KINDReD Final Report: Publishable summary

Executive summary

The Kinetoplastid Drug Development (KINDReD) consortium was funded for three years between September 2013 and August 2016 under the final EU Framework 7 call, FP7 HEALTH.2013.2.3.4-2: Drug development for neglected parasitic diseases. The consortium was devised to include expertise in three major chronic parasitic diseases; Leishmaniasis, Chagas Disease and African trypanosomiasis. Its overall objective was to reconsider the preclinical pipeline and conventional approaches to drug discovery in a way that would mean new, cost-effective and safe drugs reach those most in need.

From its inception KINDReD was planned to be different stand. Led by a small French SME, the project took a fresh look at the problems of drug discovery in NTDs. With the aid of a parasitology group from Portugal, a consortium was created that had on one side experts in the field with decades of experience and on the other, SMEs and academic groups with exciting technological platforms but no prior experience in neglected infectious diseases. Despite the fact that few of these groups had ever worked together before, it is a testament to the success of the consortium that the new collaborations formed during its lifetime are set to continue to explore new advances together.

Because after all KINDReD was based on ambition. Its goal was to strengthen, inform and advance the current drug development pipeline in order to achieve at least one new Phase I clinical candidate for each trypanosomatid disease studied by the end of, or shortly after, the project's course.

An impossible and naïve approach according to some detractors and in any case difficult considering that the EU budget had to cover the research of 13 groups for three years from early compound screening to advanced preclinical studies. However, KINDReD concentrated on all points of the pipeline and has been able, thanks to the incorporation of the NHP model, to move hits to leads and leads to drug candidates. One particular and important example is that KINDReD is now championing the first new chemical entity for the treatment of Chagas disease in over 30 years. K777 a cysteine protease inhibitor, has at least equivalent efficacy to benznidazole in the acute animal model for disease. Completion of the 28-day chronic dosing in primates, revealed no drug-related toxicity or other safety issues, therefore, submission of the IND application with progression to a Phase 1 safety study in "normal healthy" volunteers is now foreseeable.

With the KINDReD Association, a charitable organisation set-up as a durable footprint of the project, all the hits, leads and drug candidates from this project, and any other which should wish to join, have a lobbying force to help ensure that the best amongst these can also arrive in the clinic one day.

Kinetoplastid Drug Development: strengthening the preclinical pipeline (KINDReD)

Summary description of the project context and objectives

The trypanosomatid diseases, Leishmaniasis, Human African trypanosomiasis and Chagas disease, continue to impart a heavy toll on human health, affecting millions of people worldwide particularly in the economically poorest countries[(1)]. The handful of treatments currently available to control this enormous health burden is limited by serious adverse effects (toxic and/or teratogenic), high costs, difficulties in administration, political instability and the often rapid selection of drug resistance[(2-4)].

Worldwide efforts to combat "neglected infectious diseases" from bodies such as the pharmaceutical industry, the DNDi, the European Commission, Gates Foundation and others have resulted in several potential drug candidates at the preclinical trials stage but with only a few reaching clinical trial (Phases I to IV) and a depressingly low number of new compounds reaching the market. The CNS permeable drugs fexinidazole, a 2-substituted 5-nitroimidazole and SCYX-7158, an orally available benzoxaborole, show promising efficacy with the advanced stage of HAT and recruitment of patients to the fexinidaole Phase II/III (394 patients) in 2015. (http://www.dndi.org/strengthening-capacity/hat-platform/)
Nonetheless, there has been general market inertia to translate recent scientific and technological advances into the discovery of potent, safe drug candidates against the kinetoplastid protozoan parasites a factor that we communicated around strongly during the course of KINDReD.

In this SME-led consortium which brought together several novel technologies and partners new to the domain with established world leaders in neglected infectious disease research, we aimed to take an ambitious, fresh and unconventional approach to strengthen the preclinical pipeline with the aim of predicting in advance, potential pitfalls such as toxicity or resistance at the preclinical phase to have a greater chance of turning lead candidates to drugs in a timely and cost-effective manner.

References

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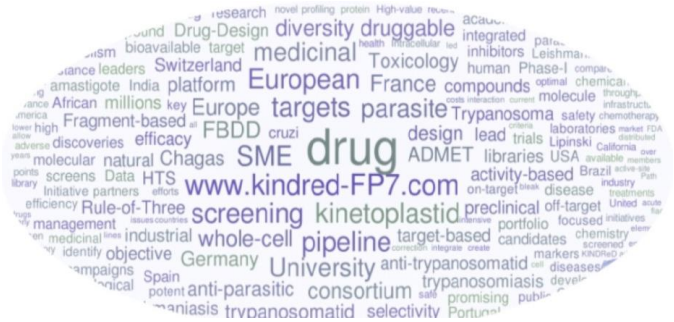
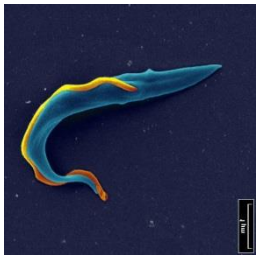


Figure 1: Word cloud representation of the KINDReD project's ethos

Objectives of the study

The aim of KINDReD is to create a unique and powerful drug discovery platform with the common objective of *advancing promising laboratory-driven discoveries into clinical utility*.

- To implement a coherent (integrated) approach to populate and advance all stages of the anti-trypanosomatid drug pipeline.
- To establish an integrated global network of academic and industrial partners united by a central objective: to develop and apply innovative cutting-edge molecular and cellular tools that will enable us to populate and accelerate the preclinical pipeline for anti-kinetoplastid chemotherapeutics.
- To advance every aspect of the preclinical pipeline, by developing new technologies where necessary, from early target discovery, validation and screening through to advanced toxicology and animal testing.
- To bring forward one new Phase I clinical candidate for each trypanosomatid disease studied at the end or within a short time after the end of the project.
- In parallel, and where appropriate in partnership with likeminded bodies such as WHO, Wellcome Trust, DNDI etc. we will set up an association that will lobby industrial groups to take an active role in the further clinical development of our registered candidates (repurposed or new chemical entities) showing potency against these parasitic diseases after the conclusion of the funding period.



Trypanosoma brucei
HAT
Human African Trypanosomiasis or
Sleeping sickness

Leishmania donovani
L. infantum
Visceral leishmaniasis (kala-azar)

Trypanosoma cruzi
Chagas disease or American trypanosomiasis

Figure 2: Our parasites and diseases

A description of the main S&T results/foregrounds

In KINDReD, originally composed of 14 partners, the scientific programme was divided over 9 of a total of 12 work packages (WPs). Our infrastructure for parasite screening integrated five leading academic laboratories in Europe (Portugal, United Kingdom and Switzerland), the United States of America (California) and South America (Brazil) with high throughput screening (HTS) facilities equally distributed between all three major kinetoplastid parasites. Follow-up medicinal chemistry and ADMET expertise was spear-headed by our industrial partners in France and Spain, respectively who interacted with the academic members of the consortium to guide their efforts. The full details of the progress of each of these WPs have been provided to the European Commission, in two periodic reports and a series of key deliverables. Below is a description of the work performed the original objective of the WP and the main results.

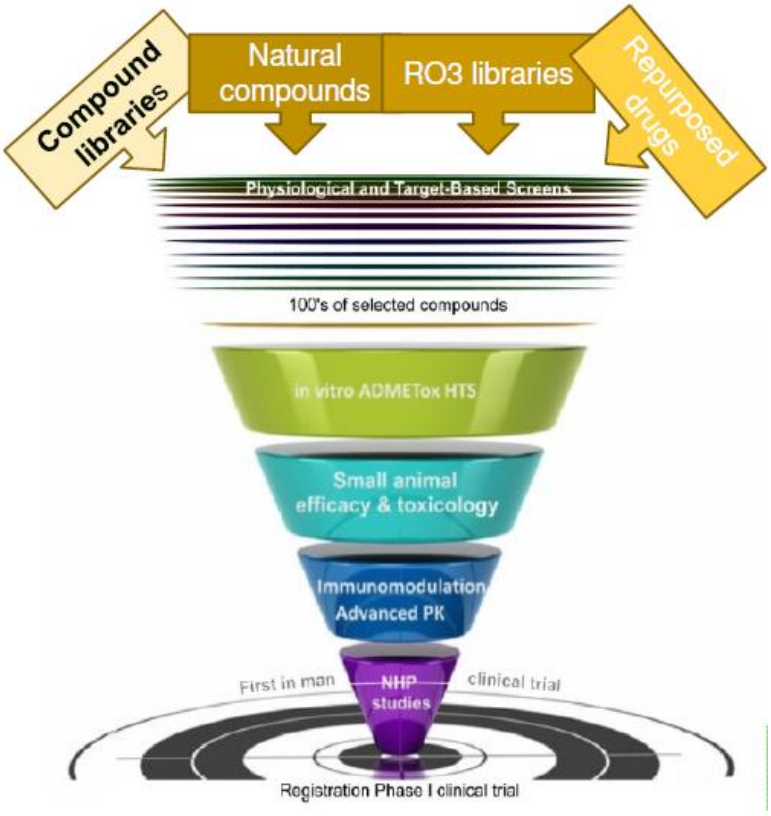


Figure 3: the central KINDReD work flow

Physiological and Target-based Screens

The largest of the work packages, WP1 “Physiological whole parasite screening assays” led by Jim McKerrow, now from UCSD, was originally planned as the driving force of the project, screening large numbers of compounds to pass hits (IC_{50} ≤ 1 µM) to WP 8 (Isbaal Ramos, Innoprot) the *in vitro* toxicology and ADME control step built into our reinforced pipeline to pinpoint potential adverse events before attempting animal experiments. This step was designed importantly to reduce the number of animals needed in preclinical testing as we were bound in our original ethical commitment to implement the 3Rs ‘Reduction, Refinement and Replacement’ under which we had agreed on a protocol to reduce the number of mice used by 59% through pooled testing of lead compounds.

We also ensured that the molecules tested were being screened in the *in vitro* system that best represented the physiologically relevant form of the disease-forming parasite. Screening assays incorporated state-of-the-art image-based screening of intracellular parasites in *T. cruzi* and *Leishmania* spp. as well as reporter-based assays of *T. cruzi* trypomastigotes, the blood-stream form of *T. brucei*, and *Leishmania* spp. intracellular amastigotes. Several whole cell parasite assays have been developed by the KINDReD partners for this purpose WP1 combined these assay systems with advances in liquid handling robotics and automated imaging to facilitate high-throughput screening of a wide collection of compounds derived from natural and synthetic sources.

The project had always been constructed with in-built flexibility which meant that over the three-year course of the programme we could change the source of molecules screened and also welcome libraries of potential hits from groups outside the original consortium to ensure our unique resources were used to full capacity.

Positive controls used in screening were melarsoprol for *T. b rhodesiense*, benznidazole for *T. cruzi*, miltefosine for *L. donovani* and *L. infantum* and podophyllotoxin for cytotoxicity in mammalian cells. All compounds were subjected to (a) single-point high-throughput screens in the physiologically-relevant parasite assay seeking > 80% inhibition at 10 µM. Then (b) EC_{50} values were obtained from 16-point sigmoidal dose-response curves performed in triplicate (range 100 to 0.001 µM). (c) Hits with EC_{50} ≤ 5 µM and ideally < 1 µM were retested to confirm long-term cidal versus static effects on cell growth. For *T. cruzi* and *Leishmania* spp. these were performed systematically on intracellular parasites where the screen identifies compounds that give complete parasitological cure in cultures of myoblasts (*T. cruzi*) or macrophages (*Leishmania*). Each promising hit compound was systematically retested in all three parasites and cytotoxicity tested in mammalian cell culture, e.g. 72h incubation with L-6 rat skeletal myoblasts, to define selectivity.

The move of the McKerrow laboratory from San Francisco to San Diego substantially reduced the whole-cell screening capacity of the consortium which endured for over 18 months this meant that alternative sources of hits needed to be considered. One of the most valuable resources has been the natural compound libraries that KINDReD has access to and formed the basis of the work in WP4. These are primarily from Helge Bode's laboratory, in Frankfurt, (WP4). The group works mainly with exotic natural product producers such as entomopathogenic bacteria (*Photorhabdus* and *Xenorhabdus*), which are usually underrepresented in natural product libraries. These were screened against *T. cruzi, T. brucei and L. donovani* by Pascal Mäser's group at the Swiss Institute for Tropical Health and interesting hits returned to the Bode laboratory for chemical total synthesis or derivatisation of the active components.

Another source of natural compounds was the Global Health Library of marine derived natural products which also produced numerous hits that have been passed on to the other WPs to study AMETox and *in vitro* immunomodulatory activity (WP7). A chance encounter at an International meeting on NTDs, led to KINDReD gaining access to a unique library of plant extracts from Nigeria which has now been screened by the McKerrow group, against all three parasites. This has revealed some very interesting activities especially against *T. brucei* and larger quantities are being synthesised in Nigeria for chemical identification of the active components in USCD. The table below summarises the screening efforts from KINDReD.

Libraries		Primary Hits			Confirm./Selected Hits		
	# Cpds	*Leish*	*T. cruzi*	*T. brucei*	*Leish*	*T. cruzi*	*T. brucei*
FDA library	1,640	21	23	121	0	4	1
Sanford Burnham Bioactive Library	2,561	89	132	75	58	65	38
BNIPs derivatives from IBMC (1st gen.)	12	2	2	12	0	1	1
BNIPs derivatives from IBMC (2nd gen.)	10	1	2	8	1	1	0
Naphtalinamide series from IBMC	60	0	2	20	0	0	0
Naphtalimide series (VEGRF2 inhibitors)	13	1	6	5	0	0	0
Nitrofuranes from USTAN (KD series)	28	4	0	9	4	0	9
Nat. Prod. from UCSC (marine)	3,020	137	102	265	21	33	125
Nat. Prod. from UCSD (marine)	120	0	1	1	0	1	1
Natural Products from Helge (fungi)	274	72	37	52	22	5	6
Nat. Prod. from Africa (plants)	114	0	4	15	0	1	12
NIH Natural Products Collection	120	6	18	7	0	7	0

Table 1: results from the KINDReD screening programme

Another potential source of hits that has been explored is the 1 600 strong list of FDA approved drugs. Now screened against all three parasites, this collection has shown a handful of compounds of interest for further study that will be passed along the KINDReD pipeline because their previous approval status could speed registration. The compounds have now been studied in the other WPs for AMETox and *in vitro* immunomodulatory activity (WP7) and snap-shot PKs have been performed where considered of interest. One of these, candicidin, is currently being tested in an animal model of cutaneous leishmaniasis in the NMTrpI consortium a model available to KINDReD as part of our "synergy" activities. Unfortunately, we still do not have the results from this study so have no update on its status. If successful, our Partner AIIMS is well-positioned to run a clinical trial in India. The results from the other FDA-approved compounds have been less interesting as although the AMETox was promising, in animal testing the efficacies were generally poor.

Additional whole-cell screens have been performed using in-house molecules (Anabela Cordeiro da Silva, Porto) and fragments from a Maybridge rule-of-three library screened against *L. major* and *T. cruzi* by Terry Smith in St Andrews.

The continued use of the CDD data base has ensured all molecules have been correctly catalogue and all our data is in one place ready for further exploitation.

One in-built strengthening mechanism in the KINDReD pipeline was to test promising leads against clinical isolates in addition to the laboratory strains used in the screening platforms. This precaution was put in place as laboratory strains are known to lose virulence after multiple passages. This work was performed by our partners at AIIMS (*L. donovani*) and FIOCRUZ (*T. cruzi*). All the compounds tested to date were found to be effective on clinical isolates as well as on laboratory strains of parasites.

Alternative approaches to whole-cell screening for the identification of hits were always a major part the project. WP2, "Target identification and screening", led by Terry Smith (USTAN) has been particularly successful in identifying numerous hits from screening several parasite protein deacetylase and kinase activities, TbUAP, TbINO1/TbIMpase 1 and 2, Tb, neutral sphingomyleinase and TbIDI. Many of these of been repurchased or synthesised and retested, validated hits have moved forward through iterative rounds of medicinal chemistry to evolve potency and/ selectivity using SAR and several are entering ADMET. These include the nitro-heterocycle-amides the vast majority, ~20, of which have a better IC_{50} than nifurtimox against *T. brucei.* Of these, one KD011 was seen as the best candidate to take forward for ADMET and animal testing.

The consortium also had access to a unique SPR array screening platform from NovAliX that features a diverse collection of library compounds (>116k compounds including >25k fragments with MW< 300Da) immobilised on gold chips. These high density chemical microarrays (9,216 sensor fields/array) enable the rapid screening of any target against one

of the largest fragment libraries commercially available. Early selectivity information can be provided on the primary screening level. This platform was used to screen three of the consortium's chosen targets. The first proteins analysed in this way was the Silent Information Regulator 2 protein, SIR2, a NAD-dependant tubulin deacetylase and which has been genetically and chemically validated as an anti-Leishmania drug target in extracellular, intracellular and animal models prior to the start of the project. The use of the purified protein in the SPR-arrays has identified several molecules of interest, one of which active against the *L. infantum* protein has now had its potential confirmed in high concentration bioassays at PHX and in intracellular amastigotes at the IBMC.

