Targeting the Leishmania kinome for the development of novel anti-parasitic strategies

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Final Report Summary - LEISHDRUG (Targeting the Leishmania kinome for the development of novel anti-parasitic strategies)

Executive Summary:
The LEISHDRUG consortium aims to exploit the Leishmania kinome for anti-parasitic drug development. The major strength of the consortium lies in the highly multi-disciplinary approach combining complementary expertise in imaging and cell biology, peptide biochemistry, genomics and proteomics, drug development and structural biology, and finally in vivo assessment of leishmanicidal activity and pharmacokinetics of lead compounds. The FP7 program provided an important platform to establish for the first time a collaborative network combining this expertise. The LEISHDRUG consortium is based on three clusters with each two interactive scientific work packages that together follow the major stages of the drug development process. Our main objectives over the past three years were (i) to identify novel anti-leishmanial hit compounds utilizing phenotype- as well as target-based screening approaches in combination with kinase-biased compound and peptide libraries (WP1 and WP2), (ii) to initiate hit-to-lead validation using mouse and macrophage infection models, and peptide-based drug delivery strategies (WP3 and WP4), and (iii) to discover novel drug targets involved in parasite-specific signal transduction using complementary genetics, proteomics, bio-informatics and structural approaches (WP5 and WP6). The final goal of the LEISHDRUG consortium was to translate basic research results into application through the establishment of a robust technical platform that will coordinate current and future European efforts in anti-trypanosomatid drug discovery.
During the first granting period (month 0 □ 18), the consortium made important progress towards the achievement of this final goal. Across all work packages, a series of novel techniques and bioinformatics tools have been developed, which include high content image analysis, conditional null mutant analysis, phosphoprotein, phosphopeptide, and phospho-signature analyses, inhibitory peptide delivery systems, activity-based kinase screens, protein kinase and phosphoprotein annotation pipelines, and phosphorylation site and structure prediction algorithms. In addition, LEISHDRUG has established a unique collection of compounds, covering more than 35,000 commercial (Prestwick and ChemDiv), national as well as in-house developed molecules with synthetic or medicinal chemistry traceability, which was applied in our kinase-biased screening campaigns but are also available for other drug development projects on the Institut Pasteur campus and beyond.

The second granting period (month 19 – 42) successfully applied the novel tools developed by LEISHDRUG to identify anti-leishmanial hit compounds and druggable target kinases. In particular, applying the phenotypic screen using over 2000 compounds of a highly selected, kinase-biased inhibitor library, LEISHDRUG has identified fifteen novel anti-leishmanial hit compounds that were able to efficiently kill intracellular parasites without affecting host cell survival, which include bisindolyl maleimides, betulins, isophtalates, azacarbazoles and quinolines. In a complementary target-based assay, over 4000 natural and synthetic compounds were screened on recombinant Leishmania casein kinase 1 alpha, identifying 11 hit compounds, including PP2, 5-iodotubercidin and gefitinib. Our durg discovery pipeline was further supplemented by (i) the genetic demonstration of LmaMPK4 as an essential protein kinase in Leishmania, thus defining this protein kinase as drug target, (ii) the identification of highly parasite-specific regulatory residues with good druggability in LmaMPK10 through genetics, crystallographic and bio-informatics approaches, and (iii) the discovery of parasite-specific essential phosphorylation events in Leishmania stress proteins, which defines respective stress kinases as interesting new drug targets.
In conclusion, the LEISHDRUG consortium established a novel, throughput capable drug development platform with complementary expertise in assay development, proteomic drug target identification and genetic validation, phenotypic and target-based compound screens, and in vitro as well as in vivo assessment. The LEISHDRUG consortium represents today a highly interactive collaborative network with unique know how, which will leave a permanent footprint in the field of neglected parasitic disease drug development through ongoing and future collaborative actions that will be sponsored by national and international funding agencies.
Project Context and Objectives:
Visceral Leishmaniasis (VL), also known as kala-azar, is caused by the protozoan parasites Leishmania donovani and Leishmania infantum (= Leishmania chagasi), and is a potentially fatal disease with a worldwide distribution in Asia, East Africa, South America and the Mediterranean region (including Southern Europe). Almost all VL patients die within months if untreated. Leishmaniasis persists in poor and remote areas, where there is limited health care and patients have little access to preventive measures and affordable drugs. For VL, almost all of the 500,000 new cases arising from recurrent epidemics each year occur in the rural areas of the Indian subcontinent (India, Nepal, Bangladesh), Brazil, Sudan and Ethiopia. The real burden of VL is unknown, but it is estimated that only 20% of cases are reported in India.The parasites are transmitted through the bite of female phlebotomine sand flies and in the human host are obligate intracellular parasites of the mononuclear phagocyte system, surviving and multiplying in different macrophage subsets in many different tissues. Sub-clinical infection in partially immune humans may be an important source of parasites when sand flies are active. Other mammals, often canids, either domesticated or wild, act as an additional zoonotic reservoir of Leishmania infantum. Patients with VL develop splenomegaly, irregular febrile episodes, anaemia, pancytopaenia, weight loss and weakness progressively over a period of weeks or even months.
The incidence of Leishmaniasis shows an important increase over the last decades due (i) to failing preventive and therapeutic measures in developing countries as a result of insufficient/inefficient control of vector and reservoir, and emergence of drug resistant parasites, (ii) to urbanization and climate changes that expose naïve populations to infected Sand flies in previously unaffected areas, and (iii) to Leishmania-HIV co-infections and anthroponotic transmission by needle sharing in the industrialized world [4]. In consequence, Leishmaniasis has been declared by the World Health Organization (WHO) as a category I neglected disease for which no vaccine and no efficient, safe, and affordable treatment is available. The need to search for more effective medicine to treat Leishmaniasis was highlighted by the WHO during the

60th World Health Assembly in March 2007. The 193 Member States recognized Leishmaniasis as one of the most neglected tropical diseases and approved a resolution on the control of this disease. Member States were urged to find alternative safe, effective and affordable medicines to treat VL, which translated into specific FP7 calls on anti-trypanosomatid drug development.
During the infectious cycle, trypanosomatid pathogens of the genus Leishmania alternate between the insect promastigote stage and the vertebrate non-motile amastigote stage that proliferates inside infected host macrophages provoking the pathology of the disease. Amastigote development and growth are highly dynamic events regulated by signaling cascades involving (i) extracellular signals encountered in the host cell phagolysosome such as pH and temperature, (ii) stage-specific signaling molecules that are able to sense these cues such extracellular-regulated kinases, and (iii) downstream phosphorylated substrates that are potentially implicated in virulence and pathogenicity. LEISHDRUG used a highly interdisciplinary approach to reveal Leishmania signaling molecules associated with amastigote virulence, with the major aim to exploit parasite-specific pathways for anti-leishmanial drug development. Translating results to clinical applications was our ultimate goal.
We proposed to build upon our complementary expertise in cutting edge bio-imaging and phospho-proteomic analysis to develop and use innovative drug screening concepts that have not been applied previously on parasitic systems. LEISHDRUG was based on 6 interactive scientific work packages that together propose a dual strategy for anti-leishmanial drug development. First, we utilized visual high-content screening to discover compounds capable to kill intracellular Leishmania amastigotes without deteriorating the host cell. This phenotype-based strategy relies on fluorescent parasites and macrophages as read-outs and allowed simultaneous assessment of anti-leishmanial activity and host cell toxicity under physiological conditions. Second, we applied a target-based strategy utilizing recombinant Leishmania protein kinases for inhibitor identification and structure-guided drug design. The identification of appropriate target kinases, with only limited homology to their mammalian counterparts relied on (i) in silico analysis by applying novel bioinformatic tools developed by consortium members, and (ii) in vitro assay based on their phospho-transferase activity towards recombinant Leishmania phospho-proteins.

The major objectives of this proposal were

 to screen small molecule and peptide libraries for hit compounds with leishmanicidal activity using phenotype- and target-based strategies.

 to identify anti-parasitic lead compounds and assess their pharmacokinetic profiles using cell-culture and experimental infection models for Leishmaniasis.

 to establish a novel drug target discovery pipeline with focus on Leishmania protein kinases through phosphoproteomic and genetic assessment.

 to initiate lead optimization by structure-based drug design.
Project Results:
The success of LEISHDRUG is based on the interdisciplinarity of its partners and the deployment of the research program in two phases, i.e. a first phase dedicated to the development and validation of a series of cutting edge genetics, proteomics, bio-informatics, and imaging technologies, which then were applied during the second funding period to their full potential for the discovery of anti-leishmanial hit compounds

and novel drug targets. The foreground generated by our project during the 42 months funding period is summarized below for each work package.

