Activity of anti-cancer protein kinase inhibitors against Leishmania spp.

Lisa Sanderson, Vanessa Yardley* and Simon L. Croft

Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK

*Corresponding author. Tel: +44-20-7927-2462; Fax: +44-20-7927-2131; E-mail: vanessa.yardley@lshtm.ac.uk

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Objectives: There is an urgent need to develop new and effective treatments for poverty-related neglected diseases. In light of the time required to bring a new drug to market and the cost involved (10–15 years, >1 billion US$), one approach to identifying new treatments for diseases like leishmaniasis is to evaluate drugs that are already registered for the treatment of other diseases. This paper describes the anti-leishmanial activities of 10 FDA-approved protein kinase inhibitors already available for the treatment of human cancers.

Methods: In vitro and in vivo models of Leishmania infection were used to evaluate the potency of selected protein kinase inhibitors.

Results: Sunitinib, sorafenib and lapatinib were identified as active against Leishmania donovani amastigotes in cultured murine macrophages with IC_{50} values of 1.1, 3.7 and 2.5 μM, respectively, a level of potency similar to that of miltefosine (IC_{50}=1.0 μM), and were not toxic to mammalian cells. In addition, some of the protein kinase inhibitors were active against L. donovani in the BALB/c mouse model of infection; dosing on days 7–11 with a 50 mg/kg oral dose of sunitinib, lapatinib or sorafenib reduced liver amastigote burdens by 41%, 36% and 30%, respectively, compared with untreated control mice. Although less efficacious, sorafenib was also active in vitro against intracellular amastigotes of the cutaneous disease-causing species Leishmania amazonensis, Leishmania major and Leishmania mexicana.

Conclusions: This study demonstrates in vivo anti-leishmanial activity of clinically used protein kinase inhibitors and provides further evidence of the potential of drug repurposing.

Keywords: drug repurposing, sunitinib, sorafenib, lapatinib, PP2

Introduction

Today, more than 350 million people in 98 countries throughout the world are at risk of leishmaniasis. The most common, cutaneous form of the disease often manifests as a simple self-healing ulcer, but the development of diffuse or mucocutaneous lesions can leave patients with severe scarring and disfigurement of the face and limbs. There are currently 220 000 reported cases of cutaneous leishmaniasis per year, but, due to under-reporting, the real incidence has been estimated to be between 0.7 and 1.2 million. In addition, there are an estimated 0.4 million cases of the more acute visceral form, which is fatal without treatment and results in an estimated 40 000 deaths per year. Treatment currently relies on a limited number of drugs that have limitations of toxicity, long courses, restrictive costs or the need for parenteral administration. Drug efficacy against different clinical isolates is variable and the emergence of acquired resistance to the pentavalent antimonials demonstrates the need for new and effective treatments.

Early studies on Leishmania protein kinases suggested that chemical inhibition or genetic knockdown of Leishmania cyclin-dependent (CDK) and mitogen-activated (MAPK) kinases could reduce viability and inhibit the proliferation of amastigotes within infected macrophages, identifying these enzymes as putative targets for anti-leishmanial chemotherapy. Screening of known CDK inhibitors identified the indirubins and tri-substituted purines as potent inhibitors of Leishmania donovani amastigote growth in cultured murine macrophages. However, subsequent attempts to optimize selectivity for the recombinant parasite enzyme (CRK) versus the mammalian enzyme (CDK) showed a poor correlation between specificity and activity against the whole organism in vitro. These results suggested that the anti-leishmanial activity of kinase inhibitors might involve non-specific binding to alternative parasite kinases and also highlighted the possible role of interactions with mammalian kinases, such as host MAPK pathways, which regulate the production of nitric oxide (NO) and inflammatory cytokines.