Our second target ribose-5-phosphate isomerase (RpiB) failed to uncover any molecules suitable for further follow-up by SPR but the thermal shift approach in St Andrews identified three fragments with activity against the parasites that may be legitimate hits to follow up.

During the course of the project the group from USTAN was also able to genetically and chemically validate drug targets in *T. brucei* and *T. cruzi* using compounds identified from their screening efforts. With the support of IBMC working in the *Leishmania* model we now have several new targets for future therapeutic campaigns that we have characterised functionally against all three parasites these include uridine diphosphate N-acetylglucosamine pyrophosphorylase (UAP) and ribose 5-phosphate isomerase.

Structure-based drug design

Several approaches to FBDD have been developed over the past decade with widely-accepted advantages over HTS campaigns to discover lead compounds. All focus on finding a seed fragment with high ligand binding efficiency (i.e. -RTKd/Heavy atom count > 0.4). Thereafter, structure-based design coupled to iterative chemistry can allow the quick evolution of potent lead compounds with optimal 'rule of five' properties. This approach to drug discovery was explored in work packages 2 and 3 which brought together experts from academia and industry as shown in figure 4 below.

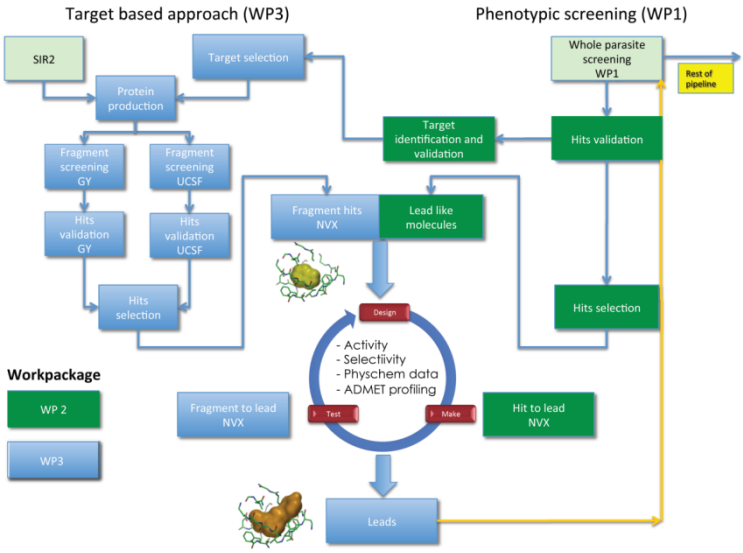


Figure 4: DMT cycle: Iterative Design, Make, Test strategy of target-based SAR and Structure-based FBDD used by KINDReD.
UCSF= UCSD; GY= NOVA

Our original target of interest was the sirtuin 2 protein from *Leishmania*, the study was enlarged to include the SIR2 proteins from all three parasites. The sirtuins proved intractable to crystallisation during the first 18-months of the project. However, in the second half of the project, SIR2 from all three parasites was successfully crystallised. Although, unfortunately the structure-based drug discovery phase that was originally planned to be performed with fragments generated e.g. from high concentration bioassays (PHX) and SPR (NOVA) was not able to be completed within the time frame of the project.

The second target chosen for crystallisation was ribose-5-phosphate isomerase (RpiB). The structure of the *T. cruzi* protein was already known and the proteins from *L. infantum* and *T. brucei* underwent crystallography studies. Although, unlike with sirtuin, crystals were quite readily obtained within the timeframe of the project we could not proceed to structure-based drug discovery. The structures are now known for future studies.

ADME-toxicity Studies

In vitro toxicology and ADME studies were the subject of WP 8. This was another SME-led work package, demonstrating the high-throughput technologies of the Spanish-based company Innoprot a specialist in HT drug toxicology testing. The platform acted as a funnel capable of filtering the compounds emerging from the physiological screens through a series of stringent tests to yield only the very best candidates for advanced animal testing. One of the reasons cited for the failure of modern HTS technologies to generate more new approved drugs is the early focus on potency as the major selection criterion. Often only a few of the most potent candidates will be selected for animal toxicology and 'snapshot' PK studies in small rodent models, inevitably reducing the chances of choosing the candidate with the best balance of efficacy, PK suitability and safety. In KINDReD we applied early high content toxicology and *in vitro* ADME assays to remove this emphasis. Our original plan had been to feedback information to our medicinal chemists so that compounds with good ADMETox parameters but lacking in potency can be fed back into the design process for potency improvements. However, it became apparent that we were limited in budget to perform this task effectively.

Nevertheless we were able to ensure that only compounds showing both anti-kinetoplastid potency and excellent ADMETox were tested in our animal models. This strategy therefore combined the optimal use of anti-parasite screening output with application of 3R guidelines on animal testing. One important feature of the work carried out in this WP has been to obtain data on the drugs currently used for the treatment of the three parasite diseases in order to create a data base of their ADME-tox characteristics and to be able to compare any new hit and eventual leads against these.

The "gold standards" that have been characterised in WPs 6, 7, 8 and 9 are amphotericin B and miltefosine for *Leishmania* and pentamidine for *T. brucei* and benznidazole and

fexinidazole for *T. cruzi.* The results from WP8 show that from the compounds tested (28 candidates and 5 gold-standards) showed hepatotoxicity, nephrotoxicity and neurotoxicity median cellular cytotoxicity concentration (CC_{50}) values 100 times lower (on average) than IC_{50} values. In terms of adsorption, distribution, metabolism and excretion (ADME), plasma protein binding assays for mouse and human have been completed for the compounds and compared to the gold standards. With additional secondary tests such as AMES, the compounds from KINDReD have now been ranked in relation to their ADMETox profiles and some in fact appear to have a more acceptable safety profile at this stage than some current treatments. This WP has also explored the effect of using PLGA nanoparticles on the delivery of potential anti-parasitic compounds and has shown controlled and effective delivery of BNIPDaoct and for *L. infantum* and *T. brucei* infection with significantly reduced toxicology as evidenced by PLGA-encapsulation of amphotericin B.

Further exploration of our "gold-standards" by Jérôme Estaquier at the CNRS has demonstrated that miltefosine, amphotericin B and pentamidine, depending on the concentration used at either micro- or at millimolar levels, induce mitochondrial depolarisation and cell death. Moreover, we have demonstrated that the effects are cell type dependent. In particular, we found that monocytes are especially sensitive to miltefosine and pentamidine. Because monocytes represent preferential targets for *Leishmania* infection, we demonstrated that infection with *L. infantum* modulates mitochondria metabolism exerting a regulatory effect on oxidative phosphorylation. Therefore, we are testing the impact of the drugs on monocyte metabolism as well as on the profile of OPA1. This work is also of particular importance to WP7 (Immunomodulation) led by Olindo Assis Martins Filho, in Brazil. Current guidance documents for preclinical filing of new drugs require evaluation of unintended immunomodulation and advocate additional immunotoxicity studies to characterise risk including *ex vivo* immunophenotyping of blood cells and *in vitro* immune function. Our experiments to date suggest it is of crucial interest to concomitantly monitor the immune response to assess the side effects of the drugs in relation to the nature of the cells that are sensitive to their effects.

Animal testing

Work package 9, animal studies of efficacy and toxicology was led by Anabela Cordeiro-da-Silva (IBMC). The strains and models used were the following; stationary phase promastigotes (visceral leishmaniasis virulent clone *L. infantum* MHOM/MA/67/ITMAP-263 and cutaneous leishmaniasis, *L. major* LV39 MRHO/SU/59/P), for *T. cruzi* trypomastigotes (the clinical strain, CAI-72, produces cardiac disease and cardiac failure similar to that seen in human Chagas disease) and for *T. brucei* blood strain trypomastigotes (e.g. clonal line of virulent *T. b. rhodesiense* STIB900). Parasites were injected i.p. at 10^8 parasites for Leishmania and *T. cruzi* or 10^4 for *T. brucei*. For Leishmania *L. infantum* and T. cruzi four

mice from each infected group were treated after 14 days of infection by i.p. injection during three consecutive days. Parasitaemia was originally monitored two to three times a week by microscopic counting of tail vein blood in a Neubauer chamber. During the project, live-imaging was introduced to follow parasitaemia (see figure 5). Mice that were aparasitaemic for 60 days after the treatment period were considered cured.

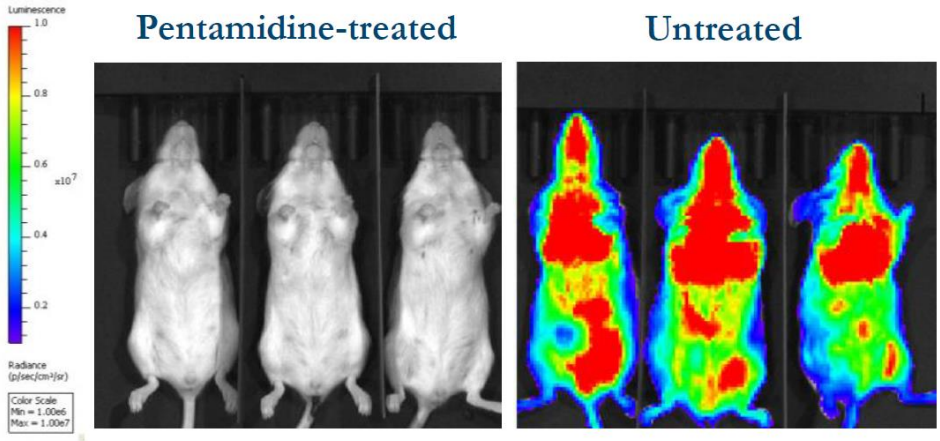


Figure 5: *In vivo* imaging of BALB/c mice infected with *T. brucei* after 5 days of treatment. Parasite load was evaluated by subcutaneous injection of 2.4 mg of luciferin and all animals were imaged using IVIS LUMINA LT groups at day 5 post-infection are represented.

The biodistribution of the reference compounds after intraperitoneal injection has been determined in our mouse models for comparison with KINDReD lead compounds. The *in vivo* efficacy of several test compounds against *Leishmania* was found to be enhanced by encapsulation in pegylated nanoparticles. However, several compounds reaching this WP and tested against the mouse model for *T. brucei* and *T. cruzi* have unfortunately, shown to have little or no *in vivo* efficacy.

Our second animal model in KINDReD to complete the final stages of evaluation of a drug candidate, which has shown good safety and tolerability in the mouse model, is the non-human primate (NHP) specifically the *Macaca Mulata* adult male. The use of NHP as animal models for the study of human diseases (including immunological studies and drug and vaccine development studies against infectious diseases) has become increasingly important. During the course of KINDReD, the NHP model has been used to perform a 28-day chronic dosing study for K777. K777 is a protease inhibitor effective against *T. cruzi* for which registration to use in the treatment of Chagas disease is being sought. Originally, part of the DNDi portfolio development of this drug as halted after a poor tolerability result (see figure 6). GMP drug ready for safety testing in the NHP model (CNRS) was provided by the McKerrow laboratory and the study was performed following an FDA approved protocol. Jérôme Estaquier has shown that the drug could be safely administered to the NHP with no

immediate or long-term side-effects. This successful study should complete the pre-IND dossier for FDA approval we are waiting to hear from the FDA for a date to discuss this data.

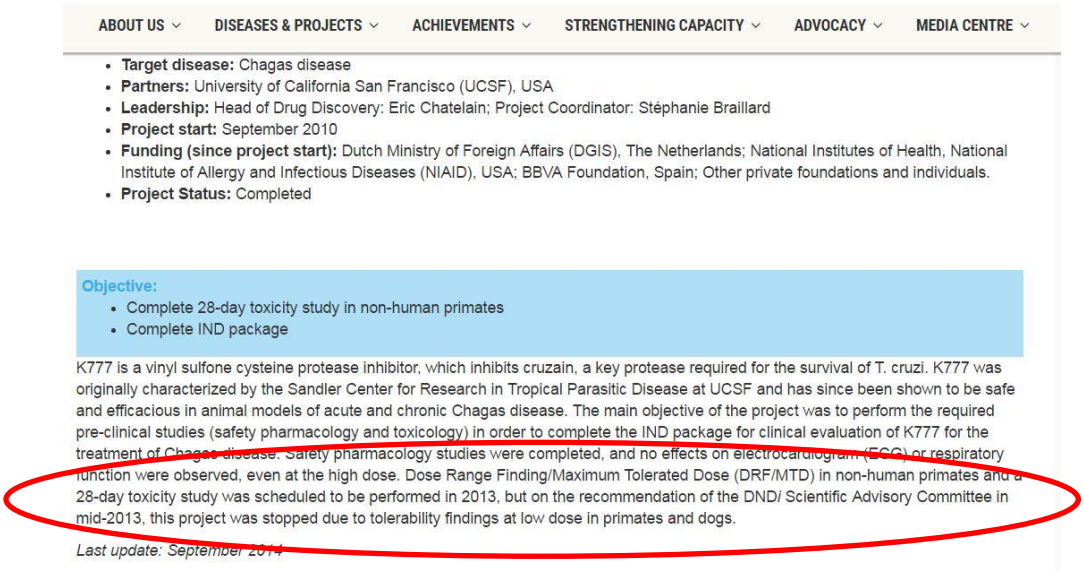


Figure 6: screen shot from the DNDi website showing their decision to discontinue K777 as a potential new treatment for Chagas disease.

<u>The unique approach of KINDReD</u>

Two work packages that were slightly to the side of the main direction of the work flow in KINDReD are WPs 5 and 6 looking at Chemoproteomics: deciphering drug “on” and “off” target interactions and parasite metabolism, respectively. These WPs are again an opportunity for “new blood” in terms of neglected infectious diseases to use their well-defined technologies and prior knowledge in the pursuit of the project’s aims. The chemoproteomics approach WP5, led by Gilbert Skorski, Phylogene, was intended as an early exploration of the potential for ABPP probes to assist the drug development workflow, requiring (i) implementation of existing methodologies and assessing their utility to drug development as well as (ii) the development of new ABPP probes and associated workflows. Whilst several important steps have been made towards these goals, the heavy demands on novel ABPP chemistry, as well as the fine-tuning of sophisticated technology to ‘read-out’ off-target interactions, has limited our analyses to a small number of the potential avenues that could realistically be explored over this three year project. Resveratrol probes (described in the mid-term report) were abandoned in favour of more promising 5-nitro-2-furancarboxamide derivatives, in direct collaboration with USTAN and NOVA. A phosphoproteomics pipeline to screen both the parasite and host proteomes was applied in order to identify new drugs targets. Infection by *T. brucei* has effects on the proteome and phosphoproteome in the mouse brain model. An impact on the immune response was shown

by differential expression level but also on the brain plasticity in our phosphoproteomics experiments. This preliminary -omics study can be used as a starting point to find a new molecular targets for treatment and also getting a better understanding of the *T. brucei* infection mechanism. Used to label proteins after co-incubation with *T. brucei* cells their subsequent identification with the new probes represents an important step in understanding the mechanism of action and cellular targets for this new class of kinetoplastid inhibitors.

Work package 6 led by Alain Pruvost from the CEA is another WP that changed course during this second reporting period. Terry Smith's group in St Andrews contributed much to this WP over the latter half of the project. In collaboration with a Canadian group, they have investigated mutations in a P-type ATPase transporter in *Leishmania* that lead to cross-resistance to two leading drugs by distinct mechanisms. Uptake of the potential anti-*T. cruzi* compound KD011 into macrophages has been investigated in collaboration with the CEA and the IBMC. Attempts have been made to gain a better understanding of the catabolic processes occurring in the lysosome of the parasites. In another approach Photeomix (PHX) have characterised proteolytic activities in leishmania promastigote cell extracts which exhibit a strong potential destroy peptide drugs.

Work package 12, synergistic activities continued to make progress towards working together. KINDReD and NMTrypI were able to exchange animal models, as close collaboration has ensured the best use of resources in the interests of advancing the kinetoplastid pipeline. The synergy meeting held in Modena in June this year was a good example of how much exceptional science and technology is contained within this European Framework call. The co-ordinators of all four projects presented an open letter to the EC to this effect and have also presented a poster at the recent COST-Action Meeting in Madrid in a similar vein.

Final results and their potential impact and use

- We have now have two potential lead candidates to move into phase I trials:
  - The FDA dossier is currently under approval for the use of K777 in Chagas disease
  - An EMA dossier is under construction for repurposing candicidin in cutaneous Leishmaniasis
- We are confident that our new techniques for use in the preclinical pathway especially our chemoproteomic probes and metabolism measurements will be of use to other researchers in the field including our synergy partners.
- Photeomix has launched a series of services based on the discovery of small molecule inhibitors of post-translational modification enzymes which is a direct result of their involvement in KINDReD.
- New products and services are available from Photeomix and other SME partners will no doubt follow over the coming months.