WP1: Development of a host-cell-based screen to identify compounds selectively killing intracellular Leishmania amastigotes. For several human neglected parasitic diseases, including the Leishmaniases, a number of factors, such as low efficacy, high cost, poor safety, poor compliance, and Leishmania resistance to the limited number of drugs, do limit the utility of existing drugs, especially in resource-poor settings. Additionally, because the evolution of Leishmania drug resistance is likely to compromise every compound delivered as single drug, there is a continuing and compelling need for new and better drugs for human Leishmaniases, including therapeutic regimens that rely on more than one drug. Between 2000 and 2006, many reports have highlighted the gaps, needs and opportunities for increased investment and activity in translational research for new product leads, one output being the FP7 call. This was a strong incentive for the different LEISHDRUG partners to synergize their efforts and know how with the objectives to control the clinically relevant Leishmania developmental stage, namely the Leishmania amastigotes within the mammal macrophages they subvert as host cells.
Within the Institut Pasteur Paris (IPP), the WP1 members have designed and validated a high-content screening approach, drawing upon (i) unique features of Leishmania amastigote propagation within primary macrophages, (ii) bio-imaging technologies, and (iii) chemical library assembly/management and screening. The IPP teams were able to integrate state of the art scientific disciplines such as developmental biology of eukaryotic parasites, from the molecular to the host organism scale, and multiple technological advances to achieve the pursued research objectives. At IPP it was established over the years that dermotropic Leishmania/L. species- e.g. L. amazonensis subvert model rodents (e.g. laboratory mice) as hosts and homogenous populations of primary macrophages as host cells for amastigote prolifertion. Homogenous macrophage populations are generated from CSF-1-responsive progenitors present in the mouse bone marrow cell population. Within these macrophages, L. amazonensis amastigotes remodel parasitophorous vacuoles (PV) where they proliferate. The resulting PVs display two features-, i.e. large size and stable acidic pH, that are tractable to live imaging-based readout assay. By selecting fluorescent probes that label the giant communal PVs and the macrophage nucleus and generating transgenic L. amazonensis expressing the DsRed 2 fluorescent reporter, the IPP WP1 members developed a powerful system to identify chemicals that selectively kill cell-cycling DsRed2 transgenic amastigotes without impairing the host cell.
After implementation of a bench-top visual screen to high content medium throughput capacity, the WP1 IPP members have developed standard operating procedures for automated sample preparation and image acquisition and established a data analysis pipeline based on robust statistical methods and tests encompassing calculation of quality metrics, phenotype classification and expert visual validation. The effective measure of leishmanicidal activity is mainly based on the ?clearance of the giant and acidic PVs?, a more or less rapid process coupled to the recovery of macrophage endosomal/lysosomal organelles? steady state features. After validation of the various processes involved in the overall screening pipeline, a series of screening campaigns on kinase-biased targeted libraries containing around 2500 compounds, provided by our LEISHDRUG partners, was conducted. SAR studies for interesting compound families including betulins, bisindolyl maleimides, isophtalates, azacarbazoles and quinolines were implemented and confirmatory screens performed for some compounds. Finally, using ex vivo amastigotes isolated from infected mice and in vitro derived promastigotes, we set up ?secondary screens? that allowed us to distinguish if intracellular parasite killing occurs through parasite or host cell

targets.
The overall procedure of the assay developed by the IPP WP1 teams has allowed selecting compounds interfering with Leishmania amastigote growth and survival within homogeneous population of model rodent primary macrophages. While closely interacting with other partners within the LEISHDRUG consortium, the IPP WP1 partners gained important new insight into Leishmania-host cell interactions at the molecular, sub-cellular and cellular levels and demonstrated how to apply cross-disciplinary research for designing robust screening assays at the level of the host organism. Thus, two main categories of related perspectives directly emerge from the integrative analysis deployed by the IPP WP1 members reflecting the dual-purpose research of the Institut Pasteur:
First, an immediate forward looking outcome of the completion of the WP1 deliverables is the selection of leishmanicidal compounds from kinase-biased libraries for future target deconvolution studies. This approach would allow the identification of the Leishmania target kinases, deciphering their functional role in intracellular infection, and developing target-specific compound optimization programs. Additionally, our understanding of the Leishmania-host cell interplay can benefit from the HCA developed by WP1 members in many ways. These includes projects based on the study of both compounds interfering with PV biogenesis and secreted molecules such as cytokines and chemokines known to act as signaling molecules on primary macrophages. The HCA assay can also serve to visualize interactions between innate and/or adaptive immune leucocytes and Leishmania infected macrophages. Altogether, these studies should provide new clues about the multilayered processes contributing to the clearance of intra-macrophagic amastigotes.
Second, another component of the collaborative studies deployed by the WP1 members, within the framework of the LEISHDRUG consortium is to promote concerted actions between chemists, physicists, engineers, computer scientists, mathematicians and biologists at the very early onset of any project aimed at reaching a better understanding of the complexity of the Leishmania developmental program in the tissues of its mammalian hosts by deciphering and characterizing complex interactions in vivo in real time. In this context, one could envision to extend our studies to viscerotropic strains of Leishmania. Indeed, it is urgent to initiate biologically sound studies to better understand how the stepwise developmental program of L. donovani, L. chagasi/L. infantum deploys over time, once skin-distant tissues such as the liver, the spleen, or the bone marrow become infected. It is now well recognized that within these three distinct tissues, the macrophage populations are much more heterogeneous than the macrophage population on which rely the dermotropic Leishmania species. In addition they experience lower oxygen tension than dermal macrophages. Thus any potential HCA involving these Leishmania species should reproduce the dynamic complexity that operates in vivo in space and time.
Teasing apart the complexity of Leishmania developmental program in its macrophage host cells allowed us translating the resulting data in a biologically sound and robust in vitro assay tractable to high content medium throughput screening. As demonstrated within the LEISHDRUG project, this innovative assay is fully adapted to the screening of small focused libraries. By combining this HCA with a global PV fluorescence-based assay, a dramatic increase in screening throughput can be achieved. This strategy will allow feeding the hit discovery pipeline for Leishmaniasis therapy with new chemical entities. Of course, the assays developed by WP1 are also suitable to detect potential synergic effects by testing the combination of more or less active compounds.
In the future, an expansion of studies relying on laboratory sand flies and rodents would provide an even better framework for unveiling and characterizing how the viscerotropic Leishmania amastigote population establishes itself in skin and distant tissues, each tissue displaying unique subsets of macrophages.

Imaging the process of inoculation and dissemination of viscerotropic Leishmania species using model rodents and laboratory-reared sand flies should indeed be explored. Imaging signaling pathways through the lens of a eukaryotic parasite such as Leishmania, will not only generate novel knowledge but also help extending the collaborative studies with other European academic and non-academic entities who understand the importance of using novel angles of investigation, generating intellectual property with respect to technology development, drug target identification and lead compound discovery.

