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Inhibitors of casein kinase 1 block the growth of *Leishmania major* promastigotes in vitro

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Abstract

Casein kinase 1 (CK1) is a family of multifunctional Ser/Thr protein kinases that are ubiquitous in eukaryotic cells. Recent studies have demonstrated the existence of, and role for, CK1 in protozoan parasites such as *Leishmania, Plasmodium* and *Trypanosoma*. The value of protein kinases as potential drug targets in protozoa is evidenced by the successful exploitation of cyclic guanosine monophosphate-dependent protein kinase (PKG) with selective tri-substituted pyrrole and imidazopyridine inhibitors. These compounds exhibit in vivo efficacy against *Eimeria tenella* in chickens and *Toxoplasma gondii* in mice. We now report that both of these protein kinase inhibitor classes inhibit the growth of *Leishmania major* promastigotes and *Trypanosoma brucei* bloodstream forms in vitro. Genome informatics predicts that neither of these trypanosomatids codes for a PKG orthologue. Biochemical studies have led to the unexpected discovery that an isoform of CK1 represents the primary target of the pyrrole and imidazopyridine kinase inhibitors in these organisms. CK1 from extracts of *L. major* promastigotes co-fractionated with [^{3}H]imidazopyridine binding activity. Further purification of CK1 activity from *L. major* and characterization via liquid chromatography coupled tandem mass spectrometry identified CK1 isoform 2 as the specific parasite protein inhibited by imidazopyridines. *L. major* CK1 isoform 2 expressed as a recombinant protein in *Escherichia coli* displayed biochemical and inhibition characteristics similar to those of the purified native enzyme. The results described here warrant further evaluation of the activity of these kinase inhibitors against mammalian stage *Leishmania* parasites in vitro and in animal models of infection, as well as studies to genetically validate CK1 as a therapeutic target in trypanosomatid parasites.
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1. Introduction

Invasion of the vertebrate host by the trypanosomatid protozoan parasites of the genus *Leishmania* can lead to a variety of disease states, ranging from cutaneous and muco-cutaneous lesions to the fatal visceral form of leishmaniasis. An estimated 350 million people live in areas where the disease is endemic, of which 12 million in Africa, Asia, Europe and the Americas are directly affected (Guerin et al., 2002; Davies et al., 2003). *Leishmania* is currently considered an emerging infectious disease in parts of the world where it has become closely associated with human immunodeficiency virus-acquired syndrome (Dedet and Pratlong, 2000). Leishmaniasis represents a significant source of morbidity and mortality world-wide. At present, the control of leishmaniasis relies primarily on chemotherapy with pentavalent antimonials, which require long treatment periods and are often toxic (Dedet and Pratlong, 2000; Davies et al., 2003). Unfortunately, therapy with antimonials can no longer be used in certain parts of the world due to the emergence of drug resistance (Sundar et al., 2000). Recent therapeutic alternatives have included

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lipid formulations of amphotericin B (originally developed for the treatment of systemic mycoses), paromomycin (an aminoglycoside for intestinal infections in late-stage clinical trials) and the newly registered lysophospholipid analog miltefosine (reviewed in Croft et al., 2005). However, none of these newer alternatives has a sufficient therapeutic window. Moreover, vaccines as alternatives to chemotherapy against leishmaniasis are not yet available nor are they anticipated in the near future. There is, therefore, a need to discover and develop effective, safe and novel anti-parasitic agents to aid in the treatment and control of leishmaniasis worldwide.

One rational approach in the hunt for new anti-leishmanial drugs is to identify and target key intracellular signals that are vital to the organism's survival within the mammalian host. The phosphorylation of serine, threonine and tyrosine by various protein kinases is a molecular event that regulates vital cellular processes and is amenable to selective targeting with small molecule drugs in a variety of disease conditions (Gross and Anderson, 1998; Knippschild et al., 2005). Casein kinase 1 (CK1), a family of multifunctional Ser/Thr protein kinases, present in all eukaryotic cells, has recently been described in various protozoan organisms (Barik et al., 1997; Knockaert et al., 2000; Calabokis et al., 2002, 2003; Spadafora et al., 2002; Galan-Caridad et al., 2004; Donald et al., 2005). In *Leishmania*, two constitutively shed ecto-kinases were characterized biochemically and determined to have CK1-like activities (Vieira et al., 2002; Sacerdoti-Sierra and Jaffe, 1997). It has been suggested that these kinases play a role in parasite–host interaction, perhaps by phosphorylating host proteins to facilitate the invasion process. Molecular biological studies in the trypanosomatid parasite *Trypanosoma cruzi* led to the identification of two cDNAs encoding the CK1 homologues TcCK1.1 and TcCK1.2 (Spadafora et al., 2002).

