mammals
> TcCyP19 transcripts were 2-fold higher in BZ-resistant *T. cruzi* populations
> TcCyP19 protein expression was 2-fold higher in BZ-resistant *T. cruzi* populations;

GRAPHICAL ABSTRACT



ABSTRACT

Cyclophilin A (*Tc*CyP19), a peptidyl-prolyl cis/trans isomerase, is a key molecule with diverse biological functions that include roles in molecular chaperoning, stress response, immune modulation and signal transduction. In this respect, CyP could serve as potential drug target in disease-causing parasites. Previous studies employing proteomic technique have shown that *Tc*CyP19 isoform was more abundant in a benznidazole (BZ)-resistant *Trypanosoma cruzi* population than in its susceptible counterpart. In this study, *Tc*CyP19 has been characterized in BZ-susceptible and BZ-resistant *T.cruzi* populations. Phylogenetic analysis revealed a clear dichotomy between Cyclophilin A (CyPA) sequences from trypanosomatids and mammals. Sequencing analysis revealed that the amino acid sequences of the *Tc*CyP19 are identical among the *T. cruzi* samples analyzed. Southern blot analysis showed that *Tc*CyP19 is a single-copy gene, located in chromosomal bands varying in size from 0.68 to 2.2 Mb, depending on the strain of *T. cruzi*. Northern blot and qPCR indicated that the levels of *Tc*CyP19 mRNA were twofold higher in drug-resistant *T. cruzi* populations compared to drug-susceptible counterparts. Similarly, as determined by two-dimensional gel electrophoresis immunoblot, the expression of *Tc*CyP19 protein was

increased to the same degree in BZ-resistant *T. cruzi* populations. No differences in the *Tc*CyP19 mRNA and protein expression levels were observed between susceptible and the naturally resistant *T. cruzi* strains analyzed. Taken together, these data indicate that cyclophilin *Tc*CyP19 expression is up-regulated at both transcriptional and translational levels in *T. cruzi* populations that were *in vitro*-induced and in-vivo selected for resistance to BZ.

Key words: *Trypanosoma cruzi* – Cyclophilin - Benznidazole - Drug resistance

1. Introduction

Chagas disease, also known as American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi.* It is estimated that 8 to 11 million people are infected worldwide, mostly in Latin America where Chagas disease is endemic (CDC 2013). Drug treatment of chagasic patients is currently involves the 5-nitrofuran, nifurtimox (NFX) and the 2-nitroimidazole Benznidazole (BZ). However, both drugs cause severe side effects. NFX exerts its action via the generation of nitro anion radicals, a process known as redox cycle (Maya et al., 2003), while BZ acting via reductive stress, which involves the covalent modification of macromolecules such as DNA, proteins, and lipids by reduced nitro intermediates (Docampo, 1990).

A Previous study suggests that both BZ and NFX are pro-drugs activated by nitroreductases to produce nitrogenated radicals (Wilkinson et al., 2008). Interestingly, the deletion of copies of genes encoding two different nitroreductases, namely, old yellow enzyme (TcOYE; also named prostaglandin synthase or NADPH oxidoreductase) (Murta et al., 2006) and trypanosomal type I nitroreductase (NTR-1) (Wilkinson et al., 2008), has been associated with a phenotype of *T. cruzi, which is resistant* to NFX and BZ *in vitro*. Overexpression of enzymatic activity related to antioxidant response, like that of tryparedoxin peroxidase, ascorbate peroxidase and iron-superoxide dismutase has been detected in *T.*

cruzi populations resistant to BZ (Nogueira et al., 2006, 2009, 2012). In addition, the hexose transporter activity was 40% lower in a BZ**-**resistant *T. cruzi* population than in the susceptible control (dos Santos et al., 2012). Differences in susceptibility to BZ and NFX between *T. cruzi* strains (Filardi and Brener, 1987; Murta et al., 1998; Toledo et al. 2004) and / or genetic diversity of the host (Filardi and Brener, 1987) might explain in part the variations in the efficacies of antiparasitic drugs. The mechanisms underlying drug resistance in *T. cruzi* and most parasites remain poorly understood. A more in-depth understanding of these mechanisms is essential to develop efficacious chemotherapeutic strategies for fighting Chagas disease.

Several groups have used proteomic approaches in order to understand the mechanisms of drug resistance in protozoan parasites. In a previous study by our group, two-dimensional electrophoresis in combination with mass spectrometry was applied to detect differences in protein expression between BZ–susceptible and resistant *T. cruzi* populations, as well as variant clones as well (Andrade et al., 2008). Among the protein up-regulated in BZ-resistant samples, we observed that cyclophilin *Tc*CyP19 was more abundant in the BZ-resistant population (17LER) and clone (27R) than in their BZ-susceptible counterparts (17WTS and clone 9S).

Cyclophilin is considered an important player in several biological processes like signal transduction, protein-protein interaction, as well as protein folding and cellular stress response (Galat, 2003). This protein belongs to the peptidyl-prolyl isomerase family (PPIases), which catalyzes the cis-trans isomerization of prolyl-peptide bonds in biochemical pathways of cellular communication. The role of CyPs in heat shock response, in which the expression of certain class of cyclophilins was shown to be up-regulated under various stressful conditions, was been reviewed previously (Andreeva et al., 1999). The cyclophilin family comprises several isoforms, including cyclophilin *Tc*CyP19, ranging in size from 19 to 110 kDa (Potenza et al., 2006). The most abundantly expressed cyclophilin expressed in the epimastigote stage of *T. cruzi* is *Tc*CyP19. This isoform is a classical cytosolic cyclophilin with theoretical mass of 18.782 Da (19 kDa). Búa and co-workers (2001) observed that

*Tc*CyP19 is homologous to Cyclophilin from *T. brucei* (*Tb*CyP19), *L. major* (*Lm*CyP19) and *T. vivax* (*Tv*CyP19). It has been reported that *Tc*CyP19 and other cyclophilin members of *T. cruzi* (e.g., *Tc*CyP22, *Tc*CyP28 and *Tc*CyP40) are also able to bind to the immunosuppressant drug cyclosporine A (CsA), which could sere to explain their role in mediating immunosuppressive responses (Potenza et al., 2006). Despite of multiple roles, the involvement of *Tc*CyP19 in the BZ-resistance phenotype of *T. cruzi* has not yet been demonstrated.

In the present study, the phylogenetic relationship between *Tc*CyP19 and CyPs from other organisms was established. TcCyP19 nucleotide and amino acid sequences polymorphisms were evaluated, the copy number and chromosomal location of *Tc*CyP19 gene was determined, and the expression of *Tc*CyP19 gene was analyzed at transcriptional and translational levels. We have observed a positive correlation between increased expression of *Tc*CyP19 in *T. cruzi* populations with vitro-induced and *in vivo*-selected resistance to BZ.

2. Materials and methods

2.1 Trypanosoma cruzi strains

Eleven *T. cruzi* strains and clones were used in this study (Table I). The BZ-resistant *T. cruzi* population (17 LER) derived from the Tehuantepec cl2 susceptible wild-type strain (17 WTS) (Nirdé et al., 1995) was obtained by *in vitro* exposure to increasing concentrations of BZ (LAFEPE Pharmaceutical Laboratory of the State of Pernambuco, Vitória de Santo Antão, Brazil). Parasites of the 17 LER population are resistant to 220 µM BZ, a concentration that is 23-fold higher than the IC_{50} for the 17 WTS control population. The BZ-resistant *T. cruzi* population (BZR) was derived from the susceptible Y strain (BZS) following *in vivo* selection after 25 successive passages in mice treated with a single high dose (500 mg/kg body weight) of BZ (Murta and Romanha, 1998). We also used two clones derived

from these populations: one susceptible (clone 9S) and other resistant (clone 27R). The other five *T. cruzi* strains were previously characterized according to their *in vivo* susceptibility to BZ and NFX (Filardi and Brener, 1987). The VL-10 strain is naturally resistant to both drugs, while CL Brener, Romano, Buriti and Berenice are susceptible (Murta et al. 1998). All eleven strains were classified as *T. cruzi* group Tc I, TcII or TcVI according to the nomenclature for *T. cruzi* (Zingales et al., 2009). Epimastigotes forms of all *T. cruzi* strains were grown in LIT medium, washed in PBS and the parasite pellets were used for the preparation of DNA, RNA and protein samples.

2.2. In silico and phylogenetic analysis of the TcCyP19 gene

Similarity searches were carried out for complete *TcCyP19* amino acid sequence (GenBank accession no. XM_816485) within five different *T. cruzi* genomes, using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information). The genomes used for similarity search were CL Brener Esmeraldo_Like, CLBrener Non-Esmeraldo_like, SylvioX10, JRc14 and Marinkellei_B7 (available in TritrypDB - www.tritrypdb.org - version 6.0). The sequences with high identity values to *Tc*CyP19 were located within the respective genomes and the predicted proteins were identified using the ARTEMIS (version 15.0 - www.sanger.ac.uk) and BLAST. For phylogenetic analysis, the predicted protein sequences corresponding to cyclophilins were aligned using CLUSTAL-W (Larkin et al., 2007), and the phylogenetic tree was constructed using the MEGA software (Molecular Evolutionary Genetics Analysis, version 5.2.2 – Tamura et al., 2011) with bootstrap test (1000 replicates). In addition, the amino acid sequence from *Tc*CyP19 was compared with related cyclophilin sequences from four species of *Trypanosoma* (*T. brucei*, *T. congolense*, *T. evansi* and *T. vivax*), five *Leishmania* (*L. donovani*, *L. infantum*, *L. major*, *L. mexicana* and *L. braziliensis*), and two mammals (*Mus musculus*, and *Homo sapiens*). A phylogenetic tree for this second multialignment was also constructed using the MEGA software.

2.3. RNA and DNA preparations

Genomic DNA and total RNA from *T. cruzi* samples were extracted as previously described (Nogueira et al., 2006). In order to prepare the molecular probes used in the Southern and Northern blot assays, a 400 bp segment corresponding to nucleotide 68 to 468 of the *Tc*CyP19 (GenBank accession no. XM_816485) was amplified from *T. cruzi* Y strain DNA by conventional PCR using the TcCYP19 forward primer 5' GGTGGCCAATCAGCCGGACG 3' and the TcCYP19 reverse primer 5' TCCATTGCCTTAACGACTTC 3'. For Southern blot assay, about 5 µg total DNA of different *T. cruzi* strains were digested with the restriction enzyme *Sac*I in standard reaction conditions (Invitrogen, Carlsbad, CA, USA). The Southern and northern blots were carried out using protocol previously described (Murta et al., 2006).

2.4. DNA sequencing

The *Tc*CyP19 534 bp ORF from *T. cruzi* BZ-susceptible and -resistant populations (17WTS, 17LER, BZS, BZR, CL Brener and VL-10) was cloned into the TOPO PCR2.1 vector (Invitrogen) and amplified in *E. coli* TOP 10 F' competent cells. Minipreparations of plasmid DNA were done using the QIAprep Spin Miniprep kit (Qiagen). Aliquots of 500 ng DNA were sequenced using the DYEnamic WET Dye Terminator Kit (GE Healthcare) in a MegaBACE 1000 DNA Analysis System (GE Healthcare), using the following primers: M13 forward 5'-GTAAAACGACGGCCAG-3', M13 reverse 5'-CAGGAAACAGCTATGAC-3' and TcCyP19 forward 5'-ATGTCGTACAAGCCGCATCA-3' and *Tc*CyP19 reverse 5'-AGGCCTCTGGTCAACTTTAA-3' Reaction consisted of an initial denaturation at 95°C followed by 30 cycles of 15s 95°C, 20s at 55°C and 80s at 60°C. Samples were analyzed on Mega Bace 400 sequencer (Amersham) and the data were analyzed using Phred, Phrap and Consed. Sequence variability between parasites was assessed by sequencing three colonies of each *T. cruzi* population and by sequencing each colony twice with each primer. Sequences selected for analysis were those with Phred >40. Nucleotide sequences were

translated into the amino acid sequence using Transec. The nucleotide and amino acid sequences were aligned using the ClustalW 2.1 software.

5. Pulsed Field Gel Electrophoresis (PFGE)

The *T. cruzi* chromosomes were separated by PFGE in a gene navigator TM system (Amersham Pharmacia, Buckinghamshire, UK), as previously described (Murta et al. 2006). After testing different electrophoresis conditions and switch times, the best condition that provided the optimal separation for *Tc*CyP19 gene was: 70 s for 15 h, 90 s for 24 h, 200 s for 15 h, and 400 s for 15 h at 180 V. After electrophoresis, the gels were transferred onto Hybond nylon membranes (Amersham) as described by the manufacturer's instructions. The membranes were hybridized with the ^{32} P-labeled *Tc*CyP19 gene probe.

2.6. Quantitative real-time RT-PCR

The protocol employed for the preparation of first strand cDNA and the procedure for real-time RT-PCR were as previously described (Nogueira et al., 2006). An ABI Prism 7000 - Sequence Detection System SDS (PE Applied Biosystems, Foster City, CA, USA) was employed in the real-time PCR amplification of first strand cDNA (5 µl) using the specific primers: 5` TGTCGTACAAGCCGCATCAC 3` (RT CYPRTF forward) and 5` CAATGCTGACGTCGAAGAAGAC 3` (RT CYPRTR 2 reverse) selected from the complete nucleotide sequence of *Tc*CyP19 (GenBank Accession No. XM_816485). The *T. cruzi* housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (TcHGPRT) was used to normalize the amount of samples (Nogueira et al., 2006). Standard curves were prepared for each experiment using known quantities of TOPO PCR 2.1 plasmids (Invitrogen) containing the *TcCyP19* and *TcHGPRT* genes. PCR products were quantified using Sequence Detection System data analysis software and normalized to the *TcHGPRT* values for each sample.

2.7. Two-Dimensional Gel Electrophoresis (2-DE) and Western blotting analysis

Protein extracts were obtained by direct lysis of parasites in lysis buffer (Matrangolo et al., 2013) in a proportion of 100 µg for 3.5 x10^8 epimastigote forms. After extraction, protein concentration was quantified by Bradford method. Protein extracts (100 µg) were loaded on 7 cm non-linear IPG strips pH 3–10 (Bio-Rad) and isoelectric focused using the Protean IEF Cell (Bio-Rad), 50 µA/strip at 20ºC. Passive rehydration was performed for 4 h, followed by an active rehydration at 50 V for 12 h. Isoelectric focusing was increased gradually to 4,000 V and run for 16,000 V-hour. Subsequently, the second dimension electrophoretic protein separation was performed in 12% SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated for 16 h at 4°C with rabbit polyclonal antibody anti-cyclophilin (TcCyP19) of *T. cruzi* (1:5000). The blots were washed and then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG 1:2000 (GE Healthcare). The blots were washed, incubated with ECL Plus chemiluminescent substrate (GE Healthcare) and exposed to X-ray film. To confirm equivalent loading, SDS-PAGE containing the same samples were stained with Colloidal Coomassie Blue G-250 (Neuhoff et al., 1988). The gels stained with coomassie Blue G250 and the immunoblots were scanned on a GS-800 calibrated densitometer (BioRad) and submitted to comparative analysis.

3. Results

*3.1. In silico and phylogenetic analysis of Tc*CyP19 *gene*

Similarity searches were carried out between the *Tc*CyP19 sequence (GenBank accession XM_816485) and local copy of the *T.cruzi* database of five different *T. cruzi* genomes. Altogether, 79 sequences similar to that of *Tc*CyP19 were found in the five *T. cruzi* genomes (13 to 19 sequences identified from each genome). The predicted protein sequence of the gene sequences identified was analyzed and compared to NCBI non-redundant database and 61 were found to share the highest similarity with cyclophilins. Of these, 24 proteins (including *Tc*CyP19) had molecular weight ranging from 19 to 23 kDa,

which is similar to the molecular eight of *Tc*CyP19. A phylogenetic tree was constructed using these 24 cyclophilins sequences (ranging from18.7 to 23.7 kDa). A cyclophilin having a molecular weigth of 35 kDa was used as outgroup (Supplementary Fig. 1S). The results showed that these cyclophilins were grouped in two divergent branches. One of the branches included cyclophilins having molecular weights of 18.7 kDa (TCyP19); 21.4-23.7 kDa and 21.0-21.1 kDa,while the other encompassed cyclophilins having molecular weights of 21.6-21.7 and 19.7-19.8 kDa.

In order to compare the similarity of *Tc*CyP19 amino acid sequence with cyclophilin-A (CyPA) sequences of different organisms, a neighbor-joining phylogenetic tree was constructed (Fig. 1). This Phylogenetic tree shows a clear dichotomous divergence between trypanosomatids and mammalian CyPA sequences. The amino acid sequence of *TcCyP19* is closely related to sequences from other species of *Trypanosoma* and *Leishmania*, sharing identities of between 84% to 90% and 78% to 81%, respectively.

3.2. Sequencing data

DNA sequencing of *Tc*CyP19 gene from *T. cruzi* populations that were susceptible (CL Brener, 17WTS and BZS), naturally resistant (VL-10) and with *in vitro*-induced (17 LER) and *in vivo*-selected (BZS) resistance to BZ was performed in order to investigate whether point mutations could be associated with the BZ-resistance phenotype. Multi-sequence alignment of the *Tc*CyP19 gene nucleotide sequences revealed three nucleotide mutations (positions 150, 261 and 390) that do not lead to amino acid substitution (Supplementary data Fig. 2S). No association was found between nucleotide mutations and BZ-resistance phenotype. These three nucleotide mutations are strain specific.

*3.3. Copy number of Tc*CyP19 *gene*

Southern blot assays were carried out using samples of *T. cruzi* genomic DNA that had been digested with the endonuclease *Sac*I; which has one restriction site within the reference *Tc*CyP19 sequence (GenBank accession no. XM_816485). Hybridization of the blots of *Sac*I-

digested DNA with a *Tc*CyP19-specific probe revealed two fragments of 7.8 and 1.5 Kb in all *T. cruzi* samples so far analyzed (Supplementary data Fig. S3).

The copy number ratio of the *Tc*CyP19 gene per genome for the pair 17WTS/17LER and BZS/BZR was also determined by real-time PCR. Considering that *T. cruzi* contains 0.33 pg of DNA (Moser et al., 1989) and that *TcHGPRT* is a single copy gene (Allen and Ullman, 1994), *Tc*CyP19 copy numbers were estimated using 200, 100, 50 and 25 ng of genomic DNA. It was found that the copy number ratio of the *Tc*CyP19 gene was the same for 17WTS/17LER and BZS/BZR populations (data not shown), indicating that the number of *TcCyP19* gene is not different in the genome of *T. cruzi* resistant populations.

*3.4. Chromosomal location of the Tc*CyP19 *gene*

Chromosomes from *T. cruzi* strains were separated by pulsed field gel electrophoresis (PFGE) (Fig. 2B). Chromosomes hybridization with the *Tc*CyP19-specific probe showed that this gene is present in chromosomal bands that range from 680 to 2200 kb (Fig. 2C). A correlation between the chromosomal location of *Tc*CyP19 gene and *T. cruzi* group was observed for the *T. cruzi* strains analyzed in this study (Table I). However, no correlation between chromosomal location of the *Tc*CyP19 gene and the drug-resistance phenotype was established.

*3.5. Levels of Tc*CyP19 *mRNA transcript*

Levels of *Tc*CyP19 mRNA in parasite populations were first investigated by Northern blot analysis. A 1.4-kb transcript was detected in Northern blots of total RNA derived from BZ-susceptible and -resistant *T. cruzi* strains, following hybridization with a ^{32}P-labelled *Tc*CyP19-specific probe (Fig. 3A). Quantitative controls using a ribosomal RNA probe are shown in Fig. 3B. Comparative densitometric analysis revealed that *Tc*CyP19 mRNA levels were at least twofold higher in BZ-resistant 17LER and BZR populations than in their susceptible counterparts 17WTS and BZS. We did not observed any comparative difference in the levels of *Tc*CyP19 mRNA among other *T. cruzi* strains and clones analyzed.

*Tc*CyP19 mRNA levels were complementarily determined by quantitative real-time RT-PCR to confirm the northern blot results. The amount of *Tc*CyP19 cDNA in the samples of *T. cruzi* was normalized by a internal reference - the single copy housekeeping gene *TcHGPRT,* used as an internal reference. The results, shown in Fig 3C, indicate that the levels of transcription of the *Tc*CyP19 gene were twofold higher in the 17LER and BZR populations compared with the 17WTS and BZS populations. No differences in the levels of transcription of the *Tc*CyP19 gene were detected between the other *T. cruzi* BZ-susceptible and -resistant sample pairs, i.e. CL Brener *versus* VL-10.

*3.6. Levels of Tc*CyP19 *protein expression*

2-DE Western blotting analysis showed that the anti-*Tc*CyP19 polyclonal antibody recognized two spots with the expected size of 19 kDa and isoelectric point (p*I*) 7.0 to 8.5 in the pair 17LER/17WTS and three spots in the BZR/BZS pair (Fig. 4A), which coincides with that for *Tc*CyP19. These spots, with p*I*s value ranging from 7.0 to 8.5 correspond to the different isoforms of *Tc*CyP19, probably owing to post-translational modifications of the protein (Fig. 4). These findings confirm the protein identity and are suggestive of post translational modification of TcCyP19. a kind of post-translational modifications of *Tc*CyP19. According, to the releante literature, cyclophilin-A of *L. infantum* has one site of acetylation justifying the occurrence of isoforms with more acidic p*I* (Rosenzweig et al., 2008). In fact, the polyclonal antibody anti-*Tc*CyP19 might recognize *Tc*CyP19 and other cyclophilin isoforms of similar molecular weight but different isoelectric points.

Comparative analysis between Coomassie Blue-stained protein profiles of BZS/BZR and 17WTS/17LER T.cruzi population showed that the protein load as similar between the samples tested (Fig. 4A and C). We selected the same region of both the Coomassie Blue-stained gels of 17WTS/17LER and BZS/BZR to perform the densitometric analysis. Densitometric analysis of the spots showed that the levels of protein expression of the two spots were higher in the 17LER BZ-resistance population than in its susceptible counterpart,

i.e., the 17 WTS. In the BZS/BZR populations, we identified three spots of cyclophilin TcCyP19. One of them had higher expression levels in the BZS population, while the other two were more hightly expressed in the BZ-resistant population BZR than in the susceptible population 17WTS (Fig. 4).

In addition, we comparatively assessed the *Tc*CyP19 protein expression of a BZ-susceptible *T. cruzi* strain (CL Brener) and that of a *T cruzi* strain (VL-10) naturally resistant to BZ, using western blot one-dimensional gel electrophoresis (1D). No differences were observed in the expression levels of *Tc*CyP19 protein between both *T. cruzi* strains analyzed (data not shown).

4. Discussion

Cyclophilin-A is a member of the peptidyl-prolyl cis/trans isomerase class of enzymes and it is a key molecule in diverse biological processes, including molecular chaperoning, protein folding and protein trafficking (Bell et al., 2006). In the present study, the gene encoding *Tc*CyP19 was characterized from populations and strains of *T. cruzi* that are susceptible or resistance to benznidazole (BZ). The ORF of *Tc*CyP19 (TcCLB.506925.300) is 534 bp in length and encodes a protein of 177 amino acids with a predicted mass of 19 kDa. Phylogenetic analyses of the amino acid sequences of CyPs revealed a clear dichotomy between trypanosomatids and mammalian cyclophilin sequences. CyP19 protein from *Trypanosoma* spp. and *Leishmania* spp. are the closest related groups in this family. In addition, sequences analysis revealed that the amino acid sequences of the *Tc*CyP19 protein are identical among the *T. cruzi* samples analyzed.

Southern blot analysis showed that *Tc*Cyp19 gene is present in the parasite genome as a single copy in BZ-resistant and susceptible *T. cruzi* populations. These data are concordant with the results of a study by Potenza et al. (2006), who reported the presence of a single copy for each of *Tc*CyP gene analyzed in the *T. cruzi* genome (i.e., *Tc*CyP19,

*Tc*CyP20, *Tc*Cyp22, *Tc*CyP25, *Tc*CyP28, *Tc*CyP34 and *Tc*CyP40). In the present study, the *Tc*CyP19 gene was located in chromosome bands varying in size from 0.68 to 2.2 Mb, depending on the strain of *T. cruzi*. In partial agreement, Búa and co-workers (2001) observed that the *Tc*CyP19 gene is located in two chromosomal bands of sizes 2.0 and 2.2 Mb in the CL Brener *T. cruzi* clone, whereas other cyclophilin isoforms are found dispersed in *T. cruzi* genome. These differences could be due to *T.cruzi* chromosomal rearrangements or cyclophilin-like sequences that could be recognized by the TcCyP19 probe.

Northern blot and qPCR indicated that the levels of *Tc*CyP19 mRNA were twofold higher in *T. cruzi* populations with in vitro-induced and in-vivo selected resistance to BZ compared with that in drug-susceptible counterparts. In accordance with these results, analytical 2DE immunoblot showed an increase of *Tc*CyP19 protein expression level in the BZ-resistant *T. cruzi* population. However, no difeferences in TcCyP19 mRNA and protein expression levels were observed between the susceptible and naturally resistant *T.cruzi* strain analyzed, indicating different drug resistance mechanisms in these samples. Many studies evaluating drug resistance mechanisms in parasites were based on models produced by artificial induction of resistance. In contrast, there is very little information available on the biochemical mechanisms underlying drug resistance in field isolates.The data presented here show that *Tc*CyP19 protein expression level is increased in *T.cruzi* populations that were in vitro-induced and in-ivo selected for resistace to BZ, a situation that is different from that obsered I the naturally resistant population. In agreement with our results, Villarreal et al. (2005) observed that the mechanisms associated with atural resistance to drugs differ from those associated with induced resistance. The mechanism of drug resistance, such as that to BZ, is often complex ad multifactorial, incluidg molecules associated with the host immune system, which may interfere with the susceptibility of the parasite to the drug (Murta et al. 1999). Studies to determine whether oerexpression of TcCyP19 in the susceptible population will confer the BZ-resistat pheotype to these parasites are required to confirm our hypothesis that TcCyP19 may be involved in *T.cruzi* resistance to BZ.

Interestingly, in the presence of BZ or other stress conditions, an increase in the expression of several proteins of distinct biological function is observed. Consequently, TcCyP19 might contribute to counteract the chemical stress stimulus by increasing, for example, the activity of protein folding, which is necessary for efficient expression of functional polypeptides. The correlation between drug resistance and increased chaperonin actiity has been observed in *L. donovani* (Kumar et al., 2013) and *T. cruzi* (Andrade et al., 2008). Interestingly, CyPA also has a protective role in murine cells functioning as an antioxidant against oxidative stress (Doyle et al., 1999; Hong et al., 2004). Additionally, in humans CyPA binds peroxiredoxins thereby increasing the peroxidase activity by their ability to transfer electrons (Lee et al., 2001). In analogy to this role, our data are indicative that the increased expression of *Tc*CyP19 could in turn favor the expression and activity of enzymes associated with antioxidant defense (Nogueira et al., 2006, 2009, 2012). This might serve in detoxifying the parasite ad might confer BZ-resistance a hypothesis that warrants further investigation.

