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Final Report

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Selection Year	2012
Host institution	Institute for Tropical Medicine, Antwerp
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Period covered by this report	from 01/04/2013 to 31/03/2015
Title of the project	Cross-talk between *Leishmania* and its host-cell: insights from chemical genetics and antimony-resistant field isolates

1. Objectives of the proposal (1 page)

2. Methodology in a nutshell (1 page)

3. Results (6-8 pages)

4. Valorisation/Diffusion (including Publications, Conferences, Seminars, Missions abroad...)

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Summary

Leishmaniasis is a neglected tropical disease with limited therapeutic options. The causing agent of this disease is the obligate intracellular parasite *Leishmania*. This parasite develops exclusively within phagocytic cells resulting in a complex molecular interplay between the parasite and its host cell.

Antimonials, like sodium stibogluconate (SSG) have been the first line treatment against leishmaniasis for decades, but have now to be replaced due to widespread drug resistance. Antimonials directly target parasites by altering their redox metabolism and antioxidant defense system, but they also indirectly affect parasite survival by increasing host cell production of toxic oxygen and nitrogen intermediates. The host institution of this mandate extensively studied the molecular diversity of *Leishmania donovani* in the Indian Subcontinent and its relation to antimonial drug resistance. Antimonial resistant (SSG-R) clinical isolates show, among other genomic adaptations, the expansion of array of genes, in particular two loci, namely the H- and the MAPK1-locus, known to be amplified in *Leishmania* in vitro induced for resistance against various drugs. SSG-R strains have in addition been shown to present a higher fitness and to be more aggressive in vitro and in vivo compared to their antimonial sensitive (SSG-S) counterpart.

The objectives of this project are (1) to understand adaptations developed by SSG-R *Leishmania* and how these adaptations affect parasite interactions with its host-cell and (2) to analyse the mechanism of action of compounds specifically active against the intracellular stage of the parasite, hence potentially targeting a host-cell pathway.

These complementary approaches have the potential to highlight novel interactions between *Leishmania* and its host which would be essential to design novel and efficient therapeutics and in particular to evaluate the relevance of targeting host-encoded factors.

First, microarray profiling of macrophages infected with SSG-R *Leishmania donovani,* compared to macrophages infected with SSG-S parasites showed downregulation of type I interferon (IFN) signaling, in particular the Interferon Regulatory Factor 7 (IRF-7), upregulation of the anti-inflammatory interleukin 10 (IL-10), as well as downregulation of p38 MAPK signaling components. These results suggested that SSG-R parasites can actively modulate their host response in order generate a better environment for their growth. The importance for parasite virulence and drug resistance, of genes amplified within the H- and MAPK-1 loci is currently being analysed.

Second, an intracellular amastigote specific compound, naloxonazine, identified in a previous study was shown to target the host-cell and therefore to indirectly affect parasite growth. This compound was linked to increased acidification of the phagolysosome which in turn inhibited parasite development. Whether such host-cell pathway could be an appropriate drug target for anti-leishmanial therapy is still under investigation.

This project will be continued through an FWO post-doctoral mandate that started in April 2015.

Key words: Leishmania, host-parasite interactions, anti-leishmanial therapy, antimonials

1- Background and objectives of the proposal

Protozoan parasites of the genus *Leishmania* are the causative agents of a wide variety of diseases that range from self-healing cutaneous or mucocutaneous lesions, to a visceral form of the disease in which parasites disseminate to internal organs. It is one of the most significant neglected tropical diseases, with an estimated 12 million people infected.
Leishmania parasites have a digenetic life cycle: switching from an insect vector, in which parasites are in the extracellular promastigote stage, to a mammalian host, where parasites are exclusively intracellular (intramacrophage amastigote stage). We focus on *Leishmania donovani*, the main agent for visceral leishmaniasis (VL) in the Indian Subcontinent (ISC).

Antimonials like sodium stibogluconate (SSG) have been the first line treatment against leishmaniasis for several decades in the ISC but their clinical value has become compromised by the emergence of resistant parasites. Pentavalent antimonials (SbV) target two different aspects of *Leishmania* biology: on one hand, SbV is reduced by the host cell into trivalent antimony (SbIII), which directly alters parasite redox metabolism and antioxidant defense system; and on the other hand, SbV indirectly affects parasite survival by increasing host cell production of toxic oxygen and nitrogen intermediates, creating additional oxidative and nitrosative stress upon SbIII-sensitized parasites (Pathak & Yi, 2001). SSG anti-leishmanial activity is therefore partly indirect, targeting host cell pathway(s) that affect *Leishmania* intracellular development. The resistance to SSG is thought to involve both aspects of its mode of action, including modulation of the expression of parasite redox metabolism critical components, expression of transporters and pumps, but also a possible acquired capacity to modulate the host cell anti-leishmanial response.

