

COST Action CM1307

**Joint COST Action CM1307 2nd Conference
/ WG2 and WG3 Meetings**

OCTOBER 26-28, 2015

Belgrade, Serbia

www.costcm1307.org/CM1307_2/1st_Meeting.html

This Joint COST Action CM1307 2nd Conference / WG2 and WG3 Meetings has been organized by the Institute of Nuclear Sciences VINCA, University of Belgrade (UoB), Serbia. The conference and the Working Group meetings will be held in Palace Hotel, Toplicin Venac 23, Belgrade, Serbia (office@palacehotel.rs).

The meeting is focused on seven complementary topics :

- 1- Medicinal chemistry
- 2- Natural products
- 3- Biological targets
- 4- Drug targeting
- 5- Novel therapeutic approaches, tools to decipher drug mechanism of action
- 6- Drug resistance
- 7- Biology for possible therapeutic considerations

This 2nd COST CM1307 conference is followed by the meetings of two chemistry Working Groups :

WG2 dedicated to Medicinal Chemistry and WG3 dedicated to Natural Products.

Organizing Committee:

Sanja Glišić (Institute of Nuclear Sciences VINCA, UoB, RS)
Veljko Veljković (Institute of Nuclear Sciences VINCA, UoB, RS)
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Karin Seifert (LSHTM, UK)
Tom Solmajer (University of Ljubljana, SL)
Thomas Schmidt (University of Münster, GE)
Ana Tomas (University of Porto, PT)

SCIENTIFIC PROGRAM

October 25

18:30 *Registration and welcome drink at the Palace Hotel*

October 26

9:00 **Loiseau P.M.**, *COST Action CM1307 Chair*

Opening remarks

OPENING LECTURE

9:10 **Lepesheva G.**, *Vanderbilt University Nashville, Tennessee, USA (Invited speaker)*

Structure-based development of antimicrobial agents targeting sterol 14-alpha-demethylase

MEDICINAL CHEMISTRY

Chairpersons: T. Calogeropoulou, M. Botta

10:00 **Botta M.** *University of Sienna, Italy (lecture)*

Novel fungal chitinase inhibitors and their possible use in the fight against parasites

10:50 **Calogeropoulou T.**, *University of Athens, Greece*

Design, synthesis and evaluation of antiparasitic activity of heteroaryl-substituted ether phospholipid derivatives

11:15 **Mangalagiu I.** *University of Iasi, Romania*

New polifunctional nitrogen derivatives as smart versatile building blocks for multiple tasks

11:40 - 12:00 : *Coffee break*

NATURAL PRODUCTS

Chairpersons: T. Schmidt, J.M. Alunda

12:00 **Schmidt T.**, *University of Muenster, Germany*

Sesquiterpene lactones target the trypanothione system of *Trypanosoma* species in different ways

12:25 **Alunda J.M.** *Universidad Complutense, Madrid, Spain*

In vitro antileishmanial activity of silybin and related flavonolignans from milk thistle

12:50-14:15 : *Lunch and poster viewing*

BIOLOGICAL TARGETS

Chairpersons: A. Tomas, L. Krauth-Siegel

- 14:15 **Soldati-Favre D.**, *University of Geneva, Switzerland*
Fundamental roles of *Toxoplasma gondii* aspartyl protease 5 at the host-parasite interface
- 14:40 **Joachim A.**, *Institute of Parasitology, University of Vienna, Austria*
Cystoisospora suis: searching for drug targets *in vitro*
- 15:05 **Tomas A.**, *IBMC, University of Porto, Portugal*
Alternative NADH dehydrogenase of *Leishmania*: a putative drug target for leishmaniasis
- 15:30 **Krauth-Siegel L.**, *Universität Heidelberg, Germany*
Trypanothione/tryparedoxin-dependent peroxidases, essential enzymes with stage-specific roles in *Trypanosoma brucei*
- 15:55 **De Koning H.**, *University of Glasgow, UK*
Evaluation of kinetoplastid purine and pyrimidine metabolism as a target for antiparasite drugs
- 16:20-16:40 *Coffee break*

DRUG TARGETING

Chairpersons : G. Barrat, D. Gryko

- 16:40 **Gryko D.**, *Polish Academy of Sciences, Warsaw, Poland*
Vitamin B12 as a potential drug delivery vehicle
- 17:05 **Barratt G.**, *CNRS-University Paris-Sud, France*
New formulations of Amphotericin B to improve its therapeutic index for leishmaniasis treatment
- 17:30 **Loiseau P.M.**, *CNRS-University Paris-Sud, France*
New formulations of 2-*n*-propylquinoline for the treatment of visceral leish
- 18:00 *Belgrade Sightseeing - Guided Walking Tour*
- 20:00 *Gala dinner*

October 27

NOVEL THERAPEUTIC APPROACHES, TOOLS TO DECIPHER DRUG MECHANISM OF ACTION

Chairpersons : E. Davioud-Charvet, N. Fasel

- 9:00 **Costi M.P.**, *University of Modena, Italy*
Novel drug discovery pipeline to spur innovations against Trypanosomatidic infections

- 9:25 **Garcia-Sosa A.**, *University of Tartu, Estonia*
Designing new antimicrobial and anti-parasite compounds with safe chemical libraries
- 9:50 **Davioud-Charvet E.**, *CNRS-University of Strasbourg, France*
Design of fluorescent probes to image chloroquine efflux from the digestive vacuolar pH of *P. falciparum* resistant parasite
- 10:15 **Fasel N.**, *University of Lausanne, Switzerland*
High content microscope based high-throughput drug screening against double-stranded endomyxobiont *Leishmania* RNA virus containing *Leishmania guyanensis* using primary murine macrophages
- 10:40 **Viira B.**, *University of Tartu, Estonia*
Data organizing, analyzing and modeling for malaria compounds
- 11:05 **Maran U.**, *University of Tartu, Estonia*
pH-dependence of membrane permeability for anti-parasitic and infection drugs for wide range of pH: experiment and modelling
- 11:30-11:50 : *Coffee break*
- 11:50 **Gemma S.**, *University of Siena, Italy*
Development of novel therapeutic approaches against sexual and asexual malaria parasite stages

DRUG RESISTANCE

Chairpersons : F. Gamarro, L. Maes

- 12:15 **Hendrickx S.**, *University of Antwerp, Belgium*
Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in *Leishmania infantum*
- 12:40 **Maes L.**, *University of Antwerp, Belgium*
Genomic and molecular characterization of miltefosine resistance in a *Leishmania infantum* strain that acquired resistance through experimental selection at intracellular amastigote level
- 13:05 **Gamarro P.**, *CSIC, Granada, Spain*
Failure of antimonial treatment associated with drug resistance in a dog with natural *Leishmania infantum* infection
- 13:30-14:30 : *Lunch and poster viewing*

BIOLOGY FOR POSSIBLE THERAPEUTIC CONSIDERATIONS

Chairpersons : J. Lukes, S. Paessler

- 14:30 **Paessler S.**, *University of Texas Medical Branch, Galveston, USA*
(Invited Speaker)
Microbial coinfections and their impact on the immune system: incidence in therapy

- 15:20 **Eberhardt L.**, *University of Antwerp, Belgium*
Evaluation of homogeneity of *L. infantum* and *L. donovani* infection in the hamster by real-time DNA qPCR and Giemsa-stained imprints
- 15:45 **Lukes J.**, *Institute of Parasitology, Ceske Budejove, Czech Republic*
The trypanosomatid flagellate *Paratrypanosoma confusum* has a unique morphology and complex life cycle
- 16:10 **Silaghi-Dumitrescu R.**, *University of Cluj-Napoca, Romania*
(Per)oxidation cascades induced by globins and related proteins: towards analytical tools of possible use for antiparasitic drugs
- 16:35-16:55 : *Coffee break*

CLOSING CONFERENCES

- 16:55 **Frézard F.**, *Federal University of Minas Gerais, Belo Horizonte, Brasil*
(Invited speaker)
Innovative nanocarriers for improved therapy of leishmaniasis
- 17:45 **Ioset J.R.**, *DNDi, Geneva, Switzerland*
(Invited speaker)
Drug discovery against kinetoplastid diseases: the DNDi perspective
- 18:30 *Concluding remarks and closure of the annual plenary COST conference*
- 20:00 *Dinner (to be paid by the participants)*

October 28

- 9:00-12:00 Meetings of the COST Working Groups in two separate rooms then common discussion: WG2 (Medicinal chemistry) and WG3 (Natural products)
- 12:00 *Quick lunch for WG participants and MC members*
- 12:30-14:30 **COST Management Committee meeting** only for MC members and MC substitutes

OPENING LECTURE

Structure-based development of novel antimicrobial agents targeting sterol 14 α -demethylase

Galina I. Lepesheva

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Sterols, such as cholesterol in humans, stigmasterol in plants, and ergosterol in fungi, are essential components of eukaryotic cells. They serve as vital constituents of cellular membranes and also as precursors for various regulatory molecules that modulate cellular growth, development, and multiplication. Sterol biosynthesis involves multiple catalytic steps, yet only two of them represent the major targets for clinical drugs. Statins, which act upstream the pathway inhibiting production of mevalonate, are currently the most widely prescribed cholesterol-lowering drugs, and azoles, which inhibit 14 α -demethylation of the cyclized sterol precursors, have been used for decades as the most efficient clinical and agricultural antifungals and are presently under consideration as potential herbicides and as new drug candidates for human infections with protozoan parasites from the family Trypanosomatidae.

The reaction of sterol 14 α -demethylation is catalyzed by the cytochrome P450 enzyme (CYP51, 1.14.30.70) that is found in all biological kingdoms and is known to preserve its strict catalytic role at a very low (20-30%) amino acid sequence identity across phylogeny. Comparative structural characterization of protozoan and fungal CYP51 orthologs, crystallized in the ligand-free state and complexed with a variety of different inhibitory chemotypes, provides new insights into the CYP51 functional conservation and inhibition, suggesting that sterol 14 α -demethylases maintain their conserved catalytic role by preserving high rigidity of their substrate binding cavity. High rigidity of the binding cavity makes CYP51 an attractive subject for structure-based drug design. This talk will present some examples and experimental details, particularly outlining the results of our first efforts in applying the structural information to enhance the potency of novel experimental CYP51 inhibitors aimed at treating Chagas disease and visceral leishmaniasis.

MEDICINAL CHEMISTRY

Novel fungal chitinase inhibitors and their possible use in the fight against parasites

Maurizio Botta

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Invasive fungal infections represent a serious issue in developed countries due to the increase of the population at risk, exemplified by immunocompromised patients and those hospitalized with serious underlying diseases. We reported that single components of guazatine (an antifungal mixture of polyamines and polyguanidines used mainly in agriculture) are able to act towards *albicans* and *non-albicans* *Candida* strains, laying the foundation for designing new agents endowed with antifungal activity. The optimization of the hit compounds discovered, allowed us to identify a new class of macrocyclic amidinoureas with potent antifungal activity. Compounds were obtained with a multi-step synthetic pathway, involving an innovative macrocyclization reaction developed in our lab.¹ The lead compound of these antifungals is macrocycle **1**, which is the starting point for other analogue series. In particular the introduction of phenyl rings into the macrocyclic chain improved the antifungal potency (compounds **2-3**).² Recently we investigated about the mechanism of action of this new class of macrocycles. Computational studies, involving a target-phishing protocol, allowed us to identify chitinase as a potential target. Enzymatic assays on a commercial available protein with a high degree of identity with *Candida albicans* chitinase, confirmed the inhibitor activity of these molecules. This enzyme, belonging to the family of glycosidases, responsible for the digestion of chitine during cell division, is also crucial in the life cycle of different species: *Onchocerca volvulus* (responsible of river blindness), *Wuchereria bancrofti* and *Brugia malayi* (responsible of elephantiasis), *Plasmodium falciparum* and *Plasmodium vivax* (responsible of malaria), etc. Chitinases are produced by the different organisms in different moments of their life cycle. This consideration lead us to think about a common scaffold that can be optimized depending upon the species we want to deal with, and have a new class of cross-active molecules.