- KINDReD has uncovered rich source of new targets which are worthy of further research as are the new scaffolds, fragments and natural products we have shown to be active.
- The KINDReD Association is a new and experimental model focused initially on lobbying funding bodies to bridge the gap between late-preclinical and first-in-human trials.
- KINDReD has been unique in bringing together partners new to the field of parasite research all of whom have integrated well into the project. It is a source of new and potentially powerful partnerships in a field that perhaps has suffered from dogmatic direction. We plan to continue the open and encompassing approach we have taken to studying the pipeline and difficulties experienced in this field after this funding period finishes.

The potential impact of KINDReD

Impact on world health and quality of life

Protozoan parasites are amongst the most common infectious agents in the tropics and subtropics. The respective diseases are a major cause of morbidity and mortality in many developing countries and have serious consequences for socio-economic development in these regions. Primary infections are not the only concern; secondary infections in HIV-infected and immuno-compromised patients also pose grave health risks. A major problem associated with these diseases is their prevalence in third world countries which are the least well-equipped to develop new drugs and invest in R&D.

- Leishmaniasis is a worldwide disease, affecting 88 countries. The annual incidence is estimated at 1-1.5 million cases of CL and 500 000 cases of VL. The overall prevalence of the disease is 12 million people and the population at risk is 350 million.
- Chagas disease occurs throughout Mexico and Central and Southern America, and continues to pose a serious threat to health in many countries of the region. The overall prevalence of human *T. cruzi* infection is estimated at 16-18 million cases with an annual burden of 196 million € in healthcare costs. Approximately 120 million people, i.e. 25% of the inhabitants of Latin America, are at risk of contracting the infection.
- For African trypanosomiasis, estimates indicate that over 60 million in 250 foci are associated with the risk of contracting the disease, and there are about 300 000 new cases every year. However, less than 4 million people are under surveillance and only about 40 000 are diagnosed and treated, due to difficulty of diagnosis and remoteness of affected areas. These figures are relatively small compared to other tropical diseases, but African trypanosomiasis, without intervention, has the propensity to develop into epidemics, making it a major public health problem with a case fatality rate in untreated patients of 100%.

Control of trypanosomatid protozoan family is a major challenge not only in the third world, but increasingly in the western hemisphere, including Europe as migration and climate change move both people and the disease-bearing vectors, respectively into previously infected areas. The KINDReD consortium has responded to this global threat through the development of two new treatments for trypanosomal diseases. These are a completely novel reagent against Chagas disease and a potential therapy from repurposing candicidin for use in cutaneous Leishmaniasis that ultimately will convey significant benefits for human health in countries where these diseases are endemic.

Impact on the anti-trypanosomatid drug pipeline- turning pipe dreams into clinical reality

Our screening, drug design and lead optimisation strategies have amassed a comprehensive portfolio of fully characterised drug leads, with emphasis placed from the beginning of the project to having candidates for early Phase I clinical trials. Our understanding of the fact that the regulatory approval process is a long, expensive and meticulous procedure of controlled clinical testing, with a low average success rate led us to perform extensive preclinical toxicity testing including introducing novel 'critical path initiatives' such as the introduction of preclinical genomic and chemoproteomic profiling. By passing the current treatments available to treat these diseases through the same platforms we have built a comprehensive picture of ADME Tox profiles that are acceptable to further development.

All our promising lead compounds will be carefully scrutinised for cellular markers of oxidative stress, mitochondrial damage, genotoxicity, DNA damage, apoptosis, cell viability and morphological dysfunction, again compared directly with current treatments. This way we have a clear preclinical profile for any future registration dossier.

Novel chemoproteomics techniques using active site probes have been developed to signal unwanted drug-protein interactions in host cells, potentially allowing identification and modification of off-target effects by further iterative drug remodelling. This work is still in a preliminary stage but is worthy of further investigation.

Impact on innovation and competitiveness of the larger pharmaceutical market

One of the major distinctions of the multi-disciplinary KINDReD consortium from other consortia has been the integration of multiple technologies on a common platform. The success of our innovative drug development procedures is equally applicable to the larger pharmaceutical market. Within this consortium we were driven by a need for cost-effective research, procedures to be developed by our members, such as differential drug fragment -selectivity-profiling and chemoproteomic 'off-target'-toxicology-profiling, these techniques have the potential to increase preclinical research efficiency, reduce drug candidate attrition and reinforce the competitiveness of the European pharmaceutical market in general. Some of the effective targets discovered by the consortium may also be investigated independently for clinical utility in other infectious or chronic diseases, such as malaria or cancer.

The linear decline in pharmaceutical research productivity over the past six decades suggests that new knowledge, technologies and increased resources, however powerful, do not translate directly into the approval of more new drugs. This is particularly the case observed with the adoption of high throughput screening techniques. The research productivity paradox was treated in the US Food and Drug Administration's landmark 'Critical Path Initiative' launched in 2004 which called for an investigation into efficiency improvements that could be made at all stages in the development path for new medicines. It is here that the most recent technology developments can have their biggest impact on

productivity. Tools developed specifically to evaluate the efficacy and safety of medicines at early stages in the process can improve pharmaceutical research productivity allowing informed decisions can be made earlier in the process. The KINDReD consortium has put the development and testing of such tools at the heart of the preclinical development of antiparasitic agents. Indeed, is a fascinating and sobering thought that a FP7-led initiative, gathering experts together to tackle neglected parasitic diseases that plague some the world's poorest nations, could provide solutions central to the revitalisation one of the world's most wealthy industries.

Impact on European SMEs

Another focus central to the KINDReD initiative was the strength of the SME involvement, each one of the which took a leading role in the work packages. Individual European countries encourage SMEs to perform internal research into new products and services aided by regional, national and tax credit initiatives. It has long been recognised that these activities are at the heart of the European economy, where SMEs account for 99% of private sector businesses and employ two thirds of the European workforce. The 2008 Small Businesses Act for Europe created a policy framework that recognises the central role of SMEs in the European economy. This 'think small first' policy can be applied equally to SMEs in the biotechnology sector, where SMEs bring innovation and expertise but can suffer from being a small player in the larger market. To flourish, SMEs need to become more competitive and more entrepreneurial. KINDReD offered a risk sharing environment for SMEs to develop the innovative products that underscore their business activities whilst contributing to the greater effort of eradicating the global burden of kinetoplastid parasite diseases. This initiative provides SMEs with both financial and intellectual support within a European framework of focused drug centred research. Each SME has used their research efforts to strengthen their market competiveness by expanding their research capabilities, developing their contract research and product ranges and developing new expertise in previously untapped areas.

Impact on ICPC countries

Eradication of neglected infectious diseases in poor countries is a global health priority, a decision supported by the leaders of the world's richest economies at several G8 summits and recently endorsed by the London Declaration on Neglected Tropical Diseases inspired by the World Health Organisation's 2020 roadmap on NTDs. Two of the partners in the KINDReD consortium are from ICPC countries of low income (Brazil) and low-middle income (India) where kinetoplastid diseases are endemic. Biotechnology efforts are underway in these countries to develop diagnostic and medicinal products. These efforts will be assisted by strengthening their existing research infrastructure and challenging the problem internally rather than relying only on external academic/bio-pharmaceutical efforts. Thus KINDReD has

had a major impact by focusing funding on ICPC research groups, helping them employ teams of researchers working in clinical environments at the source of the health burden. Their researchers have benefited from the application of many of the technological advances of the KINDReD programme and they are ready to help us with the regulatory procedures needed to be followed in order to perform clinical trials in these countries.

Impact on future EU-directed neglected infectious disease research.

The EU has long been a leader in the initiatives against neglected infectious disease supporting NID initiatives throughout several framework programmes. For these initiatives to achieve a major impact by Horizon 2020, the challenge to provide a link between prior efforts and the establishment of a clinical trial must be met. The KINDReD programme has shown how the scientific community can come together to make this a reality, leaving a permanent footprint through the establishment of clear operating procedures and workflows that will facilitate effective preclinical research throughout the current programme and beyond. The founding of these procedures in written, open access form will have a major impact on future research initiatives. Furthermore, research into neglected infectious diseases having demonstrable applications to the general pharmaceutical pipeline should aid healthcare funders and stakeholders with future funding initiatives.

Impact on society in general.

Society should be aware of how the taxpayer's money is spent on issues of great health and socioeconomic importance. In addition to the consortium agreement, we have created an independent non-profit association to convey information relating to KINDReD's activities to the research community and the public in general, to leave a durable footprint at the end of the 3 year project and to ensure the necessary continuity for the clinical development of the most promising lead compounds and the associated regulatory approval processes. Our activities on social media have had positive returns particularly from people living in endemic areas and we are continuing to communicate our progress to them even although the initial funding period is over.

Main dissemination activities and exploitation of results

Our dissemination measures were run by WP10. Throughout the period of the grant, dissemination activities have been closely monitored and communicated as much as possible via the KINDReD public website. The majority of partners have been active in disseminating their activities in KINDReD at different meetings both National and International with poster and oral presentations as shown in the lists below.

The Scientific Co-ordinator partner 4 (PHX) has presented KINDReD at several International meetings and discussed with several major parties interested in NTDs including the Wellcome Trust and the French MTN network.

Members of the consortium have continued to publish numerous scientific articles with 30 peer reviewed articles shown here. Work is still on-going to publish more articles and numerous publications citing KINDReD are already planned and will continue to appear from the work performed in KINDReD. Wherever possible publications are in line with the Commission's wishes Open Access.

The KINDReD Association, an organisation of charitable status, has been set up, initially to support the KINDReD consortium. The Association has been promoting the research undertaken by the KINDReD consortium with links to press-releases and social media and provides a durable footprint of the FP7 funded research. It operates under open-source principles, providing a community-driven forum (1) to share data and views on research objectives and results, (2) provides access to materials and techniques of the KINDReD consortium for NID drug development, (3) has established metrics to judge the success of project goals versus the international NID objectives and (4) to collaborate across organisational boundaries.

It has will continue to produce press-releases targeting the local, national and international news media. Social media including popular resources such as Twitter and Facebook provide information and link the KINDReD website and press-releases with associations and blogs.

Our work through synergy with the other four projects financed under the FP7- call HEALTH 2013.2.3.4-2 Drug Development for Neglected Parasitic Diseases we are combining our efforts to continue dissemination of the EU's contribution to funding neglected infectious disease research by amongst other channels the KINDReD Association which has the potential to continue the research funded here for many years to come, ensuring that new, affordable treatments are provided to those in the most need.

Project public website
www.kindred-fp7.com
Contact details: Dr Jane MacDougall, Photeomix, 34 rue Carnot, 93160 Noisy le Grand, France email: jmacdougall@photeomix.com.

4.2 Use and dissemination of foreground

Section A

Our dissemination measures were run by WP10. Throughout the period of the grant, dissemination activities have been closely monitored and communicated as much as possible via the KINDReD public website. The majority of partners have been active in disseminating their activities in KINDReD at different meetings both National and International with poster and oral presentations as shown in the lists below. The European Commission emblem has been included on all scientific presentations, to acknowledge Community research funding.

The Scientific Co-ordinator partner 4 (PHX) has presented KINDReD at several International meetings and discussed with several major parties interested in NTDs including the Wellcome Trust and the French MTN network.

Members of the consortium have continued to publish numerous scientific articles with 30 peer reviewed articles shown here. Work is still on-going to publish more articles and numerous publications citing KINDReD are already planned and will continue to appear from the work performed in KINDReD. All scientific publications have acknowledged European Community financial support by specifying the associated FP7 grant agreement. Wherever possible publications are in line with the Commission's wishes for Open Access.

Section B (Confidential or public: confidential information marked clearly)

Final Report

Annex

Evaluation of the *in vivo* efficacy of bisnaphthalimidopropyl-derivative compound against *Trypanosoma brucei*

Inês Loureiro[1,2], David Costa[1,2], Luís Gaspar[1,2], Nuno Graça[1,2], Catarina Baptista[1,2], Helena Ribeiro[1,2], Joana Faria[1,2], Joana Tavares[1,2] & Anabela Cordeiro da Silva[1,2,3]

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[2] Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal;
[3] Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal.
•Corresponding author ✉ cordeiro@ibmc.up.pt

ABSTRACT

Human African trypanosomiasis (HAT) is a parasitic disease caused by the protozoan *Trypanosoma brucei* (*T. brucei*) that if left untreated is usually fatal. Disease control is dependent on drug therapy, since no human vaccine is available. Once the actual chemotherapy against HAT is far from satisfactory due to the emergence of resistances, toxicity and its limited efficacy, there is an urgent need for new drugs.

Three bisnaphthalimidopropyl-derivative (BNIP) compounds were assessed for activity against *T. brucei* bloodstream form and human macrophage cell line as toxicity control. Using a resazurin-based assay, the effect of bisnaphthalimidopropyl-derivatives exposure on the parasites was performed. The compounds displayed promising anti-parasitic activity and selectively against *T. brucei* with IC50 of 2.35 ± 0.99 nM for the most active compound, namely, Bisnaphthalimidopropyldiaminobutane (BNIPDabut). This compound was tested *in vivo* against *T. brucei* infected BALB/c mice using whole animal bioluminescent imaging. *In vivo* bioluminescence imaging is a non-invasive technique that can be used to monitor infections in real time and is a powerful approach for studying drug effectiveness. For this purpose, parasites were genetically modified to express luciferase (plasmid containing codon-optimised red-shifted *Photinus pyralis* luciferase kindly provided by Dr. Martin Taylor Laboratory from Connecticut College; McLatchie et al, 2013). The different clones obtained after transfection were screened and the ones with higher levels of bioluminescence were selected. *In vitro* growth curves of these cell lines were comparable to the parental wildtype and no growth defects were detected.

The course of infection of BALB/c mice treated with 10mg/Kg/day of BNIPDabut (intravenously) was assessed following the intraperitoneal injection of 10^4 *T. brucei* bloodstream forms. Additionally, highly infected mice were treated with the same dose of BNIPDabut and parasitaemia was cleared. Untreated and pentamidine treated mice were used as negative and positive controls, respectively.

Reference:
McLatchie AP, Burrell-Saward H, Myburgh E, Lewis MD, Ward TH, Mottram JC, Croft SL, Kelly JM, Taylor MC (2013) Highly sensitive in vivo imaging of Trypanosoma brucei expressing "red-shifted" luciferase. PLoS neglected tropical diseases 7: e2571

CONCLUSIONS

- All three BNIP derivatives showed antiparasitic activities *in vitro*.
- Although the three compounds showed an inhibitory effect on *T. brucei* SIR2RP1, these must have additional targets as Sir2 has already been proven not to be essential for this trypanosomatid.
- BNIPDabut was chosen for *in vivo* studies due to its superior antiparasitic activity and selectivity.
- For the purpose of *in vivo* bioluminescence assays transgenic parasites were generated and showed no growth differences compared to the wt parasites. Moreover, this approach allows the detection of parasites in infected mice whose parasitaemias are below the detection limit.
- BNIPDabut presents an antiparasitic activity against *T. brucei*, although it was not as effective as the reference drug. Experiments with the goal of confirming these preliminary results and finding the most appropriate treatment scheme are underway.

Financing and acknowledgements:
The research leading to these results has received funding from the European Community's Seventh Framework Programme under grant agreements No.602773 (Project KINDRED) and No.603240 (project NMTrypI) under the synergy activity.

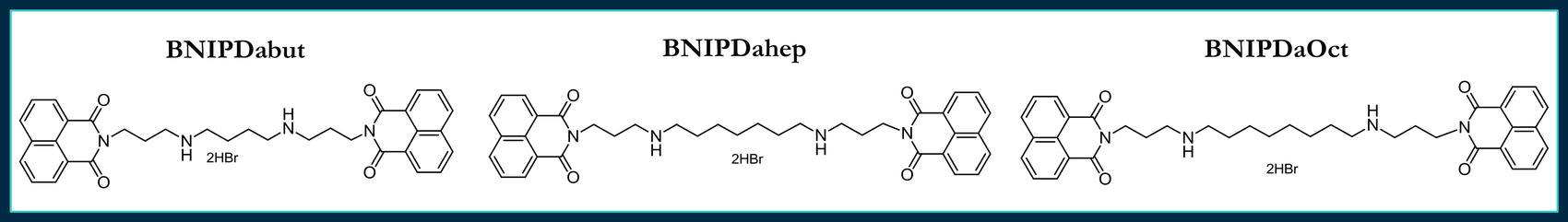


Figure 1. Chemical structures of bisnaphthalimidopropyl (BNIP) derivatives.