Cyclophilin-A is also implicated in biological processes such as tumor resilience and progression. For instance, microarray analysis has show that CyPA can upregulate the expression of cytokines and genes related to drug resistance (Chen et al., 2008). Furthermore, it was observed that the elevated CyPA expression contributes to drug-resistance phenotype in cancer cells (Yang et al., 2011). Another study revealed that the overexpression of CyPA could promote cancer cell proliferation, cell migration/invasion, apoptose inhibition and drug-resistance phenotype in various cancer cell types (Obchoei et al., 2009). These data suggested that CyPA is a good target for cancer chemotherapy. Interestingly, some lines of investigation also evidenced that *Tc*CyPs are promising targets for the treatment of Chagas disease (Búa et al., 2008; Carraro et al., 2007). Cyclophilins bind cyclosporin A (CsA), an immunosuppressive antimicrobial drug and non-immunosuppressive CsA analogues possess higher activity against *T. cruzi* (Búa et al., 2008). In conclusion given that our results show that TcCyP19 is upregulated in BZ-resistant *T. cruzi* populations and that several lines of evidence implicate CyPs as a promising chemotherapeutic target against

cancer and parasite disease, our work constitutes a starting point for further investigation into the role of *Tc*CyPs in the mechanism of drug resistance and as target for Chagas disease chemotherapy.

Acknowledgements The authors are grateful to the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for the use of facilities. Financial support for the study was received from the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Universal 475782/2012-7), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG – CBB-PPM 00536/11 and CBB - PPM-00196-13) and P3D-Programa de descoberta e desenvolvimento de drogas (PROEP/CNPq/FIOCRUZ 401988/2012-0).

Appendix: Suplementary material

Suplementary data to this article can be found online at doi:10.1016/j.exppara. 2014.11.007.

References

Allen, T., Ullman, B., 1994. Molecular characterization and overexpression of the hypoxantine-guanine phosphoribosyltransferase gene from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 65, 233–245.

Andrade H.M., Murta S.M., Chapeaurouge A., Perales J., Nirdé P., Romanha A.J., 2008. Proteomic analysis of *Trypanosoma cruzi* resistance to benznidazole. J. Proteome Res. 7, 2357-2367.

Andreeva L., Heads R, Green C.J., 1999. Cyclophilins and their possible role in the stress response. Int. J. Exp. Pathol. 80, 305-315.

Bell A., Monaghan P., Page A.P., 2006. Peptidyl-prolyl cis-trans isomerases (immunophilins) and their roles in parasite biochemistry, host-parasite interaction and antiparasitic drug action. Int. J. Parasitol. 36, 261-276.

Búa J., Aslund L., Pereyra N., Garcia G.A., Bontempi E.J., Ruiz A.M., 2001. Characterisation of a cyclophilin isoform in *Trypanosoma cruzi*. FEMS Microbiol. Lett. 200: 43-47.

Búa J., Fichera L.E., Fuchs A.G., Potenza M., Dubin M., Wenger R.O., Moretti G., Scabone C.M., Ruiz A.M., 2008. Anti-*Trypanosoma cruzi* effects of cyclosporin A derivatives: possible role of a P-glycoprotein and parasite cyclophilins. Parasitol. 135, 217-228.

Carraro R., Búa J., Ruiz A., Paulino M., 2007. Modelling and study of cyclosporin A and related compounds in complexes with a *Trypanosoma cruzi* cyclophilin. J Mol Graph Model. 26:48-61.

CDC - Centers for Disease Control and Prevention [database on the Internet] 2013. American trypanosomiasis. Available from, cdc.gov/parasites/chagas/.

Chen S., Zhang M., Ma H., Saiyin H., Shen S., Xi J., Wan B., Yu L., 2008. Oligos-Microarray analysis reveals the role of cyclophilin a in drug resistance. Cancer Chemother. Pharmacol. 61, 459-469.

dos Santos P.F., Ruiz J.C., Soares R.P., Moreira D.S., Rezende A.M., Folador E.L., Oliveira G., Romanha A.J., Murta S.M., 2012. Molecular characterization of the hexose transporter gene in benznidazole resistant and susceptible populations of *Trypanosoma cruzi*. Parasit. Vectors 5,161.

Docampo R., 1990. Sensitivity of parasites to free radical damage by antiparasitic drugs. Chem. Biol Interact. 73, 1-27.

Doyle V., Virji S., Crompton M., 1999. Evidence that cyclophilin-A protects cells against oxidative stress. Biochem. J. 341, 127-132.

Efron, B., Halloran, E., Holmes, S., 1996. Bootstrap confidence levels for phylogenetic trees. Proc. Natl. Acad. Sci. U.S.A. 93, 13429–13434

Filardi L.S., Brener Z., 1987. Susceptibility and natural resistance of *Trypanossoma cruzi* strains to drugs used clinically in Chagas Disease. Trans. R. Soc. Trop. Med. Hyg. 81, 755-759.

Galat A., 2003. Peptidylprolyl cis/trans isomerases (immunophilins): biological diversity--targets--functions. Curr. Top. Med. Chem. 3, 1315-1347.

Hong F., Lee J., Piao Y.J., Jae Y.K., Kim Y.J., Oh C., Seo J.S., Yun Y.S., Yang C.W., Ha J., Kim S.S., 2004. Transgenic mice overexpressing cyclophilin A are resistant to cyclosporin A-induced nephrotoxicity via peptidyl-prolyl cis–trans isomerase activity. Biochem Biophys. Res. Commun. 316, 1073–1080.

Kumar A., Sisodia B., Misra P., Sundar S., Shasany, A.K., Dube, A. 2010. Proteome mapping of overexpressed membrane-enriched and cytosolic proteins in sodium antimony gluconate (SAG) resistant clinical isolate of *Leishmania donovani*. Br J Clin Pharmacol. 70, 609-617.

Larkin, M. A. Blackshields,G.,Bron, N. P., Chena, R., McGettigan, P.A., Mcilliam, H., et al. 2007. Clustal W and Clustal X version 2.0. Bioiformatics 23, 2947-2948.

Lee S.P., Hwang Y.S., Kim Y.J., Kwon K.S., Kim H.J., Kim K., Chae H.Z., 2001. Cyclophilin a binds to peroxiredoxins and activates its peroxidase activity. J. Biol. Chem. 276, 29826-29832.

Matrangolo F.S., Liarte D.B., Andrade L.C., de Melo M.F., Andrade J.M., Ferreira R.F., Santiago A.S., Pirovani C.P., Silva-Pereira R.A., Murta S.M., 2013. Comparative proteomic analysis of antimony-resistant and -susceptible *Leishmania braziliensis* and *Leishmania infantum chagasi* lines. Mol Biochem Parasitol. 190, 63-75.

Maya J.D., Bollo S., Nunez-Vergara L.J., Squella J.A., Repetto Y., Morello A., Pèrie J., Chauviere G., 2003. *Trypanosome cruzi*: effect and mode of action of nitroimidazole and nitrofuran derivates. Biochem. Pharmacol. 65, 999-1006.

Moser, D.R., Kirchhoff, L.V., Donelson, J.E., 1989. Detection of *Trypanosoma cruzi* by DNA amplification using the polymerase chain reaction. J. Clin. Microbiol. 27, 1477–1482.

Murta S.M., Krieger M.A., Montenegro L.R., Campos F.F., Probst C.M., Avila A.R., Muto N.H., De Oliveira R.C., Nunes L.R., Nirdé P., Bruna-Romero O., Goldenberg S., Romanha A.J., 2006. Deletion of copies of the gene encoding old yellow enzyme (TcOYE), A NAD(P)H flavin oxidoreductase, associates with *in vitro*-induced benznidazole resistance in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 146, 151-162.

Murta S.M., Romanha A.J., 1998. *In vivo* selection of a population of *Trypanosoma cruzi* and clones resistant to benzinidazole. Parasitol. 116, 165-71.

Murta, S.M., Gazzinelli, R.T., Brener, Z., Romanha, A.J., 1998. Molecular characterization of susceptible and naturally resistant strains of *Trypanosoma cruzi* to benznidazole and nifurtimox. Mol. Biol. Parasitol. 93, 203-214.

Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W., 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis. 9, 255–262.

Nirdé P., Larroque C., Barnabé C., 1995. Drug resistant epimastigotes of *Trypanosoma cruzi* and persistence of this phenotype after differentiation into amastigotes. C. R. Acad. Sci. Ser. III 318, 1239-1244.

Nogueira F.B., Krieger M.A., Nirdé P., Goldenberg S., Romanha A.J., Murta S.M., 2006. Increased Expression of Iron-containing superoxide dismutase-A (TcFESOD-A) enzyme in *Trypanosoma cruzi* population with*in vitro*-induced resistance to benznidazole. Acta Trop. 100, 119-32.

Nogueira F.B., Rodrigues J.F., Correa M.M., Ruiz J.C., Romanha A.J., Murta S.M., 2012. The level of ascorbate peroxidase is enhanced in benznidazole-resistant populations of *Trypanosoma cruzi* and its expression is modulated by stress generated by hydrogen peroxide. Mem. Inst. Oswaldo Cruz 107, 494-502.

Nogueira F.B., Ruiz J.C., Romanha A.J., Murta S.M.F., 2009. Molecular characterization of cytosolic and mitochondrial tryparedoxin peroxidase in *Trypanosoma cruzi* populations susceptible and resistant to benznidazole. Parasitol. Res. 104, 835-844.

Obchoei S., Wongkhan S., Wongkham C, Li M., Yao Q., Chen C., 2009. Cyclophilin A: potential functions and therapeutic target for human cancer. Med. Sci. Monit. 5(11), RA221-32. Review.

Potenza, M., Galat, A., Mining, T.A., Ruiz, A.M., Duran, R., Tarleton, R.L., Marin, M., Fichera, L.E., Búa, J., 2006. Analysis of the *Trypanosoma cruzi* cyclophilin gene family and identification of cyclosporin a binding proteins. Parasitol. 132, 867-882.

Rosenzweig D., Smith D., Myler P.J., Olafson R.W., Zilberstein D., 2008. Post-translational modification of cellular proteins during *Leishmania donovani* differentiation. Proteomics. 8, 1843-1850.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731-2739.

Toledo M.J., Bahia M.T., Veloso V.M., Carneiro C.M., Machado-Coelho G.L., Alves C.F., Martins H.R., Cruz R.E., Tafuri W.L., Lana M., 2004. Effects of specific treatment on parasitological and histopathological parameters in mice infected with different *Trypanosoma cruzi* clonal genotypes. J. Antimicrob. Chemother. 53, 1045-1053.

Villarreal, D., Nirde, P., Hide, M., Barnabe, C., Tibayrenc, M., 2005. Differencial gene expression in benznidazole-resistant Trypanosoma cruzi parasites. Antimicrob. Agents Chemother, 49, 2701-2709.

Wilkinson S.R., Taylor M.C., Horn D., Kelly J.M., Cheeseman I., 2008. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. Proc. Natl. Acad. Sci. U.S.A. 105, 5022-5027.

Yang J., Li A, Yang Y., Li X., 2011. Identification of cyclophilin A as a potential prognostic factor for clear-cell renal cell carcinoma by comparative proteomic analysis. Cancer Biol. Ther. 11, 535-46.

Zingales B., Andrade S.G., Briones M.R., Campbell D.A., Chiari E., Fernandes O., Guhl F., Lages-Silva E., Macedo A.M., Machado C.R., Miles M.A., Romanha A.J., Sturm N.R., Tibayrenc M., Schijman A.G.; Second Satellite Meeting, 2009. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem. Inst. Oswaldo Cruz 104,1051-4.

TABLE I

Trypanosoma cruzi populations and clones used in this study

T. cruzi strain	Origin[a]	Host[b]	Sus[c]	*T. cruzi* group[d]	TcCYP19 chromosomal location (Kb)
17WTS	Mex	Triatomine	S	I	1640, 1120, 945, 610, 680
17LER	Mex	Triatomine	R	I	1640, 1120, 945, 610, 680
BZS	SP	Human A. P.	S	II	1640, 1120, 945
BZR	SP	Human A. P.	R	II	1640, 1120, 945
Clone 9S	SP	Human A. P.	S	II	ND[e]
Clone 27R	SP	Human A. P.	R	II	ND[e]
VL-10	MG	Human C. P.	R	II	ND[e]
Berenice	MG	Human C. P.	S	II	1640, 1120, 945
Cl Brener	RS	*T. infestans*	S	VI	2200, 2000, 1640, 1120, 945
Romano	Arg	Human A. P.	S	VI	2200, 2000, 1640, 1120, 945
Buriti	RS	*T. infestans*	S	VI	2200, 2000,1640, 1120, 945

[a] Origin - Mex, Mexico; Arg Argentina; MG, Minas Gerais; RS, Rio Grande do Sul; SP, São Paulo; MG, RS and SP are different states of Brazil.

[b] A.P., acute phase and C.P. chronic phase.

[c] Sus, *in vivo* susceptibility to BZ and NFX as previously reported (Filardi and Brener, 1987; Murta *et al.,* 1998); S, susceptible; R, resistant.

[d] *T. cruzi* group classification as previously described (Zingales *et al.,* 2009);

[e] ND Not determinate;

Legends to Figures

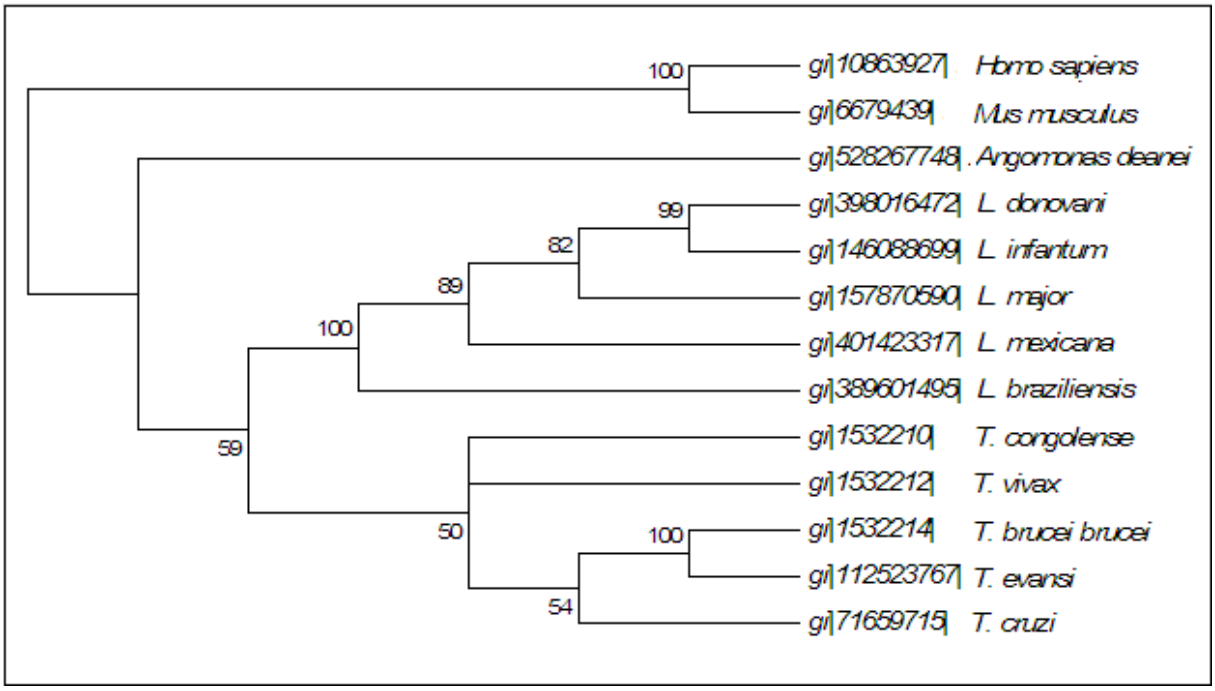


Fig. 1: Neighbour-joining phylogenetic tree of CyPA sequences of *Trypanosoma cruzi* and other organisms with a high similarity degree. The numbers shown are bootstrap values. Bootstrap is a method that provides assessments of confidence for each clade of an observed tree, based on the proportion of bootstrap trees showing that same clade (Efron et al., 1996).

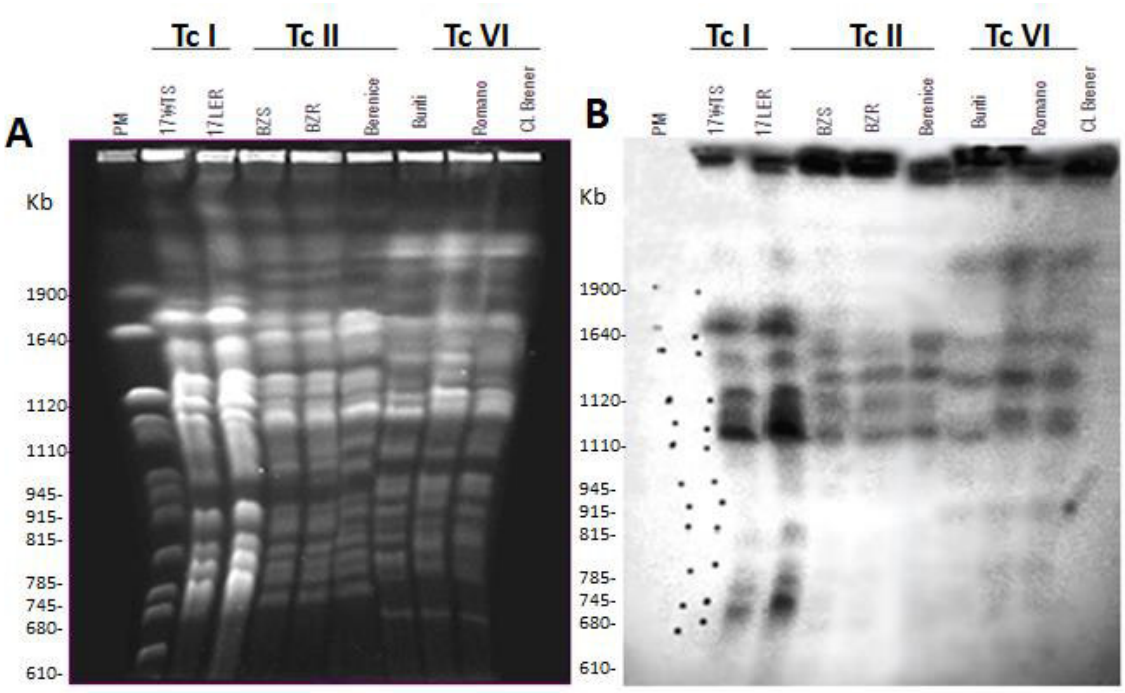


Fig. 2: Chromosomal location of the *TcCyP19* gene in the BZ-resistant and susceptible *T.cruzi* strains. (**A)** Chromosomal bands from the *T.cruzi* strais were separated by PFGE and stained with ethidium bromide. (**B)** Southern blots of the chromosomes were hybridized with a ^{32}P-labeled *TcCyP19*-specific probe. Whole chromosomes from *Saccharomyces cerevisae* were used as molecular weight markers.

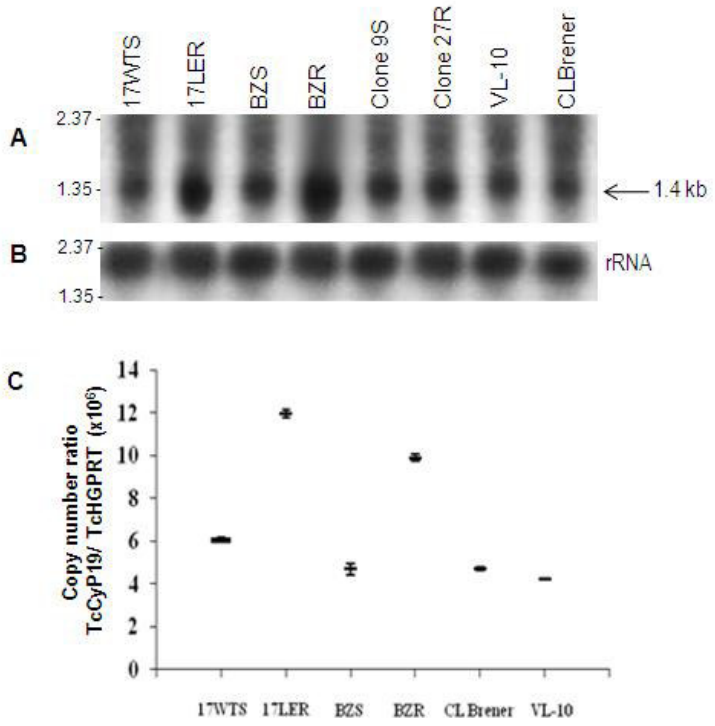


Fig. 3: Levels of *TcCyP19* mRNA in BZ-resistant and susceptible *T. cruzi* strains. (A) Northern blot profile of total RNA extracts from the *T. cruzi* strains obtained using a ^{32}P-labeled *TcCyP19*-specific probe.(B) The quantitative control was used agarose gel containing the RNA, stained with ethidium bromide. (C) Number of cDNA molecules (copy number ratio) of *TcCyP19* (x 10^6). Values were normalized to those obtained for the *TcHGPRT* and are presented as the means (±S.D.M.) of triplicate real-time RT-PCR analyses from three independent experiments.

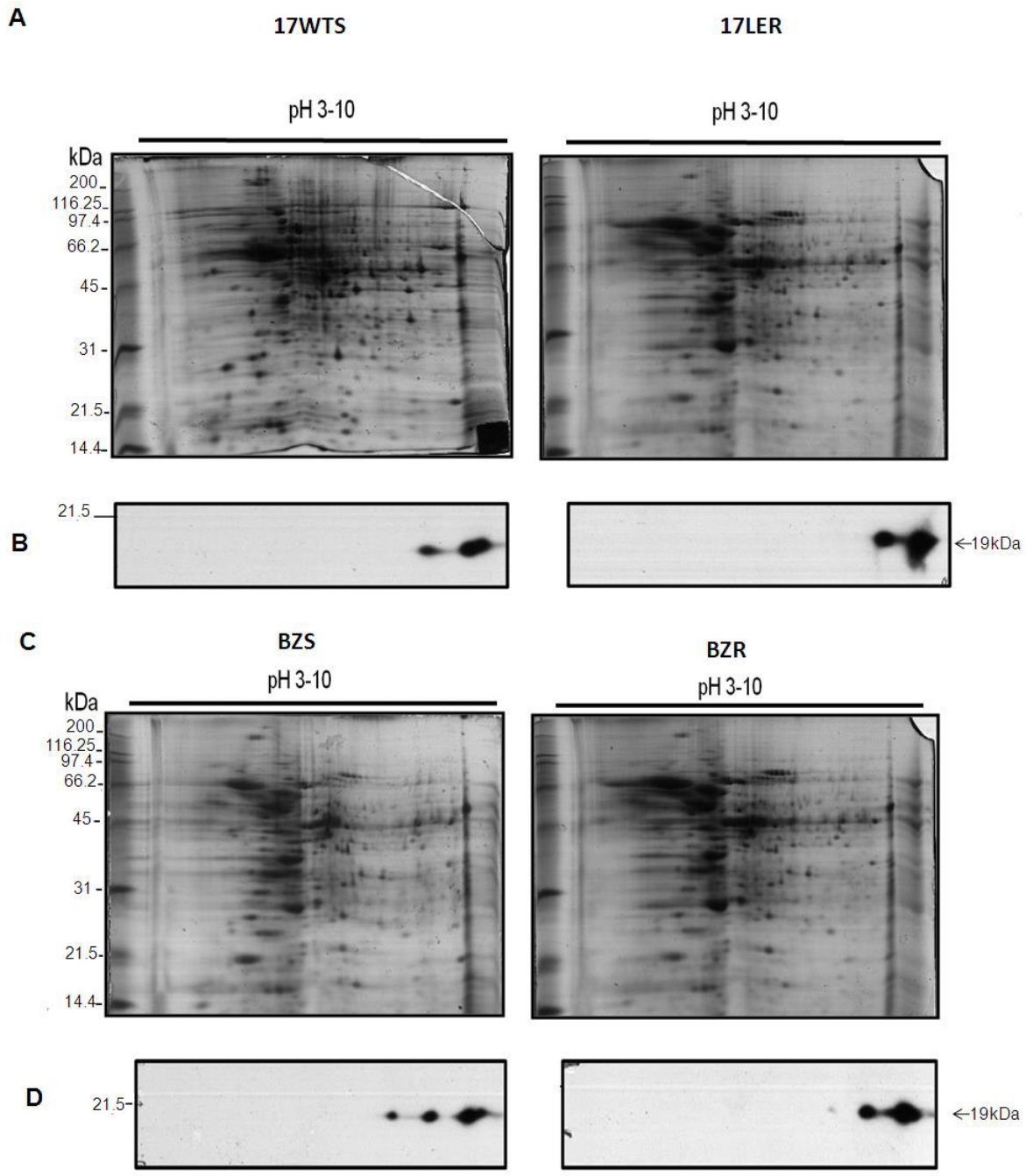


Fig. 4: 2-DE gels of proteins and Western blot analysis of TcCyP19 expression in the benznidazole-susceptible and -resistant *T. cruzi* populations. Proteins (100 µg) were loaded on 7 cm, non-linear IPG strips of pH 3–10, submitted to isoelectric focusing and separated on 12% SDS-PAGE. The gels were stained with Colloidal Coomassie BlueG250 (A and C) or blotted onto nitrocellulose membranes (B and D). The blots were probed with a rabbit polyclonal antibody anti-cyclophilin-A of *T. cruzi* (1:5,000) an developed using ECL.

Characterization of Cyclophilin A (TcCyP19) in *Trypanosoma cruzi* populations with *in vitro*-induced and *in vivo*-selected resistance to benznidazole (BZ)

Juciane Vaz Rêgo, Ana Paula Duarte, Daniel Barbosa Liarte, Francirlene de Carvalho Sousa, Humberto Medeiros Barreto, Jacqueline Búa, Alvaro José Romanha, Gandhi Rádis-Baptista, Silvane Maria Fonseca Murta.