The host laboratory of this Return Mandate (Molecular Parasitology Unit, Institute for Tropical Medicine in Antwerp) has extensively studied *Leishmania donovani* molecular diversity in the ISC and its relation to antimony drug resistance. Whole genome sequencing on 204 clinical isolates differing in their response to SSG was recently completed: a clonal population of SSG-resistant parasites was identified (further called ISC005), which emerged in the 1970s and is now the most prevalent genotype in the ISC. Different genomic adaptations have been associated to the ISC005 population including non-synonymous SNPs, indels, gene amplifications and a high level of aneuploidy (Imamura et al, submitted). Several cellular functions are affected by these adaptations, potentially leading to modified parasite fitness and virulence. Some SSG-R strains from the ISC005 group have indeed been shown to be more aggressive in vitro and in vivo (Vanaerschot et al., 2011).

1- The first question addressed by this project is to understand whether the increased capacity of SSG-R *L.donovani* to colonize its host is due to an intrinsic better fitness of the parasite or to the acquisition of novel tools for modulating the host immune response. It is therefore crucial to better **understand the relationship between *Leishmania donovani* and its host-cell in general, and more particularly the specific interactions developed during infection by SSG-R strains.**

2- Anti-leishmanial treatment is jeopardized in the ISC because of resistance to antimonials, but also because of increased treatment failure observed with the more recent drug miltefosine. Alternative therapeutics are thus urgently needed. In this context and in light of the observed adaptations developed by SSG-R parasites, the second question of this proposal is to **evaluate the relevance of targeting host-encoded factors for anti-leishmania therapy**.

The two initial objectives of this project therefore were (1) to study the infection strategies developed by a drug-resistant parasite (SSG-R *L.donovani*) compared to its sensitive counterpart, and (2) to analyse the mechanism of action of compounds specifically active against the intracellular stage of the parasite, hence potentially targeting a host-cell pathway.

In addition, another question was addressed during this 2-year period, related to the initial objectives of this project as well as the ongoing activities of the host laboratory. Strains from the

ISC005 group show, among other genomic adaptations, the expansion of array of genes, in particular two loci, namely the H- and the MAPK1- locus, known to be amplified in *Leishmania* in vitro induced for resistance against various drugs (Beverley, Coderre, Santi, & Schimke, 1984; Ubeda et al., 2008). Both these amplicons contain several genes potentially involved in virulence and drug resistance. The additional objective covered within this project is to understand **why these particular sets of genes are amplified and what advantages could they confer to the parasites in terms of infectivity/virulence and drug resistance.**

2- Methodology

2-1 Cells and culture conditions

The host institution possesses a unique collection of *Leishmania* field isolates. Two *L. donovani* strains were selected for this study: SSG-S 'MHOM/NP/02/BPK282/0cl4 and SSG-R 'MHOM/NP/03/BPK275/0cl18' (further referred to as SSG-S BPK282 and SSG-R BPK275); They have been respectively isolated from a patient cured following SSG treatment and from an SSG non responder patient. These strains were shown to be genetically highly homogeneous differing by only 160 SNPs. SSG-S BPK282 belongs to the ISC006 subpopulation and SSG-R BPK275 to ISC005.

Parasites were maintained as promastigotes at 26°C in HoMEM medium supplemented with 20% fetal bovine serum. Same numbers of stationary phase promastigotes of SSG-S BPK282 and SSG-R BPK275 were used to infect the THP-1 macrophage cell line in vitro (ATCC TIB-202). After 24h or 48h of infection, infected cells were harvested for further analysis or fixed with methanol and Giemsa stained to monitor the number of intracellular amastigotes.
Promastigote parasites were transfected by electroporation with 1ug DNA (pLexsy-GFP or pLexsy-mCherry), transfected parasites were selected by addition of a selective drug (hygromycine) to the culture media.

2-2 RNA extraction and microarrays

RNA was extracted from THP-1 infected macrophages with the RNAqueous-Micro Kit (Ambion), samples were DNAse treated and additionally purified using a Clean and Concentrator kit (Zymo Research). The quality of the RNA was verified using Agilent Bioanalyzer RNA Integrity Number as well as 260/280 and 260/230 nanodrop ratios.
RNA samples were analysed on affimetrix Prime view human Gene Expression Arrays at the Nucleomics Facility (VIB, Leuven).

2-3 Confocal microscopy and flow cytometry

Images were taken on an LSM 700 confocal microscope (Zeiss). Flow cytometry experiments were performed on a BD-FACSVerse.

2-4 Pulsed Field Gel Electrophoresis (PFGE) and southern blot hybridization

Intact chromosomes were prepared from logarithmic phase cultures of SSG-R BPK275 (ISC005), SSG-S BPK282 (ISC006) and SSG-S BPK026 (ISC001) promastigotes and separated by PFGE using a Bio-Rad CHEF-Mapper XA at 6V/cm, 120° separation angle and a range of separation from 100 kb to 2,5 Mb. Gels were transferred by capillarity and hybridized with [α-^{32}P]dCTP-labelled DNA probes specific for MAPK1 or HTBF (H-locus) according to standard protocols.

