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# BOOK OF ABSTRACT

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Medicinal Chemistry including (Natural products)
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Parasitology Ecotoxicology Pharmacology

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# Structural insights in the catalytic mechanism of pteridine reductase from *Trypanosoma* parasites for the development of antiparasitic agents

## Subject area: Drug design & structural biology

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The World Health Organization has identified 17 neglected tropical diseases (NTDs) posing health burden to over 1.4 billion people. Trypanosomatid parasites are responsible for threatening insect-vector borne NTDs, as human African trypanosomiasis (HAT, sleeping sickness) caused by *Trypanosoma brucei*, Chagas disease caused by *Trypanosoma cruzi*, and leishmaniasis caused by *Leishmania* spp. Current therapeutics are limited by toxicity, poor efficacy, and parasite resistance, thus underlining the need for novel antiparasitic agents, that can be developed by target-based drug design strategies [1,2]. The folate pathway enzyme dihydrofolate reductase (DHFR) is crucial for parasite survival, but, in trypanosomatids, its inhibition is ineffective due to the metabolic bypass provided by the biopterin-reducing pteridine reductase (PTR) [1,2]. Indeed, when DHFR is inhibited, PTR is overexpressed and sustains sufficient reduced folate levels to ensure parasite survival. Thus, both DHFR and PTR need to be considered for the effective targeting of the folate pathway in *Trypanosoma* parasite.
Here, we have investigated the mechanism of action of PTR from *Trypanosoma brucei*, to unveil the main active site features involved in the sequential two-step catalytic process reducing both unconjugated pterins and folate, firstly to their di-hydro forms, and then to the tetra-hydro products. Different snapshots of the catalytic reaction have been obtained through X-ray crystallography, allowing us to provide new insights in the PTR mechanism of action. This structural information gives pivotal contribution for the rational development of potent and selective antitrypanosomatidic inhibitors, as those obtained by us within the EU-FP7 project *New Medicines for Trypanosomatidic Infections* (*NMTrypI*) [2-6].

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Acknowledgment

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# Antimalarial Strategies: Designing Synthetic Peroxides as Promising Alternatives to Combat Drug Resistance

## Subject area: Medicinal Chemistry including (Natural products)

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Malaria is still a globally distributed infectious diseases causing high mortality and morbidity in the most affected countries, such as sub-Saharan Africa, South-East Asia and South America.
The protozoan causing the infection is part of the Plasmodium genus. More than 200 species are known, but only five cause Malaria in humans (P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi). [1]
The transmission in vertebrate host occurs through a mosquito, more specifically the anopheline mosquitoes.
Malaria causes symptoms that typically include fever, fatigue, vomiting and headaches. In severe cases it can cause yellow skin, seizures, coma, and death.[1]
Natural cycloperoxides such as artemisinin and its semi-synthetic derivatives represent well-established alternatives to quinoline antimalarials, indeed they are used in combination with other antimalaria drugs (Artemisinin-based Combination Therapies of ACT). However low bioavailability and short half-life, and high cost of these drugs are major drawbacks of their use. Moreover P. falciparum resistance to artemisinins has now been detected in five countries which manifest in the form of delayed parasite clearance. [2][3]
Our work deals with the design of suitable synthetic strategies for the preparation of antimalarial endoperoxides. Research efforts are directed towards the identification of simpler, but still potent peroxide-based molecular scaffolds. Inspired by the antimalarial potential of ART and its derivatives, several synthetic peroxides were developed with interesting antiplasmodial activity against both chloroquine-sensitive and chloroquine-resistant parasites. [2][3]

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# Pyrimido[5,4-*d*]pyrimidines: a novel class of antitrypanosomal and antileishmanial agents

## Subject area: Medicinal Chemistry including Natural products

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The scientific community have been deeply involved in the search for new antimicrobials, motivated either by the lack of efficient treatments or by the emergency of drug resistance to existing drugs. Leishmaniasis and sleeping sickness are examples of diseases in need of new medicines. Researchers have been responding to this need by discovering new chemical entities to develop as new drugs [1-4].

Our research group joined this effort and recently discovered a new class of antitrypanosomal and antileishmanial agents, the pyrimido[5,4-*d*]pyrimidines [5,6]. The *in vitro* activity against *T. brucei* and the extracellular stage (promastigotes) and intracellular stage (amastigotes) of *L. infantum* parasites was assessed. The cytotoxicity of the compounds was also assessed using the THP1 cell lines. The results from biological assays allowed the identification of several compounds that combine low toxicity against THP1 cell lines and high activity against *T. brucei* ($IC_{50}$ < 0.5 mM, SI > 100 ), *L. infantum* promastigotes ($IC_{50}$ < 1 mM, SI > 100) and *L. infantum* amastigotes ($IC_{50}$ < 5 mM, SI > 30).

New derivatives are being synthesised and *in vitro* tested to increase solubility, maintaining high activity and selectivity.

The compounds' biological target(s) and mechanism(s) of action are unknown, and ADMET studies have not yet been done.

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# High-throughput phenotypic screening and machine learning methods enabled the selection of broad spectrum low-toxicity anti-trypanosomatidic agents. Subject area: medicinal chemistry

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Recent analysis reports that 75% of all emerging human infectious diseases in the past three decades worldwide originated in animals. Poor and disadvantaged populations (sub-tropical regions, and, in Europe, Mediterranean countries) may spread new infectious agents. Infections caused by Trypanosomatidae account for 17% of the estimated global burden of all infectious diseases (70,0000 deaths/year). All the afore mentioned infections cause severe population burden if the drug candidate pipeline is not enriched. Therefore, there is an unmet medical need for novel medicinal chemistry efforts to develop new treatments.

In our effort to identify broad-spectrum anti-infective chemotherapy agents with activity against Trypanosomes, Leishmania and *Mycobacterium tuberculosis* species, important hits were identified from a high-throughput phenotypic screening program of the 456 compounds belonging to the Ty-Box, in house industry database. Active compounds were identified against *T. brucei*, *L. infantum* and *T. cruzi*, as well as replicant and non-replicant strains of *M. tuberculosis*. Simultaneously, compound characterization using machine learning approaches, enabled the identification and synthesis of 44 compounds with broad spectrum antiparasitic activity and minimal toxicity against *T. brucei, L. infantum* and *T. cruzi.* Subsequent *in-vitro* characterization confirmed the predictive models identified a promising and innovative chemical scaffold, **Ty-40**, with low micromolar activity against two parasites and low toxicity. Given the volume and complexity of data generated by the diverse HTS assays performed on the compounds of the Ty-Box library, the chemoinformatic and machine learning tools enabled the selection of compounds eligible for further evaluation of their biological and toxicological activities and aided in the decision-making process toward the design and optimization of the identified leads.