SUPPLEMENTARY DATA

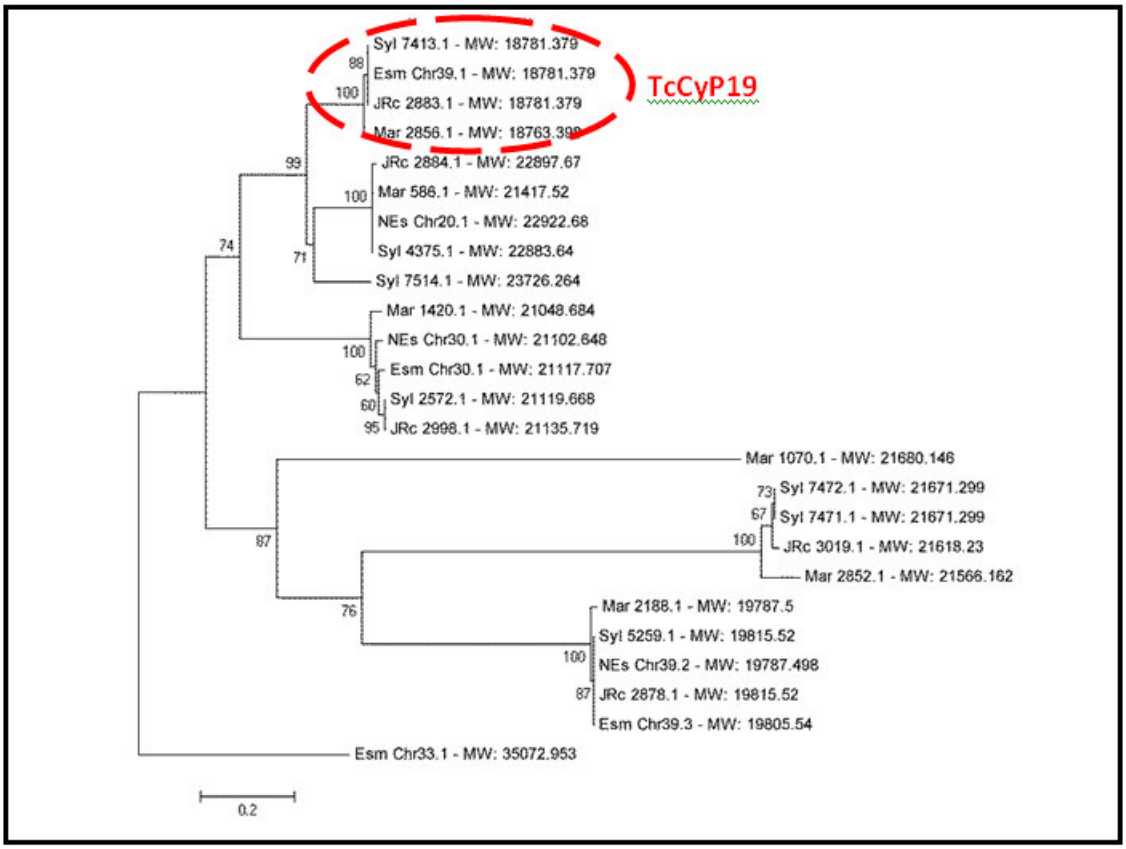


Fig. 1S. Neighbour-joining phylogenetic tree of twenty-four TcCyPs sequences of five different *Trypanosoma cruzi* genomes: NEs, Non Esmeraldo CL Brener like; Esm, Esmeraldo CL Brener like; Syl, SilvioX10; JRc, JRc14 and Mar, Marinkellei-B7. MW, predicted molecular weight (Daltons). *T. cruzi* cyclophilin amino acid sequence of 35 kDa was used as outgroup. The numbers shown are bootstrap values. Bootstrap is a method that provides assessments of confidence for each clade of an observed tree, based on the proportion of bootstrap trees showing that same clade (Efron et al., 1996). Circle red indicates cyclophilins of 18.7 kDa (TcCyP19).

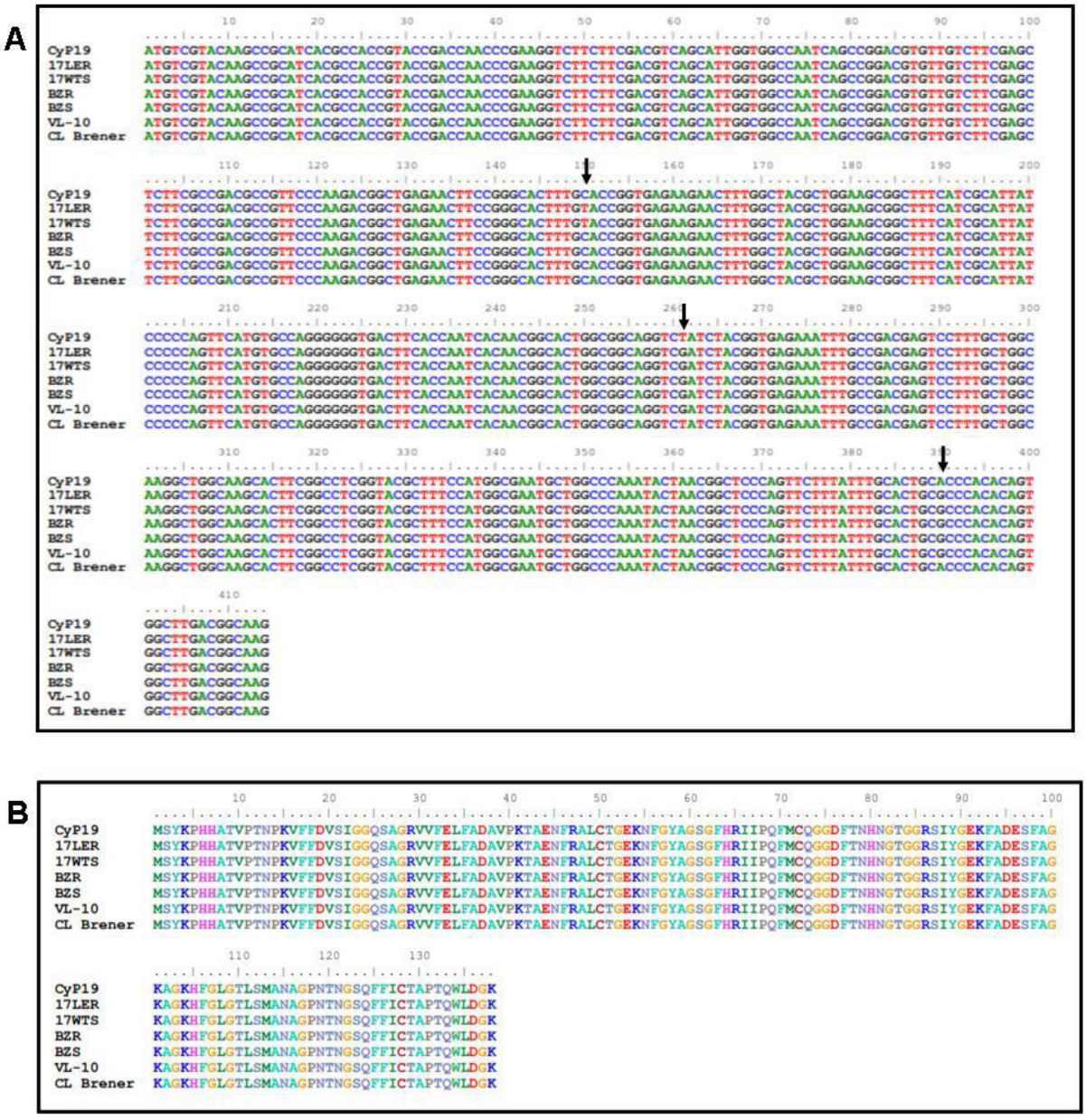


Fig. 2S. Multiple sequence alignment of TcCyP19 nucleotide (A) and amino acid (B) sequences from six BZ–resistant or –susceptible *T. cruzi* strains. The sequences were aligned against the TcCyP19 nucleotide sequence from the *T. cruzi* CL Brener clone (TcCyP19, GeneBank accession number XM_816485). Arrows indicate nucleotide mutations (position 150: T (17WTS/17LER) and -C (all samples); position 261: T (CyP19/CL Brener) and-G (all samples); position 390: A (CyP19/CL Brener) and-G (all samples).

CAPÍTULO II

Peptídeos antimicrobianos, vipericidinas e sua atividade contra *T. cruzi*

Artigo em fase de elaboração

SHORT COMMUNICATION

Activity of Antimicrobial peptides isolated from the venom of snakes (Vipericidins) against *Trypanosoma cruzi*

***Juciane Vaz Rego*[1],[3][4], *Clarissa P. Mello*[3], *Izabel Cristina J. Bandeira*[3], *Alvaro José Romanha*[4] *Alice M. C. Martins*[3], *Silvane Maria Fonseca Murta*[4] *and Gandhi Rádis-Batista*[5]**

[1] Federal University of Piauí, Teresina, PI. [2] Department of Clinical and Toxicological Analysis, Pharmacy Faculty, Federal University of Ceará, Fortaleza, CE. [3] Laboratory of Cellular and Molecular Parasitology, Research Center René Rachou -CPqRR / FIOCRUZ, Belo Horizonte, MG. [4] Laboratory of Biochemistry and Biotechnology, Institute for Marine Sciences, Federal University of Ceará, Fortaleza, CE.

Authors for correspondence:

Gandhi Rádis-Baptista,
Laboratory of Biochemistry and Biotechnoloy, Institute for Marine Sciences, Federal University of Ceará, Av. Da Abolição, 3207, 60165-081 Fortaleza, CE, Brazil. Phone: +55 85 3366 7007. FAX: +55 85 3366 7002. E-mail: gandhi.radis@ufc.br

ABSTRACT

Introduction: Given the urgent need to develop an effective drug for Chagas disease. Thus, we proposed to evaluate the *in vitro* activity of peptides vipericidins against *T. cruzi.* **Methods:** *In vitro* assay using epimastigotes forms, and mixture of trypomastigote and intracellular amastigote of *T. cruzi* by expression of β-galactosidase. **Results:** crotalicidin vipericidin had a dose-dependent cytotoxic effect for epimastigote form of *T.cruzi* (IC_{50} 24h = 4.72 µM; IC_{50} 48h = 4.37 µM)**.** For trypomastigote and amastigote forms only vipericidin GA645 crotalicidin have activity followed by cell cytotoxicity and the other samples were inactive. **Conclusions:** The vipericidins shows indications of being an interesting target of search against Chagas disease with the possibility of designing new therapeutic synthetic peptides effective starting from natural peptides.

Keywords: Vipericidins, *Trypanosoma cruzi*, Chagas disease

.

Chagas' disease (American trypanosomiasis), caused by the protozoan *Trypanosoma cruzi*, is one of the world's 13 neglected diseases and represent a public health problem. According the World Health Organization an estimated about 8 million people are infected

worldwide and more than 10,000 deaths are thought to occur annually[1]. The drugs nifurtimox (NFX) and benznidazole (BZ) are the medications presently available for the treatment of Chagas disease. The effectiveness of these drug nitroheterocyclic is observed only in patients treated in the acute phase [2].However, these drugs are toxic, require prolonged therapy, neither are active in the chronic phase of infection. The side effects are observed in up to 40% of patients, which include nausea, vomiting, abdominal pain and several neurological effects[3]. Furthermore, the appearance of naturally resistant populations in *T. cruzi* to NFX and BZ, hampers the treatment of Chagas disease[4,5]. Thus, the development of alternative therapeutic drugs effective against *T. cruzi* is urgently needed.

Antimicrobial peptides (AMPs) have been shown as potential pharmacological components against various target bacteria, fungi, protozoa, and virus[6-8]. This peptide, called peptides defense, are conserved components of the innate immune response of all organisms, including plants, animals, and humans[9]. Cathelicidin- related antimicrobial peptides (CRAMPs) derived from the venom gland of snake called Vipericidin (crotalicidina, batroxicidina, lutzicidina and lachesicidina) and the snake Elapide comprise peptides of *Bungarus fasciatus* (BF-CRAMP), *Naja atra* (Na- CRAMP) and *Ophiophagus hannah* (Oh-CRAMP) have been described[10-12]. These molecules have shown activity against bacteria Gram-negative and Gram-positive[12] fungi and cancer cells[10]. Research shows that CRAMPs have little hemolytic activity and cytotoxicity to human cells which makes interesting use of peptides as therapeutic potential[10,12].

Thereby, aiming the biotechnological use of vipericidins against *T. cruzi* and verify their anti-parasitic activity, was proposed evaluate the *in vitro* activity of peptides vipericidins against *T. cruzi* forms.

This study, the Benznidazole (BZ) was donated by LAFEPE (Phamaceutical Laboratory of Pernambuco State). Chemicals and reagents used in this work were purchased from Sigma and Aldrich (Sigma Chemical Co., St. Louis, MO), Applied Biosystems, Pierce and Bio Rad (USA).

Epimastigotes effects: Epimastigotes forms of *T. cruzi* were grown at 28 °C in Liver Infusion Tryptose (LIT) medium supplemented with 10% fetal bovine serum, penincilin and streptomycin. The epimastigotes used for all experiments were from the log growth phase (6th day). The experiments were performed in 96-well plates at a density of $1x10^6$ parasites/mL treated with of different concentrations (12.5; 6.25; 3.12 and 1.56 µM/mL) of crotalicidin peptide. The plates were incubated for 24, 48 and 72h and after that, the viability was determined by quantification in a Neubauer Chamber and the results were expressed as the percentage of inhibition in relation to the control cultured in medium alone. Cultures of the parasite without treatment were considered as 100% growth and inhibitory concentration (IC_{50}) was determined by logarithm regression analysis of the data obtained[13]. (Veiga-Santos

et al., 2010). Benznidazole was used as the reference drug. Each experiment was conducted in triplicate and repeated at least 3 times.

In vitro assay in trypomastigote and intracellular amastigote *T. cruzi* forms: The assay was performed according to the protocols established by Buckner et al. (1996)[14], with reported modifications[15]. The β-galactosidase Tulahuen strain used was courtesy of Dr Frederick Buckner, University of Washington. Initially, the L929 mouse fibroblast line was seeded into 96 well plates and incubated for 24 hours at °37 C for adhesion on the plate and infection with 10µg parasites / well. After 2 h, the medium containing the extracellular parasites is replaced with fresh medium again and the plate incubated at °37 C for 48 hours. After this period, the culture medium was replaced with fresh medium in addition to the compounds in decreasing concentrations from 10 ug/mL until reaching the IC_{50}. After 96 hours of incubation, the substrate CPRG was added, the plate incubated at C°37 and reading taken after 16-20 h in a spectrophotometer using a 570 nm filter. The benznidazole in its IC_{50} (1 ug / ml = 3.81 uM) was used as positive control. The results are expressed as the percentage of inhibition of parasite growth.

Cytotoxicity Assay: *In vitro* cytotoxicity tests were performed using alamarBlue ® to determine the toxicity of the compounds against L929 cells. The conditions were the same as those described for the β-galactosidase assay. After 96 h of exposure to the compounds, alamarBlue ® was added to each well and the absorbance at 570 and 600 nm was measured after 4-6 h. The lethal concentration (LC_{50}) and IC_{50} values were calculated using linear interpolation and the selectivity index (SI) was determined based on the ratio of the LC_{50} value for the host cell to the IC_{50} value for the parasite (LC_{50} /IC_{50} ratio) (Table I). The activity and toxicity of benznidazole as the positive control were included for comparison.

For the antiparasitic effect epimastigote forms of *T. cruzi* Y strain were treated with different concentrations of crotalicidin-vipericidin (12.5; 6.25; 3.12 and 1.56 µM) for 24, 48 and 72 hours (Figure 1) shows that crotalicidin vipericidin had a dose-dependent cytotoxic effect (IC_{50} 24h = 4.72 µM; IC_{50} 48h = 4.37 µM) while the IC_{50} of the reference drug benznidazole was greater (IC_{50}48h = 117 µM)**.**

In this present study, we demonstrated vipericidin-crotalicidin activity against epimastigote, trypomastigote and amastigotas forms of *T. cruzi*. Our data showed that crotalicidin vipericidin peptide had a dose-dependent activity against the epimastigote form after 24 and 48h of treatment, exhibiting an IC_{50} of 4,72 µM and 4,37 µM respectively, while benznidazole showed greater IC_{50} in 48h of treatment (117 µM). The activity assay of vipericidin- GA645-crotalicidin against forms of *T. cruzi* trypomastigotes and intracellular amastigotes showed activities IC_{50} 10 ug / mL followed by cell cytotoxicity, however, the other samples vipericidins did not inhibit parasite growth (Table I).

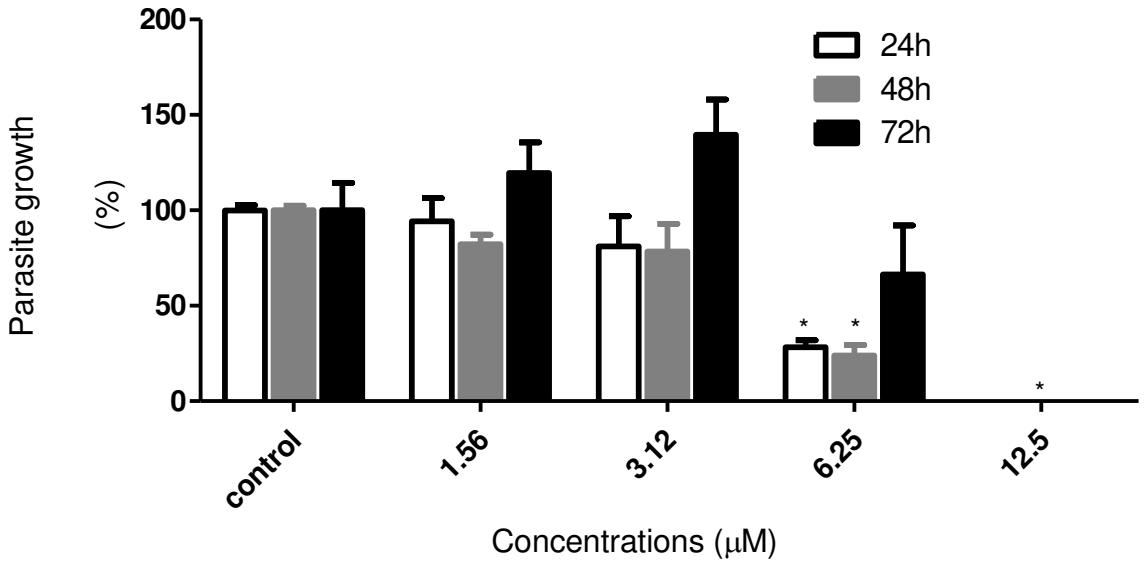


Figure 1. Effects of crotalicidina- vipericidin against the epimastigote form of *trypanosoma cruzi* Y strain. The protozoa were treated with different concentrations (12.5; 6.25; 3.12 and 1.56μM) for 24h, 48h and 72h. Each experiment was conducted in duplicate and repeated at least 3 times. The results were expressed as mean ± E.P.M and analyzed by Student's t-test. *$p<0.05$ compared to the corresponding control group.

Table I: Activity of vipericidins againt trypomastigote and intracellular amastigote *T. cruzi* forms

Types vipericidins	concentration (μg/ mL)	activity[1] (%)	IC_{50} on the parasite[2]	IC_{50} on cell[3]	Selectivity[4]
GA648	20	4	Inativo	-	-
GA646	20	0	Inativo	-	-
GA649	20	1	Inativo	-	-
GA650-lutzicidin	5	cell death	Inativo	-	-
	2,5				
GA647	20	7	Inativo	-	-
GA645-crotalicidin	20	cell death	10 μg/mL	10 μg/mL	1
	10	68			
	5	4			
Benzonidazole	1 μg/mL (3,81 μM)	-	1 μg/mL	625 μg/mL	625

[1] Percentage reduction of amastigotes and trypomastigotes under the action of vipericidins
[2] Concentration of vipericidins that reduces by 50% the parasitic growth.
[3] Concentration of vipericidins that induces 50% cell death (L929).
[4] IC_{50} of vipericins on the cells divided by the IC_{50} of vipericidin of the parasite.

The bioactive substances derived from animal products are foci of science in the search for new drugs and biotechnology applications[16-17]. Side effects of drug associated with the lack of sensitivity specific to some strains of *T. cruzi* and low clinical efficacy of benznidazole, evidences the urgent need to develop effective novel drug for the acute and chronic phases of Chagas disease. The peptide antimicrobiano of snake are atractive, because several components of poisons Viperidae have demonstrated potent anti-bacterial and anti-fungal activity[10,18,19].

In our study, the peptide vipericidin- GA645 crotalicidin was active in trypomastigotes and intracellular amastigotes, however, their therapeutic potential as anti-parasitic agent was restricted to cell cytotoxic activity, and other peptides tested did not inhibit the growth of *T. cruzi*. Furthermore, the vipericidin crotalicidin peptide was more active against epimastigotes of *T. cruzi*. corroborating these findings, in *T. brucei*, the antiparasitic action of peptides can deregulate membrane integrity and lead to instability causing osmotic swelling and cell lysis and death of the parasite. Researchers suggest that due to the presence of a variety of proteins to the surface of the membrane (GPI) present in trypanosomes affinity and may allow the communication between the cell such peptides and molecules[20].

On the other hand, the differences observed in the activities of viperecidin regarding the different types of *T. cruzi* can be due the specific constitution of each peptide or the surface charge of parasite be specific for each species and vary with the developmental stages, including their protein profiles[21]. In addition, the constitution of each peptide tested which interacts with the structure of the parasite influence the outcome. *In silico* analysis can provide a wide range of information about AMPs and their structures, suggesting which residues could be important for membrane interactions[22]

Lastly, the vipericidins shows indications of being an interesting target of search against Chagas disease. New alternatives can expand the possibilities in therapy against Chagas disease. In order to develop a more effective peptide can synthesize another peptide from the natural structure. With that, we reduce the hydropathicity the molecule and increase its amphipathicity which, according to some authors collaborate to increase the therapeutic index of the peptide[23]

REFERENCES

1. World Health Organization, 2014. Chagas' disease (American trypanosomiasis) fact sheet (revised in March 2014).
2. Andrade DV, Gollob KJ, Dutra WO.Acute chagas disease: new global challenges for an old neglected disease. PLoS Negl Trop Dis. 2014 Jul 31;8(7):e3010
3. Marin-Neto JA, Rassi A Jr, Avezum A Jr, Mattos AC, Rassi A, Morillo CA, Sosa-Estani S, Yusuf S; BENEFIT Investigators.The BENEFIT trial: testing the hypothesis that trypanocidal therapy is beneficial for patients with chronic Chagas heart disease. Mem Inst Oswaldo Cruz. 2009 Jul;104 Suppl 1:319-24.
4. Filardi L, and Brener Z. Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. Trans R Soc Trop Med Hyg. 1987; 81: 755-759.
5. Murta SM and Romanha AJ. *In vivo* selection of a population of *Trypanosoma cruzi* nd clones resistant to benznidazole. Parasitology. 1998. 116: 165-171.
6. Torrent, M., Pulido,D., Rivas,L. Andreu D. Antimicrobial peptide action on parasites. Curr. Drug Targets,2012, 13, 1138–1147.
7. Meiyalaghan S, Latimer JM, Kralicek AV, Shaw ML, Lewis JG, Conner AJ, Barrell PJ. Expression and purification of the antimicrobial peptide GSL1 in bacteria for raising antibodies. BMC Res Notes. 2014 Nov 4;7:777.
8. Maróti G, Kereszt A, Kondorosi e, Mergaert P. Natural roles of antimicrobial peptides in microbes, plants and animals. Res Microbiol, 2011,162:363–374.
9. Kościuczuk EM, Lisowski P, Jarczak J, Strzałkowska N, Jóźwik A, Horbańczuk J, Krzyżewski J, Zwierzchowski L, Bagnicka E. Cathelicidins: family of antimicrobial peptides. A review. Mol Biol Rep. 2012 Dec;39(12):10957-70.
10. Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, Wang A, Lin D, Lai R. Snake cathelicidin from Bungarus fasciatus is a potent peptide antibiotics. PLoS ONE, 2008, 16;3(9):e3217.

11. Zhao H, Gan TX, Liu XD, Jin Y, Lee WH, Shen JH, Zhang Y. Identification and characterization of novel reptile cathelicidins from elapid snakes. Peptides, 2008, 29:1685–1691

12. Falcao CB, de La Torre BG, Pérez-Peinado C, Barron AE, Andreu D, Rádis-Baptista G. Vipericidins: a novel family of cathelicidin-related peptides from the venom gland of South American pit vipers. Amino Acids. 2014 Nov;46(11):2561-71.

13. Veiga-Santos P, Pelizzaro-Rocha KJ, Santos AO, Ueda-Nakamura T, Dias Filho BP, Silva SO, Sudatti DB, Bianco EM, Pereira RC, Nakamura CV. *In vitro* anti-trypanosomal activity of elatol isolated from red seaweed *Laurencia dendroidea*. Parasitology. 2010 Sep;137(11):1661-70.

14. Buckner F, Verlinde CLMJ, la Flamme AC, van Voorhis WC 1996. Efficient technique for screening drugs for activity against *Tr ypanosoma cruzi* using parasites expressing galactosidase. *Antimicrob Agents Chemother 40*: 2592-2597.

15. Romanha AJ, de Castro SL, Soeiro MNC, Lannes-Vieira J, Ribeiro I, Talvani A, Bourdin B, Blum B, Olivieri B, Zani C, Spadafora C, Chiari E, Chatelain E, Chaves G, Calzada JE, Bustamante JM, Freitas-Junior LH, Romero LI, Bahia MT, Lotrowska M, Soares M, Andrade SG, Armstrong T, Degrave W, Andrade ZA 2010. *In vitro* and *in vivo* experimental models for drug screening and development for Chagas disease. *Mem Inst Oswaldo Cruz 105*: 233-238.

16. Sanchez EF, Eble AJ. P-III metalloproteinase (leucurolysin-B) from Bothrops leucurus venom: isolation and possible inhibition. In: Drug Design of Zinc-Enzyme Inhibitors, Supuran C., Winum JY (Eds), Chapter 30, John Wiley and Sons, Inc., pp. 789-812, 2009

17. Mortari MR, Cunha AO, Ferreira LB, Dos Santos WF. Neurotoxins from invertebrates as anticonvulsants: from basic research to therapeutic application. Pharmacol Ther, v.114, n.1, p.171-183, 2007.

18. Samy RP, Stiles BG. Gopalakrishnakone P, Chow VT. 2011. Anti-microbial proteins from snake venoms: direct bacterial damage and activation of innate immunity against *Staphylococcus aureus* skin infection. Curr Med Chem, 18, 5104–5113

19. Conlon JM, Mechkarska M, Lukic ML, Flatt PR. 2014. Potential therapeutic applications of multifunctional host-defense peptides from frog skin as anti-cancer, anti-viral, immunomodulatory, and anti-diabetic agents. Peptides, 57, 67–77

20. McGwire BS, Olson CL, Tack BF, Engman DM. Killing of African trypanosomes by antimicrobial peptides. J Infect Dis. 2003 Jul 1;188(1):146-52.

21. Okubo BM, Silva ON, Migliolo L, Gomes DG, Porto wF, Batista CL, Ramos CS, Holanda HH, Dias SC, Franco OL, Moreno Se. evaluation of an antimicrobial l-amino acid oxidase and peptide derivatives from Bothropoides mattogrosensis pitviper venom. PLoS One, 2012, 7(3):e33639.

22. Tyler KM, Fridberg A, Toriello KM, Olson CL, Cieslak JA, Hazlett TL and Engman DM. 2008. Flagellar membrane localization via association with lipid rafts J. Cell Sci.122: 859866.

23. Fjell AM, Westlye LT, Grydeland H, Amlien I, Espeseth T, Reinvang I, Raz N, Dale A, Walhovd K (2012) Accelerating cortical thinning: unique to dementia or universal in aging? Cereb Cortex. Advance online publication. Retrieved Dec. 12, 2012.