While the molecular and biochemical properties of CK1 isoforms from trypanosomatids show overall similarity to the enzymes from other eukaryotes, the opportunity for selective inhibition of parasite versus host enzyme might exist. In a recent study, CK1 from *Leishmania mexicana* was identified as the molecular target of the cyclin-dependent protein kinase (CDK) inhibitor purvalanol B (Knockaert et al., 2000). The observation that purvalanol B is a more potent inhibitor of parasite than mammalian (Gray et al., 1998) CK1 activity emphasized the potential for exploitable host–parasite differences in this protein. Selective inhibition of parasite CK1 activity has also been demonstrated in a study describing the cloning, expression and biochemical and pharmacological characterization of two CK1 genes from *Toxoplasma gondii* (Donald et al., 2005). Several protein kinase inhibitors were evaluated, including purvalanol analogs as well as compounds that inhibit cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and calmodulin like domain protein kinase (CDPK) (Donald et al., 2002, 2005; Donald and Liberator, 2002; Gurnett et al., 2002; Nare et al., 2002; Salowe et al., 2002; Wiersma et al., 2004; Biftu et al., 2005; Liang et al., 2005). Compounds initially described as PKG inhibitors have potent activity against recombinant expressed *T. gondii* CDPK1 and CK1 enzymes.

In the present study, we demonstrate that representative pyrrole and imidazopyridine PKG protein kinase inhibitors have potent in vitro activity against *Leishmania major* promastigotes and bloodstream forms of the related trypanosomatid protozoa *Trypanosoma brucei*. Orthologs of PKG and CDPK have not been identified in the genomes of kinetoplastid protozoans *Leishmania* and *Trypanosoma* (http://www.ebi.ac.uk/parasites/leish.html; http://www.sanger.ac.uk/Projects/T_brucei/). In binding studies using a radiolabeled imidazopyridine ligand to probe a crude promastigote protein extract, *L. major* CK1 isoform 2 has been identified as the primary high-affinity binding protein. These results predict an essential biochemical function for CK1 in insect stage promastigotes and warrant further evaluation of the activity of these kinase inhibitors against mammalian stage *Leishmania* parasites in vitro and in animal models of infection.

2. Materials and methods

2.1. Parasite cultures

Insect stage promastigotes of *L. major* (clone CC-1 of LT252 strain) were used in all experiments. Parasites were maintained at 26 °C in M199 medium supplemented with 10% FCS, hemin, adenine, biotin and biopterin as previously described (Iovannisci and Ullman, 1983). *Trypanosoma brucei* bloodstream-forms (Laboratory adapted 221 variant) were cultured (37 °C) in HMI-9 medium containing 10% FCS and appropriate supplements (Hirumi et al., 1997).

2.2. Parasite growth inhibition assay

Susceptibility of trypanosomatid parasites to test compounds was evaluated using the CellTiter-Glo® luminescent cell viability assay kit (Promega). The CellTiter-Glo® assay is a homogeneous method of determining the number of viable cells in culture based on quantitation of the adenosine 5′-triphosphate (ATP) present, an indicator of metabolically active cells. *Leishmania major* promastigotes (2×10^5/mL) or *T. brucei* bloodstream form trypomastigotes (1×10^4/mL) were incubated in 100 μL of M199 and HMI-9, respectively, in opaque walled, clear bottom 96-well plates (Corning Incorporated) in the presence of increasing concentrations of test compounds. After 48 h (*T. brucei*) or 120 h (*L. major*), culture plates were equilibrated at room temperature for 30 min followed by addition of an equal volume (100 μL) of CellTiter-Glo® reagent into each well. The luminescence signal was allowed to stabilize for 10 min and plates were read using a 1450 Microbeta Trilux luminescence detector (Perkin-Elmer).

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