WP2: Small/medium throughput screens of a small molecules library using recombinant Leishmania protein kinases. The aim of WP2 was to set up a target based drug discovery program. The project involved the production of selected kinases, the screening of compound libraries in order to identify molecules that would inhibit their kinase activity and have anti-leishmanial properties. Choosing kinases on the basis of their essential function does not necessarily imply that they will be easily purified or active. We encountered numerous difficulties with the purification of active MAPKs produced in bacteria. For instance, Mpk7 is a very promising target selected for its unusual structure but was impossible to purify from recombinant bacteria (see WP6). Although Mpk4 can be produced, the recombinant protein is inactive. Finally Mpk10, inactive as a full length protein, can be partially activated by truncation of the C-terminal domain. This shorter version (Mpk10-?C) shows an increased auto- and substrate phosphorylation. Nevertheless, using Mpk10-?C autophosphorylation as a mean to measure kinase activity in a drug screening failed because of the weakness of the signal. There are various ways to activate theses kinases, for example by purification of active MAPKs directly from Leishmania. As a pilot experiment, we purified FLAG tagged Mpk10 from Leishmania tarentolae amastigotes and obtained an active kinase. However given the time constraint, we could not assess whether the activity would be sufficient to develop a medium throughput assay for Mpk10. Alternatively, the identification of the upstream Mpk10 activating kinase would likely allow to generate in vitro activated Mpk10 suitable for drug screening.
In contrast, LmCK1a showed robust activity when produced in E.coli and thus was suitable for a drug screening campaign. Using similar conditions to those used for mammalian CK1, we obtained a strong activity for LmCK1a, with a specific activity of 9.8 pmol of incorporated ATP per ?g of kinase and per min. We adapted the kinase assay protocol to medium throughput drug screening and chose to screen in parallel LmCK1a with its mammalian ortholog to assess specificity. Out of 4028, we identified 128 compounds (3.7%) that inhibited LmCK1a, among which 91 showed increased potency on LmCK1a compared to mammalian CK1. This finding is surprising considering the high level of identity between the two protein kinases. The IC50 of the 45 selected compounds vary from 0.066 to 9.5 ?M (IC50: compound concentration required to decrease the kinase activity by 50%). In conjunction with partner 13, we selected eleven compounds, six with an IC50 in the nanomolar range and five with specificity for LmCK1a. Additionally, for three compounds (NSC146771, purvalanol B and indirubin 3? monoxime) we tested analogue libraries. NSC146771 and its analogues were discarded as we could not detect any anti-leishmanial activity on extracellular or intracellular parasites . The purines were abandoned as well despite their efficiency toward rLmCK1a because of their strong affinity for mammalian CK1. We showed that the purines were only weakly active on cultured parasites and highly toxic for host cells. The indirubins represent the most promising family of compounds. Out of 400 analogues tested, 202 were active against rLmCK1a, from which 103 were more or exclusively specific toward the parasite kinase. We tested 33 compounds with an IC50 ranging from 0.08 to 10 ?M on cultured and intracellular parasites. We selected several analogues that were very efficient toward cultured and intracellular parasites without being toxic for host cells. From other libraries, we identified seven compounds that affect parasite growth: 5-

Iodotubercidin, PP2, GW 5074, Rottlerin, BML-265, Gefitinib and Sunitinib. Most of them affected promastigote rather than amastigote growth, only 5-Iodotubercidin, GW 5074 and Rottlerin affect both forms. Most of the compounds were toxic for the host cells at 10 ?M and thus four compounds could be selected for further characterisation. PP2, despite its inability to block extracellular parasites growth, affected intracellular parasites very efficiently at 1 ?M. Gefitinib did not prevent axenic amastigote growth but was toxic for intracellular amastigotes at 10 ?M. This compound is particularly interesting as it has already undergone clinical trials. NSC 699479, described as a topoisomerase inhibitor, is extremely efficient at 1 ?M on both extra- and intracellular parasites. Our best compound is 5-Iodotubercidin, a general kinase inhibitor that is very potent on intracellular parasites at low concentration where it is no longer toxic for host cells. Based on these data, PP2, 5-Iodotubercidin, gefitinib, indirubins 42, 94 and 102 should be characterised further in the future.
In parallel to the screening, we used biochemical approaches to improve our knowledge on LmCK1a. We showed that recombinant Leishmania CK1a is sensitive to environmental pH and temperature. We also showed that CK1 kinase activity, detectable in L. donovani promastigotes as well as in axenic amastigotes, could account for about 40% of the total protein kinase activity. Inhibition of CK1 or CK1-like kinase activities by D4476 blocked promastigote and axenic amastigote growth and decreases the number of intracellular amastigotes in infected macrophages. This result underlines the potential role of CK1 kinases in extra- and intracellular survival during the parasite infectious cycle. The overexpression of LmCK1a tagged with v5 in L. donovani, did not affect parasite growth or cell cycle, therefore we could determine the localisation of CK1a. We found that in promastigotes and in amastigotes, CK1a shows a punctuated cytoplasmic localization, which could correspond to its localisation inside vesicles. Additionally, LmCK1a is also located along the flagella in promastigotes. We are currently generating a knockout mutant to confirm the essential function of CK1a.
Our results considerably increased our knowledge on CK1a (LmjF35.1010). Most of the existing data were obtained on promastigotes. We showed for the first time, using a chemical approach, that CK1a could play an important role for survival of intracellular and axenic amastigotes. This project also confirms that using CK1a as a drug target can lead to the identification of hit compounds that could be developed further into lead compounds. From the literature, we know that this kinase carries important virulence function as it phosphorylates human CK1 substrates to control host cell functions. However the major drawback is the high level of identity between CK1a and human CK1s. Our work clearly demonstrates that despite this homology, small molecules can discriminate between LmCK1a and mammalian CK1. For instance, the indirubin family is more potent toward LmCK1a than toward mammalian CK1, whereas the opposite is true for the purine family. The future of this project lies in the assessment of the specificity of the hit compounds we selected and their off-target effects. We will use drug deconvolution approaches to identify the targets of each compound. CK1a crystal structure analysis, which is necessary to perform drug design, is currently carried out by partner 4.

WP3: Sequence-based MAPK inhibitors and peptide carriers for drug delivery. The main goal of WP3 was to enhance the effectiveness of Leishmania kinase inhibitors discovered by the consortium through in vitro screening but acting poorly on the parasite in vivo due to either deficient membrane permeability or lack of adequate translocators. The strategy chosen to overcome these problems was to use cell-penetrating peptides (CPPs) as intracellular delivery agents, capable of entering cells by crossing their membranes in the absence of dedicated transporters. Coupling of CPPs to drugs with known leishmanicidal action but poor transcytosis properties was expected to overcome these difficulties. A second goal of WP3 was to

explore / exploit kinase inhibition by targeting sites other than the ATP-binding one, in order to achieve higher specificity. The activation loop (ALP) appeared promising in this regard, by means of peptide sequences that would compete with the native substrates, hence interfere with the MAPK signal transduction cascade. The two goals were further intertwined in the sense that, as ALP sequences are ca. 15 amino acids-long, hence intrinsically membrane-impermeable, they would need to be delivered into the intracellular space by conjugation to CPP shuttles.
The development of WP3 has allowed defining which of the CPPs described in the literature are best able to enter the Leishmania parasite, despite the hurdles posed by the special characteristics of its plasma membrane and endocytosis systems. Using Tat(48-60) and miltefosine (hexadecylphosphocholine) as typical examples of CPP and poorly absorbed leishmanicidal drug in current clinical use, respectively, the feasibility of CPP-mediated drug delivery, hence of overcoming miltefosine resistance due to poor drug accumulation inside the parasite, has been demonstrated. The approach can be easily extended to other leishmanicidal drugs with similar poor membrane absorption profiles. In a similar way, WP3 has explored the possibility of using CPP-based constructs in a pepducin-like fashion, namely to interfere with the signal transduction pathways of Leishmania. The constructs allow the internalization of peptide sequences corresponding to the activation loops (ALPs) of described Leishmania MAPKs, and can find use as new and specific reagents with applications in basic science and clinics
The results from the first goal of WP3 should hopefully have impact not only within the Leishmania community but beyond, by encouraging researchers in areas where chemotherapeutically useful drugs face difficulties due to poor absorption to develop CPP-based strategies to ferry such drugs into the target cell or microorganism. Results from the second WP3 goal are mostly applicable to Leishmania, but support the hypothesis that MAPK signaling cascades can be efficiently and specifically interfered with by means of intracellular delivery of the pertinent peptide kinase substrates.