Inhibition of *T. brucei* SIR2RP1 by BNIP derivatives

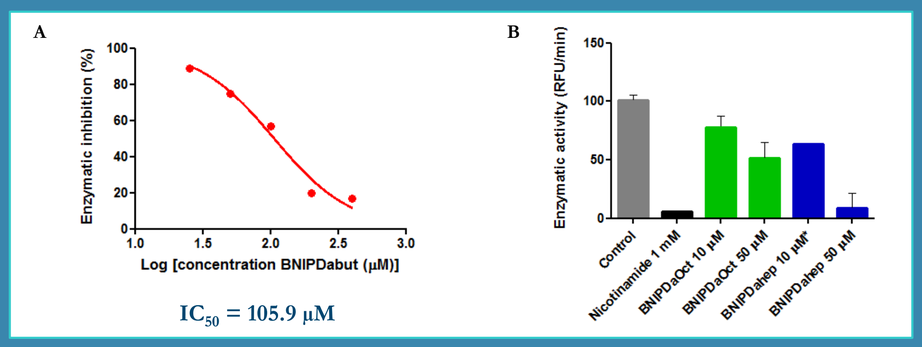
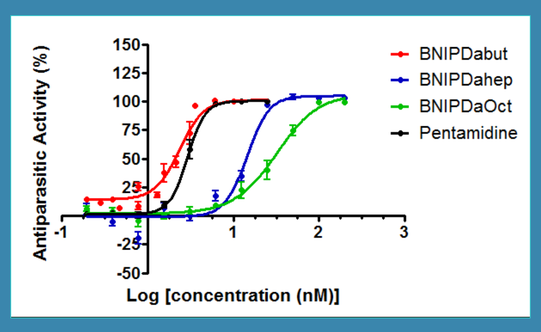


Figure 2. Inhibitory activity of BNIP derivatives on the *T. brucei* NAD-dependent deacetylase Silent Information Regulator 2 Related Protein 1 (SIR2RP1). Dose-response curve of the inhibition of *T. brucei* SIR2RP1 by BNIPDabut (A). Effect of BNIPDaOct, BNIPDahep and 1 mM nicotinamide versus control (buffer). These assays were performing using the CycLex SIRT1/Sir2 Deacetylase Fluorimetric Assay Kit and according to the manufacturers instructions. Fluorescence was measured using Synergy 2 Multi-Mode Reader (Biotek).
*Only one replicate represented.

In vitro effect of BNIP derivatives against *T. brucei* bloodstream forms



Compound	Cytotoxicity* (µM)	Antiparasitic Activity (nM)		Selectivity Index CC_{50}/IC_{50}
	CC_{50} ± SD	IC_{50} ± SD	IC_{90} ± SD	
Pentamidine	47.73 ± 3.32	2.97 ± 0.61	5.39 ± 0.55	16071
BNIPDabut	5.90 ± 0.40	2.35 ± 0.99	3.82 ± 1.40	2511
BNIPDahep	3.34 ± 0.11	14.30 ± 1.59	23.15 ± 1.41	234
BNIPDaOct	3.88 ± 0.59	29.90 ± 12.66	74.60 ± 11.85	130

Figure 3. Antiparasitic activity against *T. brucei* and cytotoxicity of BNIP derivatives. Dose-response curves of BNIP derivatives against *T. brucei* (A). Parasites were seeded in triplicates at 10^3 cells/well in 96-well plates containing compounds serially diluted in HMI-9 medium, resazurin was added at a finally concentration of 45.45 µM after 72 h and following an incubation period of 4 h fluorescence was measured using Synergy 2 Multi-Mode Reader. Table: *In vitro* antiparasitic and cytotoxicity profiles of BNIP derivatives (B). 10^5 THP1 cells/well were seeded in 96-well plates and differentiated by addition of 20 ng/ml PMA in RPMI medium, 1:2 serial dilutions of compounds were added 48 h later, and after 24 h cells were incubated for 4 h with 0.5 mg/ml MTT. Absorbance was read using Synergy 2 Multi-Mode Reader.

In vivo efficacy of BNIPDabut

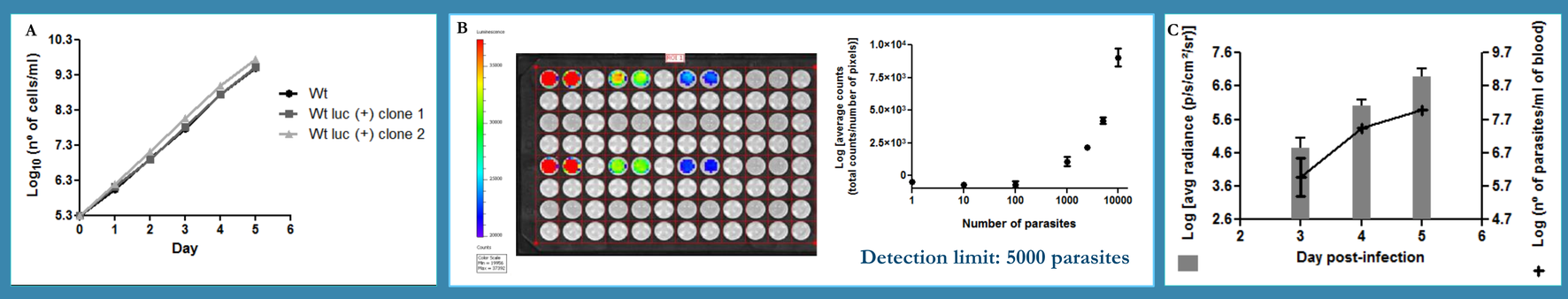


Figure 4. Validation of the method. *In vitro* growth curves of two luc(+) *T. b. brucei* Lister 427 monomorphic strains versus the parental wt: parasites were seeded at 2×10^5 cells/ml of complete HMI-9 medium and every 24 h, until day 5, cell growth was quantified microscopically (A). *In vitro* detection limit: both clones were serially diluted from 10^4 cells/well in a 96-well plate and imaged using IVIS LUMINA LT (Perkin Elmer) (B). Infected BALB/c mice were monitored daily for bioluminescence and parasitaemia. Graphic representation of the mean ± SD of average radiance (p/s/cm2/sr) and parasitaemia [log (nº of cells/ml of blood)] (C).

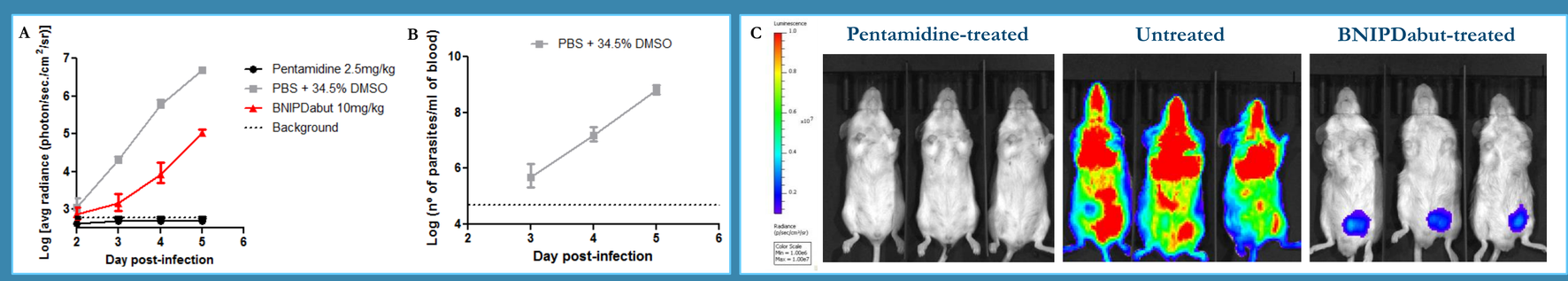


Figure 5. Monitoring the course of infection of BALB/c mice treated with BNIPDabut. Three animals per group were inoculated i.p. with 10^4 parasites and treated i.v. 24 h later with 10 mg/kg/day BNIPDabut for 5 days, 2.5 mg/kg/day pentamidine for 4 days or DMSO in PBS at 34.5%/day for 5 days. Total parasite load was determined by whole animal bioluminescence imaging (A) and parasitaemia was assessed for 7 days by parasite counting with Neubauer chamber in blood diluted with ammonium chloride (B). In the groups treated with BNIPDabut and pentamidine parasitaemia remained bellow detection limit. *In vivo* imaging of BALB/c mice after 5 days of treatment (C). Parasite load was evaluated by subcutaneous injection of 2.4 mg of luciferin and all animals were imaged using IVIS LUMINA LT. groups at day 5 post-infection are represented

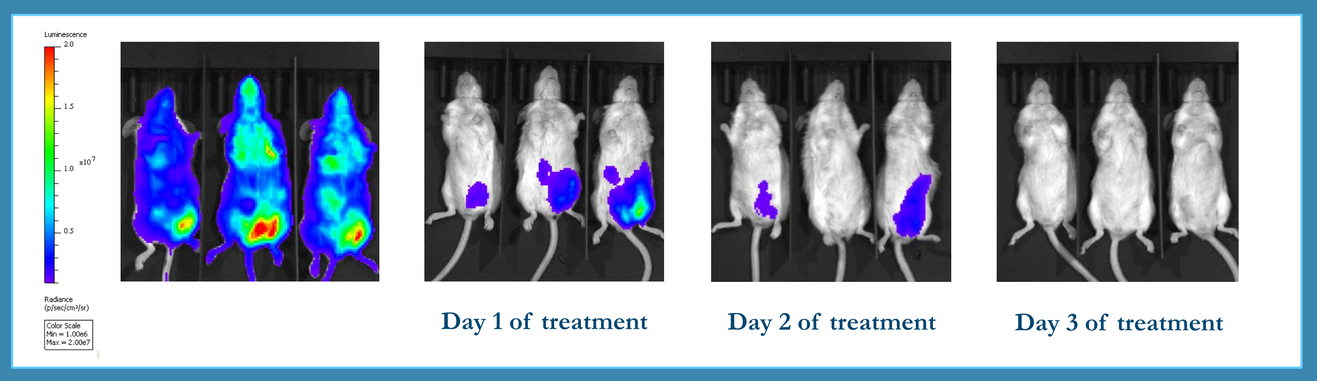
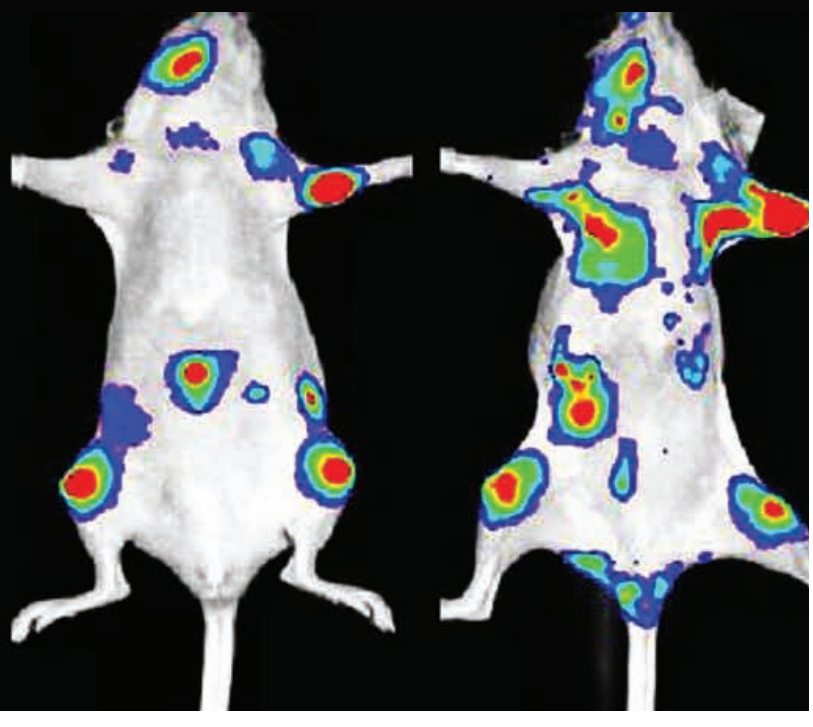


Figure 6. Effective reduction of high parasite loads following treatment with BNIPDabut. Animals presenting a parasitaemia ranging from $5x10^7$ to $1.6x10^8$ bloodstream trypomastigotes/ml of blood were treated intravenously with 10 mg/kg/day of BNIPDabut for 3 consecutive days and imaged using IVIS LUMINA LT.

WORKSHOP ON LIVE IMAGING

Anti-Parasitic drug discovery: from in vitro screening to animal validation

15 SEPTEMBER 2014 | IBMC | PORTO | PORTUGAL



PROGRAMME

08.30 – 09.00 Welcome Participants

09.00 – 09.20 Opening Talk

THEORICAL SESSIONS: MAIN AUDITORIUM

09.20 – 10.10 **Sarman Singh, New Delhi, India**
Overview of current affairs in terms of clinical leishmaniasis and medical aspects as well as drug treatments, drug resistance in the field

10.10 – 11.00 **James McKerrow, UCSF**
Drug discovery: High-throughput screening.

11.00 – 11.30 Coffee-Break

11.30 – 12.30 **Rogerio Amino, Institut Pasteur, Paris**
In vivo imaging to discover new anti-parasitic approaches

12.30 – 14.00 Lunch

PARALLEL PRACTICAL SESSIONS

14.00 – 15.45 **SESSION A: Joana Tavares, IBMC and Jair Neto, UCSF**
Evaluating anti-parasitic drugs efficiency using bioluminescent whole animal imaging system.

SESSION B: Andre Maia, IBMC
High-throughput drug screening using the IN Cell Analyzer (GE Healthcare)

16.15 – 18.00 **REPETITION OF BOTH SESSIONS**

The event is free but the registration is mandatory: http://www.ibmc.up.pt
Practical sessions registration deadline: 27th August
Practical sessions will be available for a limited number of participants. The participants selected to the practical sessions will be contacted by e-mail on 3rd September.

SCIENTIFIC AND ORGANIZING COMMITTEES:
ANABELA CORDEIRO, FFUP AND IBMC, PORTUGAL | JOANA TAVARES, IBMC, PORTUGAL | ANDRÉ MAIA, IBMC, PORTUGAL

KINDReD

FUNDED BY THE SEVENTH FRAMEWORK PROGRAMME OF THE EUROPEAN UNION

A new target for a novel class of nitro-heterocyclic-amides with potent trypanocidal activity

Terry K. Smith[1], Nathalie Trouche,[2] Paola Ciapetti[2], Isbaal Ramos[3]

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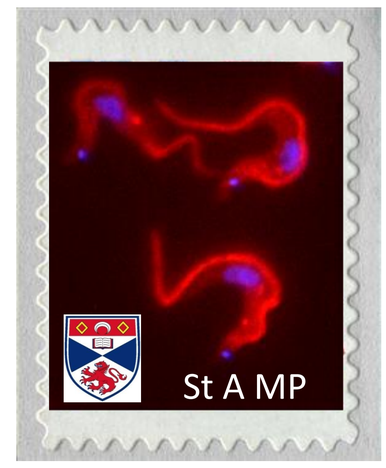
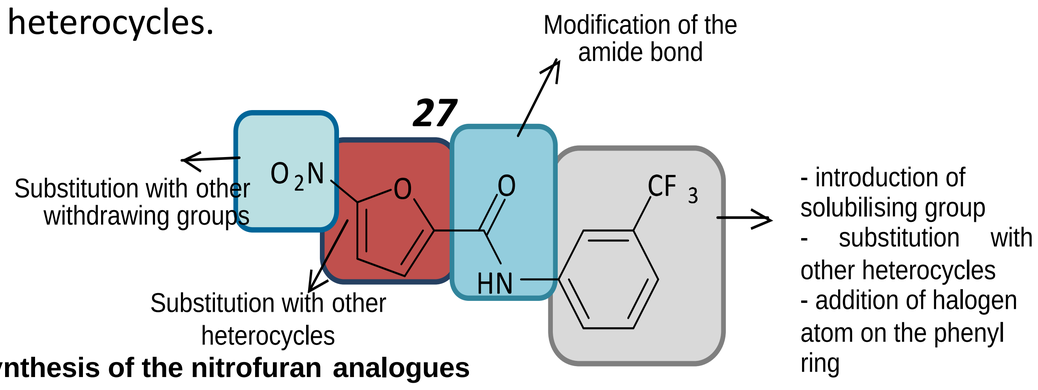
[3]Innoprot S.L Parque Tecnológico, Bizkaia, Spain
tks1@st-andrews.ac.uk



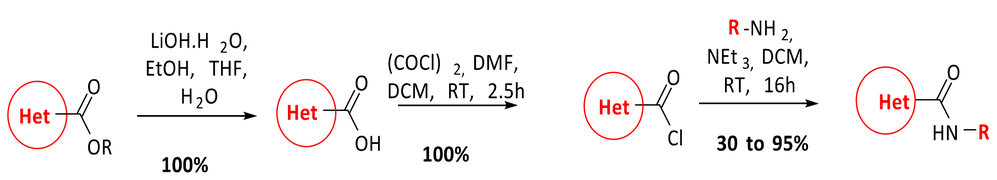
Since the World Health Organization approved the nifurtimox-eflornithine combination therapy of Human African Trypanosomiasis, there has beenrenewed interest in nitro-heterocycle therapies for this and associated diseases. We previously identified a series of novel 5-nitro-2-furancarboxamides that show potent trypanocidal activity, ~ 1000 fold more potent than nifurtimox against *in vitro T. brucei* and with very low cytotoxicity against HeLa cells. Most importantly, the most potent analogue, compound **27**, showed very limited cross-resistance to nifurtimox resistant cells and *vice versa*.