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# A basic celastrol carboxamide derivative acts as a middle-domain, potent and selective allosteric inhibitor of *Leishmania braziliensis* 90kDa Heat Shock Protein"

Area: Drug Design & Structural Biology

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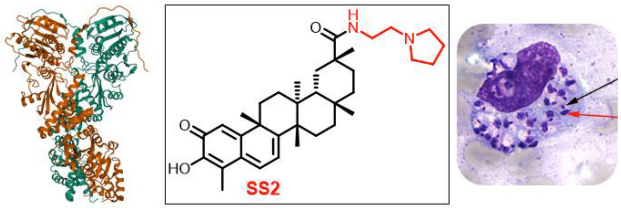
The 90 kDa heat-shock protein (Hsp90) is an ATP-dependent molecular chaperone that guarantees the folding and the homeostasis of cytoplasmic proteins stabilizing them against heat stress while also aiding proteins'degradation. HSP as a class are among the most highly expressed cellular proteins across all species: they account for 1-2% of total protein in unstressed cells and, under stress inducing conditions, they are overexpressed to the 4-6% of the proteome. For the intracellular protozoa *Leishmania* the role of Hsp90 goes far beyond protein homeostasis as it participates in crucial pathogenesis-related events. Specifically, Hsp90 has been reported to be activated by the stress-inducing events connected to *Leishmania* parasites differentiation from promastigotes to amastigotes, their macrophage-infecting form. Orthosteric Hsp90 inhibitors, which blocks its characterizing ATPase activity by binding to the protein active site located on its *N*-terminal domain (NTD), have been investigated as potential antileishmanial agents demonstrating promising profiles *in vitro*. However, their development into antiprotozoals is generally impaired by the induction of heat-shock responses (HSR) in the treated parasites cultures. In this context, the use of allosteric modulators of Hsp90, which can impair the native functionality and dynamics of the chaperone without triggering HRS, has been emerged as a promising alternative to orthosteric chaperone inhibition [2], [3].




Aiming at developing novel leishmanicidal agent targeting protozoan Hsp90 *via* an allosteric mechanism of action, synthetic derivates of the triterpene celastrol, a cytotoxic natural compound known as Hsp90 interactor, have been designed, prepared, and screened *in vitro* against cultures of *Leishmania* promastigotes and intramacrophage amastigotes. SS2, a pyrrolidine celastrol derivative, was identified as the most potent and selective leishmanicidal agent [4] and, thus, its molecular interaction with the *Leishmania* Hsp90 has been deeply investigated. By combining *in vitro* assays, high-resolution NMR experiments and *in silico* simulations, SS2 profile as Hsp90 modulator was elucidated.

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## SQ109 analogs with trypanocidal activity

## Subject area: Medicinal Chemistry including (Natural products)

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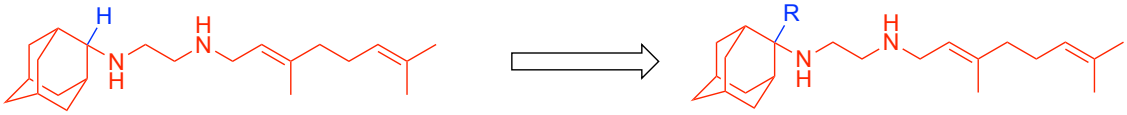
SQ109, an antitubercular drug candidate, presents activity against *T. brucei, T. cruzi* and *Leishmania* spp as a uncoupler, targeting lipid membranes, as well as calcium homeostasis. In this work, we have synthesized new SQ109 analogs bearing alkyl, aryl or heterocyclic functional groups and we have tested the latter derivatives against *Trypanosoma brucei* bloodstream forms, *T. cruzi* epimastigotes, amastigotes, and their U20S host cells, *L. donovani* promastigotes and *L. mexicana* promastigotes. Moreover, we used differential scanning calorimetry (DSC) to explore the interactions of the examined compounds with lipid bilayer membranes of the parasites. The bulkier SQ109 analogs afforded higher trypanocidal activity than the parent compound. These results were correlated with the DSC experiments, the increased size derivatives were more incorporated into the phospholipid bilayer and further fluidized the membrane, generating a structure that melted at a lower temperature [1].




**SQ109** **SQ109 analogs**

### Reference

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# Investigation of 5-nitrothiophene-2-carboxamides as Inhibitors of Trypanothione Reductase Targeting Leishmania parasites

**Subject area:** Medicinal Chemistry including (Natural products)

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Leishmaniases, caused by protozoan parasites of the genus Leishmania, are vector-borne diseases with significant global impact, resulting in up to 1 million new cases annually, according to WHO data. The prevalence of leishmaniasis is intricately linked to the human development index and environmental degradation. Notably, human and canine leishmaniasis due to *L. infantum* exemplify the intersection of animal and human health. *L. infantum* is responsible for visceral leishmaniasis (VL) in South America, the Mediterranean basin, and West and Central Asia, as well as canine leishmaniasis (CanL) in Europe.

Despite substantial efforts, existing leishmaniasis therapies have limitations, prompting an urgent need for new, more effective, and affordable treatments. A promising target for drug development is trypanothione reductase (TR), a key enzyme in trypanothione metabolism crucial for parasite survival within macrophages. This study focuses on structure-based optimization of 5-nitrothiophene-2-carboxamides as TR inhibitors. Initiated by resolving the crystal structure of TR in complex with a phenotypic lead compound, diverse derivative series were designed to explore structure-activity relationships around the 5-nitrothiazole core.

The most promising compounds underwent in vitro assays, demonstrating significant activities against both *L. infantum* promastigotes and amastigotes growth. Molecular docking studies were conducted to rationalize inhibitory activities and guide subsequent optimization efforts. These findings contribute to the ongoing quest for novel, improved therapies against leishmaniasis.

# Exploration of heterocyclic/oxygenated chalcones as antileishmanial agents and pilot investigation of a greener synthetic approach

## Medicinal Chemistry including (Natural products)

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Leishmaniasis is a group of infectious tropical diseases which is caused by a protozoan parasite, and the transmission to mammals occurs through the bite of an intermediate sandfly. This neglected disease is endemic is south of Africa, in part of the Indian peninsula and in the majority of south America. The outcome of this infectious disease varies depending on the leishmania species and on the host immune response, in fact the diversity in tropism and disease form is quite variable. Treatments are unsatisfactory, due to high-cost treatments, safety concerns and length of treatments, for these reasons natural and synthetic chalcones come into play, thanks to their interesting anti-inflammatory, antimicrobial and anti-cancer properties[1]. Thus, starting from specific natural chalcones selected as starting points, an optimized synthesis led to the preparation of a small library of derivatives showing interesting activity against promastigotes[2]. The synthetic pathway has been ultimately revised under the light of green chemistry principles, in order to use less organic solvents, reducing waste and toxicity[3].

