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Scaffold proteins LACK and TRACK as potential drug targets in kinetoplastid parasites: Development of inhibitors

Nir Qvit [a, *], Deborah Schechtman [a, b], Darlene Aparecida Pena [b], Denise Aparecida Berti [b], Chrislaine Oliveira Soares [b], Qianqian Miao [c], Liying (Annie) Liang [c], Lauren A. Baron [d], Christian Teh-Poot [d], Pedro Martínez-Vega [d], Maria Jesus Ramirez-Sierra [d], Eric Churchill [a], Anna D. Cunningham [a], Andrey V. Malkovskiy [e], Nancy A. Federspiel [a], Fabio Cesar Gozzo [f], Ana Claudia Torrecilhas [g], Maria Julia Manso Alves [b], Armando Jardim [h], Ndao Momar [c], Eric Dumonteil [d], Daria Mochly-Rosen [a]

[a] Department of Chemical and Systems Biology, Stanford University, School of Medicine, Stanford, CA 94305, USA
[b] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, SP, Brazil
[c] National Reference Centre for Parasitology, Research Institute of the McGill University, Montreal, Canada
[d] Laboratorio de Parasitología, Centro de Investigaciones Regionales "Dr. Hideyo Noguchi", Universidad Autónoma de Yucatán, Mérida, Yucatán, Mexico
[e] Biomaterials and Advanced Drug Delivery Laboratory, Stanford University, Stanford, CA 94305, USA
[f] Institute of Chemistry, University of Campinas, Campinas, SP, Brazil
[g] Departamento de Ciências Biológicas, Campus Diadema, UNIFESP, Brazil
[h] Institute of Parasitology and Centre for Host-Parasite Interactions, McGill University, Québec, Canada

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ABSTRACT

Parasitic diseases cause ~500,000 deaths annually and remain a major challenge for therapeutic development. Using a rational design based approach, we developed peptide inhibitors with anti-parasitic activity that were derived from the sequences of parasite scaffold proteins LACK (***L**eishmania*'s receptor for **a**ctivated **C**-**k**inase) and TRACK (***T**rypanosoma* **r**eceptor for **a**ctivated **C**-**k**inase). We hypothesized that sequences in LACK and TRACK that are conserved in the parasites, but not in the mammalian ortholog, RACK (**R**eceptor for **a**ctivated **C**-**k**inase), may be interaction sites for signaling proteins that are critical for the parasites' viability. One of these peptides exhibited leishmanicidal and trypanocidal activity in culture. Moreover, in infected mice, this peptide was also effective in reducing parasitemia and increasing survival without toxic effects. The identified peptide is a promising new anti-parasitic drug lead, as its unique features may limit toxicity and drug-resistance, thus overcoming central limitations of most anti-parasitic drugs.

1. Introduction

Leishmaniasis is one of the most neglected tropical diseases in terms of drug discovery and development. In over 88 countries, *Leishmania* threatens millions of the poorest people, especially those living in rural areas. Up to two million people are infected with *Leishmania* each year, and 70% of those who die from leishmaniasis are children (Chappuis et al., 2007). *Leishmania donovani* (*L. donovani*) causes visceral leishmaniasis (VL), the most severe form of the disease in humans (Hotez et al., 2007). *Leishmania amazonensis* (*L. amazonensis*) causes cutaneous leishmaniasis (CL), the most common form of leishmaniasis in the New World (Guimaraes-Costa et al., 2009). CL is endemic in 82 countries, with an incidence of approximately 1.5 million cases per year.

Available treatments for VL and CL include Amphotericin B (a toxin that forms pores in membranes with some preferences for ergosterol (Croft et al., 2006)), with some success on the Indian subcontinent (Chappuis et al., 2007). In contrast to India, the use of Amphotericin B for VL in East Africa generally results in less-effective responses; thus, in this region, pentavalent antimonials, such as sodium stibogluconate (mode of action is not clearly

* Corresponding author.
E-mail address: nirqvit@stanford.edu (N. Qvit).

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understood (Copeland and Aronson, 2015)), are the first line of treatment. However, these drugs are highly toxic and cause serious side effects including hypotension, seizures, disturbances in cardiac conduction, and phlebotoxicity (vein inflammation, which limits repeated dosing (Croft and Coombs, 2003)). Moreover, widespread resistance has developed to these drugs. In March 2014, the FDA approved miltefosine (developed in the late 1980s for cancer treatment (Vincent et al., 2014)) for treatment of CL and VL caused by specific *Leishmania* species. Although initially miltefosine demonstrated high efficacy for VL in India, numerous clinical failures have since been reported. In addition, only moderate efficacy has been observed in East Africa and the New World (Monge-Maillo and López-Vélez, 2015).

Trypanosoma cruzi (*T. cruzi*) infection causes Chagas disease, affecting ~10 million people in the Americas, and is responsible for 12,000 deaths yearly (Bonney, 2014). Moreover, increased migration has disseminated this disease worldwide (Bonney, 2014), and about 70 million people, the majority of which are children, remain at high risk for contracting this disease (Hotez et al., 2012). During the acute phase after *T. cruzi* infection, clinical signs are usually quite minimal; however, many years after the primary infection, about 30–40% of infected individuals develop the symptomatic chronic phase of Chagas disease, characterized by the presence of myocarditis and heart failure (Garcia et al., 2005). The drugs used to treat Chagas disease, benznidazole (which causes double-stranded breaks in DNA (Viotti et al., 2009)) and nifurtimox (mechanism of action is not yet fully elucidated), have limited use, as they have severe side effects and exhibit inadequate efficacy in the chronic stage (Maya et al., 2007). Several clinical studies found that although benznidazole treatment is somewhat beneficial, its side effects are still an issue (*e.g.* clinical study, NCT01162967, in which the treatment of 20% of the patients in the benznidazole group was discontinued because of severe cutaneous reactions (Molina et al., 2014)). Therefore, novel and simple strategies are needed for developing therapeutic agents that are effective and less toxic, do not trigger resistance, and are affordable for the infected populations in the developing world.