WP4: Evaluations and validation of novel anti-leishmanial compounds in macrophage and mouse models of infection. The aims of WP4 were to perform further evaluation of active compounds identified during studies arising from WP1 and WP2, in order to inform the selection of lead molecules for further development and SAR studies. Compounds were assessed using an in vitro intracellular amastigote assay against a range of visceral and cutaneous Leishmania strains and those compounds that exhibited potent and parasite-selective activity were further tested in animal models of the disease.
To date we have received 10 compounds from Partners 1, 10 and 13. These included 3 Paullone derivatives, 5 CDK inhibitors of unknown structure and 2 compounds,
PKRC013-F4 and PKRC013-F5. We have tested all of these compounds against an Ethiopian reference strain L. donovani HU3 in an intracellular amastigote assay using murine peritoneal macrophages. Only 2 candidates, PKRC013-F4 and PKRC013-F5, were confirmed as active ?Hits? and gave IC50 values of 4.24 and 2.54 µM respectively. This level of activity is comparable to that observed with the standard drug miltefosine and could be replicated against other L. donovani strains including a recent clinical isolate from the Sudan (SUKA001) and a SbV resistant strain isolated in India (BHU1). Activity against cutaneous strains was less consistent being comparable to miltefosine against L. major JISH118 (IC50 values of 4 and 7.5 µM for PKRC013-F5 and PKRC013-F4 respectively) but only moderate activity was observed against L. amazonensis DSred2 and L. mexicana M379, whilst no activity was observed against L. tropica AO21/p. PKRC013-F5 was more active than PKRC013-F4 against all of the strains tested. Further studies are now being carried out at LSHTM to investigate the efficacy of these compounds in visceral and cutaneous models of the disease.

In contrast to the PKR compounds, most of the cyclin dependent kinase (CDK) inhibitors provided by Partners 10 and 13 were only active against L. donovani HU3 at concentrations that were toxic to mouse peritoneal macrophages. The Paullone derivative KUREI 148 was not toxic to macrophages at concentrations up to 30 ?M but was only moderately active with an IC50 of approximately 40 ?M. Previous work has clearly demonstrated that CDK inhibitors such as the Paullones and indirubins, can inhibit the Leishmania-equivalent kinase, CRK and reduce Leishmania amastigote burdens in infected mammalian macrophages, however, these two classes of compounds did not appear to be selective for the parasite kinase. Our results would appear to confirm that the Paullones and indirubins are indeed targeting the mammalian rather than the parasite CDK and are therefore not good candidates for further development as antileishmanial drugs because of their probable toxicity in vivo.

In addition to the compounds identified and supplied by FP7 Partners, 12 commercially available protein kinase inhibitors have been tested for activity against L. donovani HU3 in our in vitro assay. A list of drugs that are currently approved by the FDA for use in human cancer was compiled at LSHTM and 10 of these compounds were sourced and purchased. Of these, 3 compounds, sunitinib, lapatinib and sorafenib were identified as active against L. donovani HU3 intracellular amastigotes in vitro with IC50 concentrations of 1.1 2.5 and 3.7 µM respectively. No toxic effects were observed against macrophages, indicating a good selectivity for parasite killing rather than toxicity for mammalian cells and subsequent experiments in L. donovani HU3 infected BALB/c mice, showed all 3 compounds could reduce liver amastigote burdens by 30-40% in comparison with untreated controls without any apparent side-effects. Sorafenib also showed some activity against the cutaneous strains, L. major JISH118, L. mexicana M379 and L. amazonensis DSred2 but was not active against L. tropica AO21/p.

The Rho-kinase inhibitor, Fasudil, which has been used in the clinic as a vasodilator and PP2, an Src family protein kinase inhibitor, were also tested in our in vitro screens. These compounds are both commercially available but are not approved for human use by the FDA. Neither was shown in our in vitro assay to be active against the visceral or cutaneous strains of Leishmania tested. PP2 was shown by Partner 1 to be active at 1 ?M against intracellular L. amazonensis DSred2 amastigotes in bone marrow derived macrophages using the high-throughput fluorescent imaging (OPERA) assay. These apparently contradictory results could be a reflection of our assay system which will not pick up compounds with low levels of activity that produce only a marginal reduction in the numbers of amastigotes/cell, but could also highlight the role of the host cell in determining the efficacy of a potential drug candidate, which warrants further investigation. Despite the lack of activity observed in our in vitro assays, PP2 gave a 53% reduction of liver amastigote burden compared with untreated control mice when tested against L. donovani HU3 in a BALB/c mouse model of visceral Leishmaniasis.

We have identified 4 compounds which have good levels of activity against L. donovani HU3 both in vitro and in vivo, without showing any significant toxicity towards the mammalian host. To our knowledge this is the first demonstration that protein kinase inhibitors can be used to inhibit the growth of Leishmania parasites in an animal model and 3 of these compounds, sunitinib, lapatinib and sorafenib have the advantage that they are already approved by the FDA for the treatment of human cancers. These active compounds PP2, sunitinib, lapatinib and sorafenib are structurally diverse compounds which could provide template molecules for initiating structure-activity related studies and aid in the design of more potent and selective analogues. Sunitinib and sorafenib are both inhibitors of platelet derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) and stem cell growth factor receptor (c-KIT). Both compounds contain a carboxamate group but otherwise have few functional groups in common. In contrast lapatinib is a quinazolineamine which targets epidermal growth factor receptor

(EBBR2/HER2), but two similar quinazolineamine compounds which also inhibit HER2, erlotinib and gefitinib, were not active against L. donovani HU3 in murine peritoneal macrophages.

WP5: Proteomic identification of atypical Leishmania kinases and kinases targeted by lead compounds. The ultimate goal of WP5 was to identify druggable Leishmania protein kinases through a series of complementary genetics and proteomics approaches. Three partners were involved and their respective S&T results are summarized below.

Partner 1 (Institut Pasteur, Spaeth lab) developed a novel conditional null mutant system that allows to genetically proof the essential nature of any target protein, thus establishing an important tool for drug target validation. We tested various more classical conditional knock out systems that are based on the transgenic expression of an inducible copy of the target gene. First, we obtained the constructs of the TET-inducible expression system from Jena Bioscience (Germany) and established triple-transgenic parasites expressing the T7 polymerase, the TET repressor and the target kinase (in this case LmaMPK10) under the control of the tet-operator. Unfortunately we had to abandon this system as control of expression was very leaky. We next established a conditional expression system based on the FKBP destabilization domain (ddFKBP, kindly provided by Stephen Beverley, WashU, USA), which promotes degradation of dd-fusion proteins. While expression of a ddYFP control was efficiently regulated (i.e. stabilization occurred in the presence of SHIELD or FK506 and protein degradation in the absence of this ligand), neither expression of ddMPK10 nor ddCYP40 yielded satisfactory results, i.e. protein degradation was incomplete. In addition, adding the destabilization domain bears the risk to modify activity and interaction of the target protein and thus further limits the applicability of this technology. Finally, we tested a third method based on transgenic expression of a negative selectable marker, the thymidine kinase gene of herpes simplex virus (hsv-tk), which renders parasites susceptible to the antiviral drug ganciclovir (GCV). We used a vector containing the hsv-tk gene and a GFP reporter cassette, which allows monitoring the loss of the episome by FACS analysis during negative selection. In this system, the endogenous alleles of the target gene are deleted by homologous recombination using transgenic parasites that over-express the target gene from the negative selectable vector. Ganciclovir is added to the culture medium of these conditional KO parasites. The loss of the vector driving episomal target gene expression is assessed by the reduction of the GFP fluorescence. This provides a binary readout for the requirement of the target gene for parasite survival (i.e. persistence of the vector in the presence of ganciclovir demonstrates essential nature of the gene; loss of the vector in the presence of ganciclovir demonstrates viable null mutant phenotype) (Morales et al, PNAS 2010). This system allowed us to generate a conditional knock out for the essential MAP kinase LmaMPK4 thus validating this protein kinase as drug target (Dacher et al., in preparation). The system is currently applied on the genetic analysis of the LmaMPK7 and LmaMPK10 (see WP6) as well as the Leishmania casein kinase 1 alpha (see WP2).
In addition, Partner 1 applied a series of qualitative and quantitative phosphoproteomics analyses to reveal amastigote-specific phosphorylation events that may inform on novel drug targets. A classical 2D electrophoresis approach was used to investigate qualitative changes of the phosphoproteome of the major L. donovani life cycle stages, establishing the very first non-exhaustive repertoire of the Leishmania phosphoproteome and identifying 73 new phosphoproteins across the two life cycle stages (Morales et al, Proteomics 2008). Stage-specific protein phosphorylation events were revealed by quantifying changes in phosphoprotein abundance across the parasite life cycle stages utilizing 2D-DIGE (Morales et al., PNAS 2010). The phospho-2D-DIGE analysis revealed that 38% of the Leishmania phosphoproteome showed