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# New antiparasitic bioisosteric ether phospholipids: synthesis and biological evaluation

## Medicinal Chemistry including (Natural products)

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Neglected tropical diseases (NTD) including Leishmaniasis and Human African Trypanosomiasis (HAT) cause significant morbidity and mortality in the developing world, with more than one million people dying from complications according to the WHO. The existing medications can cause serious side effects, are expensive, require long treatment regimens and can induce parasite resistance. Thus, new drugs for NTDs are still urgently needed.
Miltefosine (hexadecylphosphocholine) an ether phospholipid analogue, is currently the only oral drug for the treatment of visceral and cutaneous leishmaniasis, however it suffers from severe gastrointestinal side effects, teratogenicity and development of resistance. We previously demonstrated that the introduction of rings in the lipid portion of alkylphosphocholines results in derivatives with increased potency against *Leishmania sp.* and reduced toxicity [1-3]. Capitalising on these results and employing the bioisosterism approach we replaced the phosphate group of alkylphosphocholines by the thiazolidinone heterocyclic moiety maintaining the quaternary ammonium group. Thus, a series of derivatives were synthesized using a multicomponent reaction as the key synthetic step, in order to optimise the structural features for anti-leishmanial and anti-trypanosomal activity using an iterative approach. Several active and non-toxic compounds were prepared enriching the drug pipeline for NTDs with novel chemical entities.

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# Web sources of information on topics related to Veterinary Parasitology

## Subject area: Parasitology

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In the World Wide Web we find several sources of information related to veterinary parasitology. The accuracy and liability of the available information varies, leading us to develop considerations on what to access and what to value. We share some websites that can be consulted as reference sources of information, considering their linkage to governmental institutions, pharmaceutical companies or scientific associations. These include the DPDx – Laboratory Identification of Parasites of Public Health Concern (https://www.cdc.gov/dpdx/index.html) website, managed by the national public health agency of the United States of America, the Centers for Disease Control and Prevention (CDC). This explores an index of human and zoonotic parasites (from A to Z), cases studies, diagnostic procedures and a training section. Another relevant source of information is the Companion Animals Vector-Borne Diseases website (CVBD; https://campaign.elanco.com/en-us/cvbd), maintained by Elanco Animal Health. Contrary to what could be expected, this is not an advertising site, but rather a scientific source subject to periodic updates and peer reviews. Besides companion animals parasitosis, zoonotic aspects of vector-borne diseases are also addressed (i.e. when humans are involved). Not all agents transmitted by vectors are parasites stricto sensu (e.g. viruses and bacteria), but all vectors are (e.g. ticks, fleas, sand flies, mosquitos, kissing bugs and other flying insects). The non-profit organizations European Scientific Counsel for Companion Animal Parasites (ESCCAP; https://www.esccap.org/) and Tropical Council for Companion Animal Parasites (TropCCAP; https://www.troccap.com/) provide relevant information regarding epidemiological data and guidelines for parasite identification, treatment and prevention, within the European region and the Tropics and sub-Tropics, respectively. The World Association for the Advancement of Veterinary Parasitology (WAAVP; https://www.waavp.org/) is a not-for-profit organization for scientists who study the parasites of non-human animals, encompassing helminthology, protozoology and entomology. This website contains antiparasitic guidelines for identification and prevention of anthelminthic resistance in ruminants, pigs, equines, dogs and cats, poultry and fish, besides the Standardized Nomenclature of Animal Parasitic Diseases (SNOPAD).

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## Rational design of nanotherapeutics using bioinspired mimetic models

### Subject area: Design of drug and drug delivery nanosystems

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Recent advances in nanomedicine include a wide range of nanocarriers ranging in composition from lipidic to polymeric, as well as hybrid systems combining materials from both natures. The great diversity of these amphiphilic materials enables their self-assembly in aqueous media in nano- and meso-structured systems capable of carrying various cargos ranging from drugs to genes for either therapeutic or prophylactic purposes (vaccines) [1]. Understanding the important pharmaceutical and galenical aspects of the formulation is critical in the development of drug delivery nanosystems (DDNs). To achieve the best therapeutic outcomes, DDNs must be designed with several factors in mind, including the biological barriers encountered by the nanosystem, the route of administration, absorption, distribution, metabolism, and elimination, the target tissue, and selective toxicity [2]. The development of DDNs will be presented as a logical sequence of steps that addresses the active ingredient, its therapeutic target, and the biological interfaces in its path to the target, all of which are critical factors to consider in the design of nanosystems. Following a brief overview of some bioinspired mimetic models, I will show how these models have been used in rational drug design and in hybrid DDNs' design for biomedical applications.

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## Enzymatic redox properties and mammalian cell cytotoxicity of fexinidazole
## Subject area: Medicinal Chemistry

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Fexinidazole (1-methyl-2-[(4-methylsulfanylphenoxy)methyl]-5-nitroimidazole) has been approved by the EMA and USFDA for the treatment of human African trypanosomiasis. In order to gain a more detailed understanding of its mechanism of action, possible side effects and repurposing, it is necessary to study its enzymatic redox properties, which determine the formation of its free radicals and/or alkylating hydroxylamines. We found that the single-electron reduction of fexinidazole by NADPH: cytochrome P450 reductase and adrenodoxin is slow, with $k_{cat}/K_m$ of $4.5x10^3$ $M^{-1}s^{-1}$ and $1.5x10^3$ $M^{-1}s^{-1}$, respectively (pH 7.0). When calculating the single-electron reduction potential ($E^1_7$) of the compound based on these rate constants [1], it was equal to -0.458 V vs. NHE. It was close to $E^1_7$ of structurally related 5-nitroimidazoles. Electrochemical reduction of fexinidazole proceeds irreversibly with $E_{pc(7)}$ = -0.590 V vs. SCE (v = 50 mV/s). Two-electron reduction of fexinidazole by rat liver NAD(P)H:quinone oxidoreductase (NQO1) and *E. coli* nitroreductase-A also proceeds slowly, with $k_{cat}$ <0.1$s^{-1}$, and $k_{cat}$ = 0.6 $s^{-1}$ ($k_{cat}/K_m$ = $3.0x10^3$ $M^{-1}s^{-1}$), respectively. Taken together, low $E^1_7$ of fexinidazole and its low reactivity towards mammalian single- and two-electron transferring flavoenzymes result in its low cytoxicity ($GI_{50}$ >1000 µM in HCT-116 cells).