With this starting point, we explored the SAR around the lead compound reported in the figure below. Four different moieties in the structure of the lead compound were identified. For each of them some substitutions were envisaged with the aim to evaluate the impact of those changes on the activity. The moieties are highlighted in the figure below together with the substitutions with the different functional groups and/or heterocycles.



Synthesis of the nitrofuran analogues

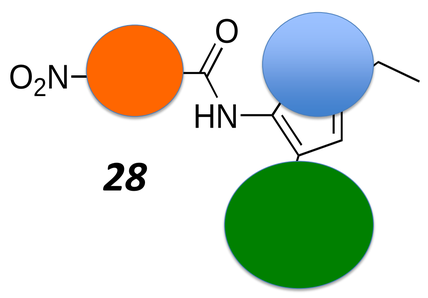
The general synthetic route for the preparation of the analogues is described below:



Scheme 1: General synthetic scheme for the preparation of nitrofuran analogues

Structure-Activity Relationships: Synthesised analogues were tested against *T. brucei brucei, T. cruzi, L. infantum* and HeLa cells. Some biological results are summarised below.

Nitro-heterocycle-amides Series 2: show even better activity against *T. cruzi* and *L. infantum*



The aforementioned nitro-furan-amides show good potency against *T.cruzi (epi)* and *L.infantum* with **EC50** ***~500 nM*** and ***selectivity >1000.***

The 2nd and a recent 3rd series are currently showing better that Nifurtimox activity for amastigote studies.
This will be followed by an vivo testing

MAY BE A MAGIC BULLET IS POSSIBLE?

ADME-T data for a selection of potent lead compounds

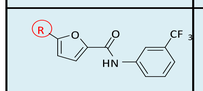
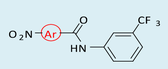
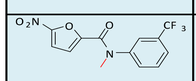
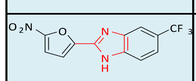
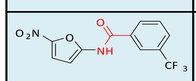
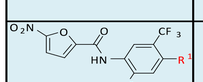
Structure	Substitution	Entry	*T. brucei* EC_{50} (nM)	HeLa EC_{50} (mM)	Selectivity
	R = Br	1	20800 ± 160	>20	-
	$R = CF_3$	2	152.9 ± 4.3	>20	>131
	R = CN	3	17600 ± 125	>20	-
	pyrrole	4	14.90 ± 0.9	>20	>1342
	4-thiazole	5	23.10 ± 1.4	>20	>866
	5-thiazole	6	535.3 ± 18.9	>20	>37
	Me-imidazole	7	1870 ± 140	>20	>11
	-	8	4740 ± 170	>20	>4
	-	9	492.8 ± 38.6	>20	>40
	-	10	71800± 640	>20	-
	$R_1 = F, R_2 = H$	11	13.90 ± 1.0	>20	>1438
	$R_1 = H, R_2 = F$	12	41.80 ± 3.7	>20	>478
	$R_1 = H, R_2 = Br$	13	47.20 ± 3.7	>20	>423
	$R_1 = H, R_2 = OCH_2iPr$	14	7.96 ± 0.18	>20	>2512
	$R_1 = H, R_2 = OCH_2cPr$	15	3.90 ± 0.59	>20	>5138
	thiazole	17	25.70 ± 0.6	>20	>778
	thiazole-CF_3	18	50 ± 3.7	>20	>400
	furane-CO_2Me	19	18.40 ± 0.6	>20	>1086
	pyrazole	20	51± 3.2	>20	>392
	3-Pyridine	21	238 ± 12.9	>20	>84
	2-Pyridine	22	416.4 ± 27.8	>20	>48
	4-Pyridine	23	814.2 ± 39.6	>20	>25
	2,4-pyrimidine	24	1090 ± 80	>20	>18
	3-pyridazine	25	459.6 ± 27.0	>20	>44
	Ph-4-CH_2-Morph.	26	701.3 ± 35.6	>20	>29
	-	27	4.69	>20	~4454
Nifurtimox	Batch 1		2356 ± 167	-	-
Pentamidine	Batch 1		0.95 ± 0.19		

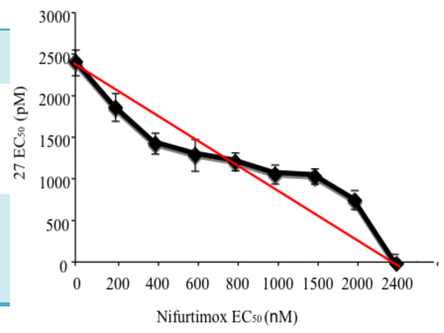
Table: Structure-activity reactivity study of analogues **1** to **27**.

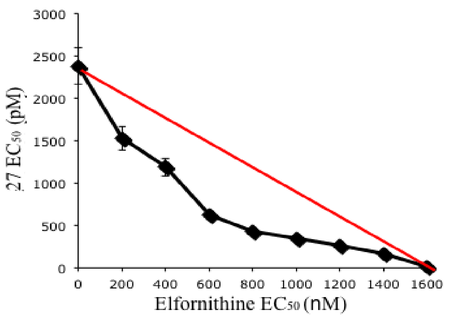
Entry	logP	Hepatotoxicity (μM)	Neurotoxicity (μM)	Nephrotoxicity (μM)	PPB (%) mice	PPB (%) human	Mouse stability %	Aqueous solub %	Chem Stab (Gastric)%
11	3.18	20.72±0.12	1.7±0.73	2.58±0.03	96	97	29	1.18	141
13	3.8	NT	4.5±0.8	2.72±0.12	56*	> 95	30.9	0.76	116.5
4	1.5	38.1±16.84	24.41±5.48	9.06±0.06	60	93	38.2	28.64	63.4
18	2.97	0.92±0.1	3.59±3.2	3.28±0.54	97	> 99	15.4	115.7	98.5
17	2.76	34.32±2.67	9.19±2.25	6.36±0.37	74	97	85.6	123.1	94.5
19	1.31	22.30±11.02	9.05±0.78	6.14±1.68	63	95	2	170.66	100.9
27	3.61	12,5*	0.99±0.08	1.96±0.05	> 99	> 97	25.3	0.99	86.9
5	3.36	>50*	>25	>25	99	> 98	32.3	13.02	100
28 (series 2)	2.69	25,6*	1.28±0.4	2.22±0.38	85	> 96*	3.1	3.41	88.5

No cross-resistance to Nifurtimox resistant cells

Cell Type	Nifurtimox (μM)[a]	Compound 27 (nM)	Pentamidine (nM)[b]
Wild-Type	2.1 ± 0.2	2.4 ± 0.3	1.0 ± 0.1
Nifurtimox Resistant	20.9 ± 1.7	7.8 ± 0.4	2.4 ± 0.2
Compound 27 Resistant	7.3 ± 0.5	29.3 ± 2.0	1.1 ± 0.1

Compound 27 shows synergy to Nifurtimox and Elfornithine

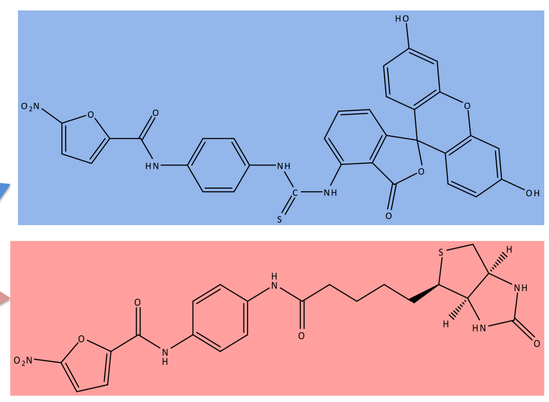




Possible new combinaional therapy with eflornithine

Target identification: If nitro-reducatse is not the target, then what is ?

On going and future experiments:
Transciptome analysis of 27-resistant strain.
SILAC and iTRAQ style experiments of cells ± treatments
Localisation/accumulation of compound inside cells
Metabolite profiling including pull-downs
UV-Cross linking and pull downs / protein ID



We are also trying to address the question…
"What makes a good/bad nitro-reductase substrate?"

(Chemical probes made)

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KINDReD

Characterising the trypanosomatid lysosome and its essential role in host lipid catabolism

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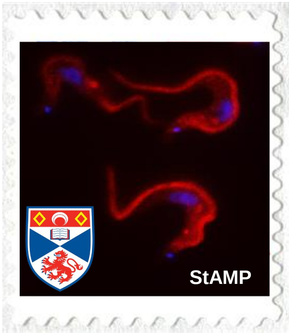


KINDReD

wellcome trust

Introduction

Kinetoplastid related diseases are a huge percentage of the world's communicable disease burden. Current drug treatments are inadequate and there is an urgent need for novel therapeutic targets and lead compounds that can be translated into safe, cheap, and easy to administer drugs. The causative agent of Human African Trypanosomiasis, *Trypanosoma brucei*, relies upon a rapid endocytosis and degradation of macromolecules from the mammalian bloodstream to provide metabolites essential for its proliferation and survival[1]. The terminus of the endocytic pathway, the lysosome, must be central to this macromolecular digestion. **However very little is known about this organelles' function and biogenesis.** To successfully study proteins, metabolites and digestive processes of the *T. brucei* lysosome, this organelle must be isolated from the other components of the cell. Standard techniques are ineffective due to the high buoyant density of the lysosomes preventing centrifugal separation from larger organelles.

Results

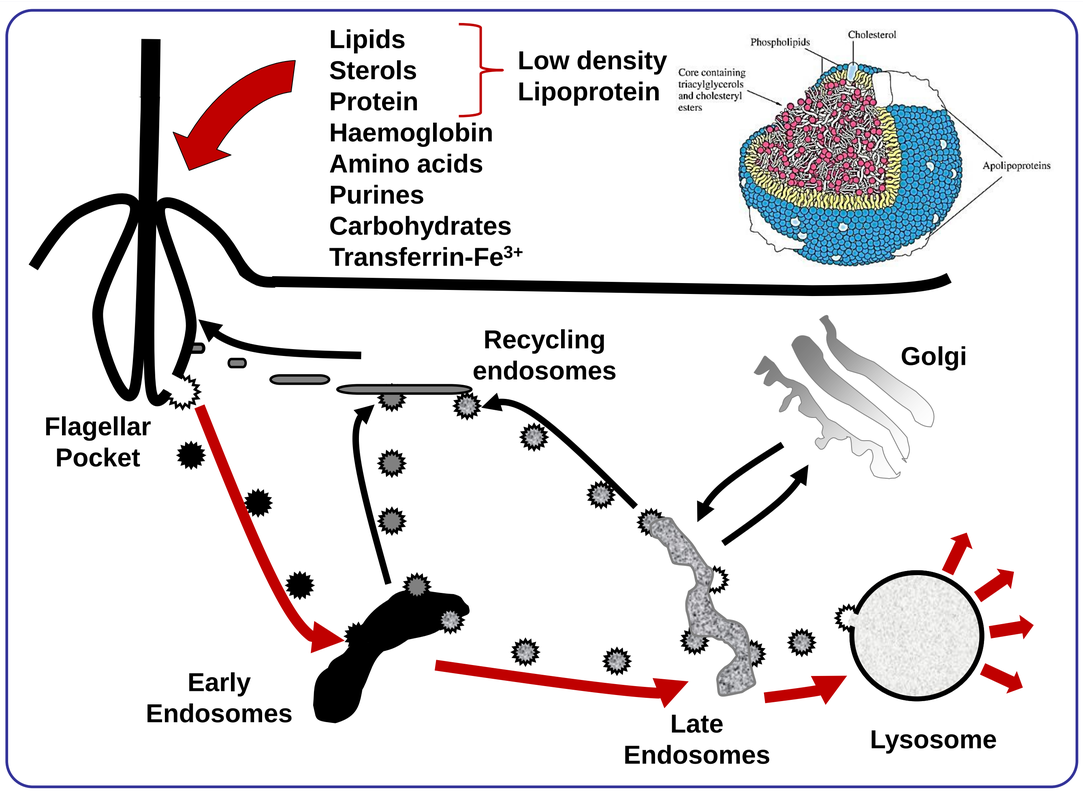
Here we have incubated insect-form procyclic *T. brucei* with a non-digestible macromolecule tyloxapol (Triton WR-1339) which accumulates in trypanosome lysosomes, reducing their density so they can be easily purified from complex cellular material[2]. Sucrose gradient centrifugation produced two fractions distinct from the dense layer of other organelles. Transmission electron microscopy (utilising a proprietary method) showed fraction 4 had electron dense particles, comparable in size and morphology to lysosomes. The lighter fraction 1 showed regular spherical particles suggestive of endosomes. nLC-ESI-MS/MS analysis of the lysosome-like fraction 4 detected known lysosomal markers and other proteins with digestive, structural and transport related functions, more than half detected in other lysosome related organelles. Importantly particles in fraction 4 showed acid phosphatase activity[3] confirming them as functioning lysosomes.

Conclusions

We are utilising this efficient subcellular purification methodology to further characterise the lysosomal proteome. Ultimately, identification of an outward cytosolic facing antigen will allow us to raise specific antibodies to purify, through immunoprecipitation, native lysosomes. In addition to *in vivo* studies, we will investigate in detail various catabolic processes in purified non-detergent disrupted lysosomes.

and test compounds that will disrupt the function of this essential *T. brucei* organelle. Furthermore we are expanding this approach to investigate the organelle in other medically important kinetoplastids

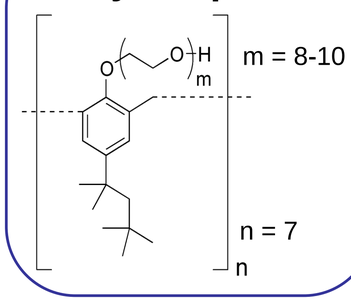
Tyloxapol is endocytosed and is trypanocidal to *T. brucei* in culture



Bloodstream form

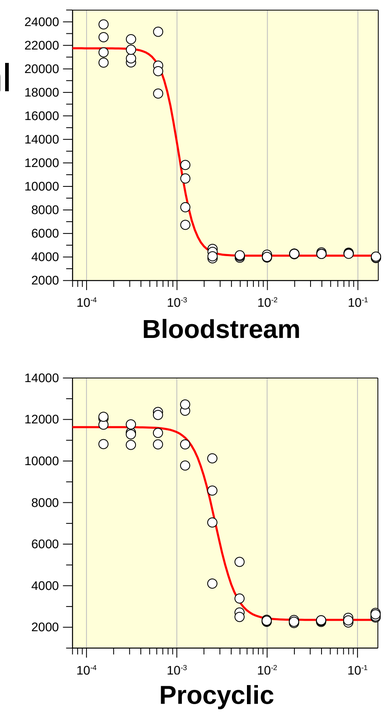
EC_{50} = **10 ± 0.4** µg/ml

Tyloxapol



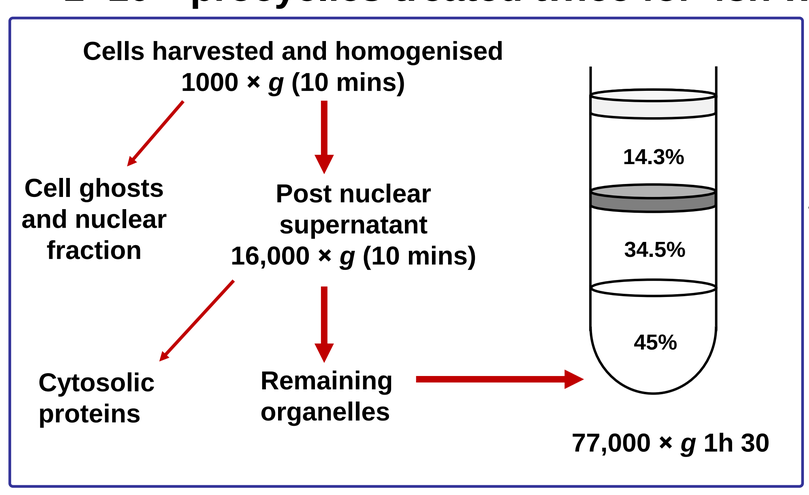
Procyclic form

EC_{50} = **27 ± 2** µg/ml



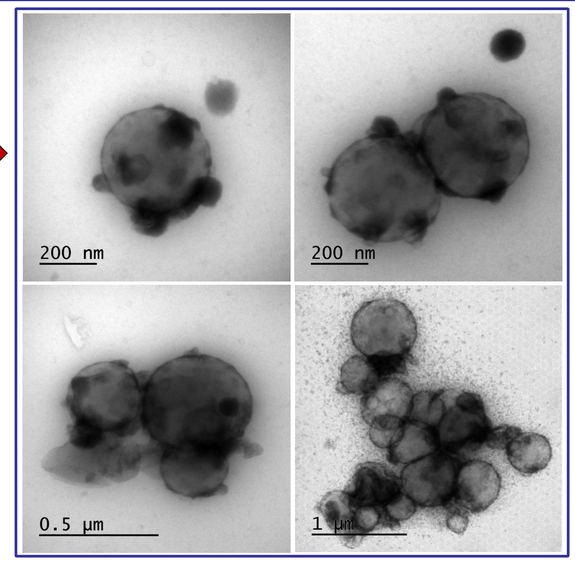
Lysosome and endosome-like structures can be purified using tyloxapol