statistically significant (p<0,05) stage-specific differences with a strong bias towards increased protein phosphorylation in amastigotes. Amastigote-specific phosphoproteins were nearly exclusively implicated in chaperone function, including the co-chaperones HOP/STI1, cyclophilin 40, and various isoforms of HSP90, HSP70, and related heat shock proteins. We confirmed the stage-specific increase in phosphorylation stoichiometry for these proteins using quantitative Western blotting analysis. We identified a unique phosphorylation site in highly conserved Leishmania HSP90 utilizing TiO2 enrichment of tryptic peptides isolated from the 2D gels and MALDI-TOF/TOF MS/MS analysis. Utilizing 2D Blue Native electrophoresis in combination with immunoprecipitation and MS-MS analysis, we revealed the presence of STI1-containing heat shock (HS) complexes, and showed their interaction with ribosomal client proteins in an amastigote-specific manner. Genetic analysis of STI1/HOP phosphorylation sites by plasmid shuffle in conditional sti1-/- null mutant parasites revealed two phospho-serine residues that were essential for parasite viability, thus defining the respective stress kinases as drug targets. We increased the resolution of our phosphoproteomics screen and revealed the phopshorylation sites of Leishmania phopshoproteins by LC-MS/MS analysis of IMAC-enriched phosphoprotein extracts, which identified 445 putative phosphoproteins shared between two independent biological experiments (Hem et al., Proteomics 2010). Functional enrichment analysis allowed us to gain insight into parasite pathways that are regulated by protein phosphorylation and revealed significant enrichment of the amastigote phosphoproteome in biological processes associated with protein turn-over, stress response and signal transduction. LC-MS/MS analysis of TiO2-enriched phosphopeptides confirmed these results and identified 157 unique phosphopeptides covering 181 unique phosphorylation sites in 126 distinct proteins. Combining phosphoproteomics and bioinformatics approaches allowed us to reveal a surprising divergence in protein phosphorylation across related trypanosomatids, suggesting the evolution of highly species-specific regulatory mechanisms and signal transduction pathways. It is conceivable that these unique signalling events have evolved in response to environmental constraints that are specific for each parasite species, such as intracellular versus extracellular proliferation or cutaneous versus visceral infection. The identification of Leishmania-specific phosphorylation sites in chaperones and HSPs, absent from highly conserved human orthologs, defines the Leishmania stress response as a prime target for drug development. This response is tightly linked to intracellular development and survival of the pathogenic amastigote stage. Hence, interference with chaperone phosphorylation through inactivation of the respective protein kinases by genetic or chemical approaches may have important consequences on Leishmania infectivity.

Finally, Partner 1 established a gel-based in gel activity assay to monitor and ultimately identify protein kinases that use these stage-specific phosphoproteins as substrates, with the aim to identify new drug targets that will feed into the LEISHDRUG pipeline (Schmidt-Arras, J Proteomics 20101). To this end, an in-gel kinase assay (IGKA) was combined with 2D electrophoresis to trace phospho-transferase activities in parasite extracts. In this assay, CyDye-labelled protein extracts are separated by IEF and SDS-PAGE using second dimension gels that have been co-polymerized in the presence of recombinant substrate. Following separation, proteins are renatured and gels are subjected to a kinase assay in the presence of ? 32P] ATP. Protein kinases that phosphorylate the embedded substrate are visualized by autoradiography and indentified by MS. This assay has been successfully applied and allowed us to identify 13 proteins with auto-phosphorylation activity (including HSP83, HSP70, LmaMPK10, and the pyruvate kinase LinJ35_V3.5450) using SDS gels without substrate. Signal intensity was improved using gels with copolymerized canonical substrates MBP and ?-casein, and the parasite-specific substrates CyP40 and LmaMPK10, and revealed various protein kinases that specifically phosphorylate these recombinant

substrates. The respective kinases are currently identified using a classical biochemical approach that combines the IGKA with FPLC fractionation of amastigote extracts in order to reduce the complexity of the protein sample and enrich for the kinases of interest.

Partner 5 (ITT, Zilberstein lab) performed LC-MS/MS analysis of phosphoprotein fractions obtained from various time points during the pro- to amastigote differentiation process with the aim (i) to gain insight into developmentally regulated processes that are necessary for intracellular infection and thus qualify as novel drug targets, (ii) to identify amastigote-specific protein kinases and substrates that qualify as potential new drug targets, and (iii) to investigate the druggability of the specific candidates. Partner 5 has used a Leishmania host-free system, developed by the Zilberstein laboratory that simulates promastigote to amastigote differentiation, to screen for stage-specific phosphoproteins. We employed quantitative proteomics approaches based on affinity tagging of peptides (iTRAQ), phosphoprotein enrichment using titanium dioxide followed by LC-MS/MS analyses. This method yielded 1700 phosphorylation sites in a few hundreds proteins. In collaboration with the bioinformatics partners of the consortium, novel Leishmania-specific phosphorylation motifs were discovered. To select for candidate amastigote-specific phosphoproteins and protein kinases, we performed differentiation phosphoproteome time course analysis during stage differentiation. Of these, we selected a few protein kinases whose phosphorylation is triggered specifically by differentiation signals (i.e. acidic pH and 37°C). To date, we have identified 4 protein kinases that meet these criteria and will further assess their value as anti-leishmanial drug targets. In addition we identified two key enzymes that are phosphorylated during differentiation and are essential for Leishmania survival; nucleolar RNA helicase and LdCDC20. The helicase is an essential enzyme whose phosphorylation changes during differentiation, but up-regulates in amastigotes. LdCDC20 is involved in regulation of entry to G1 phase in the cell cycle. Thus, this work has provided the largest database of Leishmania phosphoproteins, led to the discovery of Leishmania-specific phosphorylation motifs, and improved our knowledge of Leishmania development inside its host

Partner 11 (Photeomix, Iain Pemberton) carried out phosphopeptide signature screens, lead compound target identification and kinase druggability tests by SELDI-TOF protein array, and Activomics®analyses. Hit to target identification and validation are essential components in the pharmacological evaluation of novel drugs based on screens for cell-specific toxicity. This work sought to develop the pharmacological and molecular tools to define the mode of action of novel anti-Leishmania compounds selected from the macrophage-amastigote based screen. In particular, innovative approaches towards the identification of druggable kinases and kinase pathways were developed. The two main approaches combined (i) the use of temporal phosphoproteome signatures to study the effect of kinase inhibitors on protein phosphorylation versus markers of apoptosis and cell death and (ii) a novel kinome profiling approach (Activomics®). Activomics® was introduced as a replacement for the lead-compound target identification-protein immobilisation approach due (a) to the lack of lead compounds available for chemical coupling to agarose beads but also (b) after the preliminary development of the assay had revealed the predominance of non-kinase (ATP-binding) proteins in pull-down analyses, in part due to the formation of large protein-protein complex formation between multiple Leishmania kinases and their chaperones.
We have developed and standardized an assay for the analysis of temporal and sustained changes in protein phosphorylation patterns to define biomarker signatures that distinguish critical events affected by lead inhibitors and leading to Leishmania cell death. The assay relies on the enrichment of Leishmania phosphoproteins by Ga3+-NTA columns, phosphoproteome immobilisation on H50 SELDI protein chip

and detection by SELDI-TOF mass spectrometry (Foucher et al., 2010). We first analyzed the temporal evolution in the phosphoproteome signature associated with development and maintenance of amastigotes in a quantitative manner using differential analysis and cluster pattern analysis (Deliverable 5.4). Importantly, we demonstrated the apparent transient activation of kinase versus phosphatase activities, with kinases being significantly activated in the first 10h following induction of amastigote differentiation, whilst phosphatase activities were significantly deactivated in the following 24h hour. By contrast, differential analysis of time 0 versus 48h revealed only small quantitative differences in the phosphoprotein signatures. The analysis of the phospho-protein signatures of promastigote as they differentiate into amastigote cells confirmed the power of analyzing the phospho-proteome in a dynamic manner to quantify the activity of kinases and phosphatases, strongly supporting the need to perform kinase analysis in a temporal and differential manner.