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# Nanomedicine synergizes Repositioning: Delamanid solid lipid nanoparticles for accessible leishmaniasis treatments

## Subject area: Nanomedicine

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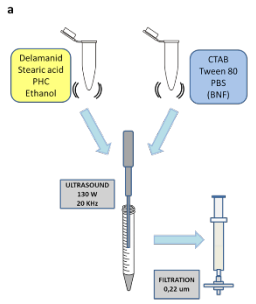
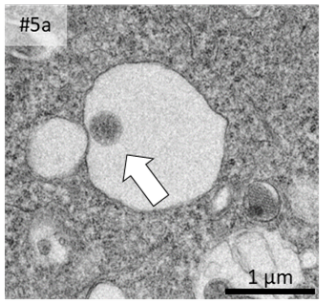
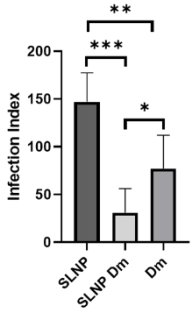
Leishmaniasis, one of the neglected tropical diseases worldwide distributed, is treated with drugs that produce serious adverse effects thus reducing patient compliance and contributing to the emergence of parasite resistance [1] [2] [3]. To reduce costs and development time for new therapeutic alternatives drugs approved for other indications can be repositioned [4] [5] . Nanomedicine could enhance activity and reduce drawbacks of left aside drugs by effectivity and safety issues [6] [7] . Nanomedicine synergizes with drug repositioning to increase the possibilities of developing safe and effective medicines. This study aims to repurpose approved drugs for an accessible (affordable and available) leishmaniasis treatment applying nanomedicine. After the screening of potential drug candidates by reviewing databases and utilizing molecular docking analysis, delamanid was chosen to be incorporated into solid lipid nanoparticles (SLNP). Both *in cellulo* and *in vivo* tests confirmed the successful payload release within macrophages and through the epidermis following topical application on murine skin. Evaluation on macrophages infected with *L. infantum* amastigotes showed that the encapsulated delamanid exhibited greater leishmanicidal activity compared to the free drug. *In vivo* studies in murine model infected with *L. major*, confirmed the SLNP leishmanicidal activity. The process of encapsulating places a strong emphasis on employing minimal technology, ensuring energy efficiency, cost-effectiveness, and reproducibility. It enables consistent, low-cost production of nanomedicines, even on a small scale, offering a promising step towards more accessible and effective leishmaniasis treatments.

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# Prevention of Leishmaniasis through Personal Protection by Use of Repellent Textile

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Vector-borne diseases is an important public health issue, the World Health Organization (WHO) estimates that every year they cause over 1 billion human cases and 1 million deaths, representing approximately 17% of total cases of communicable diseases. One of the pillar intervention to tackle with issue is the strategy of consolidating and integrating available approaches. Based on the changed epidemiological situation in Europe, the European Commission approved Implementing Decision (EU) 2018/945 of 22 June 2018 with which it updated the list of diseases to be incorporated into the community epidemiological surveillance network, extending it to several arboviruses, including Chikungunya, Dengue and Zika, which pose a threat to public health. Among vector-borne diseases, an important group is made up of arboviruses, i.e. viral diseases transmitted by arthropods. Minor attention is paid until now on control of a non-viral ones as leishmaniasis vectored by sandfly. By updated information, the number of the leishmanaisis case is rising in last years in Europe The strategy of vector control as vector eliminations- reduction is one on the reasonable activity to implement. Chemical resistance in vectors, the increased legislative restriction of pesticide and the climate change are calling new environmentally friendly solutions, innovated control measures to manage vector species and to minimize transmission of medical and veterinary disease. One of the new approaches of vector control is personal protection which is in fact prevention approach. The use of repellent to avoid the sand fly's bites is recommended as prevention protocols suggested by many international organisations. To develop and research on environmentally friendly biocides accepted by regulation the innovation options are in pipeline with the possibility to transfer to textiles the properties of a repellents. This contribution will explain the possibilities of use of fabrics to repel vectors in particularly sandflies.

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Potential Applications of Microencapsulated Essential Oil Components in Mosquito Repellent Textile Finishes
Maida Murtaza Abdullah Ijaz Hussain , Ghulam Mustafa Kamal, Saima Nazir,Shahzad Ali Shahid, Chatha Mufarreh Asmari , Jalal Uddin and Shahzad Murtaza Coatings 2023, 13, 1467. https://doi.org/10.3390/coatings13081467 Methods of imparting mosquitorepellent agents and the assessing mosquito repellency on textile. Aufa Adeela Anuar* and Nurain Yusof Fashion and textile (2016) 3:12 DOI 10.1186/s40691-016-0064-y. Microemulsification of essential oils for the development of antimicrobial and mosquito repellent functional coatings for textiles. A Soroh, L. Owen , N. Rahim, J. Masania, A. Abioye, O. Qutachi, L. Goodyer, J. Shen and K. Laird Journal of Applied Microbiology ISSN 1364-5072 Distribution and altitudinal structuring of phlebotomine sand flies (Diptera: Psychodidae) in southern. Anatolia, Turkey: their relation to human cutaneous leishmaniasis. Fatih M Simsek 1, Bulent Alten, Selim S Caglar, Yusuf Ozbel, A Murat Aytekin, Sinan Kaynas, Asli Belen, Ozge Erisoz Kasap, Mehmet Yaman, Samiye Rastgeldi Affiliations expand PMID: 18260517 DOI: 10.3376/1081-1710(2007)32[269:daasop]2.0.co;2

# Pyrimido [5,4-d] pyrimidine-based compounds as novel antimalarial and antileishmanial drugs

## Subject area: Parasitology

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Malaria and leishmaniasis are vector-borne parasitic diseases that threaten millions of people. The available therapies present several limitations including drug resistance, lack of efficacy, and high toxicity, prompting a search for novel therapeutic agents. We have been synthesizing and screening pyrimido [5,4-d] pyrimidine derivatives as novel antimalarial and antileishmanial drugs. Structure-based in vitro studies, considering the activity against *Plasmodium falciparum* and *Leishmania infantum* parasites and their cytotoxicity against human cells, guided the selective modification of the core scaffold with the selected substituent groups. For antimalarial drug discovery, compounds were tested on blood stage forms, which are responsible for symptoms and complications of malaria, of both susceptible and chemoresistant *P. falciparum* strains, 3D7 and Dd2 respectively. The most promising include molecules with IC50 below 100 nM against both strains and a selective index ranging from 100 to 1000-fold. The most active compounds against *L. infantum* were selected based on their activity against the clinically relevant intracellular amastigotes presenting superior or comparable performance to the lead oral available molecule, miltefosine. Next, we will conduct preclinical studies in mice models of malaria and visceral leishmaniasis, with the goal of identifying according to target product profiles of WHO and the leading non-profit organizations, Medicines for Malaria Venture (MMV) and Drugs for Neglected Diseases initiative (DNDi), a lead compound among derivatives of the PP class.