CAPÍTULO III

Rhodamine B- nonapeptideo Vipericidina - atividade tóxica e citotóxica contra o modelo peixe-zebra e câncer de mama

*Artigo submetido para publicação no periódico **Bioconjugate Chemistry**. As normas de publicação do periódico foram respeitadas (ANEXO B). O comprovante de submissão segue no (ANEXO D)*

Rhodamine B-conjugated encrypted vipericidin nonapeptide is a potent toxin to zebrafish and associated with cytotoxicity towards human breast cancer cells

Liang Wang[‡], Judy Y W Chan[‡], Juciane V Rêgo[§†], Tony C M Chong[‡], Nana Ai[‡], CláudioB. Falcão[†], Simon M Y Lee[‡*], Gandhi Rádis-Baptista[‡*]

[‡] State Key Laboratory of Quality Research in Chinese Medicine and Institute of Chinese Medical Sciences, University of Macau, Macao, China,

[§]Northeast Biotechnology Network (RENORBIO), Post-graduation program in Biotechnology, Federal University of Ceará, Brazil

[†]Laboratory of Biochemistry and Biotechnology, Institute for Marine Sciences, Federal University of Ceara, Brazil

Authors for correspondence:
Simon M Y Lee,
Institute of Chinese Medical Sciences, University of Macau.
Av. da Universidade, Room No N22-7003. N22 Building. Taipa, Macau. Phone/FAX: TEL: +853 8822 4695. E-mail:simonlee@umac.mo

Gandhi Rádis-Baptista,
Laboratory of Biochemistry and Biotechnoloy, Institute for Marine Sciences, Federal University of Ceará, Av. Da Abolição, 3207, 60165-081 Fortaleza, CE, Brazil. Phone: +55 85 3366 7007. FAX: +55 85 3366 7002. E-mail: gandhi.radis@ufc.br

ABSTRACT

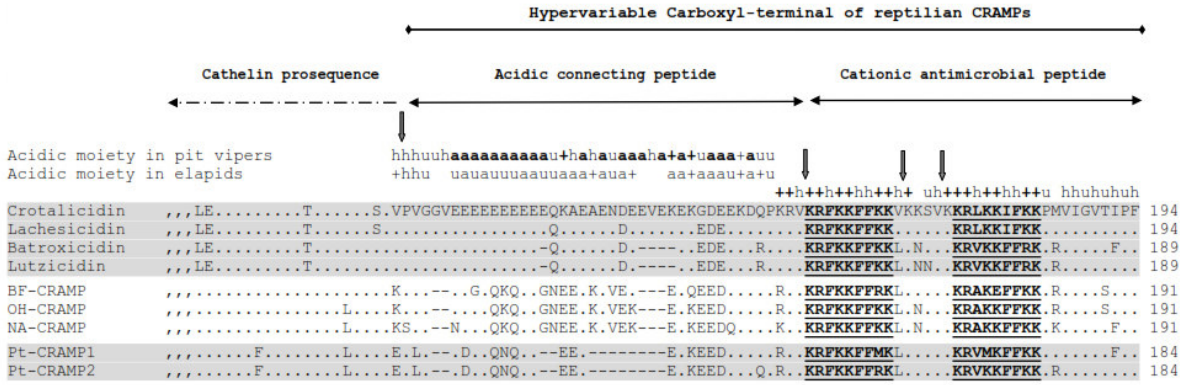
Animal venoms contain a diverse array of proteins and enzymes that are toxic towards various physiological systems. However, there are also some practical medicinal uses for these toxins including use as anti-bacterial and anti-tumor agents. In this study, we identify a nine-residue cryptic oligopeptide, KRFKKFFKK (EVP50) that is repeatedly encoded in tandem within vipericidin sequences. EVP50 displays potent lethal toxicity to zebrafish (LD= 10 µM) when the peptide's N-terminus is chemically conjugated to rhodamine B (RhoB). *In vitro*, RhoB-conjugated EVP50 exhibits a concentration-dependent antitumor effect toward MCF-7 breast cancer cells. In MCF-7 cells, the RhoB-conjugated EVP50 nonapeptide accumulated in the cytoplasm and the nucleus within minutes. In the cytoplasm, the RhoB-EVP50 induced extracellular calcium influx and intracellular calcium release. Membrane budding was also observed after incubation with micromolar concentrations of a fluorescent EVP50 conjugate. The naïve unconjugated peptide lacked toxic and cytotoxic activities in our *in vivo* and *in vitro* models. Thus, the conjugate's interference with calcium homeostasis, its nuclear function and its induced membrane dysfunction (budding and vacuolization) seem to act in concert to disrupt the cell circuitry. This synergic mechanism of toxicity was restricted to the structurally modified encrypted vipericidin nonapeptide.

Key words: vipericidins; encrypted peptides; cell-penetrating peptide; membranolytic peptide; cytotoxin; membrane budding; zebra-fish model; Rhodamine B-conjugated peptide;

INTRODUCTION

Vipericidins (crotalicidin, batroxicidin, lutzicidin, and lachesicidin) together with Bf-CRAMP, Na-CRAMP and Oh-CRAMP comprise the cathelicidin-related antimicrobial peptides (CRAMPs) from the venom gland of the pit viper and elapid snake, respectively.[1-3] The cathelicidin family of peptides encompasses thousands of sequences expressed in the tissues and cells of the immune systems of numerous species of vertebrates. Vipericidins and elapid CRAMPs are structurally arranged as prepropetides with a signal peptide of ~ 20 residues, a conserved cathelin domain (cathepsin L inhibitor protein) and a hypervariable carboxyl-terminal stretch where a range of natural antimicrobial activities are found. Mature vipericidins and elapid CRAMPs share a high level of amino acid similarity, with most of their 34 residues being identical or strictly conserved substitutions. Due to the high proportion of lysine residues alternating with hydrophobic residues, these peptides have a net positive charge and have an amphipathic character. One distinctive structural feature in reptilian CRAMPs when compared to other familial members of cathelicidins is the presence of an extra acidic patch (or acidic signature) at the C-terminal end of the prosequence, which is rich in aspartic acid and uncharged residues (Figure 1). Reptilian CRAMPs (vipericidins and elapid cathelicidins) possess effective broad-spectrum activity against clinical isolates and standard strains of bacteria and yeast.[2-5] Gram-negative bacteria[1] They are particularly active against , even rivaling organic antimicrobial compounds. Interestingly, the cathelicidin of *Bungarus fasciatus* (Bf-30) was shown to annihilate *in vitro* melanoma B16F10 cells in a dose- and time-dependent fashion and inhibit melanoma growth and metastasis in grafted mice.[7] On the same spectrum of activity, a Lys-16 mutant of BF-30 (BF30-K16) displayed selective *in vitro* cytotoxicity against cancer cell propagation in a concentration range that was not toxic to non-tumor cell lines.[8] Pre-propeptides are processed by specific proteases that release the active portion of the polypeptide responsible for a given biological effect.

Figure 1. Hypervariable C-terminal region of the reptilian CRAMPs and putative proteolytic cleavage sites. The acidic and cationic/amphiphatic domains of vipericidins and elapid CRAMPs were aligned. Identical amino acid residues are represented by dots, and gaps are represented by hyphens. The hypervariable anionic linkage sequences followed by the cationic antimicrobial peptides are preceded by the highly conserved cathelin prosequence (indicated by a slash). The *in tandem* repetitive cryptic nonapeptides embedded in the mature antimicrobial sequences are in bold and underlined. Arrows indicate putative proteolytic cleavage sites. The “a”,“h” and “u” lowercase letters and the “+” symbol represent acidic, hydrophobic, uncharged and basic amino acid residues, respectively. Numbers on the right side of each polypeptide sequence refer to the number of residues that a given cathelicidin-related pre-propeptide contains. The vipericidin (crotalicidin, lachesicidin, batroxicidin and lutzicidin) and *P. textilis* CRAMPs (PT-CRAMP1 and 2) C-termini are shown in grey.



For instance, crotamine and crotamine-like peptides from snake venom and lizards, [9,10] cytolysins from sea anemones, [11,12] apamin, melittin and mastoparan-B from hymenoptera venom, [13,14] conotoxins from the marine gastropods *Conus* [15,16] and antimicrobial and cytolytic peptides from spider venom[17] are all toxins that mature by being proteolytically cleaved. A less obvious maturation process is found in the proteolytic release of a number of bioactive peptides that are encrypted within longer polypeptides. Classic examples of cryptic peptides that contain natural physiologically active human cryptomes include the proteolytically activated prohormone precursor pro-opiomelanocortin (POMC) and the extracellular-matrix-derived crypteins.[18] In the first example, dozens of diverse active hidden opioid peptides with distinct pharmacological functions are released from POMC by tissue-specific convertases (proteases). In the second example, encrypted peptides with anti-angiogenic, anti-tumor, immunomodulation, antimicrobial and chemotaxis activities are proteolytically cleaved from proteins in the extracellular matrix, such as collagens, laminin and perlecan.[18] Thus, biological systems utilize proteolytic processing to generate encrypted peptides and to increase the structural, molecular and pharmacological diversity of the peptidome to elicit complex physiological and pathophysiological responses in mammals.

We and other groups have recently characterized vipericidins and analyzed elapid CRAMPs and observed that reptilian cathelicidins contain several proteolytic cleavage sites that can potentially produce a diverse array of smaller peptides, which are embedded and encrypted in

their corresponding precursors. It was proposed that elastase-like proteases are involved in the processing of bovine and porcine cathelicidins and potentially fowlicidins – cathelicidins from chicken.[19] Because reptilian cathelicidin-related peptides contain several proteolytic cleavage sites that may cleave to form bioactive peptides and anticipating their potential use as theranostic tools, we investigated the biological activities of select vipericidin encrypted peptides. We focused our attention on the C-terminal hypervariable region of vipericidins and prepared synthetic peptides conjugated to rhodamine-B. The biological activities and cell and

tissue compartmentalization and toxicity were evaluated *in vivo* with zebrafish and *in vitro* with human breast MCF-7 cancer cells.

RESULTS

Assessment of lethal toxicity of encrypted vipericidin peptides in zebrafish

From the reptilian CRAMPs shown in Figure 1, peptides derived from the C-terminus of the vipericidins lachesicidin and batroxicidin were chemically synthesized by standard solid phase chemistry with rhodamine B covalently linked to the peptide N-terminus. The synthesized peptides and their physiochemical properties are listed in Tabl e 1. In a screen of

the lachesicidin and batroxicidin acidic connecting peptides (ACPs, namely ACPls and ACPbs, respectively) and encrypted vipericidins peptides (EVPs), the peptides RhoB-ACP1l, RhoB-ACP1c and RhoB-EVP50 showed the highest acute lethal toxicity to the zebrafish (Table 1). RhoB-EVP50 was the most potent peptide, exhibiting an LD_{50} value <10 µM (Figure S1), and was selected for further studies

Distribution of RhoB-EVP50 in zebrafish tissue

To determine whether Rhodamine B-conjugated EVP50 is able to enter zebrafish larvae, a number of zebrafish individuals (3dpf) were incubated with Rhodamine B-conjugated EVP50 (10 µM) for 24 h, and the rhodamine B accumulation and emission was recorded using fluorescence microscopy. Free rhodamine B was used as negative control. Figure 2A and B show that free rhodamine B accumulated exclusively in the yolk of the zebrafish. In contrast, RhoB-EVP50 was distributed to the blood vessels (Figure 2E and Figure 2F) and co-localized with the green fluorescence of EGFP in *Tg(fli1:EGFP)* transgenic zebrafish (Figure 2C and 2D). Figure 2G and 2H demonstrate the distribution overlap pattern of Rhodamine B-conjugated EVP50 and EGFP expression in zebrafish. The merged images show that RhoB-EVP50 specifically accumulates in the posterior cardinal vein and in the intersegmental vessels. RhoB-EVP50 was also detected in the cardiomyocytes of the heart (Figure S2 and Video S1).

Cellular uptake of RhoB-EVP50

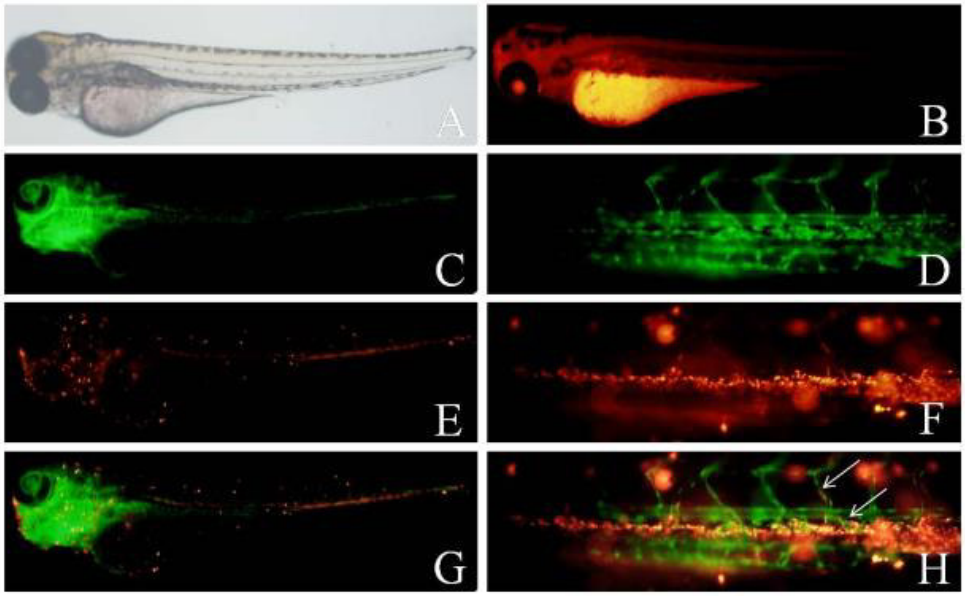
The distribution of RhoB-EVP50 in MCF-7 cells was detected in a similar manner using fluorescence microscopy. Figure 3 shows that at 10 µM, RhoB-EVP50 entered and

Table 1. Primary structures, molecular weights, physicochemical characteristics and toxicity levels of Rhodamine B-conjugated peptides derived from vipericidin hypervariable C-terminal regions.

Peptides[a]	Primary sequences[b]	MW	pI[c]	Net charge[c] (z)	LC_{50} (µM)[d]
ACP1b	RhoB-PVGGVEEEEEDEEEQKAEVEKDEEKEDEEKDRPKRVKRFKKFFKK	5935.5	4.52	-6	16
ACP1l	RhoB-PVGGVEEEEEEEEEEQKAEAENDEEVEKEKEDEEKDQPKRVKRFKKFFKK	6495.0	4.28	-10	13
ACP18b	RhoB-EEEEEDEEEQKAEVEKDEEKE	3035.1	3.59	-12	105
ACP18l	RhoB-EEEEEEEEEEQKAEAENDEEV	2978.0	3.13	-14	123
ACP41b	RhoB-EKDEEKEDEEKDRPKRV	2584.9	4.44	-3	116
ACP41l	RhoB-EKEKEDEEKDQPKRV	2312.6	4.56	-2	106
EVP26	RhoB-ENDEEV	1159.3	2.89	-4	>200
EVP50	RhoB-KRFKKFFKK	1682.2	11.85	+6	6

[a] ACP: Acidic connecting (vipericidin) peptide; EVP- Encrypted vipericidin peptide. Values of molecular weight and isoelectric point are for unlabeled peptides. Batroxicidin-derived peptides: “b”; lachesicidin-derived peptides: “l”.
[b] The fluorochrome Rhodamine-B (RhoB) is covalently linked to peptide N-terminal; the carboxyl-terminal is free. Data of toxicity are for rhodamine-B labeled peptides. Unlabeled peptides are innocuous.
[c] Values of pI and net charge are for unlabeled peptides. A pH-dependent extra charge (positive, at pH<6.0; negative, at pH>10.8) is imparted by rhodamine-B to peptide molecule. At pH between 6.0 and 10.8, rhodamine-B is neutral (uncharged).[6]
[d] LC50 denotes the concentration of rhodamine-B conjugated peptides that cause 50% of zebrafish death.

Figure 2. Fluorescent images of the distribution of RhoB-EVP50 in *Tg(fli1: EGFP)* zebrafish. Bright field microphotography (A, B) of zebrafish (3dpf) treated with 10 μM of free Rhodamine B for 24 h as a negative control. Green fluorescence pattern of EGFP expression in transgenic Tg(fli1: EGFP) zebrafish (C, D). Fluorescent images (E-F) from zebrafish (3dpf) after incubation with RhoB-EVP50 (10 μM) for 24 h. Merged fluorescent images (G, H) of blood vessels (green) and Rhodamine-conjugated EVP50 (red).



distributed uniformly around the nucleus (perinuclear region) of MCF-7 cells. At higher concentrations (40 µM), RhoB-EVP50 accumulated in both the cytosol and the nucleus of MCF-7 cells. Furthermore, our study showed that treatment with a high concentration (40µM) of RhoB-EVP50 for a longer period of incubation (60 min versus 10 min), the dye-conjugated nonapeptide damaged the cell membrane and caused MCF-7 cell lysis in cells that initiated within a 5 minute time frame (Figure 3). RhoB-EVP50 was also able to penetrate embryonic rat ventricle H9c2 cardiomyoblasts (ATCC) in culture, spread into the cytoplasm and accumulate in the nucleus (Figure S2).

Toxicity of RhoB-EVP50 toward MCF-7 breast tumor cells

The cell viability and cytotoxicity of RhoB-EVP50 toward MCF-7 cells was determined by LDH and MTT assays, respectively. As shown in Figures 4A and 4B, treatment with RhoB-EVP50 for 1 h significantly decreased the viability and increased the cytotoxicity of MCF-7 cells in a dose-dependent manner.

Rhodamine B-conjugated EVP50 promoted changes in intracellular calcium.

The dynamics of intracellular calcium levels were measured after incubating MCF-7 cells in a fixed concentration of RhoB-EVP50. Figure 5A shows that RhoB-EVP50 treatment for 90 s caused a rise in the intracellular calcium fluorescence intensity when compared to the control group. Treatment of MCF-7 cells with RhoB-EVP50 for 5 min compromised the cell membrane and caused the release of the cytoplasm from cells, as evidenced by an increased

Figure 3. Fluorescent images of the intracellular distribution of rhodamine B-conjugated EVP50 in MCF-7 cells. MCF-7 cells were treated with different concentrations of encrypted vipericidin RhoB-conjugated nonapeptide EVP50 for 10 min and 60 min.

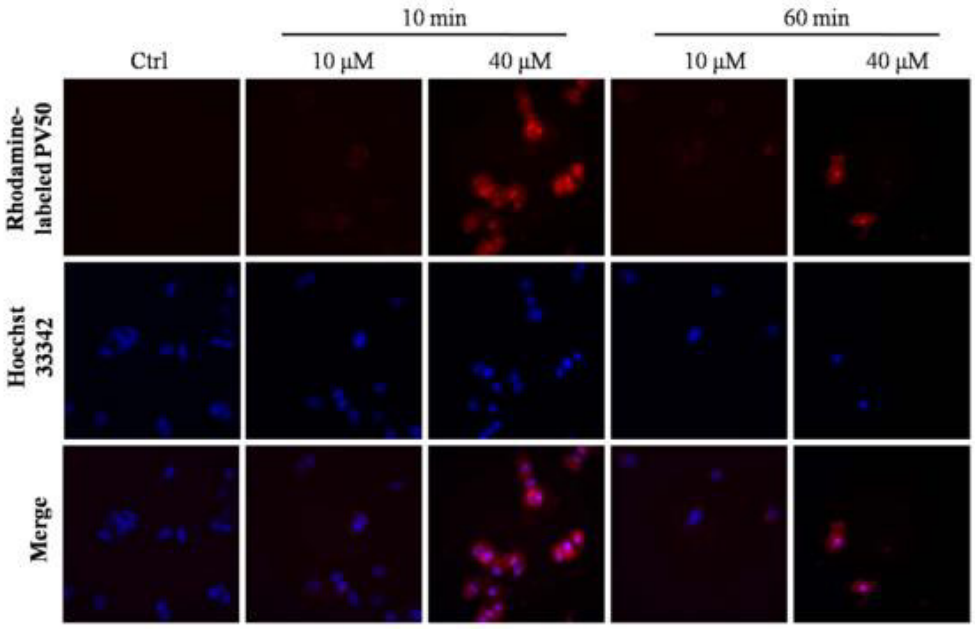


Figure 4. Rhodamine B-conjugated EVP50 induces cell death in MCF-7 cells. The cells were treated with different concentrations of RhoB-EVP50 for 1 h. Cell viability and membrane integrity were evaluated by an MTT cytotoxic assay (A) and an LDH leakage assay (B), respectively. $^{*}P$ <0.05 vs. untreated control (Ctrl).

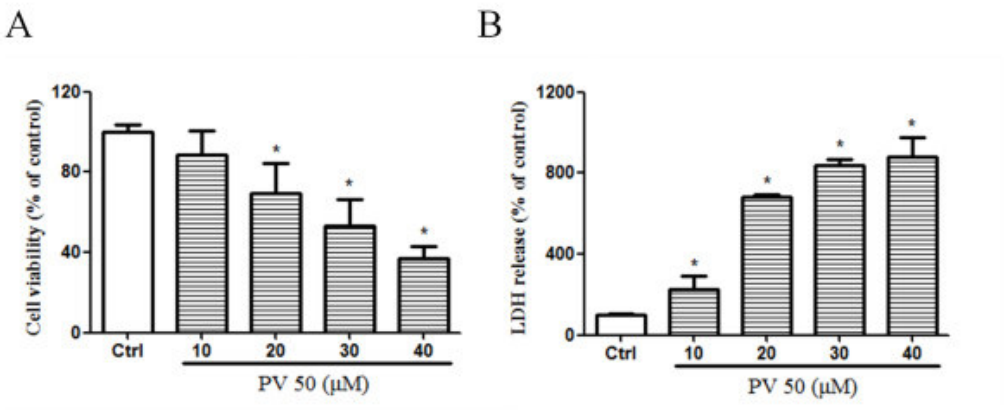
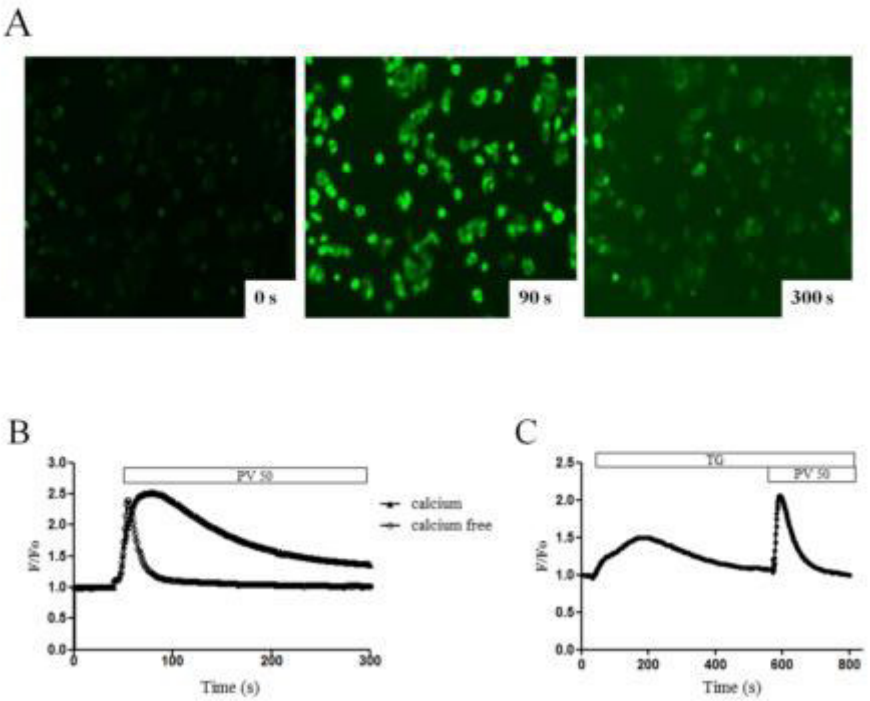


Figure 5. Rhodamine B-conjugated EVP50 induces calcium influx and intracellular calcium release in MCF-7 cells. (A) Representative images of $[Ca^{2+}]_i$ intensity in MCF-7 cells before and after treatment with EVP50 (30 μM). (B) EVP50 caused $[Ca^{2+}]_i$ elevation in the absence or presence of extracellular calcium. F/F_0 represents the fluorescence signal relative to the baseline fluorescence intensity. (C) Effect of EVP50 on $[Ca^{2+}]_i$ after intracellular calcium store depletion by thapsigargin (TG).



fluorescent background when compared to the control group. The dynamic change in intracellular calcium levels mediated by RhoB-EVP50 in the treated MCF-7 cells was also recorded (Video S2). As shown in Figure 5B, the levels of cytosolic calcium increased after addition of RhoB-EVP50 in the presence or absence of extracellular calcium, which indicated that RhoB-EVP50 caused extracellular calcium influx and intracellular calcium release from the endoplasmic reticulum into the cytosol in MCF-7 cells. This is consistent with the data presented in Figure 5C, which show that RhoB-EVP50 also induced an increase in calcium levels even if calcium stores were depleted with thapsigargin (TG).

DISCUSSION

Cathelicidin-related antimicrobial peptides (CRAMPs) are broad-spectrum antimicrobial components of tissues involved in innate immunity responses in organisms ranging from extant fossil fish (hagfish) to humans. 20,21 A recent characterization of cathelicidins from toads 22 concluded that cathelicidin genes and their peptide products are maintained in the course of biological evolution from aquatic organisms to land vertebrates. In snakes, the first members of cathelicidin family were described for elapids.2,3 Recently, four new CRAMP sequences were identified from South American pit vipers (rattlesnakes and jararacas), collectively named vipericidins, and two novel elapid CRAMPs from *Pseudonaja textilis* were characterized from venom glands.[1] These reptilian CRAMPs are expressed in the venom gland and can be purified from venom. Wang and co-workers [2] found that a *B. fasciatus* (Bf-) CRAMP is expressed in several snake tissues, including skin, trachea, stomach, muscle, heart,kidney, lung, brain, liver, and ovary, with comparable levels of transcript. Whether reptilian CRAMPs have a primary antimicrobial function for protecting the tissues of the snake venom glands or have a synergic role as a venom component in snake envenomation deserves further investigation. In *B. fasciatus* snake venom, a mature CRAMP with only 30 amino acids was purified, revealing additional post-translational proteolytic processing that removed four N-terminal residues from the predicted 34 residue CRAMP. As noted earlier, the mature reptilian CRAMPs that contain 30 to 34 residues display antimicrobial and antitumor activities against most bacterial strains and cell lines studied so far and are amenable topeptide engineering for therapeutic development.[1,4,5,7,23]

Despite the large evolutionary and geographical distances between pit vipers and elapid snakes, vipericidins and elapid CRAMPs have high structural similarities, with most of their 34 residues strictly conserved, except for a few conservative substitutions (Figure 1). The extra acidic sequence (acidic signature or acidic connecting peptide, as named here), in the propeptides is believed to help neutralize the net positive charge of the mature amphiphatic/cationic peptides that might otherwise cause undesired auto-damage to the peptide-producing cells and tissues. [24] Another possible role for the acidic stretches in the C-

terminal domains would be to assist in the correct folding of the mature amphiphatic/cationic peptides to confer full biological activity.[25] Analysis of the complete amino acid sequences of vipericidin CRAMPs reveals several putative protease cleavage sites, such as the dipeptides KK, KR and VK (Figure 1). Therefore, several different encrypted peptides can be formed after post-translational proteolytic modification,[26] releasing cryptic peptide sequences whose biological activity and potential toxicity are unpredicTable and must be experimentally ascertained. As mentioned, the proteolytic release and activation of encrypted peptides (and subsequent post-translational modifications) is a process that operates on a repertoire of long polypeptides with numerous biological functions. [17,27-29] Additionally, propeptides in certain classes of proteins with unknown biological functions can release sequences after proteolytic processing that have unpredicTabl e effects, including malignant transformation.[30] Therefore, crossing transcriptomic with proteomic data is necessary in order to understand the diversity of peptide structures in the field of venomics and is essential to characterizing functional peptidomes.

With regards to the cathelicidin-related antimicrobial peptides, Chen and co-workers[31] prepared several peptides derived from BF-30 (Bf -CRAMP) and found that a 15-mer oligopeptide (i.e., [157] VKRFKKFFRKLKKSV[172]) retains the minimal helical structurenecessary for potent antimicrobial activity. In another study, Zhang and collaborators[32] produced short synthetic analog peptides, which correspond to sequences internally embedded in *Ophiophagus hannah* cathelicidin (Oh-CATH, 34 mer), and demonstrated that they display a distinct spectra of antimicrobial activity and hemolysis toward human erythrocytes.