The quantitative analysis of temporal variation in the phosphoproteome in presence of the generic kinase inhibitor compound staurosporine, revealed an overwhelming modification of phosphotransferase and phosphatase activities affecting 82% of the detectable phosphoproteome. Phenotypic and biochemical variations were measured in parallel and showed a pleiotropic effect on Leishmania cells. These included loss of motility, elongation of the cell, activation of caspase-like activity, annexin-V binding, cell arrest, etc., demonstrating the involvement of kinases in many essential cell functions. Importantly, despite the inhibition of essential cell function by staurosporine, no cell death was observed in Leishmania. Further evaluation revealed that staurosporine-sensitive kinases are implicated in the apoptosis-like pathways of Leishmania and thus these kinases do not appear to present good drug targets.

An important further contribution came in the adaptation of the proprietary Activomics®technology to the analysis of endogenous Leishmania kinase activities. Using Activomics®, we identified phosphotransferase activities inhibited by staurosporine, which included PKA and PKB but not CK1 and CK2. On the other hand, the CK1 specific inhibitor, D4476, was able to kill Leishmania cells rapidly in a necrotic-like way and the Activomics® technology revealed inhibition of CK1, but not CK2, PKB or PKA (Foucher et al., submitted). In conclusion, the kinases PKA and PKB are less desirable drug targets than CK1. Twelve lead inhibitory compounds, selected by partners 1 and 12 and which had been screened in the macrophage infected model, were analysed using the Activomics® technology to identify their inhibitory signalling pathways. None of the phosphostransferase activities currently available on the Activomics® platform was inhibited, revealing that PKA, PKB, CK1 and CK2 were not targeted by these compounds (Deliverable 2.9). Further work remains underway to increase and optimise the Activomics® Leishmania specific kinome profiling library.

By combining genetics and proteomics tests and applying novel activity based screening procedures, including 2D in-gel kinase assay and Activomics® technology, WP5 allowed important new insight into parasite-specific signaling mechanisms that are relevant for intracellular parasite survival and thus can be exploited for anti-leishmanial drug development through the target-based screening campaigns as described in WP2. Direct and indirect evaluation of endogenous Leishmania kinase and phosphatase activities in presence of lead inhibitory compounds and generic kinase inhibitor staurosporine revealed that inhibition of a large amount of kinases did not kill the parasite rapidly, but instead led to pleiotropic effects including cell cycle arrest. The implication of kinases in the apoptosis-like pathway of Leishmania suggests also that inhibition of certain kinases might not lead to Leishmania cell death and so are not desirable drug targets. The Activomics® technology has been successfully adapted to the analysis of lead inhibitory compounds on endogenous Leishmania kinase activity to identify their targets. This technology, pioneered by Photeomix, is easily adaptable to other kinetoplastids and can be multiplexed for post-translational

modification activities incorporating specific kinases, phosphatases, proteases and caspases. Thus, WP5 represents an important drug target discovery pipeline that will feed into ongoing and future drug discovery programs.

WP6: Structural studies of the Leishmania kinome and target kinases: a complementary in silico / crystallographic approach. This WP combined bio-informatics and structural approaches and gained new insight across the following three aspects of Leishmania signal transduction:

1) Protein kinase related results:
 Determination of the crystal structure of the MAPK LmaMPK10 from Leishmania major, by X-ray diffraction techniques. Structures were obtained for the apo protein as well as for LmaMPK10 bound to the inhibitor SB203580. The refined models were subjected to comparative analyses, with particular attention to its alignment with human homologues. Interesting features that distinguish the leishmanial kinase from human kinases were identified, comforting a rational drug design strategy.
 LmaMPK10 was observed to be more similar to p38 than to other human MAPKs. However, significant differences could be identified in the catalytic pocket, as well as in potentially regulatory sites in the N-terminal lobe. The modified pocket architecture in LmaMPK10 precludes DFG-in/DFG-out regulatory flipping as observed in mammalian MAPKs. LmaMPK10-nucleotide association was also studied, revealing a potential C-terminal auto-inhibitory mechanism.
 Extensive and systematic efforts were devoted to obtaining soluble recombinant LmaMPK7 in sufficient amounts as to launch structural studies. Although this proved to be a challenging task using bacterial systems (a number of E. coli strains), protocols to transfect Drosophila S2 cells for over-expression in a eukaryotic host system have been established (expression yields are currently being optimized).
 Protocols for recombinant protein production/purification have started to be explored, aiming to obtain new kinome targets (LmaCK1 and LmaMPK4) for structural studies. Preliminary results are encouraging in the case of casein kinase 1, although sufficient material is not yet available for immediate screening of crystallogenesis conditions.
 We used the results of the in-gel kinase assay developed by partner 1(IP) to perform a GO term enrichment analysis (Schmidt-Arras et al., 2011). The experiment aims at the identification of proteins with a phosphotransferase activity. Accordingly, the analysis of the proteins identified in the assay revealed a highly significant over-representation of proteins with nucleotide or ATP-binding activity, including protein kinases, ATPases, or the guanine nucleotide-binding protein beta subunit-like protein. Likewise, our dataset was enriched for the GO term unfolded protein binding reflecting the presence of chaperones and heat shock proteins with ATPase activity.
 The kinase annotation pipeline did not reveal new atypical Leishmania specific protein kinases but gives an up-to-date description of the Leishmania kinome.
 To overcome the methodological limitations encountered for the classification of the 208 Leishmania infantum protein kinases, we developed a new method based on the concatenation of paralogous Multiple Sequence Alignments (MSA) and the construction of a phylogenetic tree with PhyML. For the task of evaluating our classification, we verified the agreement of our tree to the results of Parsons et al. [BMC Genomics, 2005]. Indeed, we believe that a classification as accurate as the previous one but extended by new sequences as is in 2010 is a valuable update for the scientific community. The single species MSAs, the concatenated MSAs, as well as the most accurate classifications are available at the following address http://www.tcoffee.org/Projects_home_page/LEISHDRUG

 A kinome comparative analysis has been realized in order to guide the prioritization effort of the consortium. By comparing the human vs Leishmania infantum against the Leishmania infantum vs Leishmania major conservation, WP6 has provided the consortium with a prioritization tool that allows rational selection of either drug targets or kinases whose analysis is likely to provide important biological insights. In the light of these results, the most promising kinases were the casein kinase 1 (LinJ.35.1030 isoform 1 and LinJ.35.1020 isoform 2), the mitogen-activated protein kinase kinase (MKK2, LinJ.13.0280) a ser/thr protein kinase (LinJ.29.2140) and the cdc2-related protein kinase (CRK3, LinJ.36.0600).
 Detailed analysis of these results shows the human kinome to be more similar to the Leishmania one as compare to the plant and the yeast kinomes. A detailed comparison of the Leishmania protein kinases and their most similar human homologues revealed that, while they are all highly conserved between Leishmania species, protein kinases belong to 3 different groups with respect to their similarity to human proteins. The first group contains housekeeping proteins very conserved across all eukaryotic species. The second group is composed of proteins showing a similarity on the average. From a drug development point of view these kinases are the most interesting ones because the conservation in Leishmania should indicate an essential function while the relative proximity with human protein kinases increase the probability that a specific inhibitor could be derived from an existing compound. The last group is composed of kinases specific to Leishmania species which therefore are good targets for drug development if their essentiality is established.