3- Results

3-1 Differential host response upon SSG-R and SSG-S Leishmania infection

3-1-a- Growth and infectivity of SSG-S BPK282 versus SSG-R BPK275

Promastigotes of SSG-R BPK275 reached higher levels at stationary phase, in accordance with previous observations in the laboratory (Fig 1A).
As previously shown, SSG-R BPK275 also showed a higher infectivity (higher number of infected cells and higher number of amastigotes per host cell, Fig 1B), although some variability could be observed on the kinetics of infection (higher infectivity of SSG-R BPK275 more pronounced either at 24h or 48h post-infection, see point 3-2-d)

3-1-b- Microarray profiling of infected THP-1 macrophages

THP-1 macrophages were infected as described above with either of SSG-S BPK282 or SSG-R BPK275 stationary phase promastigotes. Total RNA from infected macrophages was harvested 24h and 48h post-infection and analyzed on affimetrix Prime view human Gene Expression Arrays. Data were normalized by Robust Multi-array Average, and analysed using the Ingenuity Pathway Analysis software.
A principal component analysis plot of the RMA normalized data showed that gene expression in infected macrophages varied between 24h and 48h post-infection and that variation between SSG-R BPK275 and SSG-S BPK282 infected cells was more pronounced at 24h post-infection than at 48h post-infection (Fig 2A). This is not unexpected given the infectivity profile of SSG-S BPK282 and SSG-R BPK275 48h after infection during this experiment (Fig 2B – see point 3-2-d).
Although the expression profile between SSG-R BPK275 and SSG-S BPK282 infected cells was similar (Fig 2C), interesting differentially expressed genes could be identified (Table 1).

Table 1: Pathways modified in THP-1 infected with SSG-R BPK275 compared to SSG-S BPK282 24h post infection

Pathways	Genes involved	
Interferon Regulatory Factor Type I Interferon signaling	IFIT3; IFIT1; MX1; IRF7	downregulated
IL-10	IL-10	upregulated
p38 MAPK signaling	TRAF2; TGFBR1; STAT1	downregulated
Protein Kinase C, epsilon (PRKCE)		upregulated

After 24 hours of infection, Interferon (IFN) signaling and IFN regulatory factors were downregulated in SSG-R BPK275, whereas the anti-inflammatory IL-10 was slightly upregulated. This confirms previous findings that linked the higher infectivity of SSG-R strains to increased levels of IL-10 in mouse macrophages (Haldar et al., 2010; Mukherjee et al., 2013). Interestingly recent publications showed the importance of type I IFN signaling, and particularly IRF7 for the control of *L.donovani* infection (Favila et al., 2014; Phillips et al., 2010). Downregulation of the expression of molecules from this pathway, including IRF7, in cells infected with SSG-R strains might therefore contribute to their increased infection rate. Similarly, p38 MAPK signaling and the STAT1 transcription factor are involved in macrophage activation and IFNg-dependent proinflammatory response, and their role to control *Leishmania* infection has long been recognized. *Leishmania* interferes with this pathway to prevent macrophage activation and promote its growth (reviewed in (CecÃlio et al., 2014)). The downregulation of p38 MAPK signaling component observed in SSG-R BPK275 infected THP-1 compared to SSG-S infected cells is therefore also indicative of the establishment of an environment favorable for the parasite growth.

Altogether these encouraging preliminary results suggest that SSG-R parasites possess different abilities than SSG-S parasites to actively modulate their host response and achieve better

infection. This is also in accordance with previous publications, that demonstrated the presence in an SSG-R strain but not in SSG-S strains, of a particular glycan responsible for induction of IL-10 (Mukherjee et al., 2013). Whether this glycan is present on the SSG-R BPK 275 strain used in this study has not yet been verified.

Analysis of THP-1 response upon infection with SSG-R and SSG-S parasites will now be repeated using a subset of strains (3 strains from the ISC005 group and 3 strains from the ISC006 group), to validate the observations made with BPK275 and BPK282.

3-1-c- Co-infections with SSG-S BPK282 and SSG-R BPK275

Co-infections with SSG-R BPK275 and SSG-S BPK282 were planned to further investigate the increased capacity of SSG-R parasites to infect their host and to evaluate the ability of SSG-R parasites to take over the SSG-S strain.

SSG-S BPK282 and SSG-R BPK275 were respectively transfected with pLEXSY-GFP and pLEXSY-mCherry plasmids. The pLEXSY vectors (Jena Bioscience) used for transfection allow constitutive expression of the target proteins following integration of the expression cassette into the chromosomal 18S rRNA locus (Fig 3).
Co-infections in THP-1 macrophages are currently being performed but technical problems have slowed the experiment down (see point 3-2-d). In addition to these in vitro experiments, co-infections in vivo will now be performed (mouse and hamster models of visceral leishmaniasis available in the laboratory).