Several mammalian protein kinase inhibitors have already been approved by the FDA and are commercially available for the treatment of human cancers. These compounds have received little interest in anti-leishmanial discovery programmes since their primary targets are mammalian receptor tyrosine kinases.
kinases, which are not expressed in the trypanosomatids. Nevertheless, protein phosphorylation does occur in these parasites and many of the commercial inhibitors have been shown to interact with multiple off-target kinases so could have some therapeutic efficacy against *Leishmania*. The pharmacokinetic and toxicity profiles of commercial inhibitors are already known, which would significantly accelerate any future development and reduce costs for a disease endemic in some of the poorest regions of the world. The repurposing of these existing drugs could provide a more rapid and cost-effective approach to drug discovery.

We report here the activity of 10 commercially available and one experimental protein kinase inhibitor(s) on *Leishmania* parasites in cultured macrophages and in a murine model of visceral disease.

**Materials and methods**

**Parasite strains**

*Leishmania* promastigotes and KB cells were maintained under standard culture conditions in RPMI (KB cells), M199 (*Leishmania amazonensis* MPRO/BR/72/M1841 LV79-D5red2) or Schneider’s Insect Medium (*Leishmania mexicana* MNYC/BZ/62/M379, *Leishmania major* MHO/M/SA/85/JISH118 and *Leishmania tropica* A021/p). Parasites were sub-cultured every 7 days and only stationary-phase cultures were used for experiments. *L. donovani* MHOM/ET/67/HU3 was maintained in Rag1.B6 mice.

**Drugs**

Ten commercially available FDA-approved protein kinase inhibitors (imatinib, nilotinib, dasatinib, erlotinib, gefitinib, lapatinib, sorafenib, sunitinib, pazopanib and crizotinib) were purchased from Sequoia Research Products Ltd, UK. PP2, an Src family kinase inhibitor used in cancer research, but not licensed for human use, was purchased from Calbiochem, Merck Millipore, UK. For *in vitro* testing, 20 mM stock solutions were prepared in DMSO and were diluted to the appropriate concentration in RPMI medium. Active compounds were prepared for testing in a murine model of visceral leishmaniasis by suspension of 5 mg/mL in a mixture of Tween 80/ethanol/ddH2O (7:3:90).

**In vitro activity and cytotoxicity of commercial protein kinase inhibitors**

Primary macrophages that were harvested from starch-induced peritoneal exudates in CD1 mice were seeded in 16-well glass slides and infected with *Leishmania* promastigotes or amastigotes as previously described. The infected macrophages were exposed to test drug for 5 days, after which the percentage of infected macrophages was determined microscopically.

KB cells were added to 96-well plates at a density of 4 × 10^4 per well and were incubated under standard conditions for 24 h to allow the cells to adhere. Cells were exposed to test drugs for 72 h before measuring percentage survival using the Alamar blue assay. Data were analysed with MS Excel and Prism to determine IC50/IC90 values.

**Activity of commercial protein kinase inhibitors against *L. donovani* in mice**

BALB/c mice were infected as previously described. Test drugs (50 mg/kg/day) and the standard miltefosine (12 mg/kg/day) were administered to groups of five mice as a 0.2 mL oral bolus on days 7–11 post-infection. Ambisome™ (1 mg/kg/day) was prepared in 5% dextrose and administered intravenously as a 0.2 mL bolus on days 7, 9 and 11 post-infection. Liver smears were prepared at day 14 post-infection as previously described. The amastigote burden in the liver was determined microscopically and expressed as Leishman–Donovan units. Percentage inhibition was calculated for drug-treated groups compared with untreated controls.

All in vivo experiments were carried out under licence according to UK Home Office regulations [Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63/EU]. The licensing process includes ethics approval for any procedure used.