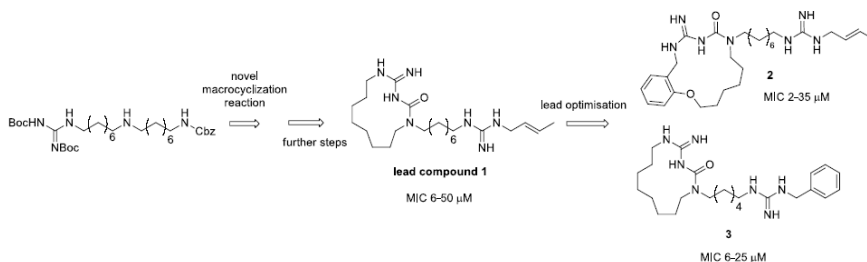


Figure 1. MICs are determined on a panel of 176 *Candida* strains

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1. Castagnolo, D., Raffi, F., Giorgi, G., Botta, M. Macrocyclization of Di-Boc-guanidino-alkylamines Related to Guazatine Components: Discovery and Synthesis of Innovative Macrocyclic Amidinoureas. *Eur. J. Org. Chem.* **2009** (3), 334-337
2. Sanguinetti, M., Sanfilippo, S., Castagnolo, D., Sanglard, D., Posteraro, B., Donzellini, G., Botta, M. Novel Macrocyclic Amidinoureas: Potent Non-Azole Antifungals Active against Wild-Type and Resistant *Candida* Species. *ACS Medicinal Chemistry Letters* **2013** 4(9), 852-857

Design, synthesis and evaluation of antiparasitic activity of heteroaryl-substituted ether phospholipid derivatives

Pantelis Afroudakis¹; Marina Roussaki¹; Kyriakos C. Prousis¹; Chiara Borsari²;
Anabela Cordeiro-da Silva³; Sheraz Gul⁴; Joachim Clos⁵; Maria P.
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Ether phospholipid derivatives possess a broad pharmacological spectrum including anticancer, antifungal and antiprotozoal activity. Miltefosine (hexadecylphosphocholine) is an alkylphosphocholine with demonstrated activity against various parasite species and cancer cells, as well as some pathogenic bacteria and fungi. Miltefosine is currently the only oral drug available for the treatment of visceral (VL) and cutaneous leishmaniasis (CL), a neglected tropical infection caused by unicellular parasites. The drug has been rolled out as first-line treatment for VL in India (28 day regimen, 2.5 mg/kg/day) and has been adopted in several national VL elimination programmes (e.g. in India, Bangladesh and Nepal). However, at the therapeutically effective doses, severe gastrointestinal side effects and serious weight loss were observed while teratogenicity remains a problem.

As a continuation of our studies on ring-substituted ether phospholipid derivatives¹⁻³ we investigated the presence of various heteroaromatic rings in the lipid portion. More specifically we introduced 1,2,3-triazolyl, isoxazolyl, 1,2,4-oxadiazolyl and 1,3,4-oxadiazolyl moieties and we studied the distance from the head group and the substituents on the heteroaromatic ring with respect to antiparasitic activity against *T. brucei* and *L. infantum*, *L. donovani* and *T. cruzi* amastigotes as well as the cytotoxicity in vitro.

Acknowledgement

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement n° 603240 (NMTrypI - New Medicine for Trypanosomatidic Infections). <http://www.nmtrypi.eu> and by COST CM1307 (STSM for Chiara Borsari).

References

1. US 8,097,752 (issued 17-1-2012) "Antiprotozoal ring-substituted phospholipids"
Inventors: T. Calogeropoulou, M. Koufaki, N. Avlonitis, A. Makriyannis², Papanastasiou I., Prousis, K.C., Georgikopoulou, K., Pavlidis, T., Scoulica, E., Kolocouris, N., Calogeropoulou, T. *Bioorg. Med. Chem. Lett.* **2010** 20 (18), 5484-5487.
3. Calogeropoulou, T.; Angelou, P.; Detsi, A.; Fragiadaki, I.; Scoulica, E. *J. Med. Chem.* **2008**, 51, 897-908.

New polifunctional nitrogen derivatives as smart versatile building blocks for multiple tasks

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Nitrogen derivatives are "privileged structures" in drug design, optoelectronics, etc., the azaheterocycle scaffold being a core skeleton for multiple purposes.

The emphasis of this work consist in design and synthesis of new polifunctional nitrogen derivatives (nitrogen podants and compounds containing \square reach and/or \square deficient heterocycles) as smart versatile building blocks for multiple tasks: biologically active molecule (anticancer, antimicrobial), chemosensors, logic gates and so on.

Acknowledgements. Authors are thankful to CNCS Bucharest, Romania, project PN-II-DE-PCE-2011-3-0038, no. 268/05.10.2011, for financial support. Part of this work (antimycobacterial tests) was supported by National Institutes of Health and the National Institute of Allergy and Infectious Diseases, Contract No. HHSN272201100012I.

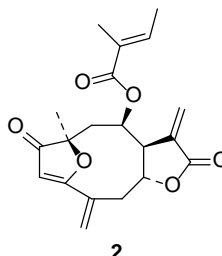
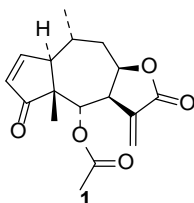
NATURAL PRODUCTS

Sesquiterpene lactones target the trypanothione system of *Trypanosoma* species in different ways

Mairin Lenz¹; R. Luise Krauth-Siegel²; Thomas J. Schmidt¹

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Sesquiterpene lactones (STLs) are a large group of plant secondary metabolites with a wide spectrum of biological activities. It has been shown by our group in cellular phenotypic assays that STLs of the pseudoguaianolide type (especially helenalin and its esters such as the acetate **1**) and of the furanoheliangolide type (especially 4,15-isoatriplicolide esters, e.g. the tiglate **2**) possess strong antitrypanosomal activity, particularly against *T. brucei*, with IC₅₀ values in the nanomolar range [1,2].



In an attempt to identify potential molecular targets responsible for this activity, we investigated the possibility that STLs interfere with the trypanothione system. Trypanothione, i.e. bis-glutathionyl spermidine, plays a similar role in redox homeostasis of trypanosomatids as glutathione in most other organisms. Unlike GSH it possesses two thiol groups per molecule and thus exists in the reduced dithiol state (T(SH)₂) and the cyclic oxidized disulphide state (TS₂). A high cellular T(SH)₂/TS₂ ratio is maintained by the NADPH-dependent action of trypanothione reductase (TR). The trypanothione/TR system provides the reducing equivalents for several vital pathways including the detoxification of hydroperoxides by tryparedoxin (Tpx)-dependent peroxidases (Px). Inhibition of TR or its co-acting enzymes will inevitably exert damage to the parasite and is thus a potential target for drug action.

Tests with isolated trypanothione reductase (TR) from *T. brucei* and *T. cruzi* showed that this enzyme is indeed a target for 4,15-isoatriplicolide esters whereas it is not significantly inhibited by helenalin acetate. Interestingly, **1** was found to nevertheless show inhibitory activity in a multiple enzyme assay composed of TR, Tpx and Px [3]. Helenalin acetate must hence act against Tpx or Px, or both.

It can thus be concluded that the two classes of STLs under study exert their activity, at least in part, via different targets within the trypanothione/TR system.

References:

- Schmidt TJ et al., 2009, *Molecules* 14, 2062-2076.
- Schmidt TJ et al., 2014, *Antimicrob. Agents Chemother.* 58, 325-332.
- Fueller F et al., 2012, *J. Biol. Chem.* 287, 8792-8802.

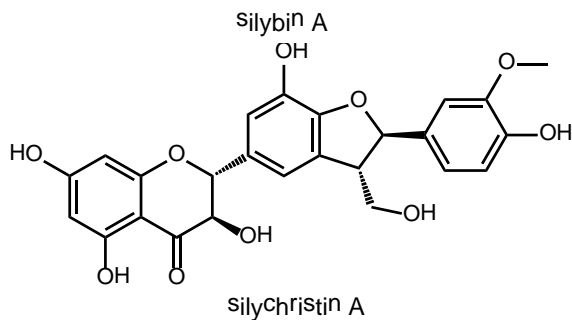
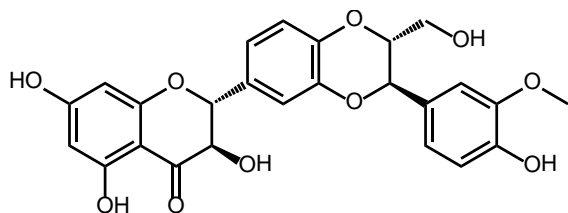
In vitro antileishmanial activity of silybin and related flavonolignans from milk thistle (*Silybum marianum*)

Ana Isabel Olfas¹, David Biedermann², M^a Dolores Jiménez-Antón¹, Catarina Baptista³, Chiara Borsari⁴, Anabela Cordeiro-da-Silva³, Maria Paola Costi⁴,
María J. Corral¹, José M^a Alunda¹

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Leishmaniasis is a vectorial parasitic disease caused by the infection with a number of *Leishmania* species. Leishmanial infections are present in all inhabited continents and provoke a range of disease conditions, from cutaneous self-healing processes (CL) to fatal unless treated visceral leishmaniasis (VL). It is considered the second most lethal parasitic disease in humans. In addition canine infections are common in the Mediterranean and Brazil. Control relies on chemotherapy although clinical failures and emergence of resistance are frequently reported. This scenario and the lack of human vaccine have fueled the identification of new potential drugs. Several flavonoids have shown notable antimicrobial and antiparasitic activities. On these grounds the effect of some synthetic and natural flavonoids against *Leishmania* was considered worth of being determined.

The synthetic compounds exhibited high toxicity and low selectivity index compared to the non infected cell line. The most promising ones revealed low stability in mice blood. Nanoformulations have been established to avoid this inconvenience. By its part, silymarin, silybin and related molecules from *Silybum marianum* (milk thistle), extensively used as hepatoprotectants, have shown additional properties (e.g. interaction with drug transporters) potentially useful for antileishmanial chemotherapy. Therefore, the effect of silybin and a series of derivatives on the multiplication of *L.infantum*, causative agent of VL and canine leishmaniasis have been tested. In addition, the potential synergistic effect of these molecules and well established antileishmanial agents has been explored.



Partial funding by European Commission FP7 project “New Medicines for Trypanosomatid Infections (NMTrypl)”, under the contract number 60324 is acknowledged

BIOLOGICAL TARGETS

Fundamental roles of *Toxoplasma gondii* aspartyl protease 5 at the host-parasite interface

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In *Plasmodium falciparum*, the Plasmepsin V (PfPMV) is an endoplasmic reticulum resident aspartyl protease that cleaves HT/PEXEL motif containing proteins. The proteolytic processing exposes an N-terminal sequence, recognized by the translocation machinery at the parasitophorous vacuole membrane (PVM) that ensure the export of effector proteins into the infected red blood cell to hijack cellular functions (Hsiao et al., 2013).