3-2 Identification of host cell pathways essential for Leishmania intracellular development through analysis of anti-leishmanial small molecule activity.

Screening of 2,300 small molecules using the intracellular amastigote assay described in (De Muylder et al., 2011) has led to the identification of a unique set of compounds showing activity against the intracellular stage of *L.donovani* but inactive against the extracellular promastigote stage or axenic amastigotes. We have analyzed the mode of action of one of the identified compounds, naloxonazine, against the intracellular stage of *Leishmania donovani*.

3-2-a Analysis of naloxonazine activity against L.donovani

Naloxonazine is a Mu-opioid receptor antagonist (Fig 4A). This compound was shown to be active against the intracellular amastigote stage of *L.donovani*, but inactive against the promastigote stage or axenic amastigote forms (Fig 4B) (De Muylder et al., 2011). The IC50 of naloxonazine against intracellular amastigotes was shown to be 2.6 uM.
Interestingly, incubation of THP-1 macrophages with 10uM of naloxonazine for 1h following infection was sufficient to inhibit parasite growth, suggesting that naloxonazine affects an early step in the infection process (Fig 4C).

3-2-b Microarray profiling of naloxonazine treated macrophages – characterization of naloxonazine's effect on phagolysosome acidification

Uninfected THP-1 macrophages were treated for 4h either with 10uM of naloxonazine or with 10uM of naloxone, another Mu-opioid receptor antagonist that do not show anti-leishmanial activity. After 4h incubation, drugs were removed and macrophages were further incubated for 16h before RNA extraction. Transcriptional profiling of THP-1 treated with naloxonazine versus naloxone was analyzed on Human Exonic Evidence Based Oligonucleotide (HEEBO) arrays (Stanford University). This microarray analysis identified naloxonazine-induced upregulation of vATPases as well as actin beta genes (this upregulation in THP-1 treated with naloxonazine was confirmed by qPCR (Fig 5A)).
This observation suggested an effect of naloxonazine on phagolysosome formation or maturation.

Infected macrophages, treated or not with either naloxonazine or naloxone were incubated with the lysotracker DND-99. Fluorescence intensity was monitored by confocal microscopy (Fig 5B) and flow cytometry (Fig 5C). Naloxonazine treated cells showed an increased DND-99 signal compared to untreated or naloxone-treated cells indicating an increased number of acidic vacuoles or a decreased vacuole pH, which correlated with reduction of parasite growth.
Treatement of infected macrophages with the vATPase inhibitor concanamycin A (ConcA 100 nM) reverted naloxonazine activity: decrease of DND-99 intensity indicating a decreased number of acidic vacuoles or increased vacuole pH, which was associated to a normal infectivity (Fig 5D).

Imatinib, a compound previously shown to also decrease lysosomal pH was tested against *Leishmania* infected THP-1: Imatinib is an inhibitor of Abelson tyrosine kinase, which among other cellular functions regulates phagosomal acidification (Bruns et al., 2012). Imatinib has been shown to strengthen the antimicrobial activity of macrophages against the intracellular *Mycobacterium tuberculosis* (Bruns et al., 2012). As shown in Fig 5E, Imatinib was also active against *L.donovani* intracellular amastigotes, confirming a role for increased lysosomal acidification on the control of *Leishmania* intracellular growth.

Naloxonazine activity was therefore linked to an increased acidification of the phagolysosome that inhibited parasite development.

3-2-c Induction of parasites resistant to naloxonazine

The objective of this experiment is to analyze adaptations of naloxonazine-resistant parasites in light of the phenotype of antimony-resistant *L.donovani*, suspected to be more infective, and evaluate if a better fitness would be a general consequence observed for parasites acquiring resistance to compounds targeting host-cell pathways.

Several attempts to generate *L.donovani* parasites resistant to naloxonazine were carried out, by exposing intracellular amastigotes to stepwise increasing concentrations of the drug. Naloxonazine was shown to affect the THP-1 host cell at 25uM, this dose was therefore selected as the upper range for resistance induction. However, this induction experiment was so far unsuccessful mainly due to technical limitations observed during in vitro infections of macrophages (described in point 3-2-d).

3-2-d Technical limitations during Leishmania in vitro infections

In vitro infections are performed on macrophage cell lines or on primary mouse cells from the peritoneal cavity, using stationary phase promastigotes, which mimic the infectious metacyclics found in the sand fly vector proboscis. After 5h to 24h of contact, extracellular promastigotes that have not been internalized by macrophages are washed away, and infected cells are incubated for 24h to 96h to allow promastigote to amastigote differentiation and amastigote development. Infection levels are then estimated by counting host cells and parasites for each condition.
False positive signals may arise if extracellular promastigotes remain, they usually stick outside of macrophages and cannot easily be distinguished from intracellular amastigotes. This issue could explain the variability observed when comparing two different strains (see point 3-1), and more importantly, it impedes in vitro induction of resistance against intracellular amastigote specific compounds, those compounds being ineffective against the residual extracellular promastigotes (see point 3-2-c).
Residual extracellular promastigotes during in vitro infections of *Leishmania* is a common problem and could be overcome by slight changes in cell culture conditions (changes in serum brands, cell culture additives etc…). We are currently testing serums and cell culture additives to improve in vitro infections results.