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## The essential role of mitochondrial type II NADH dehydrogenase in *Leishmania*

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Mitochondria are multifaceted organelles with a crucial role in energy production through the oxidative phosphorylation pathway. NADH and FADH2 electrons enter the respiratory chain (RC) and are transferred to oxygen in a process coupled to the translocation of protons into the mitochondrial intermembrane space that fuels ATP production. As in most eukaryotic cells, *Leishmania* RC is made up of complexes I through IV. Aside from these enzymes, *Leishmania* contains at least two extra and unique enzymes that cause RC bifurcations at the level of complex I: i) type II NADH dehydrogenase (NDH2), that bypasses complex I and oxidizes NADH without coupled proton pump and ii) fumarate reductase (FRD) that, together with complex II, allows electrons from NADH to enter the RC. The relative contribution of these enzymes for *in vivo* parasite survival is yet unclear.

Expression of the different enzymes was evaluated in both *Leishmania infantum* (a visceralizing species, Li) and *L. major* (a cutaneous species, Lm) through western blot analysis and oxygen consumption assays with intact parasites. Subcellular localization was addressed by immunofluorescence studies and western blot analysis. Gene deletion was generated by CRISPR Cas9 techniques and mutants' ability to prosper in animal models of infection was evaluated in mice.

NDH2 protein is expressed in both the promastigote and amastigote stages of *L. infantum*, while complex I activity was not detected. Overexpression of *Li*NDH2 was found to increase basal oxygen consumption of intact parasites, confirming the enzyme as a respiratory chain component. Moreover, we found that *Li*NDH2 is essential in *L. infantum*, including in the disease-causing stage. In fact, i) deletion of both *ndh2* alleles is only possible upon complementation with an episomal copy of the gene, ii) knockout promastigotes and amastigotes do not lose the *Lindh2* episome after multiple passages in culture in absence of drug pressure, in contrast to a control episome that is lost after few cycles of parasite replication, and iii) single knockout ndh2$^{+/-}$ parasites are less virulent than the wild type in mice. Furthermore, NDH2 is also essential in *L. major* a species expressing active complex I. FRD is expressed in promastigotes *L. major* displaying higher levels than *L. infantum*. Attempts to delete the *Lifrd* gene reveal its non-essential character even for *in vivo* infections. Mutant *Li*frd$^{-/-}$ parasites are, however, less virulent than wild type in mice.

In summary, our demonstration of the essential character of NDH2 in parasite survival *in vivo*, regardless of the presence of a functional complex I and fumarate reductase, genetically validates NDH2 – an enzyme without counterpart in mammals - as a promising target for the development of leishmanicidal drugs.

Keywords: *Leishmania*; mitochondria; respiratory chain enzymes; infection
Funding: This work was supported by National Funds through FCT - Fundação para a Ciência e a Tecnologia, I.P., under the project UIDB/04293/2020.

## Visceral Leishmaniasis in Human Immunodeficiency Virus-Coinfected Patients
## Subject area: parasitology

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Objectives: In Europe, up to 70% of visceral leishmaniasis (VL) cases occurring in adults are on HIV-infected patients. Patients with HIV-VL co-infection often display persistent parasitemia, requiring chronic intermittent anti-*Leishmania* therapies. Consequently, frequent VL relapses and higher mortality rates are common in these patients. As such, it is of paramount importance to understand the reasons for parasite persistence to improve infection management.
Methods: To outline possible causes for treatment failure in the context of HIV-VL, we followed a patient living with co-infection for nine years in a 12-month period. We characterized: HIV-related clinicopathological alterations (CD4+ T counts and viremia) and *Leishmania*-specific seroreactivity, parasitemia, quantification of pro-inflammatory cytokines upon stimulation and studied a *Leishmania* clinical isolate recovered during this period.
Results: The patient presented controlled viremia and low CD4+ counts. The subject remained PCR positive for *Leishmania* and also seropositive. The cellular response to parasite antigens was erratic. The isolate was identified as the first *Leishmania infantum* and presented decreased susceptibility to miltefosine when compared to other isolates recovered in the same period.
Conclusion: Treatment failure is a multifactorial process driven by host and parasite determinants. Still, the real-time determination of drug susceptibility profiles in clinical isolates is an unexplored resource in the monitoring of VL. The existence of parasites with altered drug susceptibility profile in this vector borne disease highlights the possibility of circulating parasites with altered susceptibility to known drugs. This represents a major challenge for the One Health vision and can also impact drug development efforts.

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# The essential role of mitochondrial type II NADH dehydrogenase in *Leishmania*

## Subject area: parasitology

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Funding: This work was supported by National Funds through FCT - Fundação para a Ciência e a Tecnologia, I.P., under the project UIDB/04293/2020.

# Implementing One Health (OH) competencies within the Veterinary & Medical Curricula

## Subject area: One Health Education

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The COVID-19 pandemic has played a significant role in enabling global governments, notably the G7 and G20 nations, to adopt the One Health framework as a tool for formulating health policies for addressing future pandemics. Antimicrobial resistance, food security, translational medicine, vector-borne zoonoses, and the impacts of climate change on public health, are key One Health issues that require collaboration by health professionals at the nexus of the animal, human, and environmental health divide. Traditionally, the siloes that have existed across the health professions, have limited the ability of transdisciplinary efforts and operationalization of One Health principles in preventing the emergence of these threats. Interprofessional education (IPE) which prepares health professions students for this collaborative approach, is an important pedagogical platform for delivering the transdisciplinary principles of One Health within the curricula of the health professions [1,2,3].
Our IPE working group consists of participants from Universities across the United States and Grenada who are focused on advancing One Health through IPE in the curricula of medicine and veterinary medicine. The group seeks to develop One Health-focused IPE core competencies, modules, and publications for dissemination, to further prepare our future graduates in medicine and veterinary medicine for a team-based approach to practice. Ultimately, the researchers seek to prepare future health professionals for a collaborative approach to clinical practice, proven to significantly reduce errors in patient care.

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# Sustainable biocatalytic derivatization of antiparasitic natural lipids and cashew nut-shell liquid (CNSL) derivatives using unspecific peroxygenase (UPOs) enzymes

## Subject area: Medicinal Chemistry including (Natural products)

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Selective oxyfunctionalization of non-activated C–H, C–C and C=C bonds is an extremely interesting chemical transformation with huge implications in the synthesis of several products, ranging from fine chemicals to pharmaceuticals. However, introducing functionalities into the inert skeletons of organic molecules is quite challenging in contemporary chemistry. Biocatalysis has proven to be a potent tool to achieve these objectives, since enzymes possess remarkable substrate specificity, chemo-, enantio-, and regioselectivity, and they can provide tens of thousands of turnovers working in mild conditions.[1] Fungal unspecific peroxygenases (UPOs) are simple and robust heme-thiolate-containing enzymes that catalyse a range of C–H oxyfunctionalization reactions, activated simply by $H_2O_2$ as the main oxygen donor and the final electron acceptor. The kaleidoscope of oxyfunctionalization reactions that UPOs are able to perform, make them result among the most promising biocatalysts in synthetic chemistry.[2] Exploring the biotransformation of food waste into high-value compounds through biocatalysis is a topic of great interest, with potential application in medicinal chemistry. Cashew nut-shell liquid (CNSL) from *Anacardium occidentale*, is an inedible oil obtained as by-product during cashew nut processing. Chemically, CNSL constitute a natural source of phenolic compounds, namely cardanols, cardols, 2-methylcardols and anacardic acids, which share a pentadecyl alkyl side chain