In *Toxoplasma gondii*, some dense granules proteins (GRAs) that assemble at the PVM or cross the PVM also exhibit similar HT/PEXEL motifs (REF). Importantly, several GRAs have recently been associated to the extravacuolar subversion of host cellular functions. The *T. gondii* aspartyl protease 5 (TgASP5) is phylogenetically related to PfPMV but localized to the Golgi. Deletion of *TgASP5* causes a significant loss in parasite fitness *in vitro* and an altered virulence *in vivo*. The PEXEL motif containing GRA19 and GRA20 are processed only in presence of TgASP5 but not in its absence or when replaced by the catalytically dead protease. Markedly, in absence of TgASP5, the intravacuolar nanotubular network is disappeared and several GRAs failed to localize to the PVM. TgGRA16 and TgGRA24 are exported into the host cell nucleus where they interact with the p53 tumor suppressor and p38 MAPK pathways, respectively (Bougdour et al., 2013; Braun et al., 2013). Importantly both GRA16 and GRA24 accumulate to the PV but are not exported anymore the host nucleus. In consequence, the absence of ASP5 dramatically compromises the parasite's ability to modulate host signaling pathways, impacting on the immune response and the subversion of dendritic cells migration. In cyst forming strains, the absence of TgASP5 does not interfere with stage conversion to bradyzoites but severely impact on cyst wall formation.

Cystoisospora suis*: searching for drug targets *in vitro

Anja Joachim, Ahmed Abd-Elfattah, Bärbel Ruttkowski, Nicola Palmieri

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Cystoisospora suis is an enteric protozoan parasites of the phylum Apicomplexa, closely related to *Toxoplasma gondii*, *Neospora caninum* and *Plasmodium* spp. It is the causative agent of neonatal porcine coccidiosis, a disease of newborn piglets characterized by transient non-haemorrhagic diarrhoea in the first weeks of life. This causes reduced intestinal absorption, growth retardation and increased susceptibility to other intestinal pathogens. Affected farms treat routinely with the triazinone toltrazuril which is the only registered effective drug. However, resistance against parasiticides already emerged in other apicomplexan parasites, e.g. *Plasmodium* and *Eimeria*, and new drugs need to be developed. As no small animal model is available for *C. suis* for pre-clinical screening, we developed a cell-culture based assay for screening using real-time PCR-based quantification of parasite stages after treatment. Currently, we are testing compounds with known efficacy against other apicomplexan parasites, such as bumped kinase inhibitors (binding partners for calcium-dependent phosphokinases), and Malariabox (www.mmv.org/malariabox). To detect additional new drug targets, we are screening genomic and transcriptomic data using bioinformatic tools. In conclusion, we show that *in vitro* cultivation of *C. suis* is a suitable tool to monitor invasion/inhibition *in vitro* on a medium scale of sample throughput. We are undertaking efforts to increase the number of samples that can be simultaneously screened, as well as to apply this *in vitro* model to development and invasion.

Alternative NADH dehydrogenase of *Leishmania*: a putative drug target for leishmaniasis

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Alternative NADH dehydrogenases (NDH2) are present in plants and a variety of microorganisms, from bacteria to protozoa, but not in mammals. These enzymes, that catalyze transference of electrons from NADH to ubiquinone without coupled proton translocation, are therefore promising drug targets. We are characterizing the NDH2 from *Leishmania infantum* (*Li*NDH2) and its therapeutic value.

We show that *Li*NDH2 is expressed in both *L. infantum* life cycle stages. Using a combination of immunofluorescence studies and western blot analysis of protease accessibility upon digitonin fractionation, we furthermore provide evidence that *Li*NDH2 is present in the parasite unique mitochondrion associated with the inner membrane. Finally, we show that overexpression of *Li*NDH2 increases the basal oxygen consumption of intact parasites, that is, *Li*NDH2 is involved in the respiratory chain.

We found that *Li*NDH2 is essential in *L. infantum*, including in the disease-causing stage, the amastigote. In fact, i) deletion of both *NDH2* chromosomal alleles is only possible upon previous complementation with an episomal copy of the gene, and ii) knockout promastigotes and amastigotes do not lose the *Li*NDH2 episome after multiple passages in absence of drug pressure, in contrast to what happens with a control episome that is lost after few cycles of parasite replication. These evidences confirm the essentiality of the protein in both forms of the parasite and genetically validate *L. infantum* NDH2 as a drug target. Present efforts are being directed towards the identification of selective inhibitors for this enzyme.

Trypanothione/tryparedoxin-dependent peroxidases, essential enzymes with stage-specific roles in *Trypanosoma brucei*

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African trypanosomes express three virtually identical non-selenium glutathione peroxidase-type enzymes (Px I-III) which preferably detoxify lipid-derived hydroperoxides. Bloodstream *Trypanosoma brucei* lacking the mitochondrial Px III show just a marginal and transient proliferation defect; and mice infected with the mutant cells display only a slightly delayed disease development compared to wild-type parasites. In contrast, parasites that lack the cytosolic Px I and Px II undergo extremely fast lipid peroxidation and cell lysis and are only viable in medium supplemented with the α -tocopherol derivative Trolox. Feeding the Px I-II knockout parasites with Alexa Fluor-conjugated dextran or LysoTracker in normal medium results in the progressive staining of the whole cell and complete loss of the signal, respectively. Supplementing the medium with iron or transferrin induces, whereas the iron chelator deferoxamine and apo-transferrin attenuates lysis of the Px I-II knockout cells (1). In the insect stage, the situation is strikingly different. Procyclic cells are fully viable if they lack either the cytosolic or the mitochondrial peroxidases. However, deletion of all three peroxidases is lethal. Flow cytometry and immunofluorescence analyses revealed that the Px I-III deficient parasites undergo cardiolipin peroxidation and lose their mitochondrial membrane potential (2). Thus, depending on the developmental stage, the lysosome or the mitochondrion is highly sensitive towards oxidative damages. In both cases, the trypanothione/tryparedoxin-dependent peroxidases are required and sufficient to protect the parasites from iron-induced membrane peroxidation.

Essentiality in the infectious bloodstream stage is a prerequisite for a putative drug target. A high-throughput screening did not reveal any peroxidase inhibitor but several compounds that inactivated tryparedoxin, the reducing substrate of the enzymes (3). Future work should reveal if the peroxidase itself could be targeted. Recently, the first inhibitor for human glutathione peroxidase 4, which is the closest related host enzyme, has been reported (4).

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Evaluation of kinetoplastid purine and pyrimidine metabolism as a target for antiparasite drugs

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Protozoan cells contain many drug targets, some well-explored, others not yet validated. Yet, the translation of potential drug targets for the development of actual therapy has a dismal track record. There are many reasons for this, including the vagaries of research funding and difficulties of advancing a promising lead towards genuine clinical development. However, among the factors contributing to the paucity of new drugs developed out of the validation of drug targets is often a lack of cellular penetration for the inhibitors developed for this target. Therefore, it is surely important to incorporate a strategy for drug uptake into the lead identification strategy.

We have studied kinetoplastid purine and pyrimidine metabolism alongside nucleobase and nucleoside transporters, as the efficient uptake of metabolic inhibitors will greatly enhance their trypanocidal and/or leishmanicidal activities. Purine transporters of *T. brucei* were characterised in detail, with emphasis on the development of models for substrate selectivity, exploring the versatility of the carriers for cytotoxic analogues. Observed transport functions were matched to specific genes of the Equilibrative Nucleoside Transporter (ENT) family. More recently, we have individually cloned, expressed and characterised each of the ENT transporter genes of *Leishmania major* and *L. mexicana*. This showed that the nucleoside transport activities of these *Leishmania* species were highly comparable to those previously reported in *L. donovani* – an essential prerequisite for the successful development of any nucleoside-based antileishmanial strategy with broad application.

While kinetoplastid parasites cannot make their own purines, they do have the pathways for de novo synthesis of all pyrimidine nucleotides, and we have shown that neither pyrimidine salvage nor pyrimidine biosynthesis are essential to bloodstream *T. brucei*. However, some pyrimidine analogues like 5-fluorouracil (5FU) display moderate trypanocidal activity and we found that potency correlates with the efficiency of uptake by the parasite. Using a metabolomics approach we further studied how 5FU impacts on the parasite's metabolism, and to what extent it is incorporated into the cellular nucleotide pool. We are currently trying to identify the genes encoding the uracil/5FU transporter, as these do not appear to belong to the ENT family or any other known nucleobase or nucleoside transporter family.

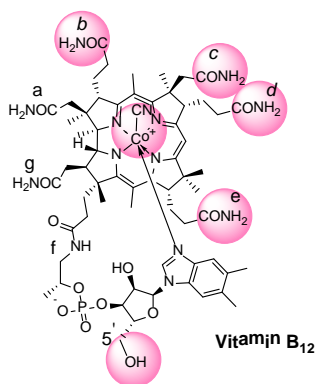
DRUG TARGETING

Vitamin B₁₂ as a potential drug delivery vehicle

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Vitamin is essential for the survival of all living cells.^[1] Recently, it has also been studied as a potential drug or imaging agent carrier^[2] due to its dietary uptake pathway assuring a proper intake, and because of its constant demand in rapidly dividing cells. In order for B₁₂ to act as a carrier its structure must be modified to allow selective coupling of biological active moieties and at the same time to maintain high affinity to transport proteins: TCI, IF and TCII. Specific sites that are known, or postulated, not be important for recognition and activity must be chosen for conjugation (highlighted on the picture).



Most, if not all, prepared conjugates so far are based on ester, amide, carbamate or carbonate bond at 5' position.^[3]

However, it would be advantageous to have an access to other conjugation methods. Recently, [1,3] azide-alkyne dipolar cycloaddition (AAC, "click" reaction) and disulphide bond forming reaction have become a powerful tool in synthetic chemistry.^[4] The following discusses an exciting new discovery in which new B₁₂ derivatives suitable for subsequent conjugations with biologically active molecules have been synthesized.

To this end, novel, stable vitamin B₁₂ derivative bearing the mesyl group in the 5' position on the ribose moiety was prepared and transformed into azide and disulphide.^[5] These novel active derivatives are easy to make and are very reactive towards various alkynes and thiols respectively. They have a big potential in the preparation of conjugates with more complex compounds, including biologically active molecules and therapeutics. Additionally, the developed methodology sidesteps difficult purification and handling. Further development of this methodology is ongoing in our laboratories and will be presented.

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New formulations of Amphotericin B to improve its therapeutic index for Leishmaniasis treatment

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Amphotericin B (AmB) is effective against visceral leishmaniasis (VL) but the renal toxicity of the conventional form, mixed micelles with deoxycholate (Fungizone®) is often dose-limiting, while the less toxic lipid-based formulations such as AmBisome® are expensive. We have used two different strategies to improve the therapeutic index of AmB with cheap ingredients: heat treatment of the commercial formulation (HF) and preparation of a microemulsion (ME).

Anforicin B®, the Brazilian equivalent of Fungizone®, was heated to 70°C for 20 min. The resulting product was characterized by UV spectrophotometry, circular dichroism and size measured by quasi-elastic light scattering (QELS). The ME was prepared from phosphate buffer pH 7.4, Tween 80®, Lipoid S100® and Myglol 810® with AmB at 5 mg/mL. The droplet size measured by QELS was about 40 nm and transmission electron microscopy confirmed a spherical shape. Rheological analysis showed low viscosity and Newtonian behavior.