3-3 Analysis of MAPK and H-locus amplification in isolates from the ISC

The amplification of the H-locus as an extrachromosomal episome has been extensively observed after in vitro induction of resistance against methotrexate or trivalent antimonials in various *Leishmania* strains (Ubeda et al., 2008). This amplification was shown to be maintained upon drug pressure but was lost in the absence of drugs.

In *L.donovani* clinical isolates from the ISC, the same locus as well as the MAPK locus were observed to be amplified in variable numbers. While one population of SSG-S parasites (ISC001) did not show amplification of these specific loci, the ISC005 population of SSG-R parasites showed the highest copy numbers of both H-locus and MAPK amplicons. A direct correlation between these loci copy numbers and SSG resistance has however not yet been demonstrated.

3-3-a Determination of the nature of loci amplification

We used pulsed field gel electrophoresis and southern blot hybridization to determine whether the amplification of the H- and MAPK loci was extrachromosomal (circular episomes) or intrachromosomal tandem duplications.

PFGE profiles of chromosomes from the ISC005 SSG-R BPK275 and ISC006 SSG-S BPK282 (both showing increased copy numbers of H- and MPK1- loci) were compared to profiles of chromosomes from the ISC001 SSG-S BPK026 (that did not present amplification of any of the loci mentioned above). No extrachromosomal copies of these loci were observed, suggesting intrachromosomal tandem duplications (Fig 6).

3-3-b Analysis of the importance of amplified genes for parasite virulence and drug resistance

The amplified H- and MAPK loci respectively contain four and three open reading frames, coding for the ABC-thiol transporter MRPA, the argininosuccinate synthase, the terbinafine resistance and a hypothetical protein (H-locus); and the MAPK, an Acidic phosphatase and a hypothetical protein (MAPK locus). Interestingly some of these genes have previously been associated with virulence or drug resistance (Lakhal-Naouar et al., 2012; Wiese, 1998).

To further characterize these genes and understand the importance of their overexpression for parasite virulence or drug resistance, they were cloned in the pLEXSY *Leishmania* expression vector and individually overexpressed in a ISC001 strain in which the amplicons are absent (BPK026). A master student under my supervision during the academic year 2014-2015 started the analysis of the overexpressing strains and found increased resistance to trivalent antimonials (SbIII) for strains overexpressing MAPK, while strains overexpressing one of the hypothetical proteins were more sensitive to SbIII. How these results translate into resistance to pentavalent antimonials is not yet known. The analysis of the infective capacity of the overexpressing strains is still ongoing, in vitro and in vivo.

4. Valorisation/Diffusion (including Publications, Conferences, Seminars, Missions abroad...)

Meetings attended:
- 1st biannual PARAFRAP conference (Alliance Française contre les maladies parasitaires), Marseille, September 2014, oral presentation entitled "Targeting host-encoded functions for anti-leishmanial therapy". This conference brings together experts in several fields of parasitology (genomics; pathogenesis and protective immunity; molecular and cellular biology as well as intervention strategies against parasites) and was an excellent opportunity for sharing our work and benefitting from the feedback of prestigious colleagues.
- WorldLeish 5 meeting, Porto de Galhinas Brazil, May 2013, oral presentation entitled "Screening against *Leishmania* intracellular amastigotes: comparison to a promastigote screen and identification of molecules targeting host-encoded functions". WorldLeish meetings cover all fields of research on Leishmaniasis –from basic research in molecular biology of the parasite to clinical

and epidemiological aspects of the disease- bringing together laboratory and field experts, which is essential to put into perspective topics such as treatment and drug resistance.
- Meetings from the Belgian Society of parasitology and protistology, November 2013 and 2014

Publications:
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Extensive and adaptive genome structure variation drives the evolution and epidemiology of visceral leishmaniasis in the Indian subcontinent. Submitted.

5. Perspectives

I was awarded an FWO post-doctoral mandate in October 2014, which effectively started in April 2015, and provides an ideal opportunity to complete the project started within this Back to Belgium mandate. The FWO post-doctoral project will also be carried out at the Institute for Tropical Medicine in Antwerp under the supervision of Jean-Claude Dujardin, and also focuses on the analysis of *Leishmania* interactions with its host, the possibility of targeting pathways involved in these interactions for treatment, and their importance when drug resistance/treatment failure is observed. This FWO post-doctoral project therefore constitutes the continuum of my Back to Belgium grant.