Phosphoproteomics results
 The experimentally defined phosphorylation sites (P-sites) revealed by LEISHDRUG have been gathered into a NoSQL database accessible at the following address: http://leishdb.crg.cat/.
 Thanks to phosphoproteomics data resulting from experiments conducted by partners 1(IP) and 5 (IIT), we developed PhosTryp, a Leishmania-specific phosphorylation site predictor (Palmeri et al., 2011). PhosTryp achieved a 17% improvement in prediction performance compared with Netphos, a non organism-specific predictor. The analysis of the peptides correctly predicted by our method but missed by a classical method (Netphos) demonstrates that PhosTryp captures Leishmania-specific phosphorylation features.
 We used known kinase-substrate interactions in higher eukaryotes (PhosphoSitePlus) to predict interactions in Leishmania. For each experimental Leishmania P-site we predicted what kind of protein kinase may catalyze the phosphorylation. All results are available at the following address http://www.tcoffee.org/Projects_home_page/LEISHDRUG. The level of phosphorylation for each P-site from partner 5 has been followed along differentiation, from promastigote to amastigote, at 6 different time points. We hypothesize that P-sites phosphorylated by the same kinase should follow similar time course profiles. Indeed, we found that 3 P-sites belonging to the proteins LinJ.26.0800 LinJ.10.0130 and LinJ.35.4450 all hypothetical proteins, have been clustered together because of the high similarity of their phosphorylation time courses and have all been predicted to be phosphorylated by the casein kinase 2.
 We performed an analysis of the conservation of Leishmania phosphorylation sites identified in experiments performed by partner 1 (IP) (Hem et al., 2010). Several interesting observations emerge from this analysis. First, a substantial number of phosphorylation sites are not conserved between L. infantum and related L. major. Second, the number of conserved phosphorylation sites is substantially reduced between L. infantum and L. braziliensis, suggesting that parasites belonging to the Leishmania viannia complex have evolved species-specific regulatory mechanisms distinct from other new world and old world Leishmania species. Third, as judged by the presence of orthologs and conserved phosphorylation sites,

L. infantum is more closely related to T. cruzi than T. brucei or T. vivax. A similar cluster analysis between L. infantum and higher eukaryotes, including human, can lead to information on regulatory sites that may be exploitable for the design of highly parasite-specific intervention strategies. More than 40% of our data set represents parasite-specific proteins that are absent from the human genome. Another source of potential drug targets is represented by the 27 experimentally validated phosphorylation sites corresponding to 19 annotated Leishmania IDs that find a highly conserved ortholog in the human genome, which, however, lacks the Leishmania phosphorylated residues.

Identification of parasite-spceific protein features
 We identify specific differences in key Leishmania proteins that can be used as drug targets. For example, Cyclosporin A (CsA) was shown to have an anti-microbial activity against a variety of protozoan pathogens, including Leishmania. CsA binds to a family of proteins termed Cyclophilins (CyPs). We therefore performed (Yau et al., 2010) the first genome-wide analysis of Leishmania Cyclophilins. Multiple sequence alignment and cluster analysis identified 17 Leishmania CyPs with significant sequence differences to human CyPs, but with highly conserved functional residues implicated in PPIase function and CsA binding. Identification of 5 out of 17 Leishmania CyPs with a highly conserved CsA binding motif strongly suggests inhibitor-binding to multiple CyPs with potentially important consequences on the biological functions of these proteins and Leishmania infectivity. Moreover structural modeling identified Leishmania CyP40 as a possible target for CsA, based on conservation of key protein-ligand interactions. This prediction was subsequently experimentally validated by partner 1(IP).
 L. major Casein Kinase 1 (CK1) reacts like a human CK1 of the type alpha. Indeed, like hsapCK1a, LmjCK1 is not sensible to the IC261 inhibitor. On the contrary, hsapCK1 delta and hsapCK1 epsilon were shown to be inhibited by IC261. In order to detect the amino acids that could explain this difference in reactivity, we performed a comparative sequence analysis of the 4 sequences searching for identical residues in LmjCK1 and hsapCK1a but different in hsapCK1d and hsapCK1e. We detected 14 substitutions respecting these criteria. One of them showed a significant difference in the physico-chemical properties of the substituted residues and is situated close to the ATP binding site. Therefore we propose that the tyrosine Y51 in LmjCK1, substituted by an isoleucine in hsapCK1 d and e, is a potential gatekeeper preventing the accession to the binding site of the inhibitor IC261.

We believe that the WP6 provided to the community a set of useful tools and results in the objective of the prioritization of protein kinases as drug targets. The 3D structures of mitogen activated protein kinases (MAPKs), basically those from human origin, have contributed to understanding kinase function, boosting drug development strategies. MAPKs are involved in environmental signal sensing, and are thus expected to play key roles in the biology of Trypanosomatid parasites, which display complex life cycles and use extracellular cues to modulate cell differentiation. Despite the relevance of MAPKs in these processes, structural data were still unavailable before the start of LEISHDRUG. We have now determined the 3D structure of LmaMPK10 from L. major, both alone as well as bound to the p38-specific inhibitor SB203580. This MAPK is activated stage-specifically during the parasite life-cycle. Its 3D structure revealed parasite-specific features, particularly within the catalytic pocket and N-terminal lobe. Nucleotide-association studies further suggest a novel auto-inhibitory mechanism. Overall, these data should accelerate the discovery of molecules interfering with LmaMPK10 functions, with relevance for anti-leishmanial drug development strategies.
Potential Impact:

In the absence of economic incentive, serious Research and Development (R&D) investments into anti-leishmanial drug development lack from the portfolio of the major pharmaceutical players. LEISHDRUG established a new public-private Partnership that responded to this limitation and aimed to improve neglected disease R&D through the translation of basic Leishmania research results into pre-clinical application. Our project responded to the FP7 call on the ?development of new tools to control infections due to parasites of the Trypanosomatidae family? and had a significant impact on the following main areas:

1) Establishing an international network of European and ICPC researchers to develop new strategies to combat Leishmaniasis

The creation of the LEISHDRUG consortium had an immediate impact on reinforcing European research on trypanosomatid control and on fostering European interactions with programs and partners in developing countries. Our proposal connected leading scientists from 5 European countries (France, Spain, UK, Italy, Germany), Israel, South Korea, and two ICPC countries, Uruguay and Tunisia. LEISHDRUG thus established an important platform that coordinated the rather fragmented and insulated research efforts in the area of anti-leishmanial drug development. In agreement with the Barcelona objectives of education, training and innovation, the LEISHDRUG consortium promoted exchange of personnel, material, and knowledge. In particular, LEISHDRUG organized various cluster meetings to foster interaction between the partners, exchange information and material (vectors, recombinant bacteria, parasite strains and transgenic lines, protein extracts) and work on joint publications. Knowledge was shared with the scientific community and industrial and governmental bodies through the organization of an international symposium by Partner 1 on drug development against neglected protozoan parasites, the participation of LEISHDRUG partners at more than 50 international meetings and the publication of over 40 manuscripts. Training actions sponsored through LEISHDRUG included short term stays of LEISHDRUG members at the Institut Pasteur (Partner 1) to transfer the technologies for Leishmania cell culture and genetics to Partners 10 and 13, and the 2D-DIGE technology to Partner 4. As part of LEISHDRUG, partner 3 proposed a training program on ?Proteomics and drug design? responding to the EU effort to strengthen European research on trypanosomatid control and to foster European interactions with programs and partners in developing countries. The objective of this course was to train the participants in bioinformatics techniques applied to drug development. The program was organized at the Institut Pasteur de Tunis on 20-24 March 2012. 7 lecturers have trained 21 participants from ICPC countries where Leishmania is endemic. The participants were selected upon their interest in strengthening their research activities and developing novel tools for the control of infections due to parasites of the Trypanosomatidae family. The course was taught in English and was organized in modules constituted by computer work, demonstrations and lectures. Criteria for candidate selection have included the description of the institutional profile, and institutional plans for applying knowledge exchanged during the courses and for stimulating collaborations across an institutional network.
Thus, by transferring scientific information and excellence to ICPC partners, the present project provided important contributions to building capacities for basic research in bioinformatics, proteomics, imaging, and structure-based drug design.