Biological activity was evaluated against *Leishmania donovani* LV9. The IC₅₀ on axenic amastigotes was 0.05 µM for HF and 0.2 µM for ME, compared with 0.05 µM for Anforicin B®. For intramacrophagic amastigotes growing in J774 cells, the IC₅₀ values for HF, ME, Anforicin B® and AmBisome® were 1.46 µM, 0.81 µM, 0.62 µM and 1.63 µM respectively. The Selectivity Index (CC₅₀ on J774/IC₅₀ on LV9) was about 20 for HF and ME, similar to the value for AmBisome® and three times that observed for Anforicin B®.

In-vivo efficacy was evaluated in Balb/C mice infected with *Leishmania donovani* LV9 after IV administration of the different formulations (three doses of 1 mg/kg AmB). HF showed a reduction on the parasite burden of 78%, compared with 72% for Anforicin B®, while ME reduced parasite burden by 78% and AmBisome® by 83%.

In conclusion, these two cheap alternative formulations for AmB showing good efficacy and selectivity for *Leishmania donovani* merit further investigation.

New formulations of 2-*n*-propylquinoline for the treatment of visceral leishmaniasis

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2-*n*-propylquinoline (2-*n*-PQ) had shown interesting antileishmanial activity in animal models after administration by the oral route. However, the lipophilic properties of this compound prevent its use by the intravenous route; however this route is indicated in cases of severe visceral leishmaniasis with vomiting.

Thus, two different formulations of 2-*n*-propylquinoline were developed: a liposomal formulation referred to as 2-*n*-PQ-LIP and a hydroxypropyl beta-cyclodextrin inclusion complex designated 2-*n*-PQ-HPC.

2-*n*-PQ-LIP was characterized as large unilamellar vesicles, and gave IC₅₀ values of 3.10±0.25 and 5.84±0.32 μM on *Leishmania donovani* axenic amastigotes and intramacrophage amastigotes, respectively, with a Selectivity Index value of 1.95. On the *L. donovani* Balb/c mouse model, 2-*n*-PQ-LIP reduced the parasite burden by 84% after an intravenous treatment regimen of 3 mg/kg/day given on five consecutive days. No synergistic activity between 2-*n*-PQ and Amphotericin B was detected either *in vitro* or *in vivo*.

The second formulation, 2-*n*-PQ-HPC was active *in vitro* on both *Leishmania donovani* axenic and intramacrophage amastigotes with IC₅₀ values at 6.22±0.82 μM and 20.01±0.52 μM, respectively, with a Selectivity Index > 5. 2-*n*-PQ-HPC did not provoke drug resistance after *in-vitro* drug pressure since the Resistance Index was less than 4. 2-*n*-PQ-HPC was also active in the *Leishmania donovani*/Balb/c mice model with an intravenous treatment regimen of 10 mg/kg/day on 10 consecutive days without hepatic, renal or blood toxicity. The pharmacokinetics data are in accordance with a treatment on 10 consecutive days. These formulations merit further antiparasitic and toxicological evaluation.

**NOVEL THERAPEUTIC APPROACHES,
TOOLS TO DECIPHER DRUG
MECHANISM OF ACTION**

A novel drug discovery pipeline to spur innovations against Trypanosomatidic infections

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According to the WHO (first WHO report on Neglected Tropical Diseases, 2010), one billion people are at risk of or are affected by Neglected Tropical Diseases (NTD), which often affect communities living in remote rural areas, in urban slums and/or in conflict zones with poor living and hygiene conditions. The conventional available therapies for common diseases caused by kinetoplastids such as Human African Trypanosomiasis, Chagas disease and Leishmaniasis continue to cause major problems in humans (Stuart *et al. J Clin Invest.* 2008). To obtain one clinical candidate starting from a characterized lead compound is a time-consuming and costly process. The NTD drug pipeline on trypanosomatidic infections is growing slowly and replacement of candidate drugs that fail in clinical trials is a painful process. Drug development in this area is much slower than in other fields due to limited resources, low revenues expected and lack of critical mass. A continuous feeding of the drug discovery pipeline is necessary to ensure back-up compounds and chemical entities with new mechanisms of action.

In this context among the many innovation actions that are in development within the existing NTD platforms worldwide, NMTrypI platform (www.nmtrypi.eu) is proposing and discussing its achievements in the actual pipeline in the field of trypanosomatidic infections. In particular, on-target studies and phenotypic screening have been performed to progress at least 5 compounds to the next stage of the drug discovery process.

Acknowledgement

This work was supported by the European Commission FP7 project New Medicines for Trypanosomatidic Infections (NMTrypI), under the contract number 60324. Thanks are due to all the researchers of the platform and to Elisabetta Airaghi, Interface Europe for her contribution.

Designing new antimicrobial and anti-parasite compounds with safe chemical libraries

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One of the most time and resource expensive failures in the drug discovery and development lifecycle is late termination due to lack of safety or lack of efficacy of a compound. The incorporation of early safety into chemical libraries would allow addressing these issues from the very initial phases of compound discovery, and inheriting benign properties to compounds that are taken forward to subsequent stages.

A strategy has been developed wherein a battery of anti-targets, *i.e.*, off-targets that are not desired for interaction with the new compound, has been assembled and scores and thresholds determined for fast assessment of the interaction of compounds with particular proteins and receptors. These are targets involved in metabolism and excretion of substances, such as cytochromes P450 2a6, 2c9, 3a4, sulfotransferase, and the pregnane X receptor. The latter is involved in the efflux of xenocompounds, while the former are involved in first-pass metabolism reactions to oxidize and render a compound easier to excrete. The anti-target battery allows generating compound libraries that can be flagged for possible interaction with an off-target, given criteria based on known antitarget inhibitors. Virtual screening has been conducted on several antiparasitic and antimicrobial targets, such as N-myristoyl transferase, HIV-1 integrase, and against Schistosomiasis. The chemical libraries include small molecule drugs, non-drugs, and commercially available compounds. The compound libraries were optimized for ADME and solubility properties, and removal of reactive groups. They are available in ready form for use in discovery and design projects.

Design of Fluorescent Probes to Image Chloroquine Efflux from the Digestive Vacuolar pH of *P. falciparum* Resistant Parasite

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Fluorescent 7-nitrobenzofurazan (NBD)-functionalized chloroquine (CQ) analogues have been developed with the aim of monitoring chloroquine drug trafficking in human pathogen malarial parasites *in vitro*. These functional molecular tools designed from the active short CQ analogue CQ1 were demonstrated to display *in vitro* the required properties (acido-basic and photophysical properties) for *in cellulo* imaging measurements in the acidic conditions of the digestive vacuole of the malaria parasite. The acido-basic properties of the dye, CQ1-SPAC-NBD, were evaluated and the influence of pH investigated on its emission properties. Coupled absorption spectrophotometric (or spectrofluorimetric) and potentiometric titrations allowed calculating the pKa of the bifunctional molecule. These studies first established that the two chromophores (i.e. 4-aminoquinoline and NBD) are not self-interacting in solution. Regardless of the experimental conditions, the global basicity significantly and gradually decreases from CQ or CQ1 to CQ1-SPAC-NBD and suggested an enhanced lysosomotropic activity for the CQ1-SPAC analogues in line with their improved accumulation and antiparasitic activities. Radioactive CQ competition experiments were conducted with the fluorescent CQ1-SPAC-NBD to measure the chloroquine resistance transporter (PfCRT) contribution in drug efflux. CQ1-SPAC-NBD behaves like CQ in terms of accumulation and is a substrate of PfCRT, in the PfCRT-expressing *Xenopus laevis* oocytes assay. Furthermore, fluorescence imaging technics were applied on both sensitive- and resistant-CQ *P. falciparum* strains. The final compound CQ1-SPAC-NBD fully reproduces the CQ accumulation phenotype of CQS and CQR parasites and was observed to discriminate between a CQS and a CQR parasite, suggesting the differential accumulation displayed by CQR parasites via PfCRT-mediated transport.

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High content microscope based high-throughput drug screening against double-stranded endosymbiont *Leishmania RNA virus* containing *Leishmania guyanensis* using primary murine macrophages

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Leishmania is a genus of obligate intracellular protozoan parasites, which is the causative agent of leishmaniasis. The metastatic types of the disease such as mucocutaneous (MCL) and disseminated (DCL) leishmaniasis spread from the symptomatic or asymptomatic primary cutaneous lesion. These metastatic forms predominantly occur in the Neotropics: an area inhabited almost exclusively by the *Leishmania Viannia* species. The discovery of a double-stranded RNA (dsRNA) *Totivirus* virus, LRV1 in some of these species (so far: *L. guyanensis*, *L. braziliensis* and *L. aethiopica*) at least partially elucidate the association of metastatic leishmaniasis and *Leishmania Viannia* species. Our group reported that the host innate sensing of endosymbiont dsRNA within *L. guyanensis* through TLR-3, an endosomal innate sensor recognizes dsRNA, render mice more susceptible to infection disease. Furthermore, we and others demonstrate that there is an increase incidence of clinical relapse in LRV-bearing *L.guyanensis* or *L.brazilnensis* infected patients, who were previously treated with pentamidine or pentavalent antimonial, respectively. AMPHOTERICIN B and Miltefosine are other currently available FDA approved drugs against leishmaniasis, which are nephrotoxic or teratogenic, respectively. There is an increase in parasite-resistance to orally available Miltefosine. Amphotericin B requires professional personnel since it needs to be carefully administered intravenously. Thus, a new drug is essential to continue the battle against leishmaniasis. Here, we established a high content microscope (HCM) based high-throughput drug screening (HTDS) protocol to measure parasitocidal activity of drugs in primary murine macrophages using the facilities of Swiss National Centre of Competence in Research (NCCR) laboratory in Geneva. We have validated our screen using Miltefosine and AMPHOTERICIN B. Currently, we are screening an FDA drug library against LRV containing *L.guyanensis* infected macrophage using HCM based HTDS protocol. We have an established mouse infection model of metastatic leishmaniasis. FDA approved drugs, identified as parasitotoxic in the drug screen, will be administered to the mice without the need of further toxicity test. Our findings can be translated into human rapidly.

Data organizing, analyzing and modeling for Malaria compounds

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We propose an analysis of the chemical structure of promising antimalarial drug candidates and approved antimalarial drugs using generative topographic mapping (GTM) method [1]. GTM is a dimensionally reduction method, and the probabilistic:) counterpart of Kohonen maps. Each *i*-th molecule in *N*-dimensional initial place is projected into the *k*-th node of 2D latent space with a probability R_{ik} . So that each compound is represented both by a mean position (a point) on a 2D map, and a probability distribution R_i , which may be used for predictions of activity (property) of new compounds. First, the in-house measured bioactivity data of possible antimalarial compounds were systematically collected. Second, these data were extended using the ChEMBL database [2]. The bioassays were critically scrutinized to identify experimental protocols that would complement each other and those that were incompatible. Third, the collected datasets were modeled quantitatively and qualitatively using support vector machine (SVM) algorithms [3] and ISIDA Substructural Molecular Fragment (SMF) descriptors [4]. The models with low performances (balanced accuracy <0.6) were discarded. The surviving 17 datasets were used to build a map, using the GTM algorithm. The built map presents the chemical space of anti-malarial compounds that is annotated by potency, putative mechanism of action and bioassay. The known drugs were positioned together with the assigned targets, on constructed GTMs. As a result regularities in the chemical structures of promising antimalarial drug candidates and approved antimalarial drugs were detected. The GTM can be used to propose new chemical structures for in vitro testing.