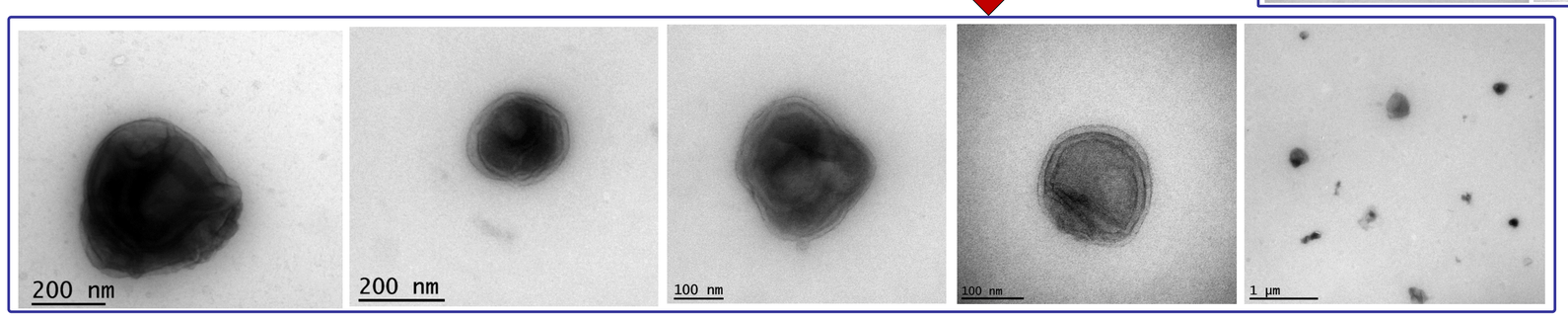
1×10[10] procyclics treated twice for 48h with 1.5 µg/ml tyloxapol



Fraction 1 "endosomes"

Fraction 4 "lysosomes"



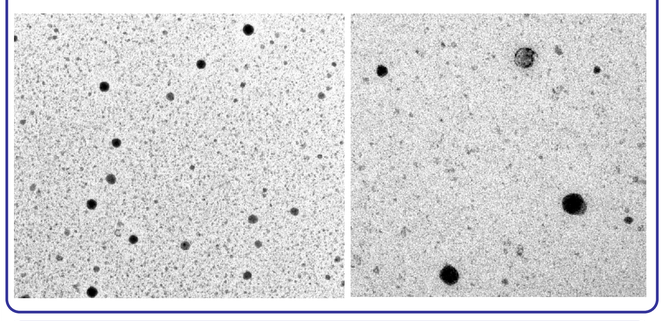


Lysosomes contain a variety of proteins and have acid phosphatase activity

Tb gene number	Protein Function	*T. cruzi*	Rat liver	
Tb927.6.960*	Cathepsin L	Y	Y	Digestive enzymes
Tb927.11.12850	Oligopeptidase B serine peptidase	Y	Y	
Tb927.1.2230	Calpain-like Cysteine proteinase	Y	Y	
Tb927.11.6590	Aminopeptidase	Y	Y	
Tb927.7.4570	Purine nucleoside hydrolase			
Tb927.8.6390	Lysophospholipase			
Tb927.10.8450	Glucose transporter	Y	Y	Transporters
Tb927.7.5950*	Carboxylate transporter (PAD)			
Tb927.2.6150*	Purine transporter		Y	
Tb927.4.4380	Vacuolar-type H^+ Pyrophosphatase	Y		
Tb927.8.7600	Amino acid transporter	Y	Y	
Tb927.5.1830	p67	Y		Structural
Tb927.1.2340*	Alpha tubulin	Y	Y	
Tb927.1.2330*	Beta Tubulin	Y	Y	
Tb927.10.10360*	Microtubule associated protein			
Tb927.11.6990	Serine/Threonine protein kinase	Y		Metabolic
Tb927.7.7420*	ATP Synthase alpha		Y	
Tb927.10.2090	Elongation factor 1 alpha		Y	
Tb927.7.4520	Thioredoxin-like protein	Y	Y	
Tb927.5.4020	T. brucei BARP related protein			GPI-anchored
Tb927.7.6850	Trans-sialidase			
Tb927.11.11290*	Hsc70[#]	Y	Y	Protein trafficking
Tb927.10.2130	VPS51 Vacuolar sorting protein[#]			
Tb927.10.3860	SNF7-like protein		Y	

Protein hits were identified using the Mascot algorithm (Matrix Science) from the MS/MS spectra based on ≥2 peptide matches to the NCBInr database (after removing false positives using a decoy database) with an identity score >25 (p-value <0.05). Homologous proteins identified in *T. cruzi* reservosomes[3] and rat liver lysosomes[4] are indicated. #Clathrin interacting proteins[5]. *Gene numbers representative of multiple homologues not differentiated by the peptide matches.

Using the Gomori technique, particles in fraction 4 are intact with acid phosphatase activity



Future work:

Determine the lysosome proteome

Characterise lysosomal enzyme activities

Lipidomic/Metabolomic studies of lysosomes

Identify chemical entities to interfere with lysosomal function

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Profiling Kinases using Chemoproteomics approach

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[(1)] **Phylogene, Bernis France.** http://ms.phylogene.com [(2)] Corresponding author (gskorski@phylogene.com)

Overview

•Purpose: Profiling Kinases expression and activity in THP-1 cell lysates.

•Methods: Adenosine tri phosphate (ATP) activity-based probes were used to specifically label and enrich Nucleotide binding proteins for mass spectrometry analysis

•Results: Using ATP probes to assess Kinases activity, we have profiled a kinases inhibitor (Purvalanol B) activity. We have identified 1422 proteins among them more than 700 nucleotides binding sites were conclusively mapped by mass spectrometry.

Introduction

•Kinases and ATP-binding proteins have major roles in numerous key biological processes ranging from control of the cell cycle to signal transduction. Parasite ATP-binding proteins are therefore an attractive target for drug discovery. However the use of ATP mimetic or nucleotides binding site blocking agents as treatment can be have dramatic side effect. As kinases are evolutionary highly conserved, this lack of host–parasite specificity is due of high sequence similarity between host and parasite. It is therefore essential to develop an early warning system to identify "off" interactions at "omics" level (most completed catalog of ATP binding proteins).

•ABPP probe for kinases coupled at high resolution mass spectrometry seems be a promising tool for this purpose. The structure of these probes consists in a modified biotin (desthiobiotin) attached to the nucleotide through a labile acyl-phosphate bond. Acyl-phosphate bond is highly reactive with primary amine radical group of lysine. It covalently modifies this amino acid and induces a mass shift of + 192.1212 Da (See fig1).

Methods

•**Cells proteins labeling and profiling**: We have used 4E7 cells as starting material, cell were lyzed to extract proteins which were purified by size exclusion SPE to remove endogenous enzyme co-factors. The purified proteome was treated or not by Puralanol B and then labeled by ABPP. At this point two strategies were applied. For the first one, we captured labeled proteins by affinity chromatography and then digested them by FASP protocol. In this case all peptides from captured proteins are identified by mass spectrometry. For the second one we digested proteins first, using trypsin, and then we captured labeled peptides by affinity chromatography and analyzed them by mass spectrometry. In parallel we digested the whole cell proteome as control to evaluate the efficiency of enrichment by ABPP probe.

•**Mass spectrometry**: The peptide digest (100 ng) was loaded onto a nanoACQUITY UPLC Symmetry C18 Trap Column in trap and elute mode with ACQUITY UPLC Peptide BEH C18 nanoACQUITY Column. The run gradient was performed by Eksigent Ultra Plus nano-LC 2D HPLC (ABSciex, Framingham) system over 90 min with a gradient from 3% to 40% buffer B (buffer A: 0.1% formic acid; buffer B: 95% acetonitrile, 0.1% formic acid) at a flowrate of 300 nl/min. The Eksigent system was coupled to a TripleTOF® 5600 (ABSciex, Framingham) mass spectrometer. The acquisition parameters were as follows: for DDA mode one 250 ms MS scan (>30K resolution). Following each survey MS1 scan, MS/MS spectra for the 30 most abundant parent ions (m/z range 350-1250) were acquired (high sensitivity mode, >15K resolution). For DIA mode one 150 ms MS scan (>30K resolution), followed by 35 fixed SWATH windows each with a 75 ms accumulation time and a 350-1250 m/z range. MS/MS SWATH scans (high sensitivity mode, >15K resolution) were set at 26 amu window, Q1 isolation windows were covering entire mass range.

•**Data analysis**: DDA spectra processing and database searching was performed with ProteinPilot (v4.5 beta, ABSciex, Framingham) using Paragon and mascot algorithms. The search parameters were as follows: sample type: identification; cys alkylation: iodoacetamide; digestion: trypsin; instrument: TripleTOF 5600; special factors: Urea denaturation. ID focus: biological modifications with special variable modification Desthiobiotin label (+192.1212 Da on Lysine) . The database was downloaded from Uniprot (June 2015), filtering for reviewed mouse proteins only. The identification peak list was loaded into Peakview® (v2.0, ABSciex, Framingham) and Skyline. Peaks from SWATH runs were extracted with a peptide confidence threshold of 99% and a false discovery rate <1%. Label-free quantification was performed by using Marker View (v1.2.1, ABSciex, Framingham). The selection of the proper peak was performed using the automated assistance of PeakView. The absolute signal of peptide or protein was calculated by summing the extracted area of all unique fragment ions. Protein contents were compared using Student T-test, differences were considered as significant for p-values lower than 0.05.

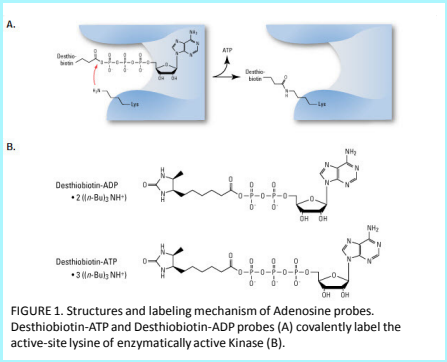

FIGURE 1. Structures and labeling mechanism of Adenosine probes. Desthiobiotin-ATP and Desthiobiotin-ADP probes (A) covalently label the active-site lysine of enzymatically active Kinase (B).

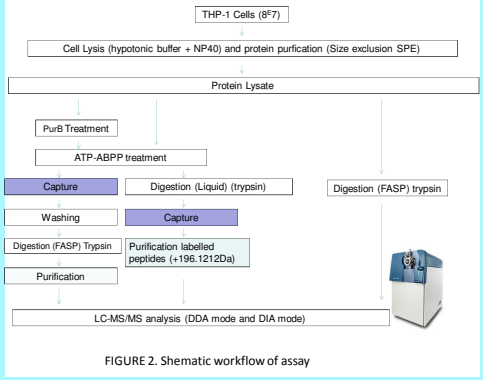

FIGURE 2. Shematic workflow of assay

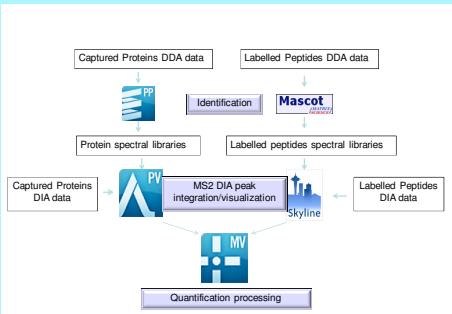

FIGURE 3. Schematic workflow of data processing

Results

•Using the MS Workflow (Figure 2), we have determined that more than 700 proteins were labeled. Among them 300 are reported as ATP-binding in mouse THP-1 cells tissues including CDK5. When comparing with unlabelled proteome, and efficient enrichment of ATP binding proteins was observed (See table 1). A specific inhibition of probe binding by inhibitor treatment was also observed for CDK5 (See fig 4).

•Analysis of labeled active sites showed a very good labeling efficiency (See fig 5), more than 95% of identified peptides were labeled by desthiobiotin.

Protein	p-value	ABPP	Total proteome	Fold Change
Tyrosine-protein kinase SYK	0,0002	1,47E+06	4,92E+05	2,98
Mitogen-activated protein kinase 14	0,0001	6,63E+05	1,56E+05	4,24
Cyclin-dependent kinase 1	0,0000	7,55E+05	3,17E+05	2,38
Serine/threonine-protein kinase 10	0,0002	7,47E+05	1,80E+05	4,14
Tyrosine-protein kinase CSK	0,0000	1,62E+06	3,85E+05	4,21
Mitogen-activated protein kinase 1	0,0002	4,52E+05	1,74E+05	2,59
Serine/threonine-protein kinase 38	0,0025	3,78E+05	1,23E+05	3,07
Adenylate kinase 2, mitochondrial	0,0009	7,19E+05	1,80E+05	3,99
[3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondrial	0,0000	3,38E+05	8,35E+04	4,04
Serine/threonine-protein kinase VRK1	0,0376	3,26E+05	1,61E+05	2,02
Tyrosine-protein kinase Fes/Fps	0,0303	1,36E+05	6,37E+04	2,14
Cyclin-dependent kinase 5	0,0001	3,47E+05	6,11E+04	5,68

TABLE 1. Relative quantification of Kinases, ABPP vs no enrichment . Examples of enriched kinases.

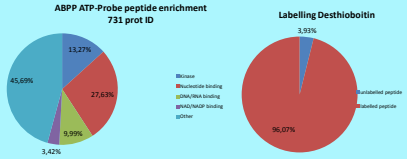

FIGURE 5. Repartition of ABPP enriched proteins by enzyme type . Labeling efficiency.

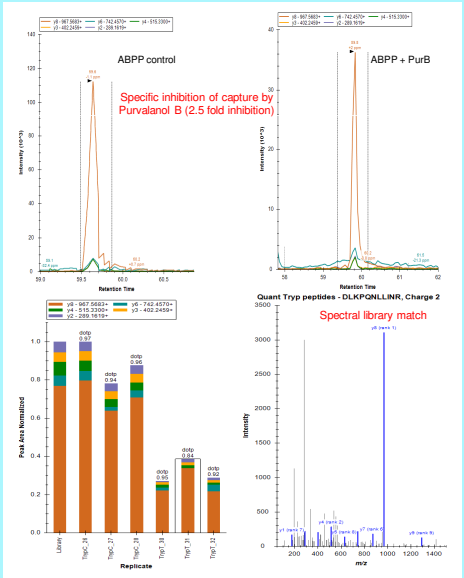

FIGURE 4. Example of CDK5 specific peptide (DLK[196.1]PQNLLINR) enriched by ABPP capture.
Upper right DIA XIC of control ; Upper Left DIA XIC of purvalanol B treatment. An ABPP probe binding inhibition was observed shown by a CDK5 decrease of activity induced by purvalanol B.

Conclusion

•ABPP ATP-Probe seems to be a potent tool to study ATP binding protein inhibitor effects at omics level. With a large number of identified proteins it is possible to observe on and off target interactions on very large data set of proteins.

•This type of assay are just one screwdriver of huge toolbox of chemoproteomics analysis as other ABPP probes are available, for instance GTP-desthiobiotin probe. Other ways for deciphering compound effect on biological model are worth to be investigated such as classical differential proteomics, or phosphosites analysis for early signal transduction.

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Kinetoplastid Drug Development: strengthening the preclinical pipeline

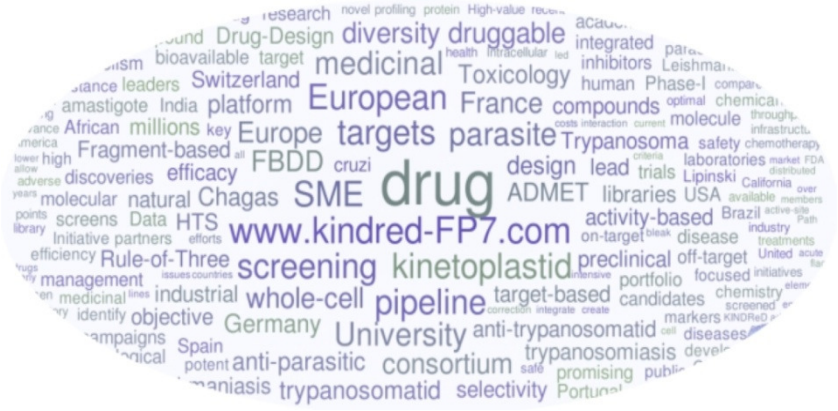
The KINDReD Consortium*

Funding - European Community's Seventh Framework Programme under grant agreement No.602773 (Project KINDRED)

Abstract

Treatments currently available for the trypanosomatid diseases; Leishmaniasis, Human African Trypanosomiasis (HAT) and Chagas Disease (CD), are limited and with few new drugs in the pipeline, any potential resistance is a threat to endemic areas. The KINDReD consortium was created with the goal to strengthen the preclinical drug development pipeline in order to achieve at least one new Phase I clinical candidate for each of these three trypanosomatid diseases.