Our study was centered on the C-terminal hypervariable acidic stretches of vipericidins and the first encrypted amphiphatic nonapeptide peptide, KRFKKFFKK. This encrypted vipericidin nonapeptide is practically invariable in all reptilian CRAMPs and appears repeatedly in tandem, with some conserved substitutions (Figure 1). Incubation of zebrafish have embryos with increasing concentrations of the Rhodamine B-conjugated vipericidin-derived peptides (RhoB-ACPs and RhoB-EVPs) indicated that both the peptides that contain the amphiphatic nonapeptide KRFKKFFKK in their sequences (i.e., RhoB-ACP1l and RhoB-

ACP1b) and the cationic nonapeptide itself (named RhoB-EVP50) are extremely toxic. The calculated LD50 for these toxic peptides is in the micromolar range of concentration (16 □M, 13µM and 10 µM, respectively) (Table 1 and Figure S1). One possible explanation for the difference in the values of LD50 for the nonapeptide-containing ACPs in relation to RhoB-EVP50 might be the presence of the anionic piece just upstream of the cationic EVP50. Thus, we focused our attention in the encrypted nonapeptide and investigated the toxicity of naïve-unconjugated EVP50 using the same animal model. Unexpectedly, the amino-terminus of unmodified EVP50 was not toxic to zebrafish even at concentrations as high as 200 µM (data not shown). Despite this fact and because peptides with covalently linked rhodamine B display improved functionalities,[33] we monitored the distribution of the RhoB-EVP50 into the organs and tissues of zebrafish. With this goal in mind, *Tg(fli1:EGFP)* transgenic zebrafish were exposed to 10 µM RhoB-EVP50 for 24 h and visualized by fluorescence microscopy.

As shown in Figure 2 (A-H), RhoB-EVP50 heavily accumulates in the cardiovascular system (posterior and intersegment vasculature) of zebrafish. After this, the embryo dies. It is interesting to note that RhoB-EVP50 easily penetrates and accumulates not only *in vivo* in the

zebrafish organs (blood vessels and heart) but also *in vitro* in rat cardiomyocytes (H9c2 cardiomyoblasts) where intense red fluorescence was observable in both the cytoplasm and nucleus of cells (Figure S2 and Video S1). Notably, free rhodamine B was not taken up and consequently was not distributed in the same tissues as the RhoB-conjugate EVP50. Indeed, it is known that free rhodamine B molecule has low bioavailability and low cell permeability in certain biological systems.[34] Consequently, the functionalization of this fluorescent dye is due to the EVP50 carrier sequence. These findings support work by El-Andaloussi and collaborators,[35] which show the cargo-dependence and the positional effect of cargo coupling on the toxicity of three different cell-penetrating peptides. Moreover, our data indicate that cells of cardiovascular system and tissues may be a target site for RhoB-EVP50 penetration; the cardiovascular system may also contain the preferential organs for peptide accumulation *in vivo* and the means by which zebrafish are poisoned.

In light of the facts that (1) RhoB-EVP50 accumulates *in vivo* in the cardiovascular system of zebrafish and in mammalian cardiomyocytes *in vitro* and that (2) elapid CRAMPs and analogues are cytotoxic against tumor cells,[5,7] we decided to investigate the specific biological activity and toxicity of RhoB-EVP50 on a selected cancer cell line. Snake venom and spider venom have previously been reported to arrest breast cancer cell growth. [36-38] In our study, human breast cancer MCF-7 cells were used to test the anti-tumor activity of EVP50. Fluorescent image analysis of MCF-7 incubated with 10 µM and 40 µM RhoB-EVP50 for 10 minutes showed that the Rhodamine B-conjugated nonapeptide rapidly internalizes into the cytoplasm and concentrates in the nucleus (and perinuclear region) of cells (Figure 3). Within 60 min of incubation, the induced toxicity led to cell death and severely decreased the number of observable cells. Standard assays of cell viability and membrane integrity showed that RhoB-EVP50 is cytotoxic to MCF-7 in a concentration-dependent manner (Figure 4). Together, these properties of fast peptide uptake, intracellular accumulation and membrane disruption impart a toxic capacity to the structurally modified encrypted nonapeptide to efficiently kill tumor cells. Again, naïve-unconjugated EVP50 was inactive and incapable of arresting cancer cells in culture (data not shown). These results imply the necessity for deeper study of the selective and specific toxicity of RhoB-EVP50 and related engineered peptide analogues against certain cancer cells and tissues. For instance, the short synthetic analog peptides produced by Zhang and collaborators,[32] which corresponded to sequences internally embedded in *Ophiophagus hannah* cathelicidin (Oh-CATH, 34 mer), displayed a distinct spectra of antimicrobial activity and hemolysis toward human erythrocytes. In the study, they found that the four N-terminal residues contribute to a hemolytic effect but not to the antimicrobial activity, whereas the ten C-terminal residues are necessary for both biological activities. Similarly, substitution of Lys for a Glu residue at position 16 (Cbf-K16) in Bf-cathelicidin improves the activity of the parental peptide against non-small lung cancer cells and antibiotic-resistant clinical isolates of *E. coli*.7,39 Most antimicrobial activities exert their effect by a single action or a combination of actions on lipid membranes, such as formation of a pore, physical disruption, and translocation, followed by

subsequent interactions with intracellular targets.40,41 Cathelicidins are multifunctional antimicrobial peptides with immunomodulatory, tissue remodeling, angiogenic and anticancer activities. They can disrupt cell membranes and interact with DNA and thus are synergistically effective in killing cells, restraining cell proliferation and recruiting components of acquired immunity.8, 42,43 However, despite their interference with lipid membranes, this general molecular mechanism of action is not the case for this class of peptides. Coincidental l y, in our present findings, a critical peptide concentration appears to play a role in triggering a cell response.[41] Specifically, low peptide/cell and peptide/membrane ratios may trigger intracellular signal transduction events, whereas excessive amounts of molecules massively intoxicate the cells. The same reasoning can be applied to cationic peptides that display cell-penetrating, antimicrobial and antitumor activity. Membrane interference/disruption and translocation followed by intracellular target interactions may play a role in cell cytotoxicity separately or in combination, depending on the peptide to cell ratio.

8,22,44,45 These facts may explain how RhoB-EVP50 exerts its action on cells. Measurements of intracellular calcium release after incubating MCF-7 cells in a micromolar concentration (30 µM) of peptide demonstrated that RhoB-EVP50 provoked an instantaneous and specific cellular calcium imbalance (increased extracellular calcium influx and intracellular calcium release) and consequent cytoplasm leakage (Figure 5 and Video S2).

Calcium homeostasis has been implicated in cell signaling for cell survival and death and is mediated by the interface between the mitochondria and the endoplasmic reticulum.[46] It is interesting to note that in some of fluorescent images from our experimental study, membrane

budding was clearly observed (Video S2 and Figure S3). Membrane budding and the formation of extracellular vesicles are associated with a plethora of intercellular messages, including pathological conditions of tumor progression, viral infection, immune responses, and microcystin-induced hepatoxicity and apoptosis.[47,48] As seen here, calcium imbalance and

outward blebbing of the cell membrane appear to be the early intracellular events in the cytotoxicity caused by RhoB-EVP50 uptake and cytoplasmic accumulation into the cytoplasm and nucleus of MCF-7 cells. In the present work, we find that the hypervariable acidic moieties of the vipericidin carboxyl-termini have reduced or are devoid of acute toxicity, whereas the lysine-rich encrypted amphiphatic nonapeptide conjugated to rhodamine B (RhoB-EVP50) displays potent toxicity toward zebrafish and tumor cells in culture. In a transgenic zebrafish model, the encrypted nonapeptide is widely distributed and massively accumulates in the zebrafish cardiovascular system, which is lethal to the organism. Additionally, RhoB-EVP50 efficiently penetrates rat ventricular cardiomyoblasts and concentrates in the cytoplasm and nucleus. Human breast cancer (MCF-7) cells are substantially sensitive to the biological effects of the vipericidin-encrypted peptide. In this cancer cell line, RhoB-EVP50 causes observable calcium imbalance, membrane budding, membrane disruption, and cytoplasm leakage. The overall effect of RhoB-EVP50 is the induction of cell death over a short period of time, ranging from seconds to minutes. However, after peptide translocation, whether vesicle budding contributes to the spread of RhoB-EVP50 toxicity to tissue and organs, as observed in zebrafish embryos, remains to be seen. The fact that rhodamine B improves the efficiency of EVP50 in the translocation of dye-peptide conjugate into the cell makes EVP50 an excellent peptide sequence platform for the development of a drug delivery system. Moreover, taking into account the findings reported here, a system of structure-activity modulation has been initiated using covalent linkage of molecules with distinct physicochemical properties to the basic core sequence of the EVP50 nonapeptide.

EXPERIMENTAL PROCEDURES

Peptide sequences and synthesis

The hypervariable C-terminus of the vipericidins, which served as template sequences for synthesis of the encrypted peptides, were from lachesicidin (GenBank accession number AGS36142.1), which shares a high degree of sequence identity with crotalicidin

(99%,AGS36138.1), batroxicidin (85%, AGS36140.1), lutzicidin (84%, AGS36141.1), Bf-CRAMP (65%, B6D434), Oh-CRAMP (65%, B6S2X2.1), and Na-CRAMP (64%, B6S2X0.1). Batroxicidin was also used for the design and synthesis of an acidic cryptic peptide (Figure 1).

All peptides were synthesized by solid phase chemistry and obtained at a purity grade over 95% and confirmed by the presence of a single peak in analytical reverse-phase HPLC and mass spectrometry analysis (Cellmano Biotech Limited, Hefei, China). The fluorescent dye Rhodamine B was covalently linked to the N-terminal end of the peptide series. Table 1 summarizes the synthetic encrypted vipericidin peptides used in this study.

Zebrafish maintenance

The enhanced green fluorescent protein (EGFP) was specifically expressed in the endothelial

cells of *Tg(fli-1:EGFP)* zebrafish embryos. The zebrafish used in our study were maintained as previously described.49 Natural pair-wise mating (3-12 months old) was used to generate zebrafish embryos. The embryos were raised and maintained in embryo medium at 28.5ºC Ethics approval for the animal experiments was granted by the Animal Research Ethics Committee in University of Macau, University of Macau, China.

Acute toxicity of encrypted vipericidin peptides toward zebrafish embryos

Zebrafish embryos at three days post-fertilization (3dpf) were separated into a 24-well plate and exposed to 2-logs concentration of Rhodamine B-conjugated ACPs (RhoB-ACP1b, RhoB-ACP1l, RhoB-ACP18b, RhoB-ACP18l, RhoB-ACP41b and RhoB-ACP41l) and Rhodamine B-conjugated EVPs (RhoB-EVP26 and RhoB-EVP50) for 24 h. The acute toxicity and mortality of zebrafish exposed to the RhoB conjugates were determined by monitoring the absence of a heartbeat, as observed under a light microscope.

Distribution of RhoB-EVP50 in zebrafish and determination of acute toxicity

Zebrafish embryos were exposed to a fixed concentration (10 μM) of RhoB-EVP50 for 24 hours and mounted on microscope glass slides. The distribution of Rhodamine B-conjugated

EVP50 in zebrafish body and tissues was visualized using an IX81 motorized inverted fluorescent microscope (Olympus Co., Tokyo, Japan).

RhoB-EVP50 cell uptake by tumor cells

Human breast cancer MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 100 IU^{-1} penicillin-streptomycin at 37°C ml in a humidified incubator containing 5% CO. Cells were seeded into a 96-well plate at a density of 5×10^3 cells per well and incubated overnight. After 24 h of seeding, MCF-7 cells were treated with 10 and 40 μM RhoB-EVP50 for 10 min or 60 min. Then, the cells were stained with Hoechst 33342 to label the nuclear DNA. The sub-cellular distribution of Rhodamine B-conjugated EVP50 was detected by an IN CELL Analyzer 2000 system (GE Healthcare Life Sciences, Piscataway NJ, USA).

Assessment of cell viability and cytotoxicity

The MCF-7 cells (5×10^3 per well) were cultured as above and treated with increasing concentrations (up to 40 μM) of RhoB-EVP50 for 1 h. The level of lactate dehydrogenase (LDH) in the medium was measured by using a Cytotoxicity Detection Kit (Roche Diagnostics Co., Indianapolis, USA) according to the protocol from the manufacturer. Cell viability was determined by a standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Measurement of intracellular calcium concentration

The MCF-7 cells were seeded on glass coverslips placed into the bottom of the wells of 12-well plate. Twenty-four hours after seeding, the cells were stained with 3 μM Fluo-3/AM for 30 min at 37°C. The cells were then washed with phosphate-buffered saline (PBS) three times, and the calcium levels in the MCF-7 cells were detected using the Cell^R imaging system of an IX81 microscope (Olympus Co., Tokyo, Japan).

ACKNOWLEDGEMENTS

Research at the University of Macao was supported by grants from the Science and Technology Development Fund (FDCT) of Macao - SAR (078/2011/A3 and 045/2007/A3 and the Research Committee of the University of Macau. Research at the Federal University of Ceará was supported by the Brazilian National Council for Scientific and Technological Development (CNPq), the Ministry of Science, Technology and Innovation (MCTI), the Program on Toxi nology (Issue 2010), the Coordination for the Improvement of Higher Education Personnel (CAPES), and the Ministry of Education of the Federal Government of Brazil. We thank Ms. Christy Kou for her assistance with pre-screening activities of some peptides.

SUPPORTING INFORMATION AVAILABLE

Video S1. A Video showing the penetration and accumulation of rhodamine B-conjugated EVP50 into the heart of zebrafish.

Video S2. A Video showing the change of intracellular calcium levels when MCF-7 cells are treated with rhodamine B-conjugated EVP50.

Figure S1. Accumulation of rhodamine-B conjugated EVP50 into rat cardiomyocytes.

Figure S2. The rhodamine B-conjugated EVP-50 causes membrane budding of MCF-7 cells.

CONFLICT OF INTEREST

The authors declare no competing financial interest

REFERENCES

(1) Falcao, C. B., de La Torre, B. G., Pérez-Peinado, C., Barron, A. E., Andreu, D., and Rádis-Baptista, G. (2014) Vipericidins: a novel family of cathelicidin-related peptides from the venom gland of South American pit vipers. Amino Acids [Epub ahead of print]

(2) Wang, Y., Hong, J., Liu, X., Yang, H., Liu, R., Wu, J., Wang, A., Lin, D., and Lai, R. (2008) Snake cathelicidin from Bungarus fasciatus is a potent peptide antibiotics. *PLoS One. 3*, e3217.

(3) Zhao, H., Gan, T. X., Liu, X. D., Jin, Y., Lee, W. H., Shen, J. H., Zhang, Y. (2008) Identification and characterization of novel reptile cathelicidins from elapid snakes. Peptides 29, 1685-1691.

(4) Wang, Y., Zhang, Z., Chen, L., Guang, H., Li, Z., Yang, H., Li, J., You, D., Yu, H., and Lai, R. (2011) Cathelicidin-BF, a snake cathelicidin-derived antimicrobial peptide, could be an excellent therapeutic agent for acne vulgaris. PLoS One 6, e22120.

(5) Wang, J., Li, B., Li, Y., Dou, J., Hao, Q., Tian, Y., Wang, H., and Zhou, C. (2013) BF-30 effectively inhibits ciprofloxacin-resistant bacteria in vitro and in a rat model of vaginosis. *Arch Pharm Res. 37*, 927-36

(6) Oh, Y. J., Gamble, T. C., Leonhardt, D., Chung, C. H., Brueck, S. R., Ivory, C. F., Lopez, G. P., Petsev, D. N., Han, S.M. (2008) Monitoring FET flow control and wall adsorption of charged fluorescent dye molecules in nanochannels integrated into a multiple internal reflection infrared waveguide. Lab Chip. 8, 251-8.

(7) Wang, H., Ke, M., Tian, Y., Wang, J., Li, B., Wang, Y., Dou, J., and Zhou, C. (2013) BF-30 selectively inhibits melanoma cell proliferation via cytoplasmic membrane permeabilization and DNA-binding *in vitro* and in B16F10-bearing mice. *Eur J Pharmacol.707*, 1-10.

(8) Tian, Y., Wang, H., Li, B., Ke, M., Wang, J., Dou, J., and Zhou, C. (2013) The cathelicidin-BF Lys16 mutant Cbf-K16 selectively inhibits non-small cell lung cancer proliferation *in vitro*. *Oncol Rep. 30*, 2502-2510.

(9) Rádis-Baptista, G., Oguiura, N., Hayashi, M. A., Camargo, M .E., Grego, K. F., Oliveira, E. B., Yamane, T. (1999) Nucleotide sequence of crotamine isoform precursors from a single South American rattlesnake (*Crotalus durissus terrificus*). Toxicon 37, 973-984.

(10) Whittington, C. M., Papenfuss, A. T., Bansal, P., Torres, A. M., Wong, E. S., Deakin, J. E., Graves, T., Alsop, A., Schatzkamer, K., Kremitzki, C., Ponting, C. P., Temple-Smith, P.,

Warren, W. C., Kuchel, P. W., Belov, K. (2008) Defensins and the convergent evolution of platypus and reptile venom genes. Genome Res. 18, 986-994.

(11) Anderluh, G., and Macek, P. (2002) Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria). *Toxicon 40*, 111-124.

(12) Norton, R. S. (2009) Structures of sea anemone toxins. *Toxicon 54*, 1075–1088.

(13) Gmach, M., and Kreil, G. (1995) The precursors of the bee venom constituents apamin and MCD peptide are encoded by two genes in tandem which share the same 3'-exon. *J Biol Chem. 270*, 12704-12708.
20

(14) Lee, V. S., Tu, W. C., Jinn, T. R., Peng, C. C., Lin, L. J., Tzen, J. T. (2007) Molecular cloning of the precursor polypeptide of mastoparan B and its putative processing enzyme, dipeptidyl peptidase IV, from the black-bellied hornet, Vespa basalis. *Insect Mol Biol. 16*, 231-237.

(15) Luo, S., Christensen, S., Zhangsun, D., Wu, Y., Hu, Y., Zhu, X., Chhabra, S., Norton, R. S., McIntosh, J. M. (2013). A novel inhibitor of a9a10 nicotinic acetylcholine receptors from *Conus vexillum* delineates a new conotoxin superfamily. *PLoS One. 8*, e54648.

(16) Yuan, D. D., Han, Y. H., Wang, C. G., Chi, C. W. (2007) From the identification of gene organization of alpha conotoxins to the cloning of novel toxins. 49, 1135-49.

(17) Kozlov, S. A., Vassilevski, A. A., Feofanov, A. V., Surovoy, A. Y., Karpunin, D. V., and Grishin, E. V. (2006) Latarcins, antimicrobial and cytolytic peptides from the venom of the spider *Lachesana tarabaevi* (Zodariidae) that exemplify biomolecular diversity. *J Biol Chem. 281*, 20983-20992.

(18) Autelitano, D. J., Rajic, A., Smith, A. I., Berndt, M. C., Ilag, L. L., Vadas, M. (2006) The cryptome: a subset of the proteome, comprising cryptic peptides with distinct bioactivities. *Drug Discov Today. 11*, 306-314.

(19) Xiao, Y., Cai, Y., Bommineni, Y. R., Fernando, S. C., Prakash, O., Gilliland, S. E., Zhang, G. (2006) Identification and functional characterization of three chicken cathelicidins with potent antimicrobial activity. *J Biol Chem. 281*, 2858-2867.

(20) Uzzell, T., Stolzenberg, E. D., Shinnar, A. E., et al (2003) Hagfish intestinal antimicrobial peptides are ancient cathelicidins. *Peptides 24*, 1655-1667.

(21) Durr, U. H. N., Sudheendra, U. S., and Ramamoorthy, A. (2006) LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim Biophys Acta 1758*, 1408– 1425.

(22) Hao, X., Yang, H., Wei , L., Yang, S., Zhu, W., Ma, D., Yu, H., and Lai, R. (2012) Amphibian cathelicidin fills the evolutionary gap of cathelicidin in vertebrate. *Amino Acids 43*, 677-685.

(23) Li, L., Wang, Q., Li, H., Yuan, M., and Yuan, M. (2014) Preparation, characterization, in vitro release and degradation of cathelicidin-BF-30-PLGA microspheres. *PLoS One 9*, e100809.

(24) Michaelson, D., Rayner, J., Couto, M., and Ganz, T. (1992) Cationic defensins arise from charge-neutralized propeptides: a mechanism for avoiding leukocyte autocytotoxicity? *JLeukoc Biol. 51*, 634-639.

(25) Tomasinsig, L., and Zanetti, M. (2005) The cathelicidins--structure, function and evolution. *Curr Protein Pept Sci 6*, 23-34.

(26) Liu, F., and Wet s, G. (2005) A neural network method for prediction of proteolytic cleavage sites in neuropeptide precursors. *Conf Proc IEEE Eng Med Biol Soc. 3*, 2805-2808.

(27) Wardman, J. H., and Fricker, L. D. (2014) ProSAAS-Derived Peptides Are Differentially Processed and Sorted in Mouse Brain and AtT-20 Cells. *PLoS One 9*, e104232.

(28) Meisel, H. (2004) Multifunctional peptides encrypted in milk proteins. *Biofactors 21*, 55-61.

(29) Rang, H. P., Dale, M. M., Ritter, J. M., Moore, P. K. (2003) *Pharmacology 5 Edition*. Churchill Livingstone, Loanhead, Scotland. pp291-293; pp237-239.

(30) Stevenson, F. T., Turck, J., Locksley, R. M., Lovett, D. H. (1997) The N-terminal propiece of interleukin 1 alpha is a transforming nuclear oncoprotein. *Proc Natl Acad Sci U S A. 94*, 508-513.

(31) Chen, W., Yang, B., Zhou, H., Sun, L., Dou, J., Qian, H., Huang, W., and Mei, Y., and Han, J. (2011) Structure-activity relationships of a snake cathelicidin-related peptide, BF-15. *Peptides 32*, 2497-2503.

(32) Zhang, Y., Zhao, H., Yu, G. Y., Liu, X. D., Shen, J. H., Lee, W. H., Zhang, Y. (2010) Structure-function relationship of king cobra cathelicidin. Peptides 31, 1488-1493.

(33) Bucki, R., Pastore, J. J., Randhawa, P., Vegners, R., Weiner, D.J., and Janmey, P. A. (2004) Antibacterial activities of rhodamine B-conjugated gelsolin-derived peptides compared to those of the antimicrobial peptides cathelicidin LL37, magainin II, and melittin. *Antimicrob Agents Chemother. 48*, 1526-1233.
th
(34) Mottram, L. F., Forbes, S., Ackley, B. D., and Peterson, B. R. (2012) Hydrophobic analogues of rhodamine B and rhodamine 101: potent fluorescent probes of mitochondria in living *C. elegans*. *Beilstein J Org Chem. 8*, 2156-2165.

(35) El-Andaloussi, S., Järver, P., Johansson, H. J., and Langel, U. (2007) Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study. *Biochem J. 407*, 285-292.

(36) Al-Sadoon, M. K., Abdel-Maksoud, M.A., Rabah, D.M., and Badr, G. (2012) Induction of apoptosis and growth arrest in human breast carcinoma cells by a snake (*Walterinnesia aegyptia*) venom combined with silica nanoparticles: crosstalk between Bcl2 and caspase 3. *Cell Physiol Biochem. 30*, 653-665.

(37) Lin, K. L., Su, J. C., Chien, C. M., Chuang, P. W., Chang, L. S., and Lin, S. R. (2010) Down-regulation of the JAK2/PI3K-mediated signaling activation is involved in Taiwan cobra cardiotoxin III-induced apoptosis of human breast MDA-MB-231 cancer cells. *Toxicon 55*, 1263-1273.

(38) Gao, L., Yu, S., Wu, Y., and Shan, B. (2007) Effect of spider venom on cell apoptosis and necrosis rates in MCF-7 cells. *DNA Cell Biol. 26*, 485-489.

(39) Hao, Q., Wang, H., Wang, J., Dou, J., Zhang, M., Zhou, W., and Zhou, C. (2013) Effective antimicrobial activity of Cbf-K16 and Cbf-A7 A13 against NDM-1-carrying Escherichia coli by DNA binding after penetrating the cytoplasmic membrane *in vitro*. *J Pept Sci. 19*,173-180.

(40) Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat Rev Microbiol. 3*, 238-250.

(41) Nicolas, P. (2009) Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *FEBS J. 276*, 6483-6496.

(42) Wong JH1, Ye XJ, Ng TB. Cathelicidins: peptides with antimicrobial, immunomodulatory, anti-inflammatory, angiogenic, anticancer and procancer activities. Curr Protein Pept Sci. 2013 Sep;14(6):504-14.

(43) Bucki, R., Leszczynska, K., Namiot, A., and Sokolowski, W. (2010) Cathelicidin LL-37: a multitask antimicrobial peptide. *Arch Immunol Ther Exp. 58*, 15-25.

(44) Wenzel, M., Chiriac, A. I., Otto, A., Zweytick, D., May, C., Schumacher, C., Gust, R., Albada, H. B., Penkova, M., Krämer, U., Erdmann, R., Metzler-Nolte, N., Straus, S. K., Bremer, E., Becher, D., Brötz-Oesterhelt, H., Sahl, H. G., Bandow, J. E. (2014) Small cationic
antimicrobial peptides delocalize peripheral membrane proteins. *Proc Natl Acad Sci U S A. 111*, E1409-418.

(45) Do, N., Weind, G., Grohmann, L., Salwiczek, M., Koksch, B., Korting, H. C., and Schäfer-Korting, M. (2014) Cationic membrane-active peptides - anticancer and antifungal activity as well as penetration into human skin. Exp Dermatol. 23, 326-31.

(46) Naon, D., and Scorrano, L. (2014) At the right distance: ER-mitochondria juxtaposition in cell life and death. *Biochim Biophys Acta. 1843*, 2184-2194.

(47) Krakstad, C., Herfindal, L., Gjertsen, B. T., Bøe, R., Vintermyr, O. K., Fladmark, K. E., and Døskeland, S. O. (2006) CaM-kinaseII-dependent commitment to microcystin-induced apoptosis is coupled to cell budding, but not to shrinkage or chromatin hypercondensation. *Cell Death Differ. 13*, 1191-202.

(48) EL Andaloussi, S., Mäger, I., Breakefield, X.O., and Wood, M.J. (2013) Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov. 12*, 347-357.

(49) Hu, G., Mahady, G. B., Li, S., Hoi, M. P., Wang, Y. H., Lee, S. M. (2012) Polysaccharides from astragali radix restore chemical-induced blood vessel loss in zebrafish. *Vasc Cell. 4*, 2.

CAPÍTULO IV

Perspectiva Biotecnológicas da aplicação das vipericidinas e peptídeos terapêuticos análogos

Perspectiva Biotecnológicas da aplicação das vipericidinas e peptídeos terapêuticos análogos

As vipericidinas e peptídeos terapêuticos análogos constituem modelos moleculares interessantes para o desenvolvimento de estratégias biotecnológicas para gerar agentes terapêuticos e / ou ferramentas experimentais para a pesquisa básica e aplicada. De modo geral as proteínas modificadas ou análogos tem intuito de alterar as propriedades das proteínas, tais como: estabilidade, eficácia, especificidade, imunogenicidade e a farmacocinética, visando a melhoria terapêutica (Buckel, 1996).