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pH-dependence of membrane permeability for anti-parasitic and infection drugs for wide range of pH: experiment and modelling

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Oral administration is preferred administration route for most of drugs. Absorption in gastrointestinal tract is first barrier for drugs to arrive blood circulation system. Drugs can move across intestinal epithelium using passive or/and active transport. It is estimated that over 90% of drugs are transported passively. Passive transport is pH-dependent property, because mainly only uncharged species are transported passively. Gastrointestinal tract includes very wide pH-range, from ~2 to ~8, which makes pH-dependence important to consider for predicting human absorption. Passive transport can be described precisely using parallel artificial membrane permeability assay (PAMPA). PAMPA method is robust and can be easily modified, which gives opportunity to measure pH- and time-dependence over very wide pH-range.

Current presentation shows how pH influences membrane permeability for anti-parasitic and infection drugs. For this PAMPA experimental values were measured for four pH (3, 5, 7.4 and 9) over 48 hours. Among total of approximately 170 measured compounds approximately 60 belong to the anti-parasitic and infectious diseases compounds according to the Anatomical Therapeutic Chemical (ATC) classification. Distribution of those compounds on the passive permeability scale will be discussed. Most of anti-parasitic and infection drugs are ionisable compounds, which indicate that absorption properties depend on pH. Therefore, considering pH-dependence allows estimating where and how much concrete compound absorbs passively through intestinal epithelium. The passive permeability data and human intestinal permeability for anti-parasitic and infection drugs are compared and analysed. Finally quantitative structure-activity relationships (QSARs) for describing and predicting passive permeability are presented and discussed

Development of novel therapeutic approaches against sexual and asexual malaria parasite stages

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Malaria is still one of the major causes of morbidity and mortality in the world. The global expansion of the disease has been attributed mainly to the failure of vector control programs and to the lack of appropriate therapeutic options. Indeed, the continued emergence of drug resistant parasites is compromising the way to the eradication of the disease and imposes an urgent need for a new generation of treatment and control measures. Control and eradication of malaria requires drugs targeting different developmental stages of the parasite life cycle. New types of antimalarials able to kill the sexual stages of the parasite (i.e gametocytes, which represent the human reservoir of malaria), or to prevent parasite development in the mosquito are absolutely required. Here we present the efforts of our group on the development of innovative antimalarial agents aimed at addressing the current therapeutic needs. In particular, we will focus on the ligand-based optimization of a novel class of heterocycles endowed with activity against both sexual and asexual parasite stages and on the rational design of SUB1 inhibitors, potentially useful against all three major parasite species affecting humans.

DRUG RESISTANCE

Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in *Leishmania infantum*

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INTRODUCTION: Miltefosine (MIL) and paromomycin (PMM) are used to treat visceral leishmaniasis for only one decade and increasing numbers of MIL-treatment failures and primary resistance against both drugs have already been reported. Since enhanced parasite fitness has been described for antimony resistant strains, former research by our group explored possible modifications in parasite fitness in *Leishmania donovani* related to PMM-resistance; however, poor promastigote infectivity hampered assessment of fitness of the intracellular amastigote stage. The present study explored the influence of both MIL- and PMM-resistance in a *L. infantum* patient isolate that had been experimentally selected for drug resistance on intracellular amastigote level and that maintained full infectivity of both parasite stages.

METHODOLOGY: *In vitro* and *in vivo* growth, metacyclogenesis, infectivity and macrophage stress responses were evaluated to compare parasite fitness between the parent wild-type and the derived PMM- and MIL-resistant strains.

RESULTS: No significant differences were observed between the parent wild-type and PMM-resistant strain on promastigote level, while clear fitness benefits could be demonstrated for PMM-resistant amastigotes in terms of *in vitro* and *in vivo* growth and intracellular stress response. MIL-resistant promastigotes showed decreased *in vitro* growth and incomplete metacyclogenesis. At intracellular amastigote level, a lesser growth and weakened stress response suggest a markedly reduced parasite fitness compared to wild-type parasites.

CONCLUSION: The use of PMM should be restricted to combination therapy and closely monitored given the rapid selection towards resistance and the fitness advantages of PMM-resistant amastigotes. Although the observed reduced fitness of MIL-resistant strains may explain the challenging nature of MIL-resistance selection *in vitro*, the growing number of MIL-treatment failures certainly requires further exploratory research.

Genomic and molecular characterization of miltefosine resistance in a *Leishmania. infantum* strain that acquired resistance through experimental selection at intracellular amastigote level.

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Miltefosine (MIL) is used as first-line treatment for visceral leishmaniasis in endemic areas with antimonial resistance, however, a decline in clinical effectiveness is now being reported. In the laboratory, phenotypic MIL-resistant *L. donovani* isolates have not yet been identified while two MIL-resistant *L. infantum* strains from HIV co-infected patients have been documented. Hence, a clear understanding of the factors contributing to increased MIL-treatment failure is necessary. Given the paucity of MIL-resistant strains, our research group succeeded in experimental selection of MIL-resistance in a *L. infantum* isolate at intracellular amastigote level. A naturally MIL-resistant clinical isolate was included to correlate both datasets. In-depth exploration of the MIL-resistant phenotype was performed by coupling genomic with phenotypic data to gain insight into gene function and mutant phenotype.

This study provides compelling evidence that the *in vitro* amastigote resistance selection model may be a good proxy for the *in vivo* field situation, since both resistant strains showed a similar genetic basis of the acquired MIL-resistant phenotype. In line with previous literature findings, our data suggest a defect in the inward translocation machinery through inactivation of the LiMT/LiROS3 protein as a main mechanism for MIL-resistance. Phenotypically, resistance was based on intracellular amastigote susceptibility *in vitro* and actual MIL-uptake. Gene sequencing analysis revealed the presence of a 2 base pair deletion in the LiMT gene of the experimentally induced strain, leading to an early stopcodon and truncation of the LiMT protein. Interestingly, the MIL-resistant clinical isolate revealed mutations in both genes LiMT/LiROS3. To verify that the mutations in LiMT/LiROS3 genes were indeed accountable for the observed acquired resistance, transfection experiments were performed to re-establish MIL susceptibility. Susceptibility of the *in vitro* resistant strain was restored by transfection with a LiMT plasmid, whereas the resistant clinical isolate was made susceptible again after transfection with the pX-ROS3 vector. Western blot experiments using anti-ROS3 and anti-LiMT antibody and [¹⁴C]MIL accumulation assays supported the restoration of MIL-susceptibility.

Failure of antimonial treatment associated with drug resistance in a dog with natural *Leishmania infantum* infection

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Visceral leishmaniasis (VL) caused by the protozoan parasite *Leishmania infantum*, is one of the most important zoonotic diseases affecting dogs and humans in the Mediterranean area. The presence of infected dogs as the main reservoir host of VL is regarded as the most important potential risk for human infection. Anti-leishmanial drugs used in dogs include meglumine antimonate (Glucantime®) and allopurinol; to date, no drug has proven to be consistently curative for visceral leishmaniasis in dogs. Although clinical improvement may occur in response to chemotherapy, relapses are common, and chemotherapeutic elimination of *L. infantum* has not been consistently achieved with any drug tested to date. New drugs, delivery systems and treatment strategies are necessary to achieve a consistent parasitological cure in infected dogs. We have studied the susceptibility profile to the recommended first-line drugs in humans (amphotericin B, miltefosine, paromomycin and pentavalent antimonials) in promastigotes and intracellular amastigotes of *L. infantum* parasites isolated from one dog diagnosed positive for leishmaniasis, before and after two treatment series with Glucantime. The dog failed to respond to the 2nd cycle of Glucantime treatment. We have observed that after the second treatment series, these parasites were significantly less susceptible to antimonials than previous isolates, presenting a Resistant Index of 6-fold and >3-fold for promastigotes and amastigotes. We have characterized this resistant *Leishmania* line and we have observed that the susceptibility profile is related to a lower antimony uptake due to lower aquaglyceroporin-1 expression levels. These results are relevant considering the low number of antileishmanial drugs available and the fact that some of these are used in human as well as veterinary medicine; consequently, it is essential to monitor drug resistance in *Leishmania* isolates from dogs with therapeutic failure.

Supported by Ministerio de Economía y Competitividad, Plan Nacional de I+D+i Proyectos SAF2012-34267 and Plan Andaluz de Investigación (Proyecto de Excelencia CTS-7282).

BIOLOGY FOR POSSIBLE THERAPEUTIC CONSIDERATIONS

Microbial coinfections and their impact on the immune system

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In the nature, animals and people can be productively infected with multiple pathogens at a time. These pathogens that have infected the host may also interact with each other and the host. This interaction determines the final outcomes of the infection. Many examples of the interactions between protozoa and viruses and helminthes and viruses, are described in the literature. In mixed infections the burden of one or both pathogens may be increased, one or both may be suppressed or one may be suppressed and the other increased. In some cases a certain co-infection increases the chance of developing a severe disease, while in other cases a pathogen-induced immunosuppression can allow other pathogens to replicate to higher levels than normally. Also, the ability of helminthes to impact the immune response, especially the parasite-induced immunosuppression, is of great importance for controlling viral diseases as well as for efficient vaccination of animals.

Evaluation of homogeneity of *L. Infantum* and *L. Donovanii* infection in the hamster by real-time DNA QPCR and Giemsa-stained imprints

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Evaluation of drug efficacy and treatment outcome in *Leishmania*-infected animals and patients is generally based on post-treatment viable parasite burdens in the various target organs. While microscopic examination of Giemsa-stained imprints is still considered as the golden standard for quantification of parasite load, DNA qPCR can be considered as an attractive alternative due to its higher sensitivity and specificity, together with its high accuracy and reproducibility. Both methods depart from a very small piece randomly taken from the target organ (liver or spleen) and total organ burdens are then extrapolated based on the assumption that the infection is homogeneously distributed throughout the organ. To our knowledge, the aspect of homogeneity using microscopy and qPCR has not yet been addressed in-depth.

Taking into account some inherent biological variation, the infection in liver and spleen was highly homogeneous in hamsters that had been infected with 10⁷ spleen-derived amastigotes of *L. infantum* and *L. donovani*. In animals treated with miltefosine, the low burdens of parasites were also equally distributed. For both techniques, organs and treatment groups, over 90% of the measurements lie within the acceptable range of 70% variation and over 70% (liver) and 85% (spleen) lie within the more stringent range of 50% variation. Related to its higher sensitivity, burdens determined by DNA qPCR show slightly more variation than by microscopy. The inter-individual variation between hamsters of a same group was found non-significant, illustrating the high reproducibility of the hamster model for both *Leishmania* species.

To focus on viable post-treatment burdens, RNA qPCR is currently being explored, as is the determination of the lowest detection level of infection using the three methods (microscopy, DNA qPCR and RNA qPCR).

The trypanosomatid flagellate *Paratrypanosoma confusum* has a unique morphology and complex life cycle

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Paratrypanosoma confusum is a recently described monoxenous trypanosomatid discovered in the gut of mosquito *Culex pipiens*. As the most basal known parasitic lineage, it represents a possible evolutionary link between the free-living bodonids and parasitic trypanosomatids. In the life cycle of *Paratrypanosoma*, three morphologically distinct stages have been observed. The swimming stage with a trypomastigote-like morphology and a long external flagellum can transform under a range of conditions (glucose-rich medium, increased pH, presence of bipterin) into an amastigote-like stage equipped with a very short internal flagellum that is firmly attached to any solid surface. Finally, on soft surface such as an agar plate, *Paratrypanosoma* transforms into an opisthomastigote-like stage with a short external flagellum. Any of these stages is capable of division, as documented by time-laps video. The dynamic changes of the flagellum during the life cycle can be followed by antibodies directed against paraflagellar rod proteins of *Trypanosoma brucei*. Preliminary data indicate that *Paratrypanosoma* engages in social motility behavior similar to that so far observed only in *T. brucei*. We are currently annotating the genome and stage-specific transcriptomes of this evolutionary unique flagellate, and expect that it will provide insight into the emergence of dixenous life style of the medically important parasitic trypanosomatids.