Building the consortium, we identified key areas which seemed to be potential bottlenecks in the preclinical pipeline and looked for novel solutions to overcome these. KINDReD integrates world renown parasitology laboratories equipped with high throughput screening (HTS) facilities for all three major kinetoplastid parasites from Europe (Portugal, United Kingdom, and Switzerland), the USA (California) and South America (Brazil) with innovative research driven SMEs whose techniques and technologies are for the first time being harnessed in the field of neglected infectious disease.



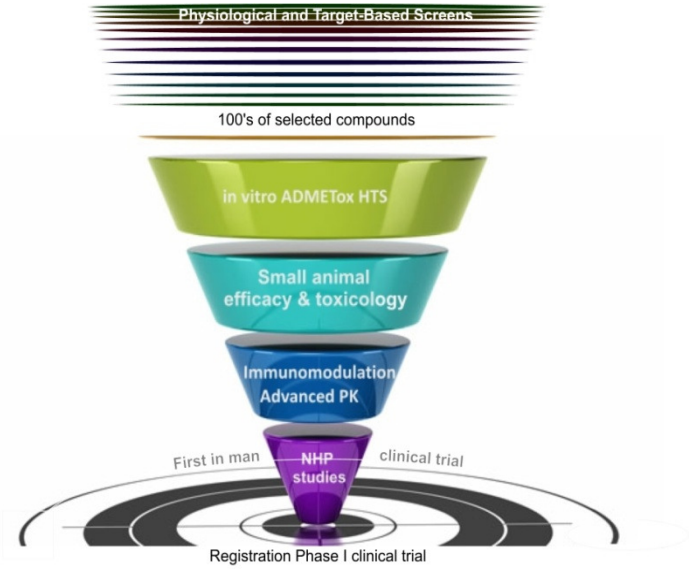
KINDReD Open Collaboration

One key feature of KINDReD's collaborative effort is to be as open as possible, making many of the consortium's platform technologies available to other researchers also interested in pre-clinical kinetoplastid drug development, including screening, toxicology and animal testing of appropriate lead candidates#. If you have a potential drug candidate that would benefit from one or more of the approaches listed in this poster or viewed on our website, please do not hesitate to contact us via the consortium website at www.kindred-FP7.com. We look forward to working with you.

Under uncomplicated, mutually acceptable CDA

Combined expertise from academia and industry

- Physiological whole-cell drug screening assays for three kinetoplastid parasites:
  - High-content imaging of intracellular parasites
  - High throughput drug screening platforms
- Novel natural compound libraries from marine, fungal and entomopathogenic bacterial sources
- Several over-lapping platforms for fragment-based drug design, including high content SPR, thermal shift, microcapillary EMS and X-ray crystallography
- Medicinal chemistry
- Multiple recombinant and chemoproteomic screens for genetically and chemically validated targets
- High throughput screening platform for *in vitro* cell-specific compound toxicity
- *In vitro* ADME bioanalytical platforms
- Coordinated multi-centre animal efficacy/toxicology screening using established mouse infection model
- Non-human primate models for the most-promising selected compound(s)
- Online project & data management (Basecamp & CDD vault)



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Trypanosoma brucei sphingomyelinase enzymes as targets for drug development

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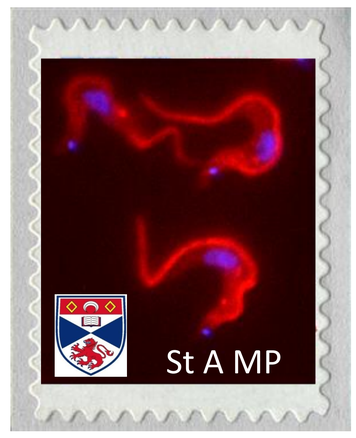


Introduction

Many novel drug targets have been identified through investigating *T. brucei* lipid biosynthesis. However, comparatively little is known about the pathways responsible for *T.brucei* lipid catabolism. My research aims to improve our knowledge of these pathways, specifically by studying the degradation of *T.brucei* sphingolipids . *T. brucei* is the only eukaryote currently known to produce all three of the major eukaryotic sphingolipid sub-species[1]: inositol phosphatidylceramide; ethanolamine phosphatidylceramide and choline phosphatidylceramide, also known as sphingomyelin. This combined with the fact the *T.brucei* sphingolipid synthase (SLSs 1-4) gene locus has been declared essential [2], suggests sphingolipids must be important to the parasite. Sphingomyelinases are the enzymes that breakdown sphingolipids in most eukaryotes. These proteins can be classified based on their pH optima, metal ion dependency and subcellular localization. *T.brucei* is thought to contain three of these enzymes – a neutral sphingomyelinase (nSMase) and two putative acidic sphingomyelinases (aSMase1 and aSMase2) (**Figure 1**). My research focuses on two of these proteins: nSMase and aSMase2.

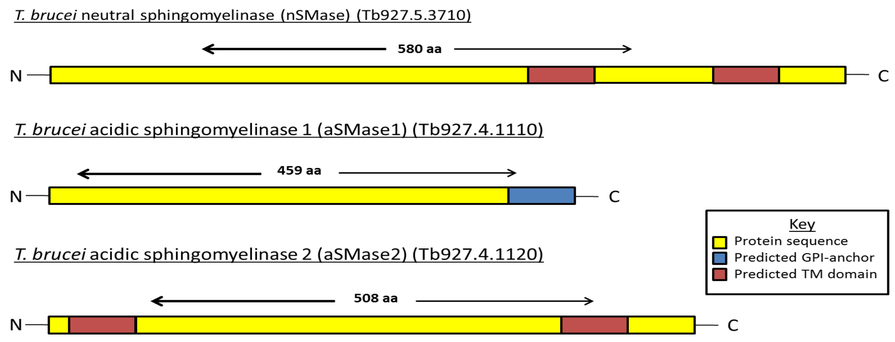


Figure 1 – Schematic representation of the three *T.brucei* sphingomyelinases. Estimated protein size and predicted modes of membrane insertion are indicated. 'N' and 'C' denote N- and C-termini respectively.

T.brucei neutral sphingomyelinase (nSMase)

Research from our lab has already shown *T.brucei* nSMase is an essential enzyme, in both bloodstream and procyclic form parasites[3]. By recombinantly expressing this membrane protein in *E.coli* , bacterial membranes are enriched with nSMase activity. As *E.coli* do not metabolise sphingolipids, nSMase activity can be assayed directly from purified *E.coli* membranes with minimal background interference. nSMase activity is monitored using the Amplex UltraRed assay system (**Figure 2**).

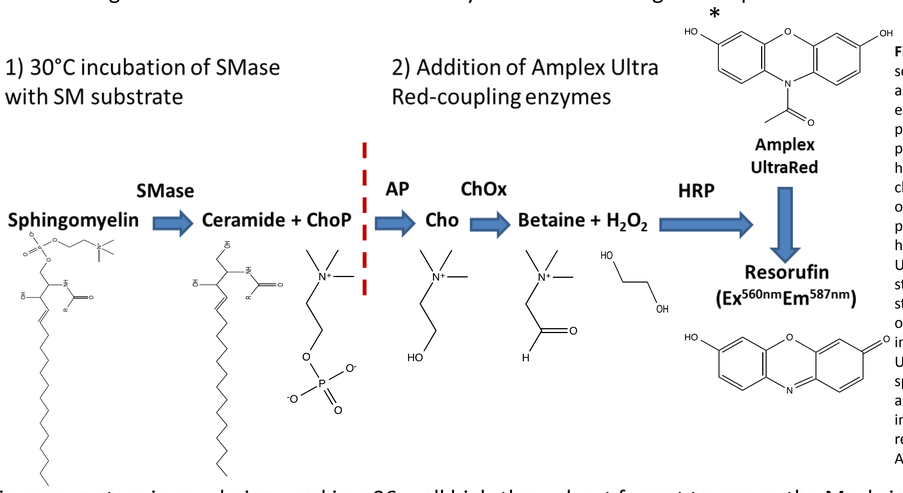


Figure 2 – Amplex UltraRed assaying of TbnSMase: schematic representation of the Amplex UltraRed assay system. Sphingomyelinase (SMase) activity is estimated by measuring choline phosphate (ChoP) production when the assayed protein is in the presence of sphingomyelin (SM) substrate. ChoP is hydrolysed by alkaline phosphatase (AP), forming choline (Cho). Choline oxidase (ChoOx) then oxidises Cho to yield betaine and hydrogen peroxide (H_2O_2). The H_2O_2 is the oxidising agent for horse radish peroxidase (HRP) that converts Amplex UltraRed to resorufin. Chemical structures for each step of the reaction are shown. *highlights that the structure shown for Amplex UltraRed is that of its originator Amplex Red, as no chemical structural information or formula is available for Amplex UltraRed. Conversion rate can be established spectrophotometrically ($Ex^{560\,nm}$, $Em^{587\,nm}$). The assay incorporates two stages:1) 30°C pre-incubation of the protein sample and substrate in reaction buffer 2) subsequent addition of standard Amplex UltraRed assay components.

This assay system is now being used in a 96-well high-throughput format to screen the Maybridge Rule of 3 fragment library, in the hope of identifying new leads for drug development (**Figure 3**). 600 fragments have been screened so far, with further screening currently in progress. In this format, the potent nSMase inhibitor Manumycin A[3] acts as the inhibitor-positive control versus uninhibited nSMase activity. These controls are used to calculate Z-factors for each fragment plate screening (Z-factors > 0.6).

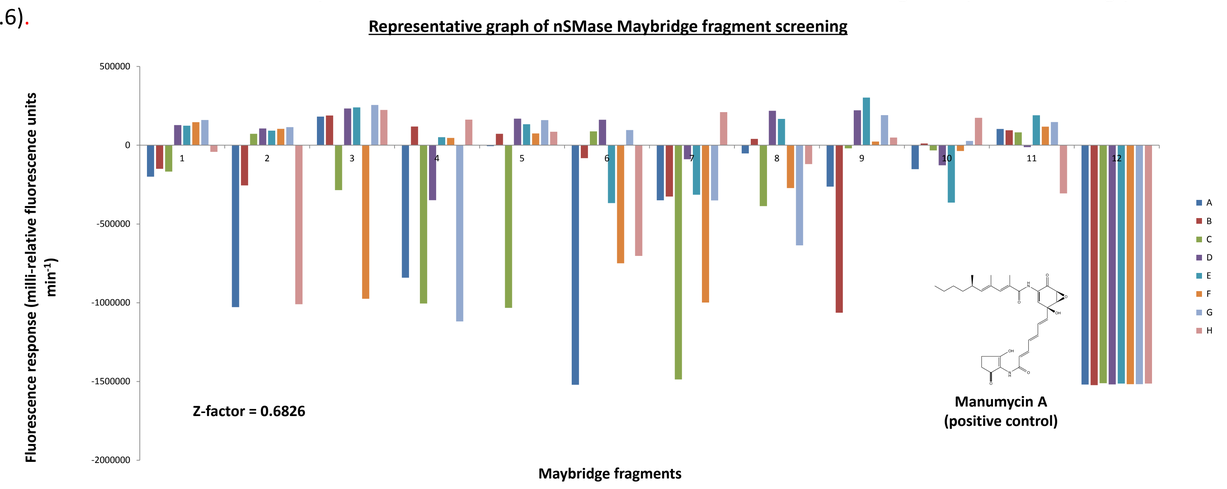


Figure 3 – Sample nSMase Fragment Library Screening Data: Graph representing the data output from the screening of nSMase activity against a 96-well fragment library plate. Fluorescence response (milli-relative fluorescence units min^{-1}) , relative to the positive control, is plotted for each fragment, identified through their positions in the plate (rows A-H; column 1-12). Column 1 is a column of 8 replicate negative control reactions, column 12 represents the Manumycin A positive control.

Based on the data obtained, a threshold of 45 % inhibition of TbnSMase activity was set to facilitate hit selection. This resulted in the selection of 6 fragments (**Figure 4**). Inhibition of TbnSMase by these fragments will be confirmed and IC_{50} values established for the assay. Preliminary EC_{50} values previously obtained by our group for these fragments against *T.brucei* and HeLa cells (**Figure 3**) also require confirmation. Studying changes in parasite morphology and lipid composition on exposure to these fragments may give an indication as to whether TbnSMase is their target *in vivo.*

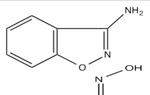
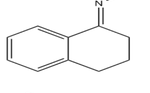
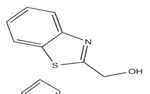
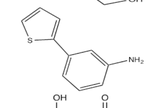
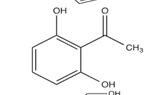
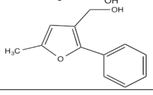
Maybridge fragment number	Fragment name	Structure	Assay Percentage Inhibition	BSF *T.brucei* EC_{50}*	HeLa EC_{50}*
107	1,2-benzisoxazol-3-amine		60	26.5 ± 5.8	76.9 ± 10.5
279	1,2,3,4-tetrahydronapthalen-1-one oxime		58	18.8 ± 5.8	19.9 ± 19.7
312	1,3-benzothiazol-2-ylmethanol		68	73.2 ± 12.2	80.7 ± 16.2
403	3-(2-thienyl)aniline		48	32 ± 5.9	49.1 ± 15.7
477	2-amino-5-phenyl-3-furonitrile		53	93.4 ± 0.3	98.3 ± 18.6
522	(5-methyl-2-phenyl-furyl)methanol		49	45.4 ± 7.9	24.3 ± 17.4

Figure 4 – Summary of hits currently established from nSMase fragment library screening. *Signifies that this data was obtained from previous preliminary tests done in our lab that are still to be replicated in light of this current work.

Future Work

- Extend current fragment screening
- Confirm inhibition of TbnSMase by hit fragments and establish IC_{50} values
- Chemically validate lead fragments in the parasites and verify EC_{50} values
- Investigate structure-activity relationships with lead fragment analogues to optimise potency and selectivity

T.brucei acidic sphingomyelinase 2 (aSMase2)

T. brucei acidic sphingomyelinase 2 (aSMase2) is one of two putatively identified acidic sphingomyelinases. Unlike aSMase1, which is predicted to employ a GPI-anchor for membrane insertion, aSMase2 is thought to have two transmembrane domains, one found at its N-terminus, the other being C-terminal. I have achieved recombinant expression of aSMase2 in TOP10 *E.coli* with a pBAD-HisA/aSMase2 expression construct (**Figure 5**).

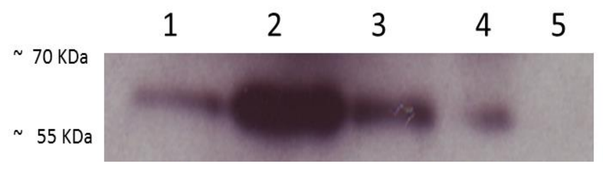


Figure 5 – pBAD His A/aSMase2 expression. Western blot for whole cell samples taken from 50 ml lysogeny broth cultures of TOP10 competent *E.coli* cells containing the pBAD His A/aSMase2 expression construct**.** An arabinose gradient (1-0.2 %; 2 – 0.02 %; 3 – 0.002 %; 4 – 0.0002 %; 5 – 0.00002 %) was set up across 5 cultures at 25 °C. Cultures were incubated for 24 hr.

I now aim to scale-up aSMase2 protein expression and carry out detergent screening for protein solubilisation, with a view to purifying enough protein for anti-aSMase2 antibody production. This antibody may then be amenable to protein localisation studies *in vivo*. We hypothesise that aSMase2 is localised to the *T.brucei* lysosome. If confirmed, this would make aSMase2 only the fourth identified *T.brucei* lysosomal protein. Assaying the purified protein for acid pH sphingomyelinase activity is also intended with the Amplex UltraRed assay system (**Figure 1**).

Ultimately, I am looking to validate aSMase2 as a drug target. To this end, production of an aSMase2 genetic knockout cell line is in progress. Although the essentiality of aSMase2 is still to be established, I have been investigating pre-existing FDA approved drugs as part of a potential 'piggy-backed' therapeutic strategy (**Figure 6**). We hypothesise that the impact of these drugs on *T.brucei* may be linked to their disruption of aSMase2 activity. Several of the drugs are well known tricyclic anti-depressants. There has long been the suggestion that part of the therapeutic value of these drugs stems from their ability to decrease the activity of human aSMases.