**Results and discussion**

Ten commercially available kinase inhibitors (Table 1) were tested twice, against both *L. donovani* and *L. amazonensis* strains cultured in mouse peritoneal macrophages in *in vitro*. For each experiment, the percentage inhibition was determined in quadruplicate at four concentrations and used to determine IC50 values. From these experiments we identified three inhibitors—sunitinib, lapatinib and sorafenib—that showed significant activity against the

| Table 1. *In vitro* activity of protein kinase inhibitors against intracellular amastigotes |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | *L. donovani* IC50 (μM) | *L. amazonensis* IC50 (μM) | *L. mexicana* IC50 (μM) | *L. major* IC50 (μM) | *L. tropica* IC50 (μM) | KB cell IC50 (μM) |
| Sorafenib                      | 4.23±0.32        | 5.36±0.28        | 4.72±0.31        | 3.77±0.21        | NA              | 7               |
| Sunitinib                      | 1.16±0.02        | 2.63±0.09        | 1.00±0.04        | NA              | NA              | 7               |
| Lapatinib                      | 3.27±0.17        | NA               | 3.27±0.17        | NA              | NA              | 6               |
| PP2                             | NA               | NA               | NA               | NA              | NA              | 42              |
| Amphotericin B                 | 0.02±0.01        | 0.11±0.01        | 0.02±0.01        | 0.09±0.01        | 0.12±0.01       | 7.08            |
| Miltefosine                     | 1.03±0.09        | 1.41±0.09        | 0.96±0.09        | 16.27±1.27       |                 |                 |

NA, not active at 30 μM.

Compounds were tested over a range of 1 in 3 dilutions between 100 and 0.14 μM.
mammalian cells as no cytotoxicity was observed against the host murine macrophages themselves, as assessed by qualitative microscopic examination, and higher IC_{50} values were obtained against the human KB cell line cultured in vitro, as shown in Table 1. Although sunitinib and lapatinib showed the greatest efficacy against L. donovani, sorafenib also exhibited a broad spectrum of activity against strains that cause cutaneous disease, including L. amazonensis, L. mexicana and L. major, with IC_{50} values of 6.9, 4.7 and 3.8 μM, respectively (Table 1). Sunitinib, sorafenib and lapatinib were also active against L. donovani in a BALB/c model, where all three compounds showed significant activity, reducing liver amastigote burdens by 30%–40% compared with untreated control mice, as shown in Table 2. No acute drug toxicity or alteration in behaviour was observed, although there was a modest reduction in body weight of 2%–3% compared with untreated control mice. To our knowledge this is the first demonstration that protein kinase inhibitors can reduce Leishmania parasite burdens in an infected animal model.

Table 2. Effect of protein kinase inhibitors on L. donovani liver burden in BALB/c mice

<table>
<thead>
<tr>
<th>Dosing regimen</th>
<th>Liver amastigote burden (LDU), mean ± SEM (n=5)</th>
<th>Percentage reduction, mean ± SEM</th>
<th>Significancea</th>
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<tbody>
<tr>
<td>Sorafenib (50 mg/kg orally×5 doses)</td>
<td>273.1 ± 74.8</td>
<td>34.5 ± 8.9</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Sunitinib (50 mg/kg orally×5 doses)</td>
<td>245.6 ± 62.0</td>
<td>41.1 ± 7.4</td>
<td>P&lt;0.05</td>
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<tr>
<td>Lapatinib (50 mg/kg orally×5 doses)</td>
<td>268.9 ± 72.5</td>
<td>35.5 ± 7.8</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>PP2 (50 mg/kg orally×5 doses)</td>
<td>194.4 ± 73.5</td>
<td>53.4 ± 7.9</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>AmBisome (1 mg/kg intravenously×3 doses)</td>
<td>8.8 ± 4.4</td>
<td>97.9 ± 1.1</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Miltefosine (12 mg/kg orally×5 doses)</td>
<td>138.5 ± 27.4</td>
<td>66.8 ± 6.6</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Untreated control</td>
<td>417.2 ± 35.7</td>
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LDU, Leishman–Donovan unit.

aLiver amastigote burdens were statistically analysed by one-way ANOVA. The P values quoted are for individual treatment groups compared with the untreated control using Bonferroni’s multiple comparison.