(Per)oxidation cascades induced by globins and related proteins: towards analytical tools of possible use for antiparasitic drugs

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Beyond the known purposefully-built enzymes that oxidatively process other organic molecules in our body (prostaglandin synthases, cytochromes P450, etc.), a few heme proteins are abundant enough for their oxidative side-reactions to become important. Such is the case of cytochrome *c*, whose pseudoperoxidase reactivity is linked to apoptosis. Similarly, several globins can engage in peroxidase reactivity *in vivo*, using hydrogen peroxide or lipid peroxide as oxidants towards small-molecule substrates such as ascorbate, urate, phenolics, lipids, thiols – but also peptides and even proteins. Such reactivity has been invoked as relevant to the manner in which severe physical effort is managed in the body of athletes, but also for certain medical conditions (e.g., rhabdomyolysis), and, last but not least, for the manner of action of certain antiparasitic drugs.

On the analytical side, the chemical reactions of readily accessible hemoproteins with peroxides can be turned into useful tools for assaying biologically relevant activities of drug candidates, be they synthetic compounds or natural extracts. Lipid peroxidation assays with cytochrome *c* or hemoglobin, as well as hemoglobin ascorbate peroxidase assays, afford insight into antioxidant activity as well as, more generally, into the ability to efficiently interact with redox proteins. A hemoglobin/copper-oxidase couple can be employed for assaying prooxidant reactivity – in a way that in fact also monitors the ability to bind and interact with proteins found in blood. On the anti/pro-oxidant side, such assays dwell on direct protein/small molecule interactions, unlike many chemically-based methods that are currently dominating the field. Our recent applications on such analyses of natural extracts or synthetic drug candidates will be illustrated with a combination of UV-vis, EPR, kinetics, and computational (docking, molecular mechanics, quantum mechanics) methods.

CLOSING CONFERENCES

Innovative Nanocarriers for Improved Therapy of Leishmaniasis

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Leishmaniasis is a complex of infective parasitic diseases that is endemic in 98 countries and affects mainly poor and marginalized populations. The clinical manifestations of the disease can involve the skin, with local (cutaneous), diffuse (diffuse cutaneous) or disfiguring lesions (mucocutaneous), or the viscera, leading to death if untreated. It is caused by parasitic protozoa of the genus *Leishmania*, transmitted to humans via the bite of sandflies. Even though pentavalent antimonials, such as meglumine antimoniate (MA) introduced in 1940s, are still the first-line drugs against all forms of leishmaniasis in most developing countries, their use in the clinical setting has several limitations. These compounds have to be given parenterally, daily, for at least three weeks. Antimony therapy is often accompanied by severe systemic side effects, requiring very careful medical supervision. More recently, two new drugs have reached the market to treat visceral leishmaniasis (VL): a liposomal formulation of amphotericin B (AmBisome), with reduced side effects, and miltefosine for oral treatment of VL. However, these drugs also present some limitations, including thermal instability and high cost for AmBisome and teratogenicity for miltefosine. The emergence of drug resistance is another critical issue of the current therapies. As dogs infected with *Leishmania infantum* represent the main natural reservoir of VL, there is major interest in an effective therapy for these animals. However, those respond poorly to existing drugs. With the aim of achieving an improved therapy of leishmaniasis, our research group has introduced and developed innovative strategies based on nanocarriers. As a first approach, a novel liposomal formulation of MA (LMA) was developed, which promoted 50% parasitological cure in dogs with VL, when used in combination with allopurinol. As an attempt to further improve this liposome formulation, long-circulating pegylated LMA was evaluated in a murine model of VL. Interestingly, this study highlighted a new concept that mixed formulations of conventional and pegylated liposomes can result in improved drug delivery for treatment of VL. Another innovative approach based on amphiphilic antimony(V) complexes was also introduced, as an attempt to promote the oral delivery of pentavalent antimonials. Such complexes were obtained from nonionic surfactant from the N-alkyl-N-methylglucamide series (L8). Because of amphiphilic character, the resulting complexes self-associate in aqueous solution, forming nanoassemblies. As major results of pharmacological evaluations, Sbl8 exhibited improved oral bioavailability of Sb and more favorable pharmacokinetic parameters, compared to conventional hydrophilic antimonial drug. The resulting amphiphilic complexes also showed efficacy by the oral route in murine models of visceral and cutaneous leishmaniasis. Interestingly, these nanoassemblies demonstrated kinetic stability following dilution below the CMC, suggesting that these nanostructures may also be used as carrier systems of antileishmanial lipophilic drugs. Work supported by CNPq, FAPEMIG, CAPES.

Drug discovery against kinetoplastid diseases: the DNDi perspective

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The Drugs for Neglected Diseases *initiative* (DNDi) is a patients' needs-driven organization committed to the development of new treatments for neglected diseases. At the discovery level, the main objective of DNDi is to build a solid portfolio of preclinical candidates using an original model based on partnership. To address this challenge DNDi has established a fully integrated process-oriented platform in close collaboration with a variety of partners, notably pharmaceutical companies. This discovery platform relies on high throughput/high content screening capacity and lead-optimization consortia supported by a pragmatic, structured and pharmaceutical-focused compound sourcing approach. The strategy adopted by DNDi to address drug discovery for Visceral Leishmaniasis and Chagas' disease will be presented and illustrated with a few concrete examples of achievements, lessons learnt and main challenges identified along the development road.

POSTERS

P1 Exploiting the Antiparasitic Activity of Naphthalimide Derivatives

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Naphthalimide and bisnaphthalimide are groups of aromatic compounds that have generated intense interest for a number of years by scientists around the world and this is due to their diverse applications in the medical area.

A set of 1,8-naphthalimido derivatives with 2, 3 and 4 carbons linking different functional groups: amino, imino, imino from furan, guanidine, urea and 1,2,3-triazole were synthesized and tested against the three protozoa, *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi*. The toxicity and the selective index was determined by quantification of the inhibition of THP1 cell line growth.

In this work we have focused our attention in studying the relationship between the length of the linker chain and the substitution of the terminal groups with their activity against the mentioned parasites and the cytotoxicity.

Among the different groups, ureas, guanidines and imines presented the best activity towards *T. brucei*. In particular, the urea derivatives group, the increase in linker chain increased activity against *T. brucei*. For *T. cruzi*, ureas and guanidines were the most active, although this activity was accompanied by an increase in cytotoxicity. No significant antileishmanial activity was found for all the groups synthesized.

In conclusion, this naphthalimides have different effects in each protozoa and the relation of structure and antiparasitic activity/cytotoxicity are, for some groups, correlated not only with the linking chain length but also with substitutions in the functional group.

P2 Monovalent ionophores as potential anti-malarial and anti-leishmanial agents

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Malaria and Leishmaniasis are major tropical protozoal diseases affecting millions of people, especially in developing countries. In the absence of an effective vaccine, chemotherapy remains the only weapon to fight these infections. However, the increased drug resistance to conventional treatments and the presence of safety issues makes very urgent the identification of new drugs.

Monovalent sodium and potassium ionophores, largely used in veterinary medicine and proposed as human anticancer agents, have antimalarial activity against asexual intraerythrocytic stages of *Plasmodium falciparum*. Recently, the transmission blocking activity of this class of molecules has been described by our group (D'Alessandro et al. Antimicrob Agents Chemother 2015, Jun 8, AAC.04332-14). We demonstrated that salinomycin and monensin killed *Pf* gametocytes, the intraerythrocytic stage responsible for transmission, and inhibited the formation of ookinetes and oocysts, the stages which develop in the mosquito vector at nanomolar doses.

Up to now, only salinomycin has been tested for anti-leishmanial activity, with micromolar IC₅₀ on promastigotes of *Leishmania donovani*. In the present work, we confirmed the activity of salinomycin on different *Leishmania* species (*L. tropica* and *L. brasiliensis*) and extended the analysis to additional ionophores. Of those, monensin and nigericin showed an inhibitory activity against promastigotes better than that of salinomycin. Experiments on *Leishmania* amastigotes are on-going.

The potential toxicity of this class of molecules is an alarming issue. All the ionophores displayed different cytotoxicity depending on the cell type tested. To reduce the dose of treatment and thus ameliorate the selectivity index, association experiments were performed on *P. falciparum* asexual parasites. Additive effects were observed when different doses of monensin were tested in association with dihydroartemisinin, the active metabolite of most artemisinins and the mainstay of antimalarial therapy. These results will be extended to *P. falciparum* gametocytes or *Leishmania* promastigotes (on going), to verify whether ionophores could be considered potential partner drugs for antimalarial or anti-leishmanial combination therapy.

P3 Loading of antimonials into nanoparticles to combat resistances

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Visceral leishmaniasis is a neglected tropical disease caused by parasites of genus *Leishmania*, with a prevalence of 1.3 million of new cases and 20,000 to 40,000 deaths annually. Current therapies include classical antimony derivatives and other drugs as paromomycin, miltefosin and amphotericin B. However, antimonials, mainly due to their accessibility and low price, are many times the only therapeutic option in many poor areas where this disease is endemic. Nevertheless, during the last decades, therapeutic failure of antimony derivatives has begun to arise as an on-growing problem, reaching therapeutic failure rates of almost 60% in some regions. This problem is mainly caused by *leishmania* parasites increasing resistance to antimony, whose intrinsic cause is essentially the reduction of intraparasitic antimony levels. As macrophages are mandatory hosts of *leishmania* parasites, and the major parasitic burden remains in liver and spleen, nanoparticles are considered as an excellent carrier to target drugs to infected cells. In this study, we have formulated an albumin nanoparticle system loaded with an antimonial derivative and coated with oxidized mannan, in order to: (i) target nanoparticles to macrophages, increasing intracellular antimony levels and (ii) activate macrophages, triggering a suitable immune response. Results indicate that potassium antimony tartrate, which had already been used as leishmanicidal, can be encapsulated in stable albumin nanoparticles coated with oxidized mannan (mean diameter $\approx 252 \pm 42$ nm). These nanoparticles are rapidly uptaken by RAW 264.7 macrophages *in vitro*, increasing 3.4 times intracellular antimony levels in comparison with the free drug. On the other hand, antimony loaded nanoparticles coated with oxidized mannan induce TNF α production and reduce IL-10 and IL-1 β secretion. However, they reduce NO production in presence of LPS, thus it is not clear how far they could induce a positive effect on immune system to combat *Leishmania* infection *in vivo*. Despite that, our results indicate that these nanoparticles could be a great weapon to combat antimony resistance. However, more studies must be carried out to evaluate the antileishmanicidal effects on infection models, both *in vitro* and *in vivo*.