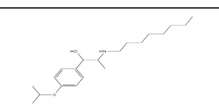
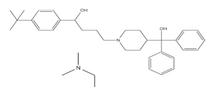
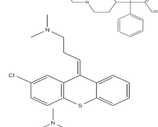
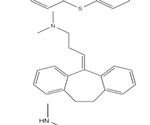
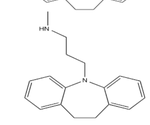
Drug	Structure	PCF *T.brucei* EC_{50} (μM)	BSF *T.brucei* EC_{50} (μM)	HeLa EC_{50} (μM)
Suloctidil		6.6	2.1	7.81
Terfenadine		7.1	1.5	6.36
Chlorprothixene		22.2	5.6	39.7
Amitriptyline		---	13.8	130.36
Desipramine		27.2	9.7	30.5

Figure 6 – Summary of FDA-approved drug EC_{50}s against procyclic and bloodstream form *T.brucei,* as well as HeLa cells.

I am currently investigating the effects of these drugs (**Figure 6**) on *T.brucei* lipid composition using mass spectrometry and I am examining changes in parasite morphology resulting from drug exposure. As an example, **Figure 7** depicts the distinctive phenotype of BSF *T.brucei* seen following a 3 hr exposure to an EC_{85} dose of desipramine. Drug exposure leads to cellular swelling and the parasites struggle to divide, with a preponderance of the 2K2N phenotype.

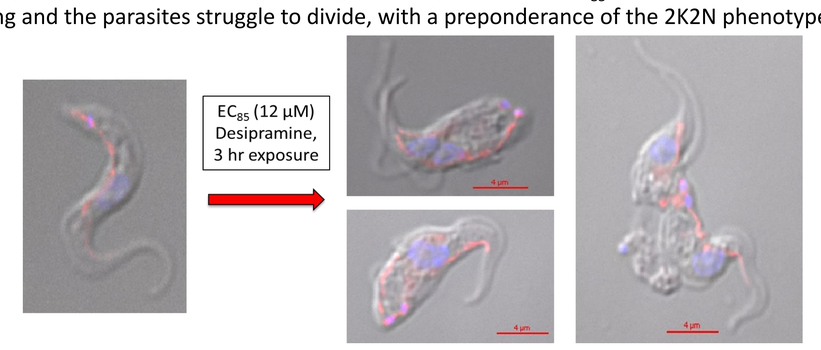


Figure 7 - Phenotypic alteration in BSF *T.brucei* resulting from high dose exposure to the tricyclic anti-depressant desipramine.

Future Work

- Production of an aSMase2 knockout cell line, in the hope of validating aSMase2 as a drug target
- Production of an anti-aSMase2 antibody for localisation studies
- Assaying aSMase2 for acidic sphingomyelinase enzyme activity
- Further investigation of selected FDA-approved drugs as a potential 'piggy-backed' therapeutic strategy

Acknowledgements and References

Thanks to Dr Simon Young for his previous work on *T.brucei* sphingomyelinases, particularly the nSMase, which provided the ground work for my project. Also to Dr Louise Major for her guidance with Maybridge fragment library screening and provision of initial EC_{50} data for the fragments against BSF *T.brucei* and HeLa cells. Further thanks to Dr Helen Denton for her guidance in processing fragment screening data.

1. Serricchio, M. and Bütikofer, P. (2011) *Trypanosoma brucei*: a model micro-organism to study eukaryotic phospholipid biosynthesis, *FEBS J.* 278: 1035-1046
2. Mina, J. G., Pan, S., Wansadhipathi, N. K., Bruce, C. R., Shams-Eldin, H., Schwarz, R. T., Steel, P. G., Denny, P. W. (2009) The *Trypanosoma brucei* sphingolipid synthase, an essential enzyme and drug target, *Mol. Biochem. Parisitol.*, 168: 16-23
3. Young, S. A., Smith, T. K. (2010) The essential neutral sphingomyelinase is involved in the trafficking of the variant surface glycoprotein in the bloodstream form of *Trypanosoma brucei, Mol. Microbiol.,* 76(6): 1451-1482

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Ribose 5-phosphate isomerase B knockdown compromises *Trypanosoma brucei* bloodstream form infectivity

INSTITUTO DE BIOLOGIA MOLECULAR E CELULAR
INSTITUTE FOR MOLECULAR AND CELL BIOLOGY

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ABSTRACT

Ribose 5-phosphate isomerase (Rpi) is involved in the non-oxidative branch of the pentose phosphate pathway and catalyses the inter-conversion of ribose 5-phosphate (R5P) and ribulose 5-phosphate (Ru5P). There are two non homologous forms, the A and the B. The presence of type B in trypanosomatids, and its absence in humans, points RpiB as a potential drug target. This study presents a functional characterization of *T. brucei* ribose 5-phosphate isomerase B (*Tb*RpiB). *In vitro* biochemical studies confirmed *Tb*RpiB isomerase activity, as it can use both R5P and Ru5P as substrates. *Tb*RpiB knockdown by RNAi affected *in vitro* growth of bloodstream forms, but more importantly *in vivo* parasites infectivity since mice infected with induced RNAi clones exhibited lower parasitemia and a prolonged survival in comparison to mice infected with control parasites. Furthermore, *in vitro* and *in vivo* phenotype was reverted when an ectopic copy of *Trypanosoma cruzi* ribose 5-phospahate isomerase was introduced. These results suggest *Tb*RpiB as a promising drug target for African sleeping sickness, since interfering with this protein represents a way to control *in vivo* parasite growth and infectivity.

MATERIAL & METHODS

A large-scale production of *T. brucei and T. cruzi* parasitic recombinant proteins under native conditions was achieved. Genes were subcloned into *E. coli* high-expression vector (pET28a), allowing the production and purification of recombinant proteins containing 6x histidine residues at its N-terminals.

RpiB *in vitro* activity was tested. To determine the *Km* for R5P and to characterize 4-PEH-inhibition mechanism, a direct spectrophotometric method at 290 nm was used in 100mM Tris/HCl, pH 7.6 and 0.5µg of enzyme. 4-PEH was tested in the presence of 25mM R5P. *Km* determination was performed at a range between 3.1 and 50mM of R5P concentrations (blank-denatured enzyme).To determine the *Km* for Ru5P, a modification of Dische's Cysteine-Carbazole method was used. A mixture contained 5µl of the diluted enzyme in buffer A [100mM Tris/HCl (pH 8.4), 1mM EDTA and 0.5mM 2-mercaptoethanol] plus 5µl of Ru5P giving final concentrations between 1 and 10mM and 5ng/µL of enzyme was used. Following a 10 min incubation at 25˚C, 15µl of 0.5% cysteinium chloride, 125µl of 75% (v/v) sulfuric acid and 5µl of a 0.1% solution of carbazole in ethanol were added. After 30min standing at 25˚C, the A_{546} was determined (blank - no enzyme).

*Tb*RpiB expression level in different stages of the life cycle by Western-blot, and localization through immunofluorescence and digitonin fractionation were assessed.

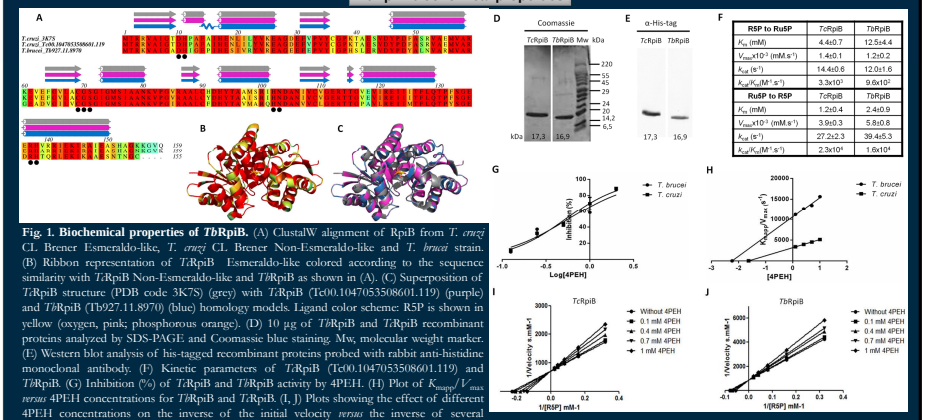
RNAi was used for *Tb*RpiB down-regulation in bloodstream forms. *TbRPIB* fragment was cloned twice in opposite direction on either sides of a 'stuffer' of the pHD1144 vector. The resulting construct was cloned into pHD1145 tetracycline (tet) inducible vector. The final construct was transfect into bloodstream forms with pHD1313. Stable individual clones were selected with 7,5 µg/ml of hygromycin. In *in vitro* growth curves, RNAi cell lines were seeded at $2x10^5$cells/ml of completed HMI9 medium, in the presence of tet. Every 24h, until day 10, cell growth was monitored microscopically and the culture diluted back to $2x10^5$cells/ml. As controls, uninduced cultures were grown in parallel. Northern blot and Western blot were performed to analysed mRNA and protein levels, respectively. In *in vivo* RNAi assays, groups of mice were intraperitoneally infected with either 1×10^4 wt and knockdown cell lines. 5µl of peripheral blood was obtained through tail blood extraction, diluted 1:5 into RBC lyses buffer and parasites were enumerated on a haemocytometer.

Functional complementation of *T. brucei* RNAi cell lines with the *T. cruzi* homologue was performed. *TcRPIB* fragment was cloned in vector pHD1034. After transfection individual clones were selected with 0.2 µg/ml of puromycin.

CONCLUSION

Our results clearly show a role of RpiB for bloodstream *in vitro* optimal growth and *in vivo* infectivity, but also a conserved function among different *Trypanosoma* species. *Tb*RpiB emerges as a novel target against African sleeping sickness

*Tb*RpiB biochemical properties



R5P to Ru5P	*Tc*RpiB	*Tb*RpiB
K_m (mM)	4.4±0.7	12.5±4.4
$V_{max}x10^{-3}$ (mM.s^{-1})	1.4±0.1	1.2±0.2
k_{cat} (s^{-1})	14.4±0.6	12.0±1.6
k_{cat}/K_m(M^{-1}.s^{-1})	$3.3x10^3$	$9.6x10^2$
Ru5P to R5P	*Tc*RpiB	*Tb*RpiB
K_m (mM)	1.2±0.4	2.4±0.9
$V_{max}x10^{-3}$ (mM.s^{-1})	3.9±0.3	5.8±0.8
k_{cat} (s^{-1})	27.2±2.3	39.4±5.3
k_{cat}/K_m(M^{-1}.s^{-1})	$2.3x10^4$	$1.6x10^4$

Fig. 1. Biochemical properties of *Tb*RpiB. (A) ClustalW alignment of RpiB from *T. cruzi* CL Brener Esmeraldo-like, *T. cruzi* CL Brener Non-Esmeraldo-like and *T. brucei* strain. (B) Ribbon representation of *Tc*RpiB Esmeraldo-like colored according to the sequence similarity with *Tc*RpiB Non-Esmeraldo-like and *Tb*RpiB as shown in (A). (C) Superposition of *Tc*RpiB structure (PDB code 3K7S) (grey) with *Tc*RpiB (Tc00.1047053508601.119) (purple) and *Tb*RpiB (Tb927.11.8970) (blue) homology models. Ligand color scheme: R5P is shown in yellow (oxygen, pink; phosphorous orange). (D) 10 µg of *Tb*RpiB and *Tc*RpiB recombinant proteins analyzed by SDS-PAGE and Coomassie blue staining. Mw, molecular weight marker. (E) Western blot analysis of his-tagged recombinant proteins probed with rabbit anti-histidine monoclonal antibody. (F) Kinetic parameters of *Tc*RpiB (Tc00.1047053508601.119) and *Tb*RpiB. (G) Inhibition (%) of *Tc*RpiB and *Tb*RpiB activity by 4PEH. (H) Plot of K_{mapp}/V_{max} *versus* 4PEH concentrations for *Tb*RpiB and *Tc*RpiB. (I, J) Plots showing the effect of different 4PEH concentrations on the inverse of the initial velocity *versus* the inverse of several concentrations of R5P for *Tc*RpiB and *Tb*RpiB, respectively.

*Tb*RpiB expression and subcellular localization

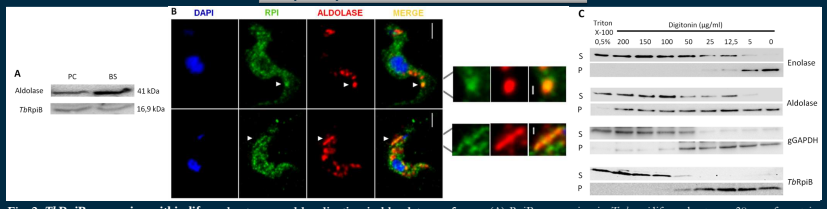


Fig. 2. *Tb*RpiB expression within life cycle stages and localization in bloodstream forms. (A) RpiB expression in *T. brucei* life-cycle stages; 30 µg of protein from bloodstream (BS) and procyclic (PC) total lysates was analysed by Western blot. (B) Immunofluorescence analysis by confocal microscopy of bloodstream forms *Tb*RpiB. Nuclear and kinetoplast DNA labelled by DAPI staining (blue). RpiB (green) and aldolase (red) were labelled respectively with rat anti-*Tb*RpiB and rabbit anti-aldolase antibodies. White arrowheads indicate RpiB and aldolase co-localization areas that are magnified in the right panels. (C) Supernatant (S) and pellet (P) fractions obtained with different concentrations of digitonin were subjected to Western blot analysis and probed with rabbit antibodies against *Tb*RpiB, enolase (cytoplasmic marker), aldolase and glycosomal GAPDH (glycosome markers).

In vitro and *in vivo* analysis of *Tb*RpiB RNAi

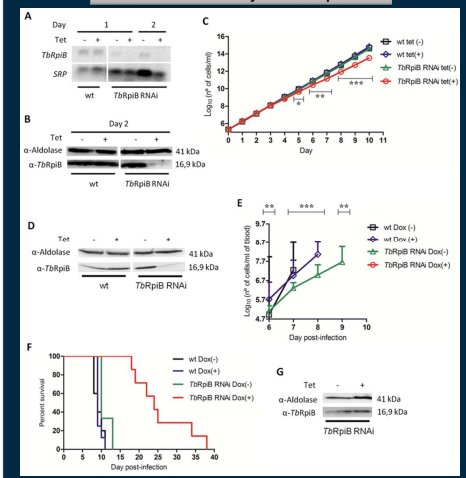


Fig. 3. *In vitro* and *in vivo* effect of RNAi-mediated RpiB downregulation on bloodstream forms. (A) Northern and (B) Western blot analysis of mRNA and protein levels, respectively, upon RpiB RNAi. (C) Growth curve of a wt *versus* a representative RpiB RNAi cell line. (D) The levels of Rpi in bloodstream forms 48h after tet induction, which were used for mice infections, were controlled by Western blot. (E) Parasitaemias of mice infected intraperitoneally with 10^4 control wt or a representative RNAi clone. The mice were either untreated or treated with 1mg/ml Dox in the water supply. (F) Kaplan–Meier survival analysis of mice infected with non-induced and induced wt cell line *versus* a non-induced and induced representative RNAi clone. (G) Western blot analysis of RpiB levels in a representative non-induced and Dox-induced RNAi clone collected from mice before being euthanized. Statistical differences between non-induced and induced *Tb*RpiB RNAi clone are depicted (* $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$).

Complementation of *Tb*RpiB RNAi phenotype

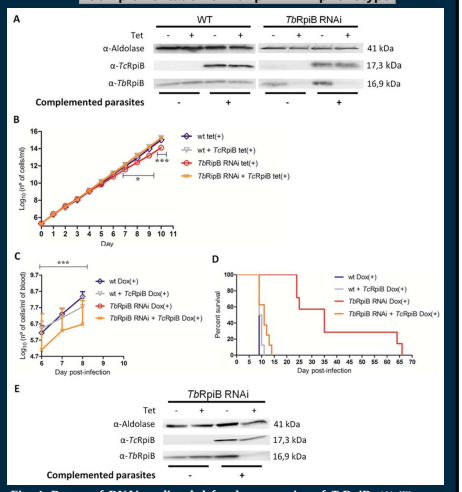


Fig. 4. Rescue of RNAi mediated defect by expression of *Tc*RpiB. (A) Western blot analysis of *Tb*RpiB and *Tc*RpiB levels in bloodstream forms 48h after tet induction. (B) *In vitro* cumulative growth of induced non-complemented and complemented wt bloodstream forms *versus* an induced non-complemented and complemented representative *Tb*RpiB RNAi clone. (C) Groups of mice were infected intraperitoneally with 1×10^4 RNAi induced non-complemented and complemented wt parental cell line *versus* non-complemented and complemented representative *Tb*RpiB RNAi clone. (D) Kaplan–Meier survival analysis of mice infected with Dox induced non-complemented and complemented wt cell line *versus* induced non-complemented and complemented representative *Tb*RpiB RNAi clone. (E) Western blot analysis of RpiB levels in a representative non-complemented and complemented *Tb*RpiB RNAi clone isolated from mice blood before being euthanized. Statistical differences between non-complemented and complemented induced *Tb*RpiB RNAi clone are depicted (* $p\leq0.05$, *** $p\leq0.001$).

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