The study of the host response to *Leishmania* in the context of antimonial resistance was also embedded in an FWO krediet aan navorsers proposal submitted in April 2015. In this project, the adaptations developed by SSG-R *L.donovani* will be analysed by an integrated and multi-level approach (parallel analysis of transcriptome, proteome and metabolome of SSG-R versus SSG-S stationary phase promastigotes, intracellular amastigotes and host macrophage response).

The possibility to continue these projects at the Institute for Tropical Medicine for three additional years will therefore allow to strengthen my profile and become highly competitive for a future permanent position in Belgium.

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Figures

Fig. 1 Growth of L.donovani SSG-S BPK282 and SSG-R BPK275 promastigotes and amastigotes
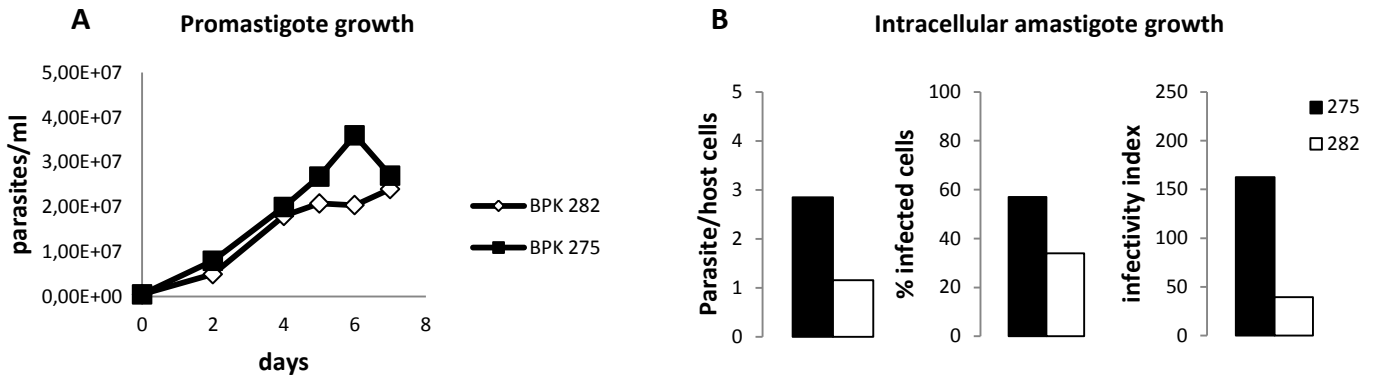
A Promastigote growth

B Intracellular amastigote growth

Fig. 2 Microarray profiling of THP-1 infected with SSG-R BPK275 versus SSG-S BPK282