P4 High throughput screening methods for antitrichomonads using fluorescent dyes

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Trichomoniasis, caused by the single-celled protozoan parasite *Trichomonas vaginalis*, is responsible for most human vaginitis. Effort to combat this disease is hampered by the existence of parasites resistant to the commonly used antitrichomonad drugs. This situation calls for the development of new drugs or the screening of existing compounds for antiparasitic activity. Drug screening efforts for antitrichomonads is limited by the lack of a high throughput and effective method. An important objective of this study is therefore to evaluate existing protocols, based on the viability indicator dye Alamar blue (resazurin) for routine drug test, develop new ones and validate these by screening small antiprotozoal libraries. The resazurin-based assay was evaluated by determining fluorescence development in *Trichomonas* media with various cell densities after various intervals and in the presence of metronidazole. Similar investigations were performed with alternative fluorescent dyes propidium iodide (PI) and resorufin. The optimized protocols were used to screen for new antitrichomonal compounds. From this study it was observed that cultures of *T. vaginalis*, under anaerobic condition, rapidly reduced the blue colour of resazurin to the red resorufin. It was also observed that ascorbic acid, a constituent of the culture medium, produced similar effects, even in the absence of cells, causing high background fluorescence and variability. Furthermore, *T. vaginalis* rapidly metabolized resorufin to the non-fluorescent and colourless metabolite dihydroresorufin, making the fluorescent signal transient and unreliable. In contrast, resorufin proved to be an excellent viability probe for *Trichomonas* due to its chemical stability in media and rapid metabolism by the parasite (to dihydroresorufin). Staining with PI after cell permeabilization was observed to be another effective method to quantify the number of trophozoites after drug exposure. Using these newly established methods, we determined EC₅₀ values of a test series of compounds and identified potent antitrichomonal compounds from a limited screen of phosphodiesterase inhibitors and phosphonium salts. We firmly conclude that the resorufin- and PI-based assays are suitable for routine and high-throughput drug screening. The establishment of these methods constitute a major advancement in efforts to screen for new antitrichomonal lead compounds.

P5 ***Trypanosoma cruzi* Phenotypic Screening using the HYPHA Mycodiverse™ Library**

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Chagas Disease is a chronic infection caused by *Trypanosoma cruzi* which affects over 8 million people in Latin America. There are no effective vaccines and very limited drug options for Chagas Disease treatment and therefore, antichagasic therapy is urgently needed for treatment of the disease. The cell-based high content screening technology allows screening of a large quantity of potential compounds, and also provides an early indication of anti-trypanosomatid activity and toxicity of the tested compounds. This assay tests molecules against the parasite as a whole without a predefined target. The present project intends to develop an image-based high content assay to screen a natural compound library for the discovery of potential drug candidates with broad efficacy against *T. cruzi* Y stain.

Acknowledgement

This work was supported by the European Commission FP7 project New Medicines for Trypanosomatid Infections (NMTrypI), under the contract number 60324 and by the Laboratório Nacional de Biociências (LNBio-CNPEM). DRBP receives a PhD scholarship from Coordenação de Aperfeiçoamento Pessoal de Nível Superior – CAPES.

P6 *In vitro* antibacterial and antiprotozoal activities of extracts from fruiting bodies of Lithuanian mushrooms: a preliminary data

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Development of the new drugs against neglected diseases is urgently needed because of arising drug-resistance and toxic side-effects associated with current drugs. Among the natural compounds, the pharmacological potential of mushrooms is still underexplored and only a few studies (mostly from China and Japan) are available. The aim of the present preliminary study was to investigate the antimicrobial and antiprotozoal activities of some crude extracts from naturally growing Lithuanian *Basidiomycota* mushrooms.

The extraction of soluble compounds of fruiting bodies was performed by extraction in 50% water-methanol. Solid residues were obtained by rotary evaporation at reduced pressure and used for *in vitro* antibacterial and antiprotozoal evaluation. Four extracts (*Suillus luteus*, *Suillus bovinus*, *Suillus granulatus* and *Phallum impudicus*) were found inactive. *Xerocomus badius* and *Tylopilus felleus* showed weak activity against *Trypanosoma cruzi* while *Suillus variegatus* showed activity against *Plasmodium falciparum*. *Picnoporus cinnabarinus* demonstrated the highest and broadest overall biological activity, starting from 1.3 µg/ml against *T. brucei*, 1.4 µg/ml against *P. falciparum* and 21.5 µg/ml against *Leishmania infantum*. Unfortunately, this extract also revealed cytotoxicity on MRC-5 cells (13 µg/ml). In the antibacterial assays, pronounced activity was found only against *Staphylococcus aureus* for *Xerocomus chrysenteron* and *Picnoporus cinnabarinus* extracts. Polyphenolic, quinonoid and steroidal compounds are known constituents and potentially active structures.

This preliminary explorative work has demonstrated that the search for novel drugs among natural compounds from the Fungi Kingdom may yield promise, and future efforts and more detailed studies should be directed to this area.

P7 In search for anthelmintic plant compounds: *Avena sativa*
against free living larvae of parasitic nematode

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In spite of many actions for good quality food production and health of animals and men, helminth control is mainly based on the use of chemical drugs and relying on existing treatment, unfortunately have lost their effectivity. Plants used from ancient times to cure disease of people and animals may be a good source of active compounds. Many of them still are waiting for detailed characterization and may be a good alternative for the drug treatment. The composting plants release the compounds into the soil where nematode developed and may also reduce infectivity of the parasites. From the ethno-medicine it is obvious that many compounds are saponins.

The bisdesmosidic steroidal saponins with two sugar chains, one at the C-3 carbon and second at C-26 are found in oat (*Avena sativa*) leaves and can be converted into the biologically active form- monodesmoside by removal of the sugar at the C-26 position by the enzyme avenacosidase. Mechanical damage of the plant causes a breakdown of compounds compartmentalization in cell, allowing the enzyme to come into contact with its saponin substrates. The C-26 glucose molecule is then removed by hydrolysis to yield the monodesmosidic 26-desglucoavenacosides A and B, which are fungitoxic. The antinematode activity of *A. sativa* 26-desglucoavenacosides B, at the molecular level has not been reported, yet.

The anthelmintic activity of avenacoside B and 26-desglucoavenacoside B were evaluated against parasitic nematode *Heligmosomoides polygyrus*, a laboratory model of gastrointestinal nematode infection in livestock and hookworm infection in men. Egg hatching, larvae development and molecular pattern of infective larvae exposed to the plant compounds were compared. Larvae infectivity with the outcome of immune induction were measured in BALB/c mice. Two ethanolic extracts of *A. sativa* e.g. avenacoside B and 26-desglucoavenacoside B differ in their antinematode action. Although both compounds affected development of larvae with greater than 40 percentage of deformed stages, only 26-desglucoavenacoside induced expression of CED-9 antiapoptotic protein and reduced infectivity of larvae. Changes in the molecular pattern of larvae proteins measured in HPLC and enhanced IL-4 but reduced IL-12 production in culture of mesenteric lymph node cells indicated the antiparasitic greater activity of 26-desglucoavenacoside: reduction in the only one residue – glucose enhanced antinematode activity of steroid saponins. *A. sativa* originated saponins are useful for cheap antiparasitic natural products.

P8 Searching for antiplasmodial compounds from Ghanaian medicinal plants

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Plasmodium is the parasite causing malaria. Species infecting humans include: *P. vivax*, *P. malariae*, *P. ovale*, *P. falciparum*, *P. knowlesi*. In Ghana, many medicinal plants are used to treat malaria, some without scientific evidence of efficacy (Asase et al., 2005). The purpose of this study is to identify medicinal plants used in Ghana to treat malaria, investigate their activity and follow-up with bioguided fractionation to isolate the bioactive compounds from promising plants. The *in vitro* antiplasmodial evaluation is carried out on *Plasmodium falciparum* and combined with an hemolysis assay leading to the determination of an *in vitro* selectivity index. From an ethnobotanical survey, aqueous, petroleum ether, ethylacetate and methanol extracts of aerial parts of *Phyllanthus fraternus* (Phyllanthaceae) and *Tridax procumbens* (Asteraceae); leaves of *Bambusa vulgaris* (Poaceae), *Tectona grandis* (Lamiaceae), *Terminalia ivorensis* (Combretaceae), *Persea americana* (Lauraceae) and *Theobroma cacao* (Sterculiaceae) and root of *Senna siamea* (Leguminosae) are considered in the present study. Five original alkaloid compounds have been purified and characterized.

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P9 Synthesis of antileishmanial puric analogues via copper nanoparticles catalyzed Huisgen cycloaddition

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Leishmaniasis are parasitic neglected diseases affecting more 80 countries around the world and responsible for about several thousand deaths a year, mostly children. The protozoan parasite *Leishmania* is transmitted by insects of the genus *Phlebotomus* or *Lutzomyia*. Currently the first line of treatment remains the antimonials compounds that have been used for over 70 years in emerging countries (a) where the resistance is becoming alarming (b). Other available drugs such as amphotericin B, miltefosine, pentamidine or paromomycin have significant side effects. The problem of the emergence of drug resistance to these compounds is also increasing or is at risk, justifying the need for the development of new drugs. Unlike the mammalian cells, *Leishmania* is not able to synthesize purines nucleotides *de novo* and must rely on their host for preformed purines (c). Among the purines derivatives, the GDP analogues play a fundamental role in the glycosylation of protein which is involved in the virulence of parasites (e,d). The design of new GDP analogues could therefore be an interesting way to develop new antileishmanial drugs. A series 5'-aryl-5'-deoxyguanosine derivatives has been synthesized by click chemistry from 5'-azido-5'-deoxyguanosine with different alkynes. The study of different catalytic systems have demonstrated the efficiency of the Cu(I) nanoparticles catalyzed azide alkyne reaction. All the synthesized compounds were screened for antileishmanial activity against *Leishmania donovani* axenic amastigotes and intramacrophage stages, the cytotoxicity was also evaluated against murine macrophages. One preliminary evaluation showed an interesting compound with an IC₅₀ of 8.6 μ M on intramacrophage amastigotes, associated with a low toxicity on macrophages.

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P10 **Anti-malarial combination therapy: synergistic effect between an antisense strategy and different anti-malarial drugs in resistant strains of *Plasmodium falciparum***

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In these days, only the Artemisinin-based Combination Therapy is still effective in fighting malaria. But a threat exists in these drug combinations due to the emergence of resistance to artemisinin derivatives in Southeast Asia which could lead to a restriction in used antimalarial drugs as increasing doses is limited to avoid toxicity. Antisense strategies represent a promising new therapeutic approach targeting nucleic acids. Antisense oligonucleotides (ODN) may be employed to treat malaria. Their limitations were mainly their low intracellular penetration to their target and their rapid degradation. New generations of drug carriers have helped to enhance the effects of ODN with reduced toxicity. During our studies, we have developed a cationic nanoemulsion (NE) in order to adsorb ODN directed against the topoisomerase II of *Plasmodium falciparum*. This NE/ODN allowed the inhibition of parasite growth. To develop a combination therapy, some anti-malarial drugs, whose resistances are proven, were associated with the NE/ODN. We tested our NE/ODN in combination with chloroquine, atovaquone and dihydroartemisinin on the 3D7 strain sensitive to all anti-malarial drugs, the W2 strain resistant to chloroquine and PAV strain resistant to atovaquone. A synergistic effect, no matter which anti-malarial drug was associated with the NE -ODN, was observed. There was also a limited reinfection in presence of the different combinations even in the resistant strains. Our perspective is to encapsulate atovaquone inside the NE/ODN due to its lipophilic properties in order to prevent or reverse the drug resistance, and reduce the dose used by increasing the bioavailability of atovaquone.