Os peptídeos terapeuticos podem ser extremamente valiosas nos problemas de infecções hospitalares, devido à sua incrível versatilidade e modos de ação que fornecem um amplo espectro de atividades, funções e exigem baixas concentrações para ser ativo (de Oliveira Junior et al., 2013), podendo ser de interesse farmacologicamente ativo na clínica-médica e conhecimento na área científica. Além disso, existem evidências de que as PAMs sejam menos suscetíveis à resistência bacteriana quando comparada como os antibióticos tradicionais utilizados, assim, podem ser considerados como uma nova alternativa de agentes terapêuticos (Hancock e Partzykat, 2002).

PAMs têm diversos efeitos biológicos, participando no controle de doenças infecciosas e inflamatórias (câncer, doenças de pele como a dermatite atópica e psoríase, doenças intestinais, na patogénese da pneumonia, doença pulmonar obstrutiva crônica, aterosclerose, fibrose cística e doenças neurodegenerativas), possuindo características que tornam estes peptídeos atraentes como ferramentas terapêuticas (Guaní-Guerra et al., 2010).

Portanto, mais estudos devem ser realizados para entendermos mais sobre os mecanismos de ação dos peptídeos antimicrobianos e como atuam nas células do corpo humano, de modo que eles possam ser melhorados para servir como novos medicamentos

para combater infecções de microorganismos resistentes como: bactérias, fungos e parasitas e/ou eliminar células cancerígenas.

Pesquisa mais aprofundada dos componentes estruturais dos peptídeos relacionados vipericidinas permitirá avaliar os locais de maior atração do peptídeo e seu alvo na célula. Foi observado que região rica em prolina com hidrofóbica resíduos (Phe e Leu) conferem 30% de taxa de hidrofóbico, o que provavelmente favorece a interação com os fosfolipídios da membrana celular. Análise *in silico* tem fornecido ampla informações sobre as estruturas dos PAMs, sugerindo que os resídeos podem ser importantes para as interações da membrana plasmática bacteriana (Okubo et al.2012).

As variantes de peptídeos sintéticos são alternativas de pesquisa que podem ser obtidas apartir dos peptídeos naturais (Deslouches et al., 2013). Os peptídeos sínteticos podem ser conduzidos para ensaios antibacteriano, antifúngico, anti-*T.cruzi e* anticancerígenos *in vivo e in vitro.* Dessa forma, poderá proporcionar melhor atividade antimicrobiana, antiparasitária e anticancerígena, e mantendo sua atividade hemolítica reduzida.

CONSIDERAÇÕES FINAIS

Nesta pesquisa seguiu duas vertentes a primeira foi possível realizar a caracterização do gene alvo molecular TcCyP19 que é regulado positivamente em populações de *T. cruzi* resistentes a BZ e que várias linhas de evidência implicam CyPs como um alvo quimioterápico promissor contra o câncer e doenças parasitárias.

A segunda vertente deste estudo foi avaliação *in vitro* a atividade antiparasitária e a antitumoral, bem como avaliação da citotoxicidade das vipericidinas e seus derivados, visando o uso biotecnológico desses peptídeos. Os ensaios de atividade anti-*T.cruzi in vitro* de formas epimastigotas foi observado que a Vipericidina (crotalicidina) teve efeito citotóxico dose-dependente contra epimastigotas de *T.cruzi*. Na avaliação a ação anticancerígena o oligopeptídeo (EVP50) conjugado com rodamina B (RhoB) exibiu uma potente toxicidade letal para peixe-zebra (Danio rerio). *In vitro*, a RhoB conjugado EVP50 exibiu um efeito antitumoral dependente da concentração, em relação às células MCF-7 de câncer da mama, demonstrando que EVP50 surge como uma plataforma de sequência de peptídeos alternativos para aplicação no desenvolvimento de nanobiomateriais.

Este estudo vem contribuir para o entendimento do alvo molecular (Ciclofilina-A) que tem a capacidade de interagir com peptídeo vipericidina e tem atuação potencial dessa classe de peptídeos em biotecnologia médica contra *T. cruzi* e atividade anticancerígena, quando o peptídeo é conjugado. Nesse contexto, essa pesquisa representa um ponto de partida para uma investigação mais aprofundada sobre a função de TcCyPs e no mecanismo de resistência aos medicamentos e como um possível alvo para Quimioterapia para doença de chagas. Além disso, as vipericidinas mostram indicações de serem interessantes alvos de pesquisa contra a doença de Chagas e com a possibilidade de se desenhar novos peptídeos sintéticos terapêuticos eficazes a partir de peptídeos naturais.

REFERÊNCIAS

Adade CM, Oliveira IR, Pais JA, Souto-Padrón T. Melittin peptide kills *Trypanosoma cruzi* parasites by inducing different cell death pathways. *Toxicon*. 2013 Jul;69:227-39.

Andersson, M., Boman, A., and Boman, H.G. (2003). Ascaris nematodes from pig and human make three antibacterial peptides: isolation of cecropin P1 and two ASABF peptides. *Cell Mol Life Sci* 60, 599-606

Andreeva L, Heads R, Green CJ. Cyclophilins and their possible role in the stress response. *Int. J. Exp. Pathol.* 1999; 80, 305-15.

Linde A, Lushington GH, Abello J and Melgarejo T. Clinical Relevance of Cathelicidin in Infectious Disease. *J Clin Cell Immunol*, 2013, S13: 003.

Atwood JA 3rd, Weatherly DB, Minning TA, Bundy B, Cavola C, Opperdoes FR, et al. The *Trypanosoma cruzi* proteome. *Science.* 2005; 309 (5733): 473-6.

Breast cancer in the personal genomics era.Ellsworth RE, Decewicz DJ, Shriver CD, Ellsworth DL. *Curr Genomics*. 2010 May;11(3):146-61.

Bell A, Monaghan P, Page A.P. Peptidyl-prolyl cis-trans isomerases (immunophilins) and their roles in parasite biochemistry, host-parasite interaction and antiparasitic drug action. *Int. J. Parasitol*. 2006; 36, 261-76.

Bechinger B, Salnikov ES.The membrane interactions of antimicrobial peptides revealed by solid-state NMR spectroscopy. *Chem Phys Lipids.* 2012 Apr;165(3):282-301.

Bhutia SK, Maiti TK. Targeting tumors with peptides fromnatural sources. *Trends Biotechnol* 2008, 26:210–217

Bond, P.J.; Parton, D.L.; Clark, J.F.; Sansom, M.S. Structural basisfor the enhanced activity of cyclic antimicrobial peptides: the case of BPC194. *Biophys. J.*, 2008, *95*, 3802-3815.

Borst P and Ouellette M. New mechanism of drug resistance in parasitic protozoa.*Annu. Rev. Microbiol.* 1995; 49: 427-460.

Brener Z. Pathogenesis and immunopathology of chronic Chagas' disease. *Mem. Inst. Oswaldo Cruz.* 1987; 82: 205-212. Brener Z. Recent advances in the chemotherapy of Chagas disease. Mem. Inst.

Brown, K.L.; Hancock, R.E. Cationic host defense (antimicrobial) peptides. *Curr. Opin. Immunol*., 2006, *18*, 24-30.

Buckel P. Recombinant proteins for therapy. *Trends Pharmacol Sci*. 1996;17(12):450-6.

Búa J, Aslund L, Pereyra N, Garcia GA, Bontempi EJ, Ruiz AM. Characterisation of a cyclophilin isoform in *Trypanosoma cruzi*. *FEMS Microbiology Letters.* 2001; 200:43-47.

Cancado JR. Long term evaluation of etiological treatment of Chagas disease with benznidazole. *Revista do Instituto de Medicina Tropical de São Paulo.* 2002; 44 (1), 29-37.

Carballar-Lejarazu R, Rodriguez MH, de la Cruz HernandezHernandez F, et al.Recombinant scorpine: a multifunctional antimicrobial peptide with activity against different pathogens. *Cell Mol Life Sci* 2008; 65: 3081-92.

Castro JA, de Mecca MM, Bartel LC. Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis). *Hum Exp Toxicol*. 2006; 25(8):471-9.

CDC - Centers for Disease Control and Prevention [database on the Internet] 2013. American trypanosomiasis (also known as Chagas Disease). Available from, cdc.gov/parasites/chagas/.

Ceron, J. M., J. Contreras-Moreno, E. Puertollano, G. A. de Cienfuegos, M. A. Puertollano & M. A. de Pablo: The antimicrobial peptide cecropin A induces caspase-independent cell death in human promyelocytic leukemia cells. *Peptides,*2010, 31, 1494-503.

Coura JR and De Castro SL. A critical review on chagas disease chemotherapy. *Mem. Inst. Oswaldo Cruz*. Rio de Janeiro. 2002; 97 (1): 3-24.

Cruz-Chamorro, L., M. A. Puertollano, E. Puertollano, G. A. de Cienfuegos & M. A. de Pablo: *In vitro* biological activities of magainin alone or in combination with nisin. *Peptides,* 27, 1201-9 (2006)

Cruz J, Ortiz C, Guzmán F, Fernández-Lafuente R, Torres.R. Antimicrobial peptides: promising compounds against pathogenic microorganisms. *Curr Med Chem*. 2014;21(20):2299-321.

De Lucca AJ, Bland JM, Jacks TJ, Grimm C, Walsh TJ. Fungicidal and binding properties of the natural peptides cecropin B and dermaseptin. *Med Mycol.* 1998 Oct;36(5):291-8.

De Oliveira Junior NG, e Silva Cardoso MH, Franco OL. Snake venoms: attractive antimicrobial proteinaceous compounds for therapeutic purposes. *Cell Mol Life Sci.* 2013 Dec;70(24):4645-58.

Delgado M, Anderson P, Garcia-Salcedo JA, Caro M, GonzalezRey E. Neuropeptides kill African Trypanosomes by targeting intracellular compartments and inducing autophagic-like cell death. *Cell Death Differ* 2009; 16: 406-16.

Devera R, Fernandes O, Coura JR. Should *Trypanosoma cruzi* be called "cruzi" complex? a review of the parasite diversity and the potential of selecting population after in vitro culturing and mice infection. *Mem Inst Oswaldo Cruz.* 2003; Jan;98(1):112.

Dias JC, Amato Neto V. Prevention concerning the different alternative routes for transmission of *Trypanosoma cruzi* in Brazil. *Rev. Soc. Bras. Med. Trop*. 2011; 44 (Suppl. 2), 68–72.

Dias JCP, Neto VA, Luna EJA: Mecanismos alternativos de transmissão do *Trypanosoma cruzi* no Brasil e sugestões para sua prevenção. *Rev Soc Bras Med Trop* 2011, 44(3):375–379.

Drummelsmith J, Brochu V, Girard I, Messier N, Ouellete M. Proteome mapping of the protozoan parasite *Leishmania* and application to the study of drugs targets and resistance mechanisms. *Molecular & Cellular Proteomics*. 2003; 2 (3): 146-155.

Du Q, Hou X, Ge L, Li R, Zhou M, Wang H, Wang L, Wei M, Chen T, Shaw C. Cationicity-Enhanced Analogues of the Antimicrobial Peptides, AcrAP1 and AcrAP2, from the Venom of

the Scorpion, Androctonus crassicauda, Display Potent Growth Modulation Effects on Human Cancer Cell Lines. *Int J Biol Sci*. 2014 Sep 21;10(10):1097-107.

Dvorak JA, Hall TE, Crane MS, Engel JC, McDaniel JP, Uriegas R. *Trypanosoma cruzi* flow cytometric analysis. I. Analysis of total DNA/organism by means of mitramycin-induced fluorescence. *J. Protozool.* 1982; 24: 430-437.

El Fakhry Y, Ouellette M, Papadopoulou B. A proteomic approach to identify developmentally regulated proteins in *Leishmania infantum*. *Proteomics*. 2002 Aug;2(8):1007-17.

Elias MC, Vargas NS, Zingales B, Schenkman S. Organization of satellite DNA in the genome of *Trypanosoma cruzi*. Mol Biochem Parasitol. 2003 Jun;129(1):1-9.

El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, et al. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science*. 2005; Jul 15; 309(5733):409-15.

Essig A, Hofmann D, Münch D, Gayathri S, Künzler M, Kallio PT, Sahl HG, Wider G, Schneider T, Aebi M. Copsin, a novel peptide-based fungal antibiotic interfering with the peptidoglycan synthesis. *J Biol Chem*. 2014 Oct 23.

Falcao CB, de La Torre BG, Pérez-Peinado C, Barron AE, Andreu D, Rádis-Baptista G. Vipericidins: a novel family of cathelicidin-related peptides from the venom gland of South American pit vipers. *Amino Acids.* 2014 Nov;46(11):2561-71.

Filardi L, and Brener Z. Susceptibility and natural resistance of *Trypanosoma cruzi* strains to drugs used clinically in Chagas disease. *Trans R Soc Trop Med Hyg*. 1987; 81: 755-759.

Finley RW, Dvorak JA. *Trypanosoma cruzi:* analysis of the population dynamics of heterogeneous mixtures. *J Protozool*. 1987 Nov; 34(4): 409-15.

Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. Nature. 2002 Oct 3;419(6906):520-6.

Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat.Rev. Immunol.,* 2003, v.3, n.9, p. 720.

Gongora R, Acestor N, Quadroni M, Fasel N, Saravia NG, Walker J. Mapping the proteome of Leishmania Viannia parasites using two-dimensional polyacrylamide gel electrophoresis and associated technologies. *Biomedica*. 2003 Jun;23(2):153-60.

Hancock, R.E.; Patrzykat, A. Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics. Curr. *Drug Targets Infect. Disord.,* 2002, 2(1), 79-83.

Hancock RE, Scott, M G. The role of antimicrobial peptides in animaldefenses. Proc Natl Acad Sci U S A. 2000 Aug 1;97(16):8856-61.

Hauschka TS. Persistence of strains-specific behavior in two strains of *Trypanosoma cruzi* after prolonged transfer through imbred mice. *J. Parasitol* 1949;35:593-599.

Hayes BM, Bleackley MR, Wiltshire JL, Anderson MA, Traven A, van der Weerden NL. Identification and mechanism of action of the plant defensin NaD1 as a new member of the antifungal drug arsenal against Candida albicans. *Antimicrob Agents Chemother*. 2013 Aug;57(8):3667-75

Hsu CH, Chen C, Jou ML, Lee AY, Lin YC, Yu YP, Huang WT, Wu SH. Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. *Nucleic Acids Res*., 2005, *33*, 4053-4064.

Ministério da Saúde. *Instituto Nacional de Câncer -INCA*. Disponível em: <http://www.inca.gov.br>, Acesso em Setembro de 2014

Jang WS, Bajwa JS, Sun JN, Edgerton M. Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity inCandida albicans. *Mol Microbiol.* 2010, Ju:77(2):354-70 12.

Jin ZG, Melaragno MG, Liao DF, Yan C, Haendeler J, Suh YA et al. Cyclophilin A is a secreted growth factor induced by oxidative stress. *Circ Res* 2000; 87: 789–796

Kavanagh K, Dowd S. Histatins: antimicrobial peptides with therapeutic potential. J Pharm Pharmacol. 2004, 56:285–289.

Kirchhoff LV. Chagas disease: american trypanosomiasis*. Infect Dis Clin North Am* 1993, 7: 487-501.

Kościuczuk EM, Lisowski P, Jarczak J, Strzałkowska N, Jóźwik A, Horbańczuk J, Krzyżewski J, Zwierzchowski L, Bagnicka E. Cathelicidins: family of antimicrobial peptides. A review. *Mol Biol Rep.* 2012 Dec;39(12):10957-70.

Kragol G, Hoffmann R, Chattergoon MA, Lovas Cudic M, Bulet P, Condie BA, Rosengren KJ, Montaner LJ & Otvos L Jr (2002) Identification ofcrucial residues for the antibacterial activity of theproline-rich peptide, pyrrhocoricin. *Eur J Biochem* 4226–4237.

Kulkarni MM, McMaster WR, Kamysz W, McGwire BS. Antimicrobial peptide-induced apoptotic death of leishmania results from calcium-de pend ent, caspase-independent mitochondrial toxicity*. J Biol Chem.* 2009, 5;284(23):15496-504.

Kulkarni MM, Karafova A, Kamysz W, Schenkman S, Pelle R, McGwire BS. Secreted trypanosome cyclophilin inactivates lytic insect defense peptides and induces parasite calcineurin activation and infectivity. *J Biol Chem*. 2013 Mar 22;288(12):8772-84.

Kumari S, Roy S, Singh P, Singla-Pareek SL, Pareek A. Cyclophilins: proteins in search of function. *Plant Signal Behav*. 2013 Jan;8(1):e22734.

Lasonder E, Ishihama Y, Andersen JS, Vermunt AM, Pain A, Sauerwein RW, et al. Analysis of the *Plasmodium falciparum* proteome by high-accuracy mass spectrometry. *Nature*. 2002 Oct 3;419(6906):537-42.

Lee SP, Hwang YS, Kim YJ, Kwon KS, Kim HJ, Kim K, Chae HZ. Cyclophilin a binds to peroxiredoxins and activates its peroxidase activity. *J Biol Chem.* 2001; Aug 10;276(32):29826-32.

Lee, DG, Kim HK, Kim SA, Park Y, Park SC, Jang SH and Hahm KS.Fungicidal effect of indolicidin and its interaction with phospholipid membranes. *Biochemical and Biophysical Research Communications*, 2003, 305: 305-310.

Liendo A, Lazardi K, Urbina JA. In-vitro antiproliferative effects and mechanism of action of the bis-triazole D0870 and its S(-) enantiomer against *Trypanosoma cruzi. J Antimicrob Chemother*. 1998 Feb;41(2):197-205.

Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*. 1991 Aug 23;66(4):807-15.

Luque-Ortega JR, van't Hof W, Veerman EC, Saugar JM, Rivas LHuman antimicrobial peptide histatin 5 is a cell-penetrating peptidtargeting mitochondrial ATP synthesis in Leishmania. *FASEB J*, 2008; 22: 1817-28.

Lynn MA, Kindrachuk J, Marr AK et al.Effect of BMAP28 antimicrobial peptides on *Leishmania major* promastigote andamastigote growth: role of leishmanolysin inparasite survival. *PLoS Negl. Trop. Dis*. 5(5),e1141 (2011).

Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev.* 2003 Mar;55(1):27-55.

Machado CR, Augusto-Pinto L, McCulloch R, Teixeira SM. DNA metabolism and genetic diversity in Trypanosomes. *Mutat Res*. 2006; 612(1):40-57.

Maróti g, Kereszt A, Kondorosi e, Mergaert P (2011) natural roles of antimicrobial peptides in microbes, plants and animals. *Res Microbiol* 162:363–374

Martins-Melo FR, Ramos Jr AN, Alencar CH, Heukelbach J. Mortality due to Chagas disease in Brazil from 1979 to 2009: trends and regional differences. *J. Infect. Dev. Ctries*. 2012; 6, 817–824.

Matsuzaki, K.; Murase, O.; Fujii, N.; Miyajima, K. An antimicro-bial peptide, magainin 2, induced rapid flip-flop of phospholipidscoupled with pore formation and peptide translocation. *Biochemis-try*, 1996, *35*, 11361-11368.

Maya JD, Bollo S, Nuñez-Vergara LJ, Squella JA, Repetto Y, Morello A, Périé J, Chauvière G *Trypanosoma cruzi:* effect and mode of action of nitroimidazole and nitrofuran derivates. *Biochem. Pharmacol*. 2003; 65: 999-1006.

McAtee CP, Hoffman PS, Berg DE. Identification of differentially regulated proteins in metronidazole resistant *Helicobacter pylori* by proteome techniques. *Proteomics* 2001;1: 516 –521.

Mcdaniel JP and Dvorak JA. Identification, isolation and characterization of naturallyoccurring *Trypanosoma cruzi* variants. *Mol. Biochem. Parasitol*. 1993;57: 213-222.

McGwire BS, Olson CL, Tack BF, Engman DM. Killing of African trypanosomes by antimicrobial peptides. *J Infect Dis.* 2003 Jul 1;188(1):146-52.

Mejia AM, Hall BS, Taylor MC, Gómez-Palacio A, Wilkinson SR, Triana-Chávez O, Kelly JM. Benznidazole-resistance in *Trypanosoma cruzi* is a readily acquired trait that can arise independently in a single population. *J Infect Dis.* 2012 Jul 15;206(2):220-8.

Meiyalaghan S, Latimer JM, Kralicek AV, Shaw ML, Lewis JG, Conner AJ, Barrell PJ. Expression and purification of the antimicrobial peptide GSL1 in bacteria for raising antibodies. *BMC Res Notes*. 2014 Nov 4;7:777.

Miles MA, Lanham SM, de Souza AA, Povoa M. Further enzymic characters of Trypanosoma cruzi and their evaluation for strain identification. *Trans R Soc Trop Med Hyg*. 1980;74(2):221-37.

Miles MA, Povoa MM, de Souza AA, Lainson R, Shaw JJ, Ketteridge DS. Chagas's disease in the Amazon Basin: Ii. The distribution of *Trypanosoma cruzi* zymodemes 1 and 3 in Para State, north Brazil. *Trans R Soc Trop Med Hyg.* 1981;75(5):667-74.

Miles MA, Souza A, Povoa M, Shaw JJ, Lainson R, Toye PJ. Isozymic heterogeneity of *Trypanosoma cruzi* in the first autochthonous patients with Chagas' disease in Amazonian Brazil. *Nature.* 1978; Apr 27; 272(5656):819-21.

Miles MA, Toye PJ, Oswald SC, Godfrey DG. The identification by isoenzyme patterns of two distinct strain-groups of *Trypanosoma cruzi*, circulating independently in a rural area of Brazil. *Trans R Soc Trop Med Hyg.* 1977;71(3):217-25.

Midura-Nowaczek K, Markowska A. Antimicrobial peptides and their analogs: searching for new potential therapeutics. *Perspect Medicin Chem*. 2014 Oct 12;6:73-80.

Mok WW, Li Y. Therapeutic peptides: new arsenal against drug resistant pathogens. *Curr Pharm Des*. 2014, 20(5):771-92. Review.

Murta SM and Romanha AJ. *In vivo* selection of a population of *Trypanosoma cruzi* nd clones resistant to benznidazole. *Parasitology*. 1998. 116: 165-171.

Murta SM, dos Santos WG, Anacleto C, Nirde P, Moreira ES, Romanha AJ. Drug resistance in *Trypanosoma cruzi* is not associated with amplification or overexpression of P-glycoprotein (PGP) genes. *Molecular and Biochemical Parasitology*. 2001; 117 (2): 223-8.

Murta SM, Krieger MA, Montenegro LR, Campos, FF, Probst CM, Avila AR, Muto NH, De Oliveira RC, Nunes LR, Nirdé P, Bruna-Romero, O., Goldenberg, S., Romanha, A.J. Deletion of copies of the gene encoding old yellow enzyme (TcOYE), A NAD(P)H flavin oxidoreductase, associates with *in vitro*-induced benznidazole resistance in *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* 2006; 146, 151-62.

Murta SM, Nogueira FB, Dos Santos PF, Campos FM, Volpe C, Liarte DB, Nirdé P, Probst CM, Krieger MA, Goldenberg S, Romanha AJ. Differential gene expression in Trypanosoma cruzi populations susceptible and resistant to benznidazole. *Acta Trop*. 2008 Jul;107(1):59-65.

Narasimhan ML, Damsz B, Coca MA, Ibeas JI, Yun DJ, Pardo JM, Hasegawa PM, Bressan RA. A plant defense response effector induces microbial apoptosis. *Mol Cell.* 2001,8(4):921-30.

Nan, Y.H., Park, K.H., Park, Y., Jeon, Y.J., Kim, Y., Park, I.S., Hahm, K.S., and Shin, S.Y. Investigating the effects of positive charge and hydrophobicity on the cell selectivity, mechanism of action and anti-inflammatory activity of a Trp-rich antimicrobial peptide indolicidin. *FEMS Microbiol Lett* 292, 2009, 134-140

Neal RA, Van Bueren J. Comparative studies of drug susceptibility of five strains of *Trypanosoma cruzi in vivo* and *in vitro. Trans. R. Soc. Trop. Med. Hyg* 1988;82:709-714.

Nicolas, P. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. *FEBS J*. 2009, 276: 6483-6496.

Nilsen TW. Trans-splicing in protozoa and helminths. *Infect Agents Dis*. 1992; Aug;1(4):212-8.

Nigro P, Pompilio G, Capogrossi MC. Cyclophilin A: a key player for human disease. *Cell Death Dis*. 2013 Oct 31;4:e888.

Nirdé P, Larroque C, Barnabé C. Drug-resistant epimastigotes of *Trypanosoma cruzi* and persistence of this phenotype after differentiation into amastigotes. *Comptes Rendus de l'Académie des Sciences*. 1995; Série III 318:1239-1244

Nogueira FB, Krieger, MA, Nirdé P, Goldenberg S, Romanha, AJ, Murta SM Increased expression of iron-containing superoxide dismutase-a (TcFeSOD-A) Enzyme in *Trypanosoma cruzi* population with*in vitro*-induced resistance to benznidazole. *Acta Trop*. 2006; 100, 119-32.

Nogueira FB, Rodrigues JF, Correa MM, Ruiz JC, Romanha, AJ, Murta SM. The level of ascorbate peroxidase is enhanced in benznidazole-resistant populations of *Trypanosoma cruzi* and its expression is modulated by stress generated by hydrogen peroxide. *Mem. Inst. Oswaldo Cruz*. 2012; 107, 494-502.

Nogueira FB, Ruiz JC, Romanha AJ, Murta, SMF. Molecular characterization of cytosolic and mitochondrial tryparedoxin peroxidase in *Trypanosoma cruzi* populations susceptible and resistant to benznidazole. *Parasitol. Res.* 2009; 104, 835-844.

Olli S, Nagaraj R, Motukupally SR. A hybrid cationic peptide composed of human beta-defensin-1 and humanized theta-defensin sequences exhibits salt-resistant antimicrobial activity. *Antimicrob Agents Chemother*. 2014, 27.

Park, C.B.; Kim, H.S.; Kim, S.C. Mechanism of action of the antimicrobial peptide buforin ii: Buforin ii kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Res. Commun.* 1998, 244, 253–257.

Pedrosa RC, De Bem AF, Locatelli C, Pedrosa RC, Geremias R,Wilhelm Filho D. 2001. Time-dependent oxidative stress caused by benznidazole. *Redox Rep* 6:265–270.

Pedroso A, Cupolillo E, Zingales B. Evaluation of *Trypanosoma cruzi* hybrid stocks based on chromosomal size variation. *Mol Biochem Parasitol*. 2003 Jun;129(1):7990.

Pereira PCM, Navarro EC. Challenges and perspectives of Chagas disease: a review. *Journal of Venomous Animals and Toxins including Tropical Diseases* 2013; 19:34.

Phillips JB and Westerfield M. Zebrafish models in translational research: tipping the scales toward advancements in human health. *Dis Model Mech*. Jul 2014; 7(7): 739–743.

Pitchai D, Roy A, Ignatius C. *In vitro* evaluation of anticancer potentials of lupeol isolated from Elephantopus scaber L. on MCF-7 cell line. *J Adv Pharm Technol Res*. 2014 Oct;5(4):179-84.

Pontes AL & Andrade SG. Response to chemotherapy with benznidazole and nifurtimox in mice inoculated with strains of *Trypanosoma cruzi* isolated from previously treated animals. *XX Congresso da Soc. Bras. Med*. Trop. 1984; p. 25.