A PCA of microarray normalized data

B SSG-R BPK275 vs SSG-S BPK282 infectivity

C Gene expression level of THP-1 infected with SSG-R BPK275 versus SSG-S BPK282 (24h and 48h post infection)
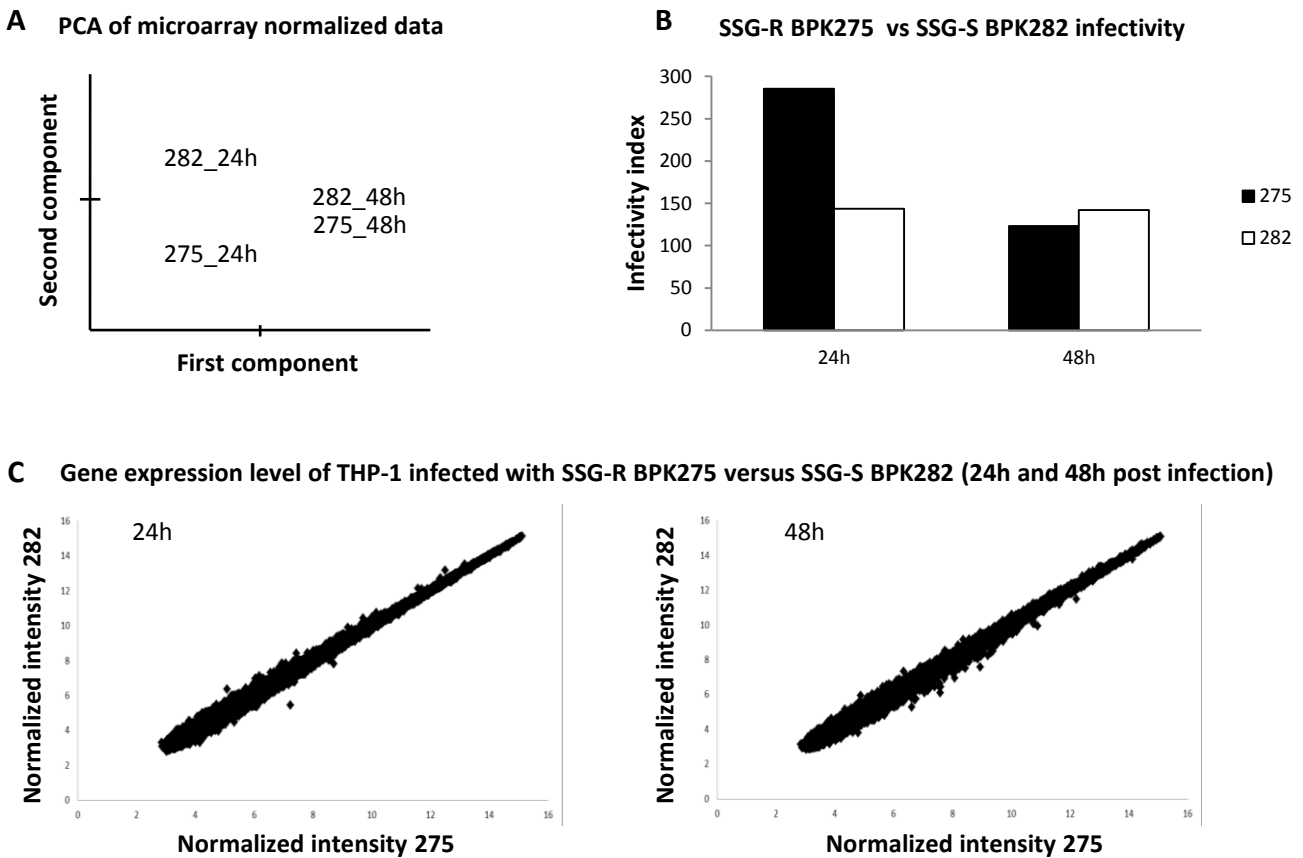
Fig. 3 GFP and mCherry expressing SSG-S BPK282 and SSG-R BPK275
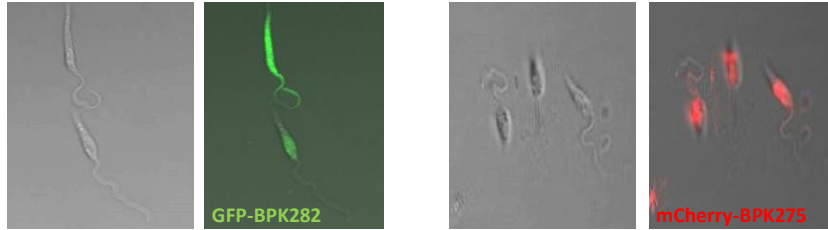
Fig. 4 Naloxonazine structure and activity against L.donovani

1- Untreated infected THP-1
2- THP-1 Naloxonazine pretreated before infection
3- THP-1 infected with *L.donovani* Naloxonazine pretreated
4- THP-1 infected then Naloxonazine treated for 1h
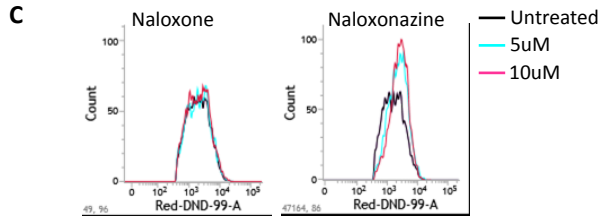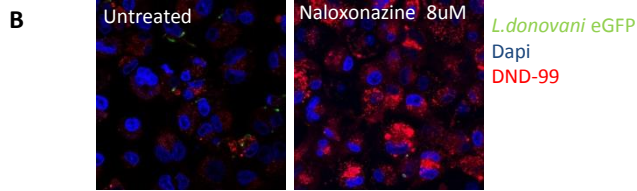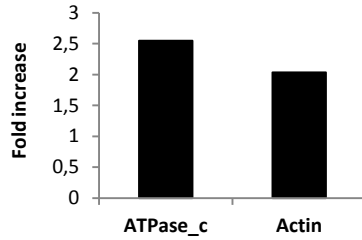5- THP-1 infected then Naloxonazine treated for 48h
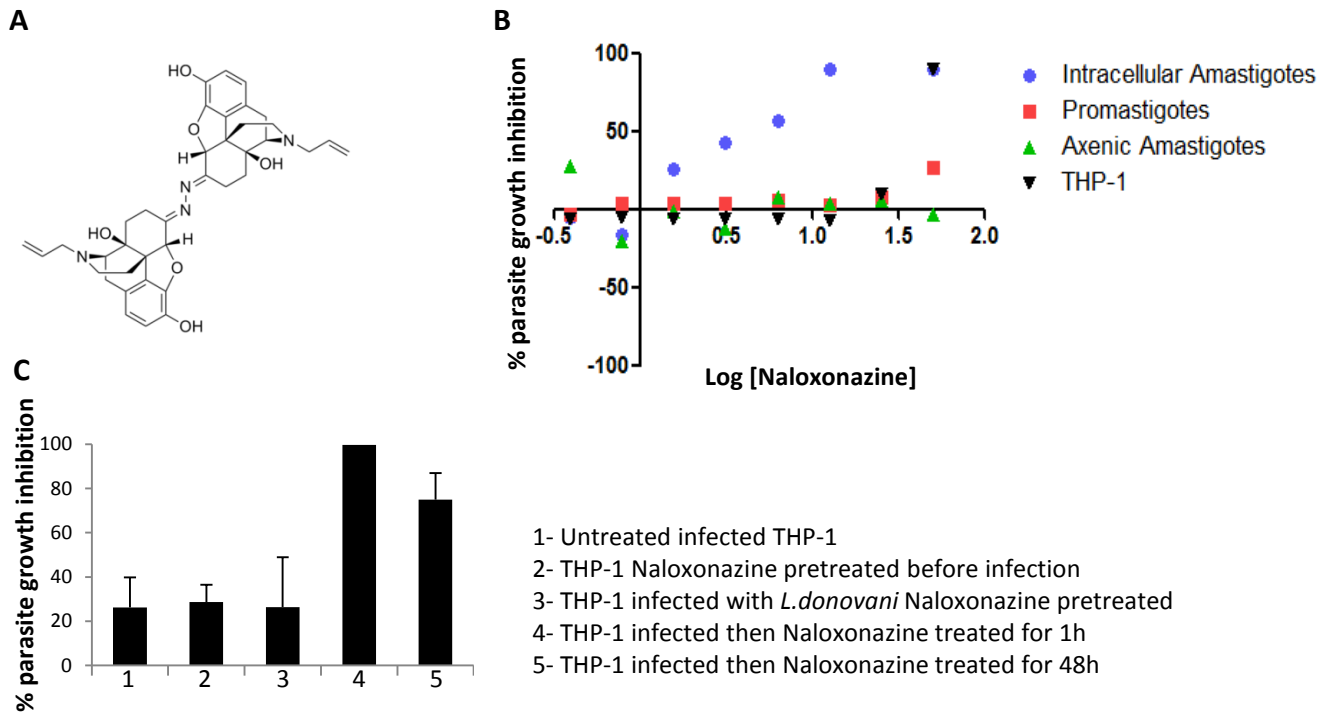
Fig. 5 Naloxonazine affects macrophage acidification of host-cell vacuoles