P11 **Growing risks due to free living amoebas in water destined for human consumption: Environmental diagnostic and Therapeutics approaches**

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Free-living amoebae (FLA) belong to a heterogeneous group of protozoa ubiquitously found in natural and artificial environment such as water towers. Some FLA are pathogenic for humans, and are responsible for eyes infections (keratitis) or central nervous system diseases: Granulomatous Amoebic Encephalitis (GAE) and Primary Amoebic Meningo- Encephalitis (PAM). These microorganisms represent a undervalued pathogenic risk in the environment. In the present work, the diversity of FLA was investigated in 3 different water towers containing water destined for human consumption. Samplings were performed at 3 different depths of the water column, at 4 different seasons giving a total of 90 liquid and 72 biofilm samples. In these samples, amoebas were studied by microscopy and by PCR on partial 18S rDNA, by using specific designed primers. All samples were positive for FLA both in molecular analysis and microscopy. The quantification of FLA observed in the samples showed a seasonal effect for liquid samples and an effect of the depth of water columns for biofilm samples. Besides this environmental project, a therapeutic approach was developed, by using an *in vitro* screening evaluation on the pathogenic FLA model, *Acanthamoeba castellanii*, to determine IC50 of different drugs commonly used in the treatment of amoebic keratitis or GAE. Clotrimazole, pentamidine and amphotericin B seem to be the most efficient molecules among the 12 analyzed. In order to determine the best drug combination that could be used for the treatment of GAE or amoebic keratitis, potential synergies between the most active drugs will also be investigated.

**P12 Enzymatic analysis of a new leishmanial therapeutic target:
the GDP-Mannose Pyrophosphorylase (GDP-MP)**

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Leishmaniasis is a parasitic disease classified as a Neglected Tropical Disease (NTD) which is transmitted by an insect vector, the sandfly. Leishmaniasis threatens 350 million people worldwide, with 2 million new cases each year. There are currently very few available antileishmanial treatments which present several drawbacks such as toxicity and emerging drug resistance. This project aims to develop new antileishmanial inhibitors from a validated target in the parasite: the Guanosine-Diphospho-D-Mannose Pyrophosphorylase (GDP-MP). This ubiquitous enzyme is involved in the glycosylation process which is of considerable importance in all living cells: it catalyzes the synthesis of GDP-Mannose from Man-1-P and GTP. With GDP-Mannose, which is the activated mannose donor, *Leishmania* parasites synthesize a range of mannose-containing glycoconjugates thought to be essential for amastigote survival. The GDP-MP has been shown to be essential for amastigote survival in macrophages *in vitro* and in infected mice *in vivo* (1). The goal of this work is to characterize and to compare the enzymatic properties of the target GDP-MP in the parasite as well as its human counterpart in order to further evaluate newly designed compounds that would specifically be directed against the GDP-MP in *Leishmania*. From the production and the purification of recombinant GDP-MPs from *L. infantum*, *L. donovani* and human, enzymatic assays were performed in order to characterize and to compare GDP-MP properties in the parasite and in human, and to evaluate inhibitors activities. Inhibitors were also evaluated *in vitro* on axenic and intramacrophage amastigotes. This analysis allowed us to select 3 compounds among 96 which present interesting activities on both the enzyme and the parasite. Crystallization analyses, which are currently in progress, will allow to design inhibitors that specifically inhibit the enzyme of the parasite.

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P13 Natural endoperoxide scaffolds from artemisinin and G-factor; elaboration of hybrid compounds with antimalarial activities.

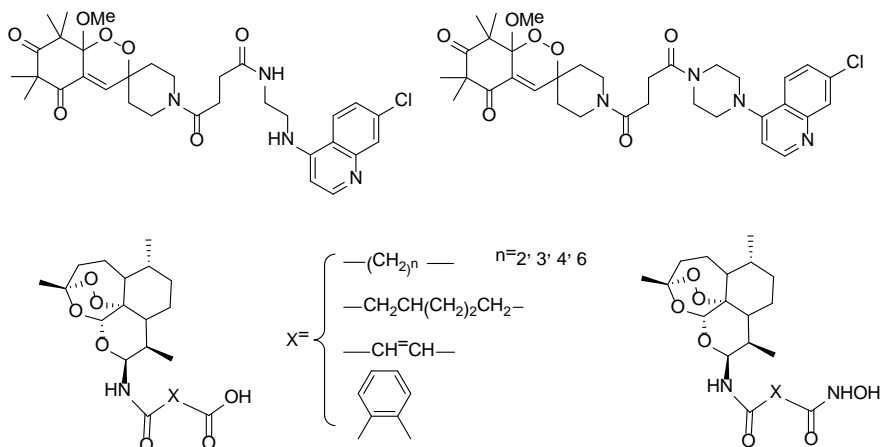
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Malaria caused by protozoan parasites of the genus *Plasmodium* is the most prevalent parasitic disease and remains a major world health problem following the emergence and spread of *P. falciparum* one of the four genuses that is the most dangerous. The discovery of the sesquiterpene lactone artemisinin and the subsequent development of potent derivatives have been a key event in the malaria treatment. Nevertheless, the emergence of artemisin- resistant *P. falciparum* strains makes it urgent for discovery of new compounds, and/or new targets. The generation of drugs that could share dual activities may represent a new therapeutic challenge in the prevention and treatment of malaria. In that latter respect, we have developed two families of compounds based on two endoperoxidic scaffolds.

The first one is based on the natural G-factors, endoperoxides found in the leaves of *E. grandis*. Through a synthetic program in the laboratory, we have optimised by two different strategies the synthesis of G factors *ie* a) synthesis of the parent syncarpic acid obtained through linear C-alkylation reactions and also from phloroglucinol b) Mannich type reaction with various aldehydes (chemical diversity) and oxygen uptake. By applying a rational approach involving “covalent bitherapy” hybrid compounds bearing streptocyanine or aminoquinoleine frames have been elaborated and evaluated *in vitro* against *P.falciparum*. One of them presented excellent activities against wild and chloroquine sensitive strains.

The second family is based on the artemisinin scaffold. The dihydroartemisinin derivative was further functionalized by introduction of various linear linkers ending to functional groups that might be considered as strong siderophores. In that respect terminal hydroxamic derivatives were synthetically studied; all steps were optimized and a series of hydroxamic acid derivatives bearing the artemisin frame were thus obtained and should be evaluated.



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P14 Malaria in Cameroon: preparation of a predictive model for studying the impact of interventions

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Malaria is a disease caused by parasitic protozoan of the genus *Plasmodium*. It is a mosquito-borne disease that affects both humans or animals, and it is transmitted by bite of an infected *Anopheles* mosquito. Malaria is endemic in tropical and subtropical regions including Asia, Africa and America. The World Health Organization (WHO) estimates that 3.3 billion people are at risk of malaria, of whom 1.2 billion people at high risk. In 2012, malaria killed an estimated 482.000 children under five years of age. The situation in Cameroon is alarming with 70% of population living in high-risk areas. The intervention policies seem to be inadequate with an insufficient coverage of the population.

In the present study, we have investigated the efficacy of different interventions for the epidemiological control of the malaria transmission in Cameroon. The simulations have been performed with the open-source program OpenMalaria, whose mathematical models is steadily updated and have been proved to be effective in predict the diffusion of the disease. The input parameters for the simulation were collected through literature searches, report of the WHO and inquiring the local administration. We have simulated the use of different pesticide for Indoor Residual Spraying (IRS), including also the use of the outlaw DDT. Results are evaluated from a social and economic point of view by means of Disability-Adjusted Year indicator (DALY).

The use of DALY have permitted the direct comparison of different scenarios.

Based on the data collected from our simulation the best result led to a DALY reduction using Insecticide-Treated Net (ITN) coverage up to 62% and an implementation (60-80%) of IRS, with an estimated annual cost of 8.5\$ per person.

From our results, several guidelines for the malaria control in Cameroon could be considered in order to help eradicating malaria. More details will be shown during the poster session.

P15 Stability of artemisinin derivatives in physiologically-relevant conditions

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Artemisinin derivatives are the most effective antimalarials available to-date: they are highly active against all *Plasmodium* species and parasite stages and constitute the backbone of malaria case management, both for severe and uncomplicated malaria. These molecules, characterized by the presence of an endoperoxide pharmacophore, are highly unstable; they degrade very in the presence of ferrous iron or organic solvents. We recently showed the stability of dihydroartemisinin (DHA), an antimalarial drug on its own and the main metabolite of other artemisinins, in a range of conditions relevant to both *in vitro* testing and clinical situations (Parapini et al, AAC, 2015).

Here we studied the stability of artemisinin, artemisone (which are not metabolized to DHA) and artesunate (extensively converted to DHA) in a range of conditions relevant to both *in-vitro* testing and *in-vivo* effects. Drugs were incubated in PBS, plasma or erythrocytes lysate at different time points, throughout a range of temperatures and pH values. The residual activity of the drugs were then evaluated with the chemosensitivity assay on *P. falciparum* measuring the pLDH activity. The role of the Fe(II)-haem was investigated by blocking its reactivity using carbon monoxide. Chloroquine, a 4aminoquinoline antimalarial, was used as control drug without endoperoxide bridge.

A significant reduction in the antimalarial activity of the drugs was seen after incubation in PBS, plasma and erythrocytes lysate. DHA was the most unstable in plasma: a 3-hour incubation reduced by half DHA activity, which was almost completely lost after 24 hours. The others artemisinins were more stable than DHA in PBS, plasma and in serum-enriched media customarily used for *in vitro* cultures. Differently from DHA, the major reduction of the antimalarial activity of artemisinin was seen after incubation in erythrocytes lysate and to a lesser extent in plasma. Antioxidants like ascorbic acid or N-acetyl cysteine further reduced artemisinins antimalarial efficacy only when added to erythrocytes lysate, but not plasma. Chloroquine activity was unaffected in any of the tested *in vitro* conditions.

The results of this study have therefore practical methodological implications for *in vitro* drug assays suggesting that particular care has to be taken in conducting *in vitro* studies, and in storing these compounds. Conditions such as fever, hemolysis, acidosis associated with malaria severity may contribute to artemisinins instability and reduce their effectiveness. Moreover, instability of the compounds seems to be related to the endoperoxide bridge but depends also on the substituent present in the C10 position of the molecules.

P16 **Discovery of the phenolic monoterpene carvacrol binding sites in the nicotinic acetylcholine receptors (ion gated channels) within parasitic nematodes**

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Carvacrol is natural anthelmintic known to act as antagonist of nicotinic acetylcholine receptors (nAChRs), within the neuromuscular system of the nematodes. The nAChRs contain several allosteric modulatory sites and carvacrol binds non-competitively within these sites, showing inhibitory effect on ligand-gated ion channels. Known features of nAChR structure were used to optimize molecular cavity of extracellular binding domain, which is the main drug target region. These receptors are studied in parasitic nematodes *Ascaris Suum* and *Caenorhabditis elegans*. Unknown binding site of carvacrol within targeted region can be found using grid map and "blind docking" methods. Blind docking is introduced for the detection of possible binding sites and in silico solutions by using docking tools focused on the (supposed) primary binding region. Finding these binding solutions is necessary in order to explain the mechanism of carvacrol inhibitory potential and further on, to use such properties in ligand-based drug design. Above all, such information is needed for developing better selectivity of anthelmintic, cholinergic drugs.

P17 Combined in silico approaches for the selection of antileishmanial inhibitors

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Arginase, polyamine biosynthesis enzyme, is promising drug target for the treatment of leishmaniasis. It was shown that flavonoids inhibit this central enzyme in *Leishmania* infection.¹ We performed in silico screening of the Tim Tec Flavonoids Database² for candidate arginase inhibitors using EIIP/AQVN filter³. 49 flavanoids and their derivatives were selected as candidate anti- *Leishmania* drugs. Further filtering of these compounds by means of 3D QSAR revealed 21 compounds as most promising candidates for arginase inhibitors which should be subjected to further experimental evaluation.

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