Potenza M, Galat A, Mining TA, Ruiz AM, Duran R, Tarleton RL, Marin M, Fichera LE, Búa J. Analysis of the Trypanosoma cruzi cyclophilin gene family and identification of cyclosporin a binding proteins. *Parasitol.* 2006; 132, 867-82.

Pukala, T. L.; Bowie, J. H.; Maselli, V. M.; Musgrave, I. F. e Tyler, M. J. Hostdefence Peptides from the glandular secretions of amphibians: Structure and activity. *Natural Product Reports* , 2006, v.23, n.3, p.368-393.

Rajão MA, Furtado C, Alves CL, Passos-Silva DG, de Moura MB, Schamber-Reis BL, Kunrath-Lima M, Zuma AA, Vieira-da-Rocha JP, Garcia JB, Mendes IC, Pena SD, Macedo AM, Franco GR, de Souza-Pinto NC, de Medeiros MH, Cruz AK, Motta MC, Teixeira SM, Machado CR. Unveiling benznidazole's mechanism of action through overexpression of DNA repair proteins in Trypanosoma cruzi. *Environ Mol Mutagen.* 2014 May;55(4):309-21

Ramos Jr AN, Martins-Melo FR, Barbosa JC, Heukelbach J. The role of operational research and the challenges for integration of care and control of Chagas disease in the Brazilian Unified Health System. *Rev. Soc. Bras. Med. Trop*. 2010; 43(Supl II):12-17.

Rassi A Jr, Rassi A, Marcondes de Rezende J. American trypanosomiasis (Chagas disease). *Infect Dis Clin North Am.* 2012; Jun;26(2):275-91.

Satoh K, Godo S, Saito H, Enkhjargal B, Shimokawa H. Dual roles of vascular-derived reactive oxygen species--with a special reference to hydrogen peroxide and cyclophilin A. *J Mol Cell Cardiol.* 2014 Aug;73:50-6.

Sanderson, J. M.Peptide–lipid interactions: insights and perspectives. *Organic & Biomolecular Chemistry*, 2005, v.3, n.2, p.201-212.

Scherl A, Francois P, Charbonnier Y, Deshusses JM, Koessler T, Huyghe A, et al. Exploring glycopeptide-resistance in *Staphylococcus aureus*: a combined proteomics and transcriptomics approach for the identification of resistance-related markers.BMC *Genomics*. 2006 Nov 22;7:296.

Schröder-Borm H, Bakalova R, Andrä J. The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine. *FEBS Lett.* 2005 Nov 7;579(27):6128-34..

Selsted, M.E., Novotny, M.J., Morris, W.L., Tang, Y.Q., Smith, W., and Cullor, J.S. (1992). Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J Biol Chem 267,4292-4295*

Shamova, O., Brogden, K.A., Zhao, C., Nguyen, T., Kokryakov, V.N., and Lehrer, R.I. (1999). Purification and properties of proline-rich antimicrobial peptides from sheep and goat leukocytes. *Infect Immun* 67, 4106-4111.

Shai Y. Mode of action of membrane active antimicrobial peptides. *Biopolymers.* 2002;66(4):236-48. Review

Shenvi S, Diwakar L, Reddy GC. Nitro Derivatives of Naturally Occurring β -Asarone and Their Anticancer Activity. *Int J Med Chem*. 2014;2014:835485.

Silveira JF. Biologia Molecular do *T. cruzi*. *In Trypanosoma cruzi e a doença de Chagas*. 2ª edição. Brener Z, Andrade ZA, Barral-Neto M. Rio de Janeiro. 2000; 127-147.

Solari A. The 3-dimensional fine structure of the mitotic spindle in *Trypanosoma cruzi. Chromosoma*. 1980; 78: 239-255.

Sørensen OE and Borregaard N. Cathelicidins – Nature's Attempt at Combinatorial Chemistry. *Combinatorial Chemistry & High Throughput Screening*, 2005, 8, 273-280

Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst*. 1973 Nov;51(5):1409-16. No abstract available.

Souto RP, Fernandes O, Macedo AM, Campbell D, Zingales B. DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi. Mol. Biochem. Parasitol* 1996;83:141152.

Suzuki J, Jin ZG, Meoli DF, Matoba T, Berk BC. Cyclophilin A is secreted by a vesicular pathway in vascular smooth muscle cells. *Circ Res* 2006; 98: 8

Tavares B e Lopes SS. A Importância do Peixe-Zebra na Investigação Biomédica. *Acta Med Port* 2013 Sep-Oct;26(5):583-592

Tanowitz HB, Kirchhoff LV, Simon D, Morris SA, Weiss LM, Wittner M. Chagas' disease. *Clin Microbiol Rev*. 1992; Oct; 5(4):400-19.

Thiel M and Bruchhaus I. Comparative proteome analysis of *Leishmania donovani* at different stages of transformation from promastigotes to amastigotes. *Med Microbiol Immunol* (Berl). 2001 Nov;190(1-2):33-6.

Tian Y, Wang H, Li B, Ke M, Wang J, Dou J, Zhou C.The cathelicidin-BF Lys16 mutant Cbf-K16 selectively inhibits non-small cell lung cancer proliferation *in vitro*. *Oncol Rep*. 2013 Nov;30(5):2502-10.

Tibayrenc M. Genetic epidemiology of parasitic protozoa and other infectious agents: the need for an integrated approach. *International Journal for Parasitology*. 1998; 28, 85-104.

Torrent, M., Pulido,D., Rivas,L. Andreu D. Antimicrobial peptide action on parasites. *Curr. Drug Targets*,2012, 13, 1138–1147.

Ullman B. Multidrug resistance and P-glycoproteins in parasitic protozoa. *Journal of bioenergetics and biomembranes*. 1995; (1), 77-84.

Urbina JA, Docampo R. Specific chemotherapy of Chagas disease: controversies and advances. *Trends Parasitol* 2003;19(11):495-501.

Van der Weerden, N.L.; Hancock, R.E.; Anderson, M.A. Permeabilization of fungal hyphae by the plant defensin nad1 occurs through a cell wall-dependent process. *J. Biol. Chem.* 2010, *285*, 37513–37520.

Vanhame L and Pays E. Control of gene expression in Trypanosomes. *Microbiological reviews.* 1995; 59: 223-240.

Villarreal D, Nirdé P, Hide M, Barnabé C, Tibayrenc M. Differential gene expression in benznidazole-resistant Trypanosoma cruzi parasites. *Antimicrob Agents Chemother*. 2005 Jul;49(7):2701-9.

Wagner W and So M. Genomic variation of *Trypanosoma cruzi:* involvement of multicopy genes. *Infect. Immun*. 1990; 58: 3217-3224.

Wang H, Lu C, Li Q, Xie J, Chen T, Tan Y, Wu C, Jiang J. The Role of Kif4A in Doxorubicin-Induced Apoptosis in Breast Cancer Cells. *Mol Cells.* 2014 Nov 5.

Wang Y, Zhang Z, Chen L, Guang H, Li Z, Yang H, Li J, You D, Yu H, Lai R. Cathelicidin-BF, a Snake Cathelicidin-Derived Antimicrobial Peptide, Could Be an Excellent Therapeutic Agent for Acne Vulgaris. *PLoS ONE*, 2011, 6(7): e22120.

Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, Wang A, Lin D, Lai R. Snake cathelicidin from *Bungarus fasciatus* is a potent peptide antibiotics. *PLoS ONE*, 2008, 16;3(9):e3217.

Wang H, Ke M, Tian Y, Wang J, Li B, Wang Y, Dou J, Zhou C (2013) BF-30 selectively inhibits melanoma cell proliferation via cytoplasmic membrane permeabilization and DNA-binding *in vitro* and in B16F10-bearing mice. *Eur J Pharmacol* 707:1–10

Wang P and Heitman J. The Cyclophilin. *Genome Biology,* 2005, 6:226.

WHO. World Health Organization. 2002.Disponível em http://www.who.gov.br.

Wilkinson SR, Taylor MC, Horn D, Kelly JM, Cheeseman I. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proc. Natl. Acad.* Sci. U.S.A. 2008; 105, 5022-5027.

World Health Organization, 2014. Chagas' disease (American trypanosomiasis) fact sheet (revised in March 2014).

Wu, M. e Hancock, R. E. W. (1999)."Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane". *Journal of Biological Chemistry*, v.274, n.1, p.29-35.

Zanh M, Berthold N, Kieslich B, Knappe D, Hoffmann R, Sträter N.Structural studies on the forward and reverse binding modes of peptides to the chaperone DnaK.*J Mol Biol.* 2013, 24;425(14):2463-79.

Zhao H, Gan TX, Liu XD, Jin Y, Lee WH, Shen JH, Zhang Y. Identification and characterization of novel reptile cathelicidins from elapid snakes. *Peptides,* 2008, 29:1685–1691

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. *Nature* 415, 389–395.

Zingales B, Andrade S G, Briones M R S, Campbell D A, Chiari E, Fernandes O, Guhl F, Lages-Silva E, Macedo A M, Machado C R, Miles M A, Romanha A J, Sturm N R, Tibayrenc M, Schijman AG. A new consensus for *Trypanosoma cruzi* intraspecific nomenclature:

second revision meeting recommends TcI to TcVI. *Memórias do Instituto Oswaldo Cruz*. 2009; 104: 10511054.

ANEXO A

Normas para publicação do periódico Experimental Parasitology

EXPERIMENTAL PARASITOLOGY

AUTHOR INFORMATION PACK

IMPACT FACTOR

2012: 2.154 © Thomson Reuters Journal Citation Reports 2013

GUIDE FOR AUTHORS

Your Paper Your Way

ypyw-gfa-banner.gifyour paper your way

INTRODUCTION

Experimental Parasitology emphasizes modern approaches to parasitology, including molecular biology and immunology. The journal features original research papers on the physiological, metabolic, immunologic, biochemical, nutritional, and chemotherapeutic aspects of parasites and host-parasite relationships.

Authors of studies on anti–microbial properties of natural substances are strongly encouraged to submit their work to other, more appropriate journals unless the studies have a strong focus on anti– parasitic activity and address the following points: the chemical characterization of the compounds likely implicated to a reasonable degree controls for the effects of vehicles and solvents if an animal model is not investigated or not available, in vitro activity studies that include at least comparative cytotoxicity data using host cells or tissues to show selective anti–parasitic activity at a level that could indicate a realistic prospect for translation some investigation into the potential mode of action or effects on likely molecular targets in parasites

BEFORE YOU BEGIN

Ethics in publishing

For information on Ethics in publishing and Ethical guidelines for journal publication see http://www.elsevier.com/publishingethics and http://www.elsevier.com/journal-authors/ethics.

Conflict of interest

All authors are requested to disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work. See also

Submission declaration

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis or as an electronic preprint, see

Changes to authorship

This policy concerns the addition, deletion, or rearrangement of author names in the authorship of accepted manuscripts:

Before the accepted manuscript is published in an online issue: Requests to add or remove an author, or to rearrange the author names, must be sent to the Journal Manager from the corresponding author of the accepted manuscript and must include: (a) the reason the name should be added or removed, or the author names rearranged and (b) written confirmation (e-mail, fax, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed. Requests that are not sent by the corresponding author will be forwarded by the Journal Manager to the corresponding author, who must follow the procedure as described above. Note that: (1) Journal Managers will inform the Journal Editors of any such requests and (2) publication of the accepted manuscript in an online issue is suspended until authorship has been agreed.

After the accepted manuscript is published in an online issue: Any requests to add, delete, or rearrange author names in an article published in an online issue will follow the same policies as noted above and result in a corrigendum.

Copyright

This journal offers authors a choice in publishing their research: Open Access and Subscription.

For Subscription articles

Upon acceptance of an article, authors will be asked to complete a 'Journal Publishing Agreement' (for more information on this and copyright, see http://www.elsevier.com/copyright). An e-mail will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult http://www.elsevier.com/permissions). If excerpts from other copyrighted works are

included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult http://www.elsevier.com/permissions.

For Open Access articles

Upon acceptance of an article, authors will be asked to complete an 'Exclusive License Agreement' (for more information see http://www.elsevier.com/OAauthoragreement). Permitted reuse of open access articles is determined by the author's choice of user license (see http://www.elsevier.com/openaccesslicenses).

Retained author rights

As an author you (or your employer or institution) retain certain rights. For more information on author rights for:

Subscription articles please see

http://www.elsevier.com/journal-authors/author-rights-and-responsibilities.

Open access articles please see http://www.elsevier.com/OAauthoragreement.

Role of the funding source

You are requested to identify who provided financial support for the conduct of the research and/or preparation of the article and to briefly describe the role of the sponsor(s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source(s) had no such involvement then this should be stated.

Funding body agreements and policies

Elsevier has established agreements and developed policies to allow authors whose articles appear in journals published by Elsevier, to comply with potential manuscript archiving

requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit http://www.elsevier.com/fundingbodies.

Open access

This journal offers authors a choice in publishing their research:

Open Access

• Articles are freely available to both subscribers and the wider public with permitted reuse

• An Open Access publication fee is payable by authors or their research funder

Subscription

• Articles are made available to subscribers as well as developing countries and patient groups through our access programs (http://www.elsevier.com/access)

• No Open Access publication fee

All articles published Open Access will be immediately and permanently free for everyone to read and download. Permitted reuse is defined by your choice of one of the following Creative Commons user licenses:

Creative Commons Attribution (CC BY): lets others distribute and copy the article, to create extracts, abstracts, and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text or data mine the article, even for commercial purposes, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, and do not modify the article in such a way as to damage the author's honor or reputation.

Creative Commons Attribution-NonCommercial-ShareAlike (CC BY-NC-SA): for non commercial purposes, lets others distribute and copy the article, to create extracts, abstracts and other revised versions, adaptations or derivative works of or from an article (such as a translation), to include in a collective work (such as an anthology), to text and data mine the article, as long as they credit the author(s), do not represent the author as endorsing their adaptation of the article, do not modify the article in such a way as to damage the author's honor or reputation, and license their new adaptations or creations under identical terms (CC BY-NC-SA).

Creative Commons Attribution-NonCommercial-NoDerivs (CC BY-NC-ND): for non commercial purposes, lets others distribute and copy The article, and to include in a collective work (such As an anthology), as long as they credit the author(s) and provided they do not alter or modify the article.

To provide Open Access, this journal has a publication fee which needs to be met by the authors or their research funders for each article published Open Access.
Your publication choice will have no effect on the peer review process or acceptance of submitted articles.

The publication fee for this journal is **$2200,** excluding taxes. Learn more about Elsevier's pricing policy: http://www.elsevier.com/openaccesspricing.

Language (usage and editing services)

Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop (http://webshop.elsevier.com/languageediting/) or visit our customer support site (http://support.elsevier.com) for more information.

Submission

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts source files to a single PDF file of the article, which is used in the peer-review process. Please note that even though manuscript source files are converted to PDF files at submission for the review process, these source files are needed for further processing after acceptance. All correspondence, including notification of the Editor's decision and requests for revision, takes place by e-mail removing the need for a paper trail.

PREPARATION

NEW SUBMISSIONS

Submission to this journal proceeds totally online and you will be guided stepwise through the creation and uploading of your files. The system automatically converts your files to a single PDF file, which is used in the peer-review process.
As part of the Your Paper Your Way service, you may choose to submit your manuscript as a single file to be used in the refereeing process. This can be a PDF file or a Word document, in any format or layout that can be used by referees to evaluate your manuscript.
It should contain high enough quality Figures for refereeing. If you prefer to do so, you may still provide All or some of the source files at the initial submission. Please note that individual figure files larger than 10 MB must be uploaded separately.

References

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s),

journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct.

Formatting requirements

There are no strict formatting requirements but all manuscripts must contain the essential elements needed to convey your manuscript, for example Abstract, Keywords, Introduction, Materials and Methods, Results, Conclusions, Artwork and Tables with Captions. If your article includes any Videos and/or other Supplementary material, this should be included in your initial submission for peer review purposes.

Divide the article into clearly defined sections. *Figures and tables embedded in text* Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file.

REVISED SUBMISSIONS

Use of word processing software Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier: http://www.elsevier.com/guidepublication). See also the section on Electronic artwork.

To avoid unnecessary errors you are strongly advised to use the 'spell-check' and 'grammar-check' functions of your word processor.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Experimental

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Theory/calculation

A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Vitae - no photos

Include in the manuscript a short (maximum 100 words) biography of each author, along with a passport-type photograph accompanying the other figures.

Essential title page information

• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.

• **Author names and affiliation.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.

• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that phone numbers (with country and área code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.**

• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.

Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Highlights

Highlights are mandatory for this journal. They consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See http://www.elsevier.com/highlights for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Database linking

Elsevier encourages authors to connect articles with external databases, giving their readers one click access to relevant databases that help to build a better understanding
Of the described research. Please refer to relevant database identifiers using the Following format in your article: (e.g., TAIR: AT1G01020; CCDC: 734053; PDB: 1XFN)
Database: xxxx http:// www.elsevier.com/databaselinking for more information and a full list of supported databases.

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

Artwork

Image manipulation

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly.

For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files. A detailed guide on electronic artwork is available on our website: http://www.elsevier.com/artworkinstructions.

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.

TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
• Supply files that are too low in resolution.
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version.

Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Illustration services

Elsevier's WebShop (http://webshop.elsevier.com/illustrationservices) offers Illustration Services to authors preparing to submit a manuscript but concerned about the quality of the images accompanying their article. Elsevier's expert illustrators can produce cientific, technical and medicalstyle images, as well as a full range of charts, tables and graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Figure captions

Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Reference links

Increased discoverability of research and high quality peer review are ensured by online links to the sources cited. In order to allow us to create links to abstracting and indexing services, such as Scopus, CrossRef and PubMed, please ensure that data provided in the references are correct. Please note that incorrect surnames, journal/book titles, publication year and pagination may prevent link creation. When copying references, please be careful as they may already contain errors. Use of the DOI is encouraged.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference formatting

There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style

Text: All citations in the text should refer to:

1. *Single author*: the author's name (without initials, unless there is ambiguity) and the year of publication;
2. *Two authors:* both authors' names and the year of publication;
3. *Three or more authors:* first author's name followed by 'et al.' and the year of publication.

Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.

Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al.

(2010) have recently shown ....'

List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2010. The art of writing a scientific article. J. Sci. Commun. 163, 51–59.

Reference to a book:

Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.

Reference to a chapter in an edited book:

Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S.,

Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.

Journal abbreviations source

Journal names should be abbreviated according to the List of Title Word Abbreviations: http://www.issn.org/services/online-services/access-to-the-ltwa/.

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation

files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect:

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article.

AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high resolution images, background datasets, sound clips and more. Supplementary files Supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

ANEXO B

Normas para publicação do periódico Bioconjugate Chemistry

ACS Publications
Most Trusted. Most Cited. Most Read.

Bioconjugate Chemistry

Bioconjugate Chemistry Guidelines for Authors

(Revised May 2014)

Contents (Click on the topic)

Bioconjugate Chemistry (http://pubs.acs.org/bc) invites original contributions on all research at the interface between man-made and biological materials. The mission of the journal is to communicate advances in fields including drug delivery, bionanotechnology, and synthetic biology. Bioconjugate Chemistry is intended to provide a forum for presentation of research relevant to all aspects of bioconjugates, including the preparation, properties, and applications of molecular conjugates. It is the expectation of the journal that chemical, structural, and biological tools be rigorously applied.

General Considerations

General information on the preparation of manuscripts for ACS journals may be found in The ACS Style Guide, 3rd ed. (2006), available from the Oxford University Press, Order Department, 201 Evans Road, Cary, NC 27513. Further insight on manuscript preparation can be obtained through the resources available through ACS Publications, Manuscript Submission. The responsibility for all aspects of manuscript preparation rests with the authors. The editors will not undertake extensive changes or rewriting of the manuscript.

Manuscript Classes

Pioneering reports of sufficient contemporary importance and general interest to justify accelerated publication should be submitted as Communications. Communications should be brief, on the order of 2000 words or the equivalent. Comprehensive accounts of significant studies should be submitted as Articles. The majority of publications are in this category.

Bioconjugate Chemistry features two classes of review article. These reviews will typically be invited, though proposals for topics will be considered. Topical Reviews are intended to introduce the readership to new topics and challenges through the personal viewpoint of the authors. These articles can describe new strategies in bioconjugation, or describe the role of synthetic/biological constructs in topics such as immunology, cancer therapeutics, and tissue engineering. Topical Reviews provide concise (~2000□3000 word) overviews of both fundamental topics and translational issues, with a target audience of advanced graduate students, post-docs, and faculty looking to extend their research in new directions. Reviews provide a forum for more in-depth coverage of an area, and likewise should be accessible to the broad audience of *Bioconjugate Chemistry*. These reviews will typically be 4000□6000 words in length. The use of effective figures is strongly encouraged for both Topical Reviews and Reviews.

Peer Review
All submissions deemed appropriate by the Editors for the journal are subject to peer review. The author may suggest the name of the appropriate associate editor as well as the names and associations of persons competent to review the manuscript. Authors may also suggest nonpreferred reviewers, with a brief explanation.

Manuscript Submission
Manuscripts must be submitted via the ACS Paragon Plus Environment (http://paragonplus.acs.org/login). Complete instructions and an overview of the electronic online (Web) submission process are available through the secure ACS Paragon Plus Web site. The Web submission site employs state-of-the-art security mechanisms to ensure that all electronically submitted papers are secure. These same security mechanisms are also active throughout the peer-review process, permitting access only to editors and reviewers who are assigned to a particular manuscript. Authors must also submit all *revisions* of manuscripts via the ACS Paragon Plus Environment. Authors must submit the manuscript as a single word-processing file, including text, tables, and graphics in the same file. Complete instructions, including information on which platforms and software packages are supported, are available on the Web submission site. Authors may provide their own manuscript PDF file along with the manuscript word-processing file, or provide the word-processing file only and allow ACS to do the PDF conversion. Supporting Information (optional) should be submitted electronically in a separate file. Any closely related manuscripts that are under consideration for publication or already in press elsewhere must be made available for the Editor and reviewers and should be submitted as Supporting Information for Review Only. The date received will be recorded as the date the complete set of properly formatted documents described above is received by the Editor's office. Manuscripts that are ready to be reviewed when first submitted will receive priority for initial processing and assignment to an Associate Editor.

Electronic Cover Letter
A cover letter must accompany the manuscript. The cover letter may be created as a separate file or may be typed into the appropriate text box in the ACS Paragon Plus Environment. It should provide the postal address, telephone number, fax number, and e-mail address of the corresponding author of the manuscript. All editorial correspondence concerning receipt, status, review, revision, and publication of a manuscript will be directed to the corresponding author, who is responsible for communicating the manuscript status to all coauthors of the paper and for obtaining the coauthors' assent to any substantial changes of content or interpretation made
during revision.

Journal Publishing Agreement
A properly completed and signed Journal Publishing Agreement must be submitted for each manuscript. ACS Paragon Plus provides an electronic version of the Agreement that will be

available on the **My Authoring Activity** tab of the corresponding author's home page once the manuscript has been assigned to an editor. A PDF version of the Agreement is also available, but **authors are strongly encouraged to use the electronic Journal Publishing Agreement.** If the PDF version is used, all pages of the signed PDF Agreement must be submitted. If for any reason the corresponding author cannot execute the Journal Publishing Agreement, then another author should complete and sign the PDF version of the form. Forms and complete instructions are available at http://pubs.acs.org/page/copyright/journals/index.html

.

Conflict of Interest Disclosure

A statement describing any conflicts of interest or lack thereof is published with each manuscript. During the submission process, the corresponding author must provide this statement on behalf of all authors of the manuscript. The statement should describe all potential sources of bias, including affiliations, funding sources, and financial or management relationships, that may constitute conflicts of interest (please see the ACS Ethical Guidelines).

The statement will be published in the final article. If no conflict of interest is declared, the following statement will be published in the article: “The authors declare no competing financial interest.”

Professional Ethics

In publishing only original research, ACS is committed to deterring plagiarism, including selfplagiarism. ACS Publications uses CrossCheck's iThenticate software to screen submitted manuscripts for similarity to published material. Note that your manuscript may be screened during the submission process. Further information about plagiarism can be found in Part B of the Ethical Guidelines to Publication of Chemical Research

.

Professional Ethics

In publishing only original research, ACS is committed to deterring plagiarism, including selfplagiarism.

ACS Publications Uses CrossCheck's iThenticate software to screen submitted manuscripts for similarity To published material. Note that your manuscript may be screened during The submission process. Further information about plagiarism Can be found in Part B of the Ethical Guidelines to Publication Of Chemical Research

.

Author List

During manuscript submission, the submitting author must provide contact information (full name, email address, institutional affiliation and mailing address) for all of the co-authors. Because all of the author names are automatically imported into the electronic Journal Publishing Agreement, *the names must be entered into ACS Paragon Plus in the same sequence as they appear on the first page of the manuscript*. (Note that co-authors are not required to register in ACS Paragon Plus.) The author who submits the manuscript for publication accepts the responsibility of notifying all co-authors that the manuscript is being submitted. Deletion of an author after the manuscript has been submitted requires a confirming letter to the Editor-in-Chief from the author whose name is being deleted. For more information on ethical responsibilities of authors, see the Ethical Guidelines to Publication of Chemical Research

.

Manuscript Preparation

The manuscript should be submitted as a single file. Authors are strongly encouraged to incorporate their figures and tables into the text as opposed to placing them at the end of the manuscript. *Figure captions should be placed under figures*; scheme titles and footnotes should be placed with schemes, etc. It is best to use the fonts “Times” and “Symbol”. Other

fonts, particularly those that do not come bundled with the system software, may not translate properly.
Ensure that all special characters (e.g., Greek characters, math symbols, etc.) are present in the body of the text as characters and not as graphic representations. Tables may be created using a word processor's text mode or table format feature. The table format feature is preferred. If the text mode is used, please separate columns with a single tab and use a return (line feed) at the end of each row.

Word Processing Packages
Information on acceptable word processing software is available from the "Submission & Review" tab of the Author & Reviewer Resource Center at http://pubs.acs.org/4authors. This website also contains links to a list of Language Editing Services, a list of free software for viewing Supporting Information, and more information on services and policies of the ACS.

Assistance with English Language Editing
Authors may want to have their manuscripts edited professionally before submission to improve clarity. The ACS ChemWorx English Editing Service can assist you in improving and polishing the language in your manuscript. You can learn more about the services offered, at http://es.acschemworx.acs.org.

Manuscript Organization
Articles should be assembled in the order Title (including full title, byline, and associated footnotes), Abstract, Introduction, Results and Discussion, Experimental Procedures (Materials and Methods), Acknowledgments, Supporting Information description, Abbreviations, References and Footnotes. For Communications, the need for brevity precludes subdivisions within the text for introduction, experimental procedures, results, and discussion; experimental procedures should be placed in Supporting Information, incorporated into the text or figure captions, or included as footnotes in Communications. Authors should also submit a small structural diagram or other informative illustration that represents or summarizes the topic for use in the Table of Contents (TOC) and abstract.

Title
A brief, accurate, and informative title will aid in the classification and indexing of the paper. Do not use trade names of drugs or abbreviations. List full names and institutional affiliations of all authors, and if differentiation is necessary, indicate the affiliations of the authors by the superscript symbols †, ‡, §, etc. The author to whom correspondence should be addressed is indicated by an asterisk. The corresponding author's address should include a street address, or post office box number if no street address is available, and an e-mail address. The
corresponding author's current telephone and fax numbers should also be included. It is implicit in listing a person as an author that this individual has agreed to appear as an author of the manuscript.