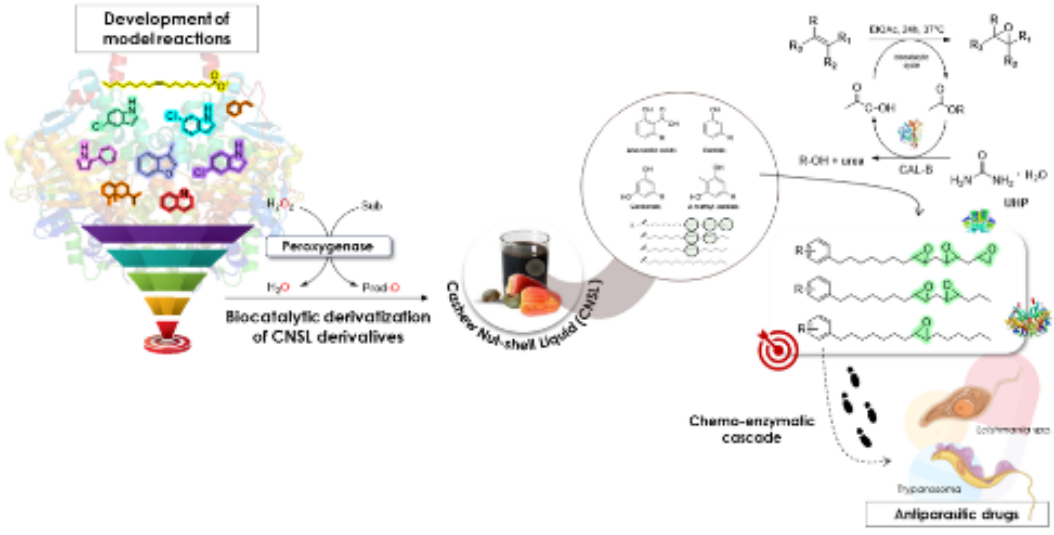


**Figure 1.** Biocatalytic derivatization of natural lipids and CNSL derivatives.

with a variable degree of unsaturation.[3] Although CNSL has potential as a source of drug precursors, there are no examples of its biocatalytic derivatization into drugs. Thus, the goal of this STSM was the development of biocatalytic reactions for the derivatization of natural lipids, CNSL derivatives, and drug building blocks exploiting enzymes (e.g. UPOs, CAL-B – UHP oxidative system[4]), with the aim of identifying and synthesizing a small library of compounds with potential improved antiparasitic activity. We have reasoned that the development of an alternative procedure to functionalise the pentadecyl alkyl side chain of CNSL derivatives using enzymes, might be particularly promising in terms of sustainability and in order to avoid the use of hazardous reagents and solvents.

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# Comparative study on a multi-parametric serological approach to feline *Leishmania* infection
## Subject area: Parasitology

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*Leishmania infantum* is a sand fly-transmitted protozoan, responsible for human and canine leishmaniasis (CanL) in the Mediterranean basin. A growing number of evidence of *L. infantum* infection among cats has been reported in this region, though feline leishmaniasis (FeL) is less prevalent than CanL. In the absence of clinical signs, FeL poses diagnostic challenges. The indirect immunofluorescence antibody test (IFAT) has been validated for its diagnosis, but not other serologic methods, such as the direct agglutination test (DAT) and enzyme-linked immunosorbent assays (ELISA), which are daily used in CanL diagnosis.

Herein, we compare serological results obtained by IFAT (cut-off 1:80), DAT (cut-off 1:100) and ELISA in detecting anti-*L. infantum* antibodies in 224 domestic cats from Portugal. Seroreactivity was evaluated by ELISA set up with six different *Leishmania*-specific antigens, including: *Leishmania* promastigote soluble antigens (SPLA); recombinant proteins K39 (rK39) and KDDR (rKDDR), *L. infantum* cytosolic peroxiredoxins (CPX); total *Leishmania* excreted proteins (EXO), and *Leishmania* secreted extra-cellular vesicles (EVS). Seropositivity against each ELISA antigen was considered for optical densities (OD) above the cut-off established by the average OD+3*Stdev of OD values obtained in 40 cats from Azores and Madeira. The study was complemented with q-PCR in 119 blood samples. Agreement between the different results was calculated by Cohen's *k* index.

Preliminary results indicate *Leishmania*-specific antigens as promising biomarkers for FeL serodiagnosis. A substantial agreement ($k$ = 0.61− 0.80) was found for SPLA/rKDDR and SPLA/CPX, and moderate agreement ($k$ = 0.61−0.60) for SPLA/rK39 and SPLA/rK28. Reactivities to *Leishmania* excreted proteins and SPLA and rKDDR agreed substantially. For DAT, PCR, and IFAT the results agreed fairly ($k$ = 0.01−0.20) but DAT presented moderate agreement with SPLA, rK39, rKDDR and CPX. Longitudinal studies are required to address feline humoral response dynamics to *Leishmania* infection, besides the impact of FIV-associated immunodeficiency in compromising anti-*Leishmania* antibodies production and the role of cross-reactivity against other parasites in impairing anti- *Leishmania* antibodies detection.

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# Novel antitrypanosomal diaminoquinazoline analogues from repurposing the Medicines for Malaria Venture Open Access Pathogen Box library (MMVPBox)

Subject area: Pharmacology

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African trypanosomiasis is a neglected tropical disease known to exert negative socio-economic impacts on human and animal populations in affected developing countries [1,2]. The drawbacks associated with the current therapies represent the main bottleneck of this disease control, highlighting the need to develop alternative treatments [3,4]. The present work was designed to identify promising starting points for trypanosomiasis drug development from repurposing the MMVPBox library. Compounds from the MMVPBox library were screened for their antitrypanosomal activity against *Trypanosoma brucei brucei* and cytotoxicity on Vero cells using the resazurin-based cell viability assay. Furthermore, a small library of analogues of one (MMV675968) of the identified hits was screened for antitrypanosomal, cytotoxicity and further studies including DMPK prediction and mode of action studies (*in vitro* and *in silico* enzymatic studies, time-kill kinetic, DNA fragmentation) were performed. The Rudimentary Structure Activity Relationship (SAR) study perform on the parent hit MMV675968 led to the identification of two diaminoquinazoline analogues which displayed approximately 40-fold and 60-fold more potency and selectivity (MMV1578445: $IC_{50}$ = 0.045 µM, SI = 1737; MMV1578467: $IC_{50}$ = 0.06 µM; SI = 412). Overall, the two analogues were strong binders of the DHFR enzyme *in silico*, in all their accessible protonation states, and interacted with key DHFR ligand recognition residues Val32, Asp54, and Ile160. They also exhibited significant activity against trypanosome protein isolate. Moreover, MMV1578445 portrayed fast and irreversible trypanosome growth arrest between 4–72 h at $IC_{99}$. Analogs MMV1578467 and MMV1578445 induced *in vitro* ferric iron reduction and DNA fragmentation or apoptosis induction, respectively. The two potent analogues endowed with predicted suitable physico-chemical and predicted ADMET properties are good candidates for further deciphering their potential as starting points for new drug development for African Trypanosomiasis [5].