2) Discovery of new classes of lead compounds for curing leishmaniasis

The major long-term impact of LEISHDRUG relies on the identification of a series of very promising novel hit compounds against Leishmania using a dual compound screening strategy. As part of the LEISHDRUG project, Partner 1 set up and validated a high content live cell-based readout assay using an advanced automated liquid dispensing facility and digital-imaging microscopy to select leishmanicidal hits. This powerful innovative drug discovery assay developed at the Institut Pasteur in a secure Biosafety Level 2 environment was successfully applied for a series of screening campaigns using kinase-biased compound libraries containing around 2500 compounds provided by LEISHDRUG partners. From initially 240 compounds identified in this screen, fifteen new hit candidates were selected after the completion of high content confirmatory screens, which display different scaffolds, including bisindolyl maleimides, betulins, isophtalates, azacarbazoles and quinolines. Thus, the WP1 team members from the Institut Pasteur Paris successfully developed and applied a miniaturized assay for high throughput screening that revealed very promising new drug candidates, which feed into current translational research projects and will jump start future drug development programs.
Applying a complementary, target-based screening procedure using recombinant Leishmania casein kinase 1a, Partner 2 identified a series of independent hit compounds that could be developed further into drug leads. From 4028 compounds screening in this assay, 11 compounds were selected for further testing as they were either very potent or very selective, including NSC146 771, Purvalanol B and Indirubin-3□-monoxime. The indirubins were the most promising compounds, showing potent and specific inhibition against recombinant LmCK1a, but also cultured and intracellular parasites without any overt toxicity toward the macrophage host cells. The variability in inhibitory activity seen with different indirubin analogues suggests that medicinal chemistry approaches applied on these compounds could dramatically improve the potency and the specificity of these compounds and therefore increase the chances to find a lead. Three promising indirubins were selected and further tested by Partner 6 in in vitro and in vivo assays, together with three other compounds that gave excellent results on intracellular parasites without showing toxicity toward the host cells: PP2, 5□-iodotubercidin and gefitinib. In addition, Partner 6 tested ten FDA-approved protein kinase drugs used in cancer treatment and the commercially available Src family kinase inhibitor, PP2 were obtained from commercial sources. These were evaluated both in vitro and where appropriate, in an in vivo model of visceral Leishmaniasis. Significant activity was demonstrated by PP2 in vivo and by sunitinib, lapatinib and sorafenib in vitro and in vivo. To our knowledge this is the first demonstration that protein kinase inhibitors can be used to inhibit the growth of Leishmania parasites in an animal model and 3 of these compounds, sunitinib, lapatinib and sorafenib have the advantage that they are already approved by the FDA for the treatment of human cancers.
In conclusion, LEISHDRUG established a very potent, multi-disciplinary screening pipeline that identified important new compounds with anti-leishmanial activity, which could translate into powerful therapeutic agents with potential broad applicability to other trypanosomatid pathogens. Efficient treatment will dramatically improve public health in disease-endemic countries. Infections due to Trpanosomatidae account for 122 000 deaths per year and over 4 million Disability Adjusted Life Years (DALY) (http://www.who.int/tdr/diseases). Reducing the burden of disease will have important consequences for the reduction of poverty and the economic development of ICPCs. This may be further enhanced by manufacture and commercialization through ICPC partners of inhibitors identified by our consortium.

3) Development of new technologies for drug target identification, validation, and characterization

LEISHDRUG developed and applied important technical tools for drug target discovery by exploiting the

multidisciplinary expertise of its consortium partners in genomics, bio-imaging, in silico biology, proteomics, peptide chemistry, and structure-based drug design.
Novel drug targets were identified using a series of complementary proteomics investigations. Quantitative gel-based and qualitative LC-based approaches allowed Partner 1 to reveal amastigote-specific over-phosphorylation of all major heat shock proteins and chaperones suggesting a post-translational regulation of the Leishmania stress response through stress-regulated protein kinases. Phospho-peptide analysis combined with multiple sequence alignment revealed that phosphorylation of these highly conserved proteins occurs on parasite-specific residues that are absent from the mammalian homolog. In a complementary, quantitative LC-based phosphoproteomic study, Partner 5 revealed 1658 phosphorylated sites representing 628 distinct proteins across both promastigote and amastigote developmental stages, and analyzed the developmental dynamics of stage-specific phosphorylation during promastigote to amastigote differentiation using titanium dioxide-enriched phosphopeptides from different differentiation stages (promastigotes at 0h, transitional stages at 2.5h 5h, 10h, 15h, 24h, and mature amastigotes at 120h). iTRAQ labeling followed by LC-MS/MS analyses identified 807 phosphorylated sites in 616 peptides that correspond to 301 distinct proteins, including six protein kinases detected at all seven time points of differentiation, some of which had multiple phosphorylation sites that showed distinct phosphorylation kinetics during differentiation and may represent interesting new drug targets. Significantly, this analysis revealed two Trypanosomatid-specific phosphorylation sites characterized by a conserved ?SF? motif, one of which appeared only in amastigotes. This novel motif might be a Leishmania-specific substrate of known kinases or a site for novel, parasite-specific protein kinases. In both cases these unique motifs might lead to the identification of novel druggable protein kinase. In addition, Partner 1 developed a novel activity-based screening approach that allows visualizing protein kinase activities on a proteomic level by combining in-gel kinase activity assay and 2D electrophoresis. This novel procedure allowed identification of proteins that are associated with amastigote ATP-binding, ATPase, and phosphotransferase activities. The two dimensional in-gel kinase assay (2D-IGKA), in combination with recombinant phospho-protein substrates identified by the previous phospho-proteomics analyses described above, provides a novel drug target discovery tool able to identify target kinases through their activity towards essential phosphorylation sites.
For drug target validation, Partner 1 developed a novel conditional null mutant system based on negative selection protocol. This approach was applied to assess the biological relevance of parasite-specific phosphorylation sites by gene deletion and complementation of the null mutant with phospho-site mutated versions of the target gene. This strategy revealed two phosphorylation sites in the co-chaperone STI1 that were essential for parasite viability validating the corresponding stress kinases as novel drug targets. The same genetic system was applied on the Leishmania MAP kinase LmaMPK4 providing the genetic proof of the essential nature of this protein, which thus is genetically defined as an important drug target.
Partner 11 developed alternative, innovative strategies for the characterization of druggable kinases and kinase pathways in Leishmania donovani cell extracts. The analysis by SELDI-TOF of the temporal quantitative variation in phosphoproteins in presence of a generic kinase inhibitor compound revealed an overwhelming inhibition of phosphotransferase activity affecting 82% of the detectable phosphoproteome. Phenotypic and biochemical variations measured in parallel showed pleiotropic effects on Leishmania cells, ranging from loss of motility to cell arrest, demonstrating the involvement of kinases in many essential cell functions. However, despite the inhibition of essential cell functions by staurosporine, no cell death was observed in Leishmania. Further evaluation revealed that staurosporine-targeted kinases are implicated in the apoptosis-like pathways of Leishmania and thus are not good drug targets.

Applying the proprietary Activomics® technology to the analysis of endogenous Leishmania kinase activities, Partner 11 identified phosphotransferase activities inhibited by staurosporine, which included PKA and PKB but not CK1 and CK2. On the other hand, the CK1 specific inhibitor, D4476, was able to kill Leishmania cells rapidly in a necrotic-like way and the Activomics® technology revealed inhibition of CK1, but not CK2, PKB or PKA. In conclusion, PKA and PKB are less desirable drug targets than CK1. Twelve lead inhibitory compounds, selected by partners 1 and 12 and which had been screened on the OPERA platform, were analysed using the Activomics® technology to identify their inhibitory signalling pathways. None of the phosphostransferase activities currently available on the Activomics® platform was inhibited, revealing that PKA, PKB, CK1 and CK2 were not targeted by these compounds.
An important contribution to validation of the Leishmania kinases as a source for parasite-specific drug target was delivered by Partners of WP6, which allowed target selection through genomic comparisons, 3D-modelling and experimental determination of 3D structures. A novel phylogenetic approach was developed based on comparative genomics in order to characterize the parasite-specific kinome and to prioritize potential drug targets in comparison with host kinomes. The resolution of the LmaMPK10 crystal structure alone or bound to the inhibitor SB203580 revealed parasite-specific structural features, which will be further tested in vitro and in vivo through structure/function studies.

In summary, LEISHDRUG synergized important national and international expertise of its academic and industrial Partners in Leishmania signal transduction and kinase-biased drug development. The project generated considerable foreground that may be exploited for intellectual property purposes, including innovative activity-based target screens, phenotypic drug screening assays, and hit compound identification using a unique kinase-biased compound library. This foreground lays the basis for our future drug development endeavors, which are aimed (i) to identify anti-leishmanial lead compounds that fulfill the major criteria of broad anti-leishmanial activity, selectivity, bio-availability, and feasible medicinal chemistry, (ii) to develop and apply novel proteomic strategies of target deconvolution to identify the parasite kinases that are targeted by these leads, and (iii) to validate the druggability of these protein kinases by biochemical and genetic approaches. LEISHDRUG thus created a powerful platform to drive translational research in the field of anti-parasitic drug treatment. It delivered considerable progress beyond the state-of-the-art with respect to the development of innovative, systems-wide drug screening concepts, and advanced our understanding on trypanosomatid signal transduction that may be exploited for the development of new therapeutic approaches.
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Documents connexes

final1-publishable-summary-and-annex-final-report.pdf

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