Abstract
The abstract should briefly present the problem and experimental approach and state the major findings and conclusions. It should be self-explanatory and suitable for reproduction without rewriting. *Footnotes or undefined abbreviations may not be used in the abstract.* Generally, the abstract will be formatted as one paragraph without any subheads or other divisions.

Introduction
The introduction should state the purpose of the investigation and its relation to other work in the field. Background material should be brief and relevant to the research described. Lengthy reviews of the literature should be avoided.

Results and Discussion
Normal formatting of the manuscript will have a combined Results and Discussion section, however authors may provide these as separate sections. The results should be presented concisely. In the interest of economy of space in the article, it is often desirable to place

supplementary data (also subject to review) in a separate document for publication as Supporting Information on the Web. Recent issues of the journal should be consulted for current practices.

Tables and figures should be designed to make it easy for the reader to understand the experimental data. The same data should not be presented in more than one figure or in both a figure and a table in the manuscript. In some cases it is appropriate to validate the significance of processed data (e.g., a graph), by including the underlying raw data (e.g., a gel or biodistribution) as Supporting Information.

Experimental Procedures

The experimental procedures should be described in sufficient detail to enable others to repeat the experiments. Names of products and manufacturers should be included only if they are not widely known or if alternate ources are deemed unsatisfactory. Novel experimental procedures should be described in detail. Care should be taken to describe any differences between published methods and methods actually used. Published procedures should be referred to by literature citation of both the original and any published modifications, unless the information is so fragmented that the reader would benefit from a unified presentation. Precautions for handling dangerous materials or for performing hazardous procedures must be stated or referenced.

Characterization of New Compounds and Bioconjugates. ACS journals require a high standard of chemical characterization, to confirm the identity and purity of the compounds under study.

Papers that explore conjugation conditions for multifunctional molecules, e.g., amino groups on proteins, should include analytical studies to identify the *sequence positions* of the residues affected. For medium-sized or larger peptides, evidence for homogeneity by separation methods of adequate resolving power and by amino acid analysis is normally required. For small organic molecules, the conventional practices of organic chemistry apply, including nuclear magnetic resonance data (for recommended practices see NMR guidelines available in the Author & Reviewer Resource Center (http://pubs.acs.org/page/4authors/tools/index.html) and elemental analyses and/or high resolution mass spectrometry of all new small molecules if practical. The numerical results for all elemental analyses should be included in Supporting Information.

Biological Data. Manuscripts generally will contain biological data such as images of cells or animal models, or other appropriate functional validations of the properties of new bioconjugates. Biological test methods must be referenced or described in sufficient detail to permit the experiments to be repeated by others. Statistical limits (statistical significance) are required for all biological data. Doses and concentrations should be expressed as molar quantities (e.g., mol/kg, nM) whenever possible.

Acknowledgments

This section should acknowledge financial support, technical assistance, advice from colleagues, gifts, etc. Sources of funding of the research should be stated. Permission should be sought from persons whose contribution to the work is acknowledged in the manuscript.

*Funding Sources***.** During manuscript submission, the submitting author is asked to select funding sources from the list of agencies included in the FundRef Registry http://www.crossref.org/fundref/

.

Web Enhanced Objects

The Journal Editors encourage the submission of Web enhanced objects (WEOs) that complement a reader's understanding of the research being reported. These types of files include color figures (including 3D rotatable figures), animations, spectra, video, and sound. Links to WEOs will appear in the Web HTML edition of the paper. Files suitable for this form

of publication should be viewable with commonly available Internet plug-ins or helper applications.
More information on WEOs is available on the Paragon Plus Web site at ttp://pubs.acs.org/page/4authors/ submission /weo.html
.
Authors should submit WEOs via the Paragon Plus Web site as part of their submissions.Descriptions of WEOs should be noted in the appropriate places within the graphic caption or text of the paper, noting the type of file and format. Example: “A 3D rotatable image in xyz format is available.”

Supporting Information
Material that is not needed for reading the paper but is needed to document the experiments or methodology should be put into a Supporting Information file. Such Supporting Information is published on the Web as a separate document. Authors are encouraged to use this resource in the interest of shorter articles, which not only save journal space but also result in clearer and more readable presentations. However, “dumping” of materials to this section is to be discouraged, and the editors reserve the right to make downward adjustments in the amounts of such material presented.
A statement of the availability of Supporting Information should be placed after the Acknowledgments, using the following format: “ Supporting Information Available: [Give description of material.] This material is available free of charge via the Internet at http://pubs.acs.org.”
Supporting Information must be submitted at the same time as the manuscript and uploaded as a separate file to the ACS Paragon Plus Environment. A list of acceptable file types is available on the Web. All Supporting Information files of the same type should be prepared as a single file (rather than submitting a series of files containing individual images or structures). In most cases, all Supporting Information will be contained in one PDF file.
Files for Supporting Information should be separately identified with authors’ names and manuscript title and should begin with a list of contents. The pages should be numbered consecutively. Figure legends, titles to tables, and other identifying captions should appear with the figure or table. Figure and table numbering should not duplicate numbering of figures/tables in the main article; the convention for numbering in Supporting Information should be “Figure S1”, “Table S1”, etc. Type size or letter size should be large enough for easy reading. As a rule, if one has difficulty reading the material as submitted, it is not acceptable. *The ACS Style Guide* has more information.

Figures and tables that are to be published in the article do not belong in the Supporting Information.
Abbreviations
Standard abbreviations should be used throughout the manuscript. Note that abbreviations are used in ACS journals without periods. The preferred forms for some of the more commonly used abbreviations are mp, bp, °C, K, kDa (for kilodalton), min, h, mL, µL, g, mg, µg, cm, mm, µm, nm, mol, mmol, µmol, nmol, ppm, HPLC, TLC, GC, NMR, LC-MS, and UV.
For lengthy technical names used *more than five times* in the manuscript, it is appropriate to employ abbreviations. Such nonstandard abbreviations should be collected and defined in a single Abbreviations paragraph at the end of the text. Excessive use of abbreviations makes a manuscript difficult to read; failure to collect the definitions of all abbreviations into a single paragraph is a disservice to the reader.
References and Footnotes
All the references and footnotes must be placed together in a list at the end of the manuscript text. In the Web edition, many of them will have links to other Web resources, such as the corresponding abstracts in *Chemical Abstracts* and the full text on publisher Web sites. Because of this electronic linking, and to aid scientific research, *it is crucial that authors verify the accuracy of all reference citations and footnotes.*

Unnecessarily long lists of references should be avoided, and excessive self-citation is strongly discouraged. However, authors *must* reference all previous publications in which portions of the present work have appeared. Literature references and explanatory footnotes must be numbered with Arabic numerals in the order of their first citation in the text and the corresponding numbers placed at the appropriate locations in the text as superscripted numerals without parentheses or bracket. For periodicals, follow the format shown: Fritzberg, A. R., Abrams, P. G., Beaumier, P. L., Kasina, S., Morgan, A. C., Reno, J. M., Sanderson, J. A., Srinivasan, A., Wilbur, D. S., and Vanderheyden, J. (1988) Specific and stable labeling of antibodies with technetium-99m with a diamide dithiolate chelating agent. *Proc. Natl. Acad. Sci. U.S.A. 85*, 4025–4029. Inclusive pagination is preferred. Titles of journals are abbreviated according to *Chemical Abstracts Service Source Index*. Serial publications such as *Methods in Enzymology* and *C.R.C. Critical*
Reviews should be listed in the same form as journals. For journal articles accessed online prior to print publication, please provide the DOI number. For books, follow the format shown: Aida, T., and Jiang, D.-L. (2000) Dendrimer porphyrins and metalloporphyrins: syntheses, structures and functions. *The Porphyrin Handbook. Volume 3: Inorganic, Organometallic and Coordination* (Kadish, K. M., Smith, K. M., and Guilard, R., Eds.) pp 369–384, Chapter 23, Academic Press, New York. Submitted manuscripts should be designated as “in press” only if formally accepted for publication; otherwise “unpublished results” should be used.
Use of URLs as references: As a publisher, ACS must be able to archive the data essential to a published article. Where such archiving is not possible, deposition of data in public databases, such as GenBank, ArrayExpress, RCSB Protein Data Bank, Unidata, etc., is acceptable. Only links to web sites that are permanent public repositories, such as self-perpetuating online resources funded by government, academia, and industry, are permitted. Links to an author’s personal web page are not acceptable.

Tables
Tabulation of experimental results is encouraged when this leads to more effective presentation. Tables should be numbered consecutively with Arabic numerals. Provide a brief title with each table and a brief heading for each column. Clearly indicate the units of measure (preferably SI).Data should be rounded to the nearest significant figure. Footnotes in tables should be givenlowercase letter designations and cited in the tables as italicized superscripts . Explanatory material referring to the whole table is to be included as a footnote to the title. All tables must be cited in the text.

Schemes and Charts
Chemical syntheses and some processes may be represented as schemes. These often do not have captions or legends. Charts are used to illustrate chemical structures rather than syntheses. All schemes and charts must be cited in the text.

Figure Legends (captions)
These should be brief but informative. The reader should not have to search through the text in order to understand the basic aspects of the figure being described. *Each figure legend should appear below its figure in the submitted manuscript.* All figures must be cited in the text.

Figures
Figures cannot be modified or enhanced by the journal production staff. Graphics should have
the following minimum resolution requirements:
Black and white line art 1200 dpi
Grayscale art 600 dpi
Color art (RGB color mode) 300 dpi

Most graphic programs provide an option for changing the resolution when you are saving the image. Best practice is to save the graphic file at the final resolution and size using the program used to create the graphic.

Only graphics to be printed in color should be submitted in color (see *Color* section below for details).

Illustrations must fit a one- or two-column format on the journal page. For efficient use of journal space, single column illustrations are preferred.

	single (preferred)	**double**
minimum width		300 pts (10.58 cm; 4.167 in.)
maximum width	240 pts (8.45 cm; 3.33 in.)	504 pts (17.78 cm; 7 in.)
maximum depth (including caption – allow 12 pts for each line of text)	660 pts (23.28 cm; 9.167 in.)	660 pts (23.28 cm; 9.167 in.)

For best results, submit illustrations in the *actual size* at which they should appear in the journal.

Original illustrations that do not need to be reduced to fit a single or double column will yield the best quality. Lettering should be no smaller than 4.5 points. (Helvetica or Arial fonts work well for lettering.) Lines should be no thinner than 0.5 point. Lettering and lines should be of uniform density; consistent sizing of lettering and labels will ensure a consistent presentation for publication. Avoid using complex textures and shading to achieve a three-dimensional effect.

Color. The editors encourage the use of color to enhance the clarity of images, molecular graphics, complex structures, spectra, schemes, figures, etc. Appropriate color reproduction Will be provided at no cost to the author.

Submit color figures electronically embedded as graphic files in the Word document. It may help to print the manuscript on a laser printer to ensure all artwork is clear and legible. Acceptable source file formats are TIFF, PDF, EPS (vector artwork), or CDX (ChemDraw file). Labeling of all figure parts should be present and the parts should be assembled into a single graphic. The color settings should be RGB (preferred) or CMYK. RGB is commonly used when creating online graphics; all graphics that are submitted in RGB color mode will retain the RGB color in all web products, and then be converted to CMYK color mode for the print version only (some color shift may occur).

Chemical Structures. Structures should be produced with the use of a drawing program such as ChemDraw. Compounds should be numbered in bold Arabic numerals. Structure drawing preferences (preset in the ACS Stylesheet “ACS Document 1996” in ChemDraw) are as follows.

(1) As drawing settings select:

chain angle	120º
bond spacing	18% of width
fixed length	14.4 pt (0.508 cm, 0.2 in.)
bold width	2.0 pt (0.071 cm, 0.0278 in.)
line width	0.6 pt (0.021 cm, 0.0084 in.)
margin width	1.6 pt (0.056 cm, 0.0222 in.)
hash spacing	2.5 pt (0.088 cm, 0.0347 in.)

(2) As text settings select:

font	Arial/Helvetica
size	10 pt

(3) Under the preferences choose:

units	points
tolerances	5 pixels

(4) Under page setup choose:

Paper	US Letter
Scale	100%

Authors using other drawing packages should, if possible, modify their program's parameters so that they reflect the above guidelines.

Table of Contents Graphic. Authors should also submit a small structural diagram or other informative illustration for use in the Table of Contents (TOC) and abstract. This small graphic element, in conjunction with the title, should capture the reader's attention and give a quick impression of the importance of the paper. For best presentation, this graphic should be no wider than 3.5 inches (9.0 cm) and no taller than 2 inches (5.1 cm), and it usually contains color.

Nomenclature

The complexity of typical macromolecular bioconjugates has so far defeated efforts to systematize their nomenclature. It is customary to name products by referring to starting materials; thus, antibody–drug conjugates are named according to the individual molecules involved. It is important that complicated or unwieldy names be avoided. Informative schemes, charts, or figures depicting the subject molecules, which can be referred to by number, are essential.

For the individual molecules assembled to form bioconjugates, it is the responsibility of the authors to provide correct nomenclature. All nomenclature must be consistent and unambiguous, and should conform to current American usage. Insofar as possible, authors should use systematic names such as those used by Chemical Abstracts Service, the International Union of Pure and Applied Chemistry (http://www.chem.qmul.ac.uk/iupac/), and the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/). *Chemical Abstracts* nomenclature rules are described in Appendix IV of the *Chemical Abstracts Index Guide*. A name generation service is available for a fee through CAS Client Services, 2540 Olentangy River Road, P.O. Box 3343, Columbus, OH 43210-0334; phone: (614) 447-3870; fax:(614) 447-3747; e-mail: answers@cas.org.

Structural Data

Atomic coordinates for structures of proteins determined by X-ray and all macromolecules

determined by NMR should be deposited with the Protein Data Bank (PDB; http://www.rcsb.org/pdb/). It is the responsibility of the author to obtain a File Name for the macromolecule; the File Name must appear in a footnote on the title page. A manuscript will be accepted only after receipt from the corresponding author of a written statement that the coordinates have been deposited. The PDB File Name must be provided before the paper can be published, and coordinates must be released immediately upon publication. Please address all inquiries about deposition to the Protein Data Bank. Crystal structures of nucleic acids should be deposited with the Nucleic Acid Database (NDB; http://ndbserver.rutgers.edu/).

Revised Manuscripts

Each revised manuscript should be submitted in final form, without edit marks, highlighting, or colored text, so that it may be transmitted directly for processing if appropriate. Careful attention should be paid to reference format and accuracy. In addition, a marked-up file showing all changes should be submitted as supporting information not for publication. Following this instruction will avoid delays in processing.

Proofs

The corresponding author of an accepted manuscript will receive e-mail notification and complete instructions when page proofs are available for review via a secure Web site. Authors will access the secure site through ACS ChemWorx and will need an ACS ID. To obtain an ACS ID or to reset your password, go to www.acschemworx.org

.

Routine rephrasing of sentences or additions are not permitted at the page proof stage. Alterations should be restricted to serious changes in interpretation or corrections of data. Extensive or important changes on page proofs, including changes to the title or list of authors, are subject to editorial review. It is the responsibility of the corresponding author to ensure that all authors listed on the manuscript agree with the changes made on the proofs. Proofs should be returned within 48 h of receipt in order to ensure timely publication of the manuscript. All changes should be incorporated in a single response and be submitted by the corresponding author.

***Just Accepted* Manuscripts**

Just Accepted manuscripts are peer-reviewed, accepted manuscripts that are published on the ACS Publications website prior to technical editing, formatting for publication, and author proofing—usually within 30 minutes to 24 hours of acceptance by the editorial office. During the manuscript submission process, authors can choose to have their manuscript published online as a *Just Accepted* manuscript. To ensure rapid delivery of the accepted manuscript to the Web, authors must adhere carefully to all requirements in the journal's Guidelines for Authors. For further information, please refer to the *Just Accepted* FAQ, at http://services.acs.org/pubshelp/passthru .cgi?action=kb&item=244 . Note that publishing a manuscript as *Just Accepted* is not a means by which to comply with the NIH Public Access Mandate. *Patent Activities and Intellectual Property Issues.* Authors are responsible for ensuring that all patent activities and intellectual property matters are satisfactorily resolved prior to first publication. Acceptance and publication will not be delayed for pending or unresolved issues of this nature.

Official Date of Publication. The date that the accepted paper **first** appears on the Web (Just Accepted, ASAP, or in issue)
is the **official date of publication** and appears on both the HTML
and PDF versions of a paper.

ASAP Publication

Accepted manuscripts, edited and formatted for publication, will be published on the "Articles ASAP" page on the Journal Web site as soon as page proofs are corrected and all author concerns are resolved. Publication on the Web usually occurs within 4 working days of

receipt of page proof corrections, and this can be anywhere from 2 to 7 weeks in advance of the cover date of the issue.

E-prints and Reprints

Under the ACS Articles on Request policy, the Society will provide (free of charge) to all contributing authors a unique URL within the ACS Web site that they may e-mail to colleagues or post on external Web sites. These author-directed links are designed to facilitate distribution of an author's published work to interested colleagues in lieu of direct distribution of the PDF file by the author. The ACS Articles on Request policy allows 50 downloads within the first year after web publication and unlimited access via the same author-directed links 12 months after web publication.

Author Choice

The ACS AuthorChoice option establishes a fee-based mechanism for authors or their research funding agencies to sponsor the open availability of their articles on the Web at the time of online publication. Effective January 1, 2014, ACS AuthorChoice will be expanded to offer authors a wider range of open access license options, such as Creative Commons licenses and provisions for immediate or 12-month embargoed open access, both coupled with a new ACS Certified Deposit service. For complete details see http://acsopenaccess.org/

.

Corrections

If errors of consequence are found in the published paper, the author should use Paragon Plus to submit corrections to the Editor for publication in the "Additions and Corrections" section. **Policy on Prior Publication**

Bioconjugate Chemistry considers for publication only original work that has not been previously published and is not under consideration for publication elsewhere. When submitting a manuscript, an author should inform the editor of any prior dissemination of the content in print or electronic form. This includes electronic posting of conference presentations, posters,

and preprints on institutional repositories and other Web sites. Any content that has already been made publicly available either in print or electronic form may jeopardize the originality of a submitted manuscript and may preclude its consideration for publication. The editor will judge these matters on a case-by-case basis.

Any closely related manuscripts must be included with the submitted manuscript, as Supporting Information for Review Only. This includes those prepared after the submitted manuscript, which should be brought to the editor's attention. Failure to do this may jeopardize publication of the submitted manuscript.

As additional features become available, these instructions will be updated on the *Bioconjugate Chemistry* Web site, http://pubs.acs.org/page/bcches/submission/index.html , select Author Guidelines. These instructions and the Journal Publishing Agreement form are always available on the Web site.

No Page Charges

There are no page charges or production charges for *Bioconjugate Chemistry.*

ANEXO C

Comprovante de aceite do Periódico "Experimental Parasitology"

Ms. No.: EP-14-114R2
Title: Molecular characterization of Cyclophilin (TcCyP19) in Trypanosoma cruzi populations susceptible and resistant to benznidazole
Corresponding Author: Dr. Silvane Maria Fonseca Murta
Authors: Juciane V Rego, M.Sc.; Ana Paula Duarte, Ph.D.; Daniel B Liarte, Ph.D.; Francirlene C Sousa, Master student; Humberto M Barreto, Master student; Jacqueline Búa, Ph.D.; Alvaro J Romanha, Ph.D.; Gandhi Rádis-Baptista, Ph.D.;

Dear Dr. Murta,

I am pleased to inform you that your manuscript referenced above has been accepted for publication. Your article has been forwarded to Elsevier's Production Department, and you should be receiving confirmation from them shortly.

When your paper is published on ScienceDirect, you want to make sure it gets the attention it deserves. To help you get your message across, Elsevier has developed a new, free service called AudioSlides: brief, webcast-style presentations that are shown (publicly available) next to your published article. This format gives you the opportunity to explain your research in your own words and attract interest. You will receive an invitation email to create an AudioSlides presentation shortly. For more information and examples, please visit http://www.elsevier.com/audioslides.

Many thanks for submitting your paper to Experimental Parasitology.

Sincerely,

Bernd Kalinna
Editor-in-Chief
Experimental Parasitology

ANEXO D

Comprovante de Submissão do periódico "Bioconjugate Chemistry"

16-Oct-2014

Dear Prof. Dr. RÁDIS-BAPTISTA:

Your manuscript has been successfully submitted to Bioconjugate Chemistry.
Title: "Rhodamine B-conjugated encrypted vipericidin nonapeptide is a potent toxin to zebrafish and associated with cytotoxicity towards human breast cancer cells"
Authors: Wang, Liang; Chan, Judy; Rêgo, Juciane; Chong, Tony; Ai, Nana; Falcao, Claudio; Lee, Simon M.Y.; RÁDIS-BAPTISTA, GANDHI
Manuscript ID: bc-2014-004789.

Please reference the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your contact information, please log in to ACS Paragon Plus at https://acs.manuscriptcentral.com/acs and select "Edit Your Account" to update that information.

You can view the status of your manuscript by checking your "Authoring Activity" tab on ACS Paragon Plus after logging in tohttps://acs.manuscriptcentral.com/acs.

Thank you for submitting your manuscript to Bioconjugate Chemistry.

Sincerely,

Bioconjugate Chemistry Editorial Office

ANEXO E

Análise *in silico* de interação das viperidinas e alvos moleculares

Análise *in silico* foi realizada identificando alvos gênicos da base de dados DrugBank que se interagem com as vipericidinas. Essa análise revelou que entre os 6 peptídeos (vipericidinas) testados (Tabela), dois GA645-crotalicidina e GA650- lutzicidina se interagem com 24 alvos moleculares que tiveram melhores hits. Desses alvos moleculares identificamos uma ciclofilina que se interage com o peptídeo GA650. Segue abaixo a tabela e as listas de alvos moleculares obtidos pela análise *in silico* das vipericidinas GA650-lutzicidina e GA645-crotalicidina , respeticvamente.

Tabela Anexo E. Peptídeos Vipericidinas utilizadas na análise *in silico*

Peptideo	Sequencias
GA645-Crotalicidin (Ctn)	KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTIPF
GA646-Ctn fragment A*	KRFKKFFKKVKKSV
GA645-Ctn fragment B*	KKRLKKIFKKPMVIGVTIPF
GA648	KKRLKKIFKKPIFKKV
GA649	KKSV
GA50-lutzicidin	KRFKKFFKKLKNNVKKRVKKFFRKPRVIGVTIPF

* Peptides derived from crotalicidin (Ctn)

Lista 1: Relação de Alvos gênicos que se interage com Peptídeo GA650- lutzicidina *in silico* utilizando a base de dados DrugBank

```
Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Query= GA650
         (34 letters)

Database: C:\BioEdit\database\all_target.fasta
           3789 sequences; 1,829,073 total letters



                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value

drugbank_target|3803 Sodium channel protein type 3 subunit alpha...    24   2.2
drugbank_target|6331 Probable ribosome biogenesis protein RLP24 ...    21     25
drugbank_target|6300 Poly [ADP-ribose] polymerase 2 (DB07232)          21     25
drugbank_target|256 Tyrosyl-tRNA synthetase, cytoplasmic (DB0013...    21     25
drugbank_target|4382 Sulfotransferase family cytosolic 1B member...    20     32
drugbank_target|2950 Inositol-trisphosphate 3-kinase A (DB01863;...    20     32
drugbank_target|21 Beta-adrenergic receptor kinase 2 (DB00171)         20     32
drugbank_target|340 Apoptotic protease-activating factor 1 (DB00...    20     42
drugbank_target|94 5-hydroxytryptamine 4 receptor (DB00604; DB00...    20     42
drugbank_target|4086 Peptidyl-prolyl cis-trans isomerase   (DB00...    20     55
drugbank_target|1939 Heat shock protein HSP 90-alpha (DB00615; D...    20     55
drugbank_target|771 Pyruvate carboxylase, mitochondrial (DB00119...    20     55
drugbank_target|6544 STE20-like serine/threonine-protein kinase ...    19     72
drugbank_target|6243 Nuclear receptor-interacting protein 1 (DB0...    19     72
drugbank_target|5239 Exopolyphosphatase (DB03382)                      19     72
drugbank_target|4343 Alpha-glucosidase (DB01769; DB01907; DB03323)     19     72
drugbank_target|1716 Tripartite motif-containing protein 13 (DB0...    19     72
drugbank_target|288 Trifunctional enzyme subunit alpha, mitochon...    19     72
drugbank_target|6552 Poly [ADP-ribose] polymerase 3 (DB07677; DB...    19     94
drugbank_target|4467 Alpha-glucosidase, putative (DB01907)             19     94
drugbank_target|4116 Dihydropteroate synthetase (DB00359; DB0066...    19     94
drugbank_target|3950 Cell division protein kinase 9 (DB03496)          19     94
drugbank_target|3273 SpoOB-associated GTP-binding protein (DB04022)    19     94
drugbank_target|222 DNA ligase 1 (DB00290)                             19     94
```

Lista 2: Relação de Alvos gênicos que se interage com Peptídeo GA645-crotalicidina *in silico* utilizando a base de dados DrugBank

```
Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
"Gapped BLAST and PSI-BLAST: a new generation of protein database search
programs",  Nucleic Acids Res. 25:3389-3402.

Query= GA645
         (34 letters)

Database: C:\BioEdit\database\all_target.fasta
           3789 sequences; 1,829,073 total letters



                                                                  Score    E
Sequences producing significant alignments:                       (bits) Value

drugbank_target|288 Trifunctional enzyme subunit alpha, mitochon...    23   5.0
drugbank_target|256 Tyrosyl-tRNA synthetase, cytoplasmic (DB0013...    22   8.5
drugbank_target|2399 Uricase (DB01875)                                 21      19
drugbank_target|662 Fatty aldehyde dehydrogenase (DB00157)             21      25
drugbank_target|2322 Genome polyprotein (DB01693; DB01752)             20      32
drugbank_target|1757 Myeloperoxidase (DB00244; DB00535; DB00583;...    20      32
drugbank_target|6069 Penicillin-binding protein 2 (DB00303; DB00...    20      42
drugbank_target|6832 Serine/threonine-protein kinase SRPK2 (DB00...    20      55
drugbank_target|774 Cysteinyl-tRNA synthetase, cytoplasmic (DB00...    20      55
drugbank_target|3 Histidine decarboxylase (DB00114; DB00117)           20      55
drugbank_target|6371 Beta-hexosaminidase (DB07432; DB08704)            19      72
drugbank_target|4539 Catalase (DB01942; DB03014)                       19      72
drugbank_target|220 Sodium channel protein type 5 subunit alpha ...    19      72
drugbank_target|215 Sodium channel protein type 11 subunit alpha...    19      72
drugbank_target|198 Sodium channel protein type 10 subunit alpha...    19      72
drugbank_target|6063 Major histocompatibility complex class I-re...    19      94
drugbank_target|5239 Exopolyphosphatase (DB03382)                      19      94
drugbank_target|408 Riboflavin kinase (DB00140; DB03247; DB03431)      19      94
drugbank_target|328 Sorbitol dehydrogenase (DB00157; DB01907; DB...    19      94
drugbank_target|222 DNA ligase 1 (DB00290)                             19      94
drugbank_target|94 5-hydroxytryptamine 4 receptor (DB00604; DB00...    19      94
drugbank_target|70 Type-1 angiotensin II receptor (DB00177; DB00...    19      94
```