A   **Upregulation in THP-1 treated with naloxonazine compared to untreated THP-1**

B

C

D
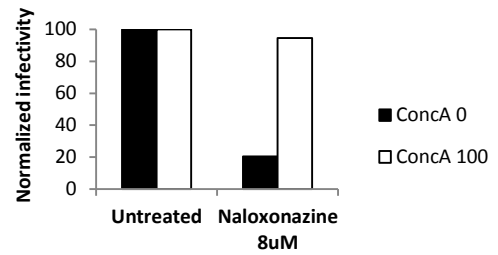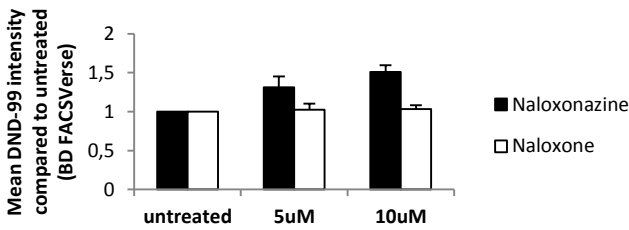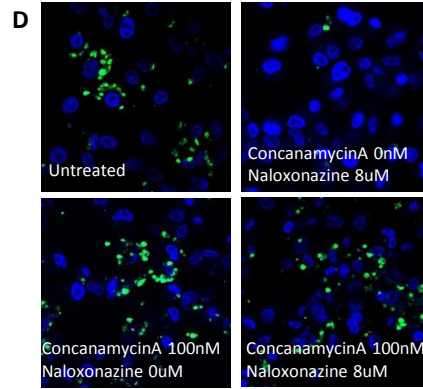
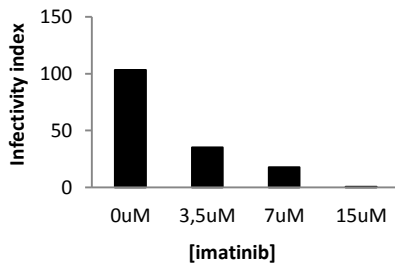**E**   **Imatinib activity on *Leishmania* intracellular amastigotes**

Fig. 6 Pulsed field gel electrophoresis (A) and southern blot hybridization (B) to detect MAPK (arrowhead) and HTBF (H-locus, arrow).

No circular extrachromosomal episomes were detected suggesting intrachromosomal amplification of both H- and MAPK loci by tandem duplications. (chromosome 36 carrying MAPK locus is 2,7 Mb and chromosome 23 carrying the H-locus is 0,78 Mb)

1: BPK 275 (ISC005)
2: BPK 282 (ISC006)
3: BPK 026 (yeti ISC001)