visceral disease-inducing strain L. donovani, with IC_{50} values of 1, 2–3 and 3–4 μM, respectively (for IC_{50} values see Table 1). This level of potency was comparable to that of miltefosine, which had an IC_{50} value of 1.0 μM. These compounds also showed a degree of selectivity against amastigotes compared with mammalian cells, as no cytotoxicity was observed against the host murine macrophages themselves, as assessed by qualitative microscopic examination, and higher IC_{50} values were obtained against the human KB cell line cultured in vitro, as shown in Table 1. Although sunitinib and lapatinib showed the greatest efficacy against L. donovani, sorafenib also exhibited a broad spectrum of activity against strains that cause cutaneous disease, including L. amazonensis, L. mexicana and L. major, with IC_{50} values of 6.9, 4.7 and 3.8 μM, respectively (Table 1). Sunitinib, sorafenib and lapatinib were also active against L. donovani in a BALB/c model, where all three compounds showed significant activity, reducing liver amastigote burdens by 30%–40% compared with untreated control mice, as shown in Table 2. No acute drug toxicity or alteration in behaviour was observed, although there was a modest reduction in body weight of 2%–3% compared with a 0.3% increase in untreated control mice. To our knowledge this is the first demonstration that protein kinase inhibitors can reduce Leishmania parasite burdens in an infected animal model.

Sunitinib and sorafenib were both originally designed as inhibitors of vascular endothelial growth factor receptor (VEGFR) and of platelet derived growth factor receptor (PDGFR). In mammalian cancer cells, these receptor tyrosine kinases are highly up-regulated and play a major role in angiogenesis, an essential process required to satisfy the elevated requirement for oxygen and nutrients during proliferation and tumour growth. Sunitinib maleate has previously been shown to block vascular remodelling and progressive spleenomegaly in an L. donovani LV9 (HU3) C57Bl/6 mouse model. Treatment resulted in an increased frequency of T cells producing interferon-γ and tumour necrosis factor, as well as enhanced NO production by splenic macrophages, but the authors were unable to demonstrate a significant reduction in splenic or liver amastigote burden when mice were treated orally with 35 mg/kg on days 21–28 post-infection. Pazopanib, another inhibitor of mammalian VEGFR and PDGFR, was not active in this study, indicating that the anti-leishmanial action exhibited by sorafenib and sunitinib was not due to inhibition of these mammalian receptors within the macrophage. Since there are no known orthologues of VEGFR and PDGFR expressed in Leishmania species, it would appear that these compounds are able to interact with other targets. Sunitinib and sorafenib have both been shown to be non-specific inhibitors that interact with many structurally diverse protein kinases. In contrast, the quinazolineamine lapatinib has been shown to be a highly specific inhibitor of the epidermal growth factor family of receptors (EGFRs). However, the anti-leishmanial activity observed cannot be attributed to inhibition of these mammalian enzymes since erlotinib and gefitinib, which have dissociation constants similar to that of lapatinib, were not active in this study. Like VEGFR and PDGFR, this family of receptors is not expressed by trypanosomatids, but lapatinib-binding protein kinases have been identified in Trypanosoma brucei brucei, which is also susceptible to lapatinib treatment in vitro. Structural optimization of the anilinoquinazoline scaffold has recently resulted in the development of more potent lapatinib analogues that can inhibit the growth of T. b. brucei by 50% at concentrations as low as 0.04 μM in vitro, and reduced death rates in T. b. brucei-infected mice. To our knowledge, there have been no reports of synthetic orthologues of lapatinib-binding protein kinases in Leishmania species; however, using a bioinformatics approach, Katjiur et al. have identified a putative glycogen synthase kinase from L. major (Uniprot ID Q4QE15) that shares 69% sequence homology with TbdPK4.

The role of the immune system in parasite killing by protein kinase inhibitors in our BALB/c model was not investigated in this study. However, treatment with PP2, which did not exhibit any direct killing effect on intracellular amastigotes in vitro, resulted in a 53% reduction in L. donovani liver amastigotes in vivo compared with those found in untreated control mice (Table 2). PP2 is an Src family kinase inhibitor that is used in cancer research, but not licensed for human use. This family of cytoplasmic tyrosine kinases is responsible for both activation and inhibition of complex signal transduction pathways in mammalian innate immune cells.

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Transparency declarations
None to declare.
References