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# A perspective on human leishmaniasis and novel therapeutic methods for diagnosis, prevention, and treatment

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Introduction: Human leishmaniasis as a complex skin infectious disease is a severe burden in several subtropical and tropical developing countries and is caused by over 20 species of Leishmania parasite and they are transmitted by several sandfly species.
Methods: By searching related keywords in databases, papers with English abstracts and titles were reviewed and chosen to create a review article. For this aim, the search was conducted in electronic PubMed, Scopus, web of sciences, and Google Scholar databases. The specific search medical subject headings (MeSH) terms include "Leishmaniasis," "Leishmaniasis Prevention," "Cutaneous leishmaniasis," "Leishmaniasis Diagnosis", "Leishmaniasis Treatment", "Leishmaniasis Drug", "Leishmaniasis Parasite", "Leishmaniasis Cutaneous", "Rural leishmaniasis", "Urban leishmaniasis", "Dry Leishmaniasis", "Wet Leishmaniasis", "Human Leishmaniasis", "*Leishmania donovani*", "Leishmaniasis trials", "leishmaniasis Vectors", "Leishmaniasis Reservoirs" were conducted and articles with English titles and abstracts were screened and selected, and finally related full-text articles were studied and used in writing a review article.
Results: Differential responses to available Leishmania treatments is the main obstacle to control the disease. No information about critical assessments of the findings of these studies is obstacle challenge to general understanding. Considering available data on treatment outcomes, drug sensitivities/resistances, the most effective doses, and treatment failures were discussed in Leishmania for combination therapy and monotherapy. Leishmaniasis is linked to disfiguring scars and serious social stigma. Its severity is associated with factors, such as host, parasite species, socioeconomic level, endemic area, and access to healthcare facilities. In spite of numerous studies of current and emerging treatments, treatment outcomes for leishmaniasis remain controversial.
Discussion: We focused on treatments for leishmaniasis caused by various Leishmania species, considering their species-specific effectiveness informing drug selection to control and treat leishmaniasis.

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# Design, Synthesis and Biological Evaluation of Antileishmanial Azaheterocyclic Compounds as Inhibitors of the Parasitic Exokinase CK1 (casein kinase 1)
## Subject area: Medicinal Chemistry

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Leishmaniasis, a neglected tropical disease, is caused by a protozoa parasite from *Leishmania* species and it is transmitted to humans by the bite of the infected female phlebotomine sandflies. There are three main forms of the disease: fatal visceral leishmaniasis (Kala-azar), cutaneous leishmaniasis and mucocutaneous leishmaniasis. The drugs used for the treatment of Leishmaniasis are limited. The first-line drugs used against leishmaniasis are pentavalent antimony (SbV) compounds despite all their side effects. Also, liposomal amphotericin B (L-AmB), miltefosine, paromomycin and pentamidine are other medications used in the treatment.
In this context, the research groups need to speed up the development of a new generation of more effective and safer antileishmanials with a new mechanism of action to limit the devastating impact of parasite resistance.
To this end, Marchand *et al.* selected and validated *Leishmania* Casein Kinase I paralog 2 (*L*-CK1.2) as a drug target. *L*-CK1.2 is essential for intracellular parasite survival and released in macrophages *via* extracellular vesicles [1,2]. Consequently, CK1 enzyme emerges as an important molecular target to tackle the disease.
Imidazo[1,2-*a*]pyrazine derivatives were reported with the structural requirements to target L-CK1.2 [1,2]. On the other hand, thiazolo[5,4-*d*]pyrimidine scaffold with promising *in vivo* antileishmanial effect was reported previously [3,4]. Based on these, thiazolopyrimidines endowed with the suitable pharmacophoric part responsible of *Leishmania* CK1 inhibition were designed and synthesized in this study. Bioactivity studies will be carried out and further research is ongoing.

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# Evaluation of antiparasitic and (eco)toxicological characteristics of advanced nucleoside-based leads against animal trypanosomiasis

## Subject area: parasitology & ecotox

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Animal trypanosomiasis (AT) is a widespread disease caused by *Trypanosoma spp.* and has a devastating effect on animal husbandry all over the world due to the scarcity of efficient drugs and development of drug resistance, hence emphasizing the need for novel treatment options. Following previous identification of 3'-deoxytubercidin as a highly potent trypanocide with curative activity in mouse models of both stage-1 and stage-2 Human African Trypanosomiasis (HAT), we now present a comprehensive preclinical evaluation of new 6-amino substituted tubercidin analogues with promising activity against a broad range of AT species. Potent hits were identified *in vitro* across all important AT species, *i.e. Trypanosoma brucei brucei*, isometamidium (ISM)-resistant and -susceptible *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma evansi* (type A and B) and *Trypanosoma equiperdum*. Selected 'hits' were further tested for *in vitro* metabolic stability (using bovine, horse and piglet liver microsomes), *in vivo* mouse models for each AT species, genotoxicity assays and mode-of-action studies (*i.e.* genome-wide RNA interference library screening, metabolomics). Analogue **3** was highly active in *T. vivax*, *T. congolense*, *T. equiperdum*, *T. evansi* and *T. brucei* curative mouse models. Furthermore, there was no indication of *in vivo* toxicity or *in vitro* genotoxicity in Vitotox®, micronucleus and comet assays. Mode-of-action studies for **3** revealed that the P1 nucleoside transporter and adenosine kinase are involved in drug uptake and activation, respectively. Ecotoxicological assessments on *Daphnia* and green alga *Desmodesmus* revealed that the compound has an acceptable ecotoxicological footprint. Given the preferred target product profile for a broad-spectrum drug against AT, analogue **3** represents an advanced lead candidate for treatment of animal trypanosomiasis, regardless of the causative species.