Here, we describe our effort to develop novel inhibitors for the parasites *Leishmania sp.* and *T. cruzi*. We concentrated on generating inhibitors that are specific for the parasites by targeting unique domains of parasite scaffold proteins, LACK (***L****eishmania*'s receptor for **a**ctivated **C**-**k**inase) and TRACK (***T****rypanosoma* **r**eceptor for **a**ctivated **C**-**k**inase).

LACK is a scaffold protein required for the viability of the parasite and for its establishment in the host (Gomez-Arreaza et al., 2011). LACK plays an important role in the early phase of *Leishmania* infection (Mougneau et al., 1995); LACK-deficient parasites are not viable (Kelly et al., 2003), and parasites expressing lower levels of LACK fail to parasitize even immune-compromised mice (Kelly et al., 2003). Similarly, TRACK in *Trypanosoma* is an essential protein and its homologs are found in several trypanosomatids, including *T. cruzi* (Rothberg et al., 2006). Although there is limited information about TRACK's functions in *T. cruzi*, in *Trypanosoma brucei* (*T. brucei*), TRACK is expressed throughout the parasite's life cycle and has a role in the final stages of mitosis (Rothberg et al., 2006). In addition, decreased TRACK expression by RNAi leads to incomplete cytokinesis in pro-cyclic blood stream trypanosomes and accelerates the elimination of parasites from peripheral blood (Rothberg et al., 2006). These data suggest that LACK and TRACK are involved in essential parasite processes and could be important anti-parasitic drug targets.

Previously we developed a rational approach to generate inhibitors of scaffold proteins, which interfere with normal and pathological signaling events in cells, in animal models, and in humans (review (Churchill et al., 2009; Mochly-Rosen and Qvit, 2010; Qvit and Mochly-Rosen, 2010)). Relevant to this study, we have identified peptides that inhibit the function of RACK (**r**eceptor for **a**ctivated **C-k**inase), the mammalian homolog of LACK and TRACK. RACK is a ubiquitous and highly conserved scaffold protein (McCahill et al., 2002) that binds several signaling enzymes, all of which are critical for cell survival, growth, and differentiation (Schechtman and Moshly-Rosen, 2001). We rationally designed peptides that interfere with RACK function; these peptides were found to be effective and selective in cells, *in vivo* (in a variety of animal models of human diseases (Inagaki et al., 2003; Kim et al., 2008)), and in clinical trials (Bates et al., 2008).

Here we apply the same rational design for development of peptides that target leishmaniasis and Chagas disease. We describe an inexpensive and fast approach that enabled the identification of novel peptides derived from the parasitic scaffold proteins, LACK and TRACK, as anti-parasitic therapeutic leads. These may ultimately provide the basis for a specific, less toxic, and more convenient treatment for people who suffer from these diseases.

2. Materials and methods

2.1. Sequence alignments

Sequences from different species were aligned using the following proteins: human RACK (P63244), *L. donovani* LACK (Q76LS6), *Leishmania braziliensis* LACK (A4HGX7), *Leishmania panamensis* LACK (Q9GUB0), *Leishmania major* LACK (Q253306), *Leishmania turanica* LACK (496205235), *Leishmania aethiopica* LACK (496205233), *Leishmania tropica* LACK (404515577), *Leishmania gerbilli* LACK (388850676), *Leishmania infantum* LACK (P62884), *Leishmania chagasi* LACK (P62884), *Leishmania mexicana* LACK (Q7KFG4), *L. amazonensis* LACK (Q95NJ3), *Trypanosoma cruzi* TRACK (Q4DTN2), *Trypanosoma brucei* TRACK (P69103), *Trypanosoma congolense* TRACK (O96653), *Trypanosoma carassii* TRACK (A6ZIC2) and *Trypanosoma vivax* TRACK (O96654). The alignment was done using the FASTA server of the University of Virginia (Pearson and Lipman, 1988), where: (:) represents identical amino acids, and (.) represents similar amino acids.

2.2. Peptide synthesis

In brief: Peptides were synthesized on solid support using a fully automated microwave peptide synthesizer (Liberty, CEM Corporation). The peptides were synthesized by SPPS (solid phase peptide synthesis) methodology (Merrifield, 1963) with a fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (tBu) protocol. The lysine side chain was protected with N-methyltrityl (Mtt), a protection group that can be deprotected selectively using acid labile conditions (Aletras et al., 1995). After completion of the synthesis of the linear peptide, an anhydride spacer was coupled to the N-terminal amino group and cyclization was performed using amide bonds between the moiety linker at the backbone N-terminus and an epsilon amino on the side chain of a C-terminal Lys residue (Gilon et al., 1991; Qvit, 2011, 2014). The final cleavage and side chain deprotection was done manually without microwave energy. Peptides were analyzed by analytical reverse-phase high-pressure liquid chromatography (RP-HPLC) (Shimadzu, MD, USA) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) and purified by preparative RP-HPLC (Shimadzu, MD, USA).

Further details: All commercially available solvents and reagents were used without further purification. Dichloromethane (DCM), N-methyl-2-pyrrolidone (NMP), triisopropylsilane (TIS), N,N-diisopropylethylamine (DIEA), O-benzotriazole-N,N,N′,N′-tetramethyl-uronium-hexafluoro-phosphate (HBTU), 1-