Animal trypanosomiasis | drug discovery | nucleoside analogues | RNAi

# Insights into host-target interaction from the proteome modulation analysis through untargeted LC-MS/MS Proteomics of drug resistant L infantum-THP1 infected cells

## Subject area: Omics

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Vector borne diseases (VDBs) are the cause of more than 75% of the emerging human infections worldwide originated from animals. One of the most diffused VDB is represented by Leishmaniasis, represented by over 12M of new clinical cases every year. Depending on the endemic region of diffusion, current therapeutic options include Miltefosine, Antimonials and Paromomycin. However, the unsupervised of these few drugs in the livestock and humans has selected specific hyper-resistant strains, determining the rapid onset of concerning resistance phenomena. This led to a decrease of drugs efficacy and increase of interspecies diffusion [1,2].
With the aim to characterize drug resistance phenomena at a cellular level, we have treated THP-1 cells with clinical isolates of drug resistant *L. infantum* strain and processed the cells for a MS based proteomics analysis. The quali-quantitative differential analysis of the samples performed with Proteome Discoverer tool vs controls (non-resistant lines) revealed the presence of 15 Differentially Expressed Proteins (DEP's), 6 of which in miltefosine sample, 8 in paromomycin and 6 in Sb(V) resistant strain. Some DEPs are mutual to more than one lines, and peroxidoxin - whose role in parasitic oxidative stress neutralization is well established - resulted up-regulated (FC >2) in all the three resistant lines. We have combined these results with the outcome of the analysis of the human proteome modulated by the guest [3], which was previously investigated with STRING, Reactome, and other bioinformatic tools to define the most involved GO's.
From a guest-host cross talking analysis, we have identified the parasitic DEPs which do not share any GOs (functional and bioprocess) with the ones from THP-1. They represent a starting point of a Medicinal Chemistry programme, whose aim is to identify a druggable guest target with a low level of homology for both human and other species. This will allow the specific inhibition of the protein to obtain an antiparasitic effect, avoiding inter species off target activity. The ecotoxicological analysis, including specie-similarity analysis, will drive the whole workflow through target validation and exploitation.

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Acknowledgment

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# Strategic Evolution of SiDOCK@HOME: From COVID-19 Drug Design to a Broad-Spectrum Drug Search

## Subject area: Drug design & structural biology

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The SiDOCK@HOME team has made an important contribution to the global fight against COVID-19 with its distributed computing project over the past three years. We are no longer focusing solely on the pandemic response, but are now addressing a broader range of health issues that align with the goals of the One Drug Health COST action. Our project, based on BOINC platform, harnesses the collective power of nearly 5,000 computers operated by some 2,100 dedicated volunteers. This massive resource provides over 58,000 gigaflops of computing power, which has been instrumental in the successful completion of 21 projects analyzing one billion candidates for COVID-19 therapeutics.

In light of the dwindling threat of COVID-19, SiDOCK@HOME is shifting its focus to a broader drug development paradigm. An important focus of our expanded area of activity is the development of drugs against parasites. Parasitic diseases, which are often overlooked in drug research, represent a significant health burden, particularly in underserved regions. SiDOCK@HOME is well positioned to offer its services in this area and to contribute to the joint efforts of the COST One Drug Health Action. This change reflects not only our adaptive strategy, but also our commitment to global health issues.

We are also in the process of improving the interactivity of our platform. The upcoming redesign of our website will introduce features such as dynamic display of targets and docking results. A groundbreaking development is our plan to allow users to design and interactively dock molecules to encourage greater community involvement in our drug discovery process.

Our transition to an overall focus on drug development, particularly antiparasitic drug discovery, underscores our commitment to addressing a broad range of health challenges and thus makes an important contribution to the goals of the One Drug Health COST Action. We invite your collaboration and support as we embark on this new phase of our journey.

Acknowledgment
This work by the Slovenian Research and Innovation Agency (ARIS) programme and project grants P1-0201, P1-0403, J1-4398, J1-4414.

# Liposomes containing amphotericin B: innovative formulation for cutaneous leishmaniasis treatment
## Subject area: Parasitology

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Leishmaniasis is a group of neglected tropical diseases, as they affect socially vulnerable populations, with difficult access to the health system and often immunocompromised. This disease is caused by different species of *Leishmania*, leading to two main clinical forms: cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). In 2022, there were 205,986 and 12,842 new cases of CL and VL, respectively (1). Among the few drugs available, amphotericin B (AmB) is considered the most potent antileishmanial. However, its low solubility, high molecular weight, and tendency to self-aggregate result in low oral and topical bioavailability, in addition to high toxicity (2). Liposomal amphotericin B or AmBisome® is considered the most effective drug, but its efficacy is limited in complicated cases of CL and in HIV/VL coinfected patients. In this context, new therapeutic strategies are of great interest. This study aims to develop and characterize innovative AmB-PEGylated liposomes (LAmB) for CL therapy. The LAmB developed had hydrodynamic diameter of 128.4 ± 4.8 nm, polydispersity index of 0.10 ± 0.02 and surface charge slightly negative, -3.6 ± 0.6 mV, desirable for *in vivo* administration. A good encapsulation rate was also obtained (94.8 ± 5.2%). The physicochemical characteristics of LAmB remained stable over 30 days in the refrigerator. Additionally, the aggregation state of AmB in the formulation was stable over time. Furthermore, it presented lower hemolytic activity compared to the commercial formulation Anforicin B® in hemolysis study. Evaluation of lesion size in BALB/c mice infected with *L. (L.) amazonensis* and treated with LAmB showed marked reduction (8.77 ± 1.60 mm) in comparison to the untreated control (11.32 ± 1.70 mm) at the end of treatment. Notably, the LAmB group had smaller lesion size than AmBisome® (9.87 ± 1.85 mm). This formulation is therefore promising for the treatment of CL.

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Acknowledgment

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# Guanidino-Containing Derivatives as Promising Agents for Targeting the Folate Enzyme Pathways in Human African Trypanosomiasis
## Subject area: Medicinal Chemistry including (Natural products)

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Human African trypanosomiasis (HAT, also known as sleeping sickness) is a vector-borne parasitic infection caused by the *Trypanosoma brucei* (*T.b.*). Over the past decade, the World Health Organization (WHO) has made significant strides in reducing the spread and contagion of the disease. However, to fully achieve the goal of eradicating the disease by 2030, there remains a critical requirement for enhanced disease tracking, intensified control efforts, and the development of innovative, safer, and more effective drugs.

One promising approach in the treatment of HAT involves targeting enzymes involved in folate metabolism: Trypanosomatids, such as *T.b.*, are unable to synthesize essential folates and pterins, which are crucial cofactors for nucleic acid and protein biosynthesis. Inhibiting key enzymes of the folate salvage pathways, such as dihydrofolate reductase (DHFR) and pteridine reductase-1 (PTR1), holds promise for an effective HAT treatment [1].

The antimalarial drug cycloguanil, a known DHFR inhibitor, has showed to inhibit *Tb*PTR1 as well [2,3]. In the present study, we synthesized two series of compounds: (2-aminotriazino)benzimidazoles, where the amino triazino motif of CYC is fused with the benzimidazole ring, and 2-guanidino benzimidazoles, as their open-ring analogues.

The dual-targeting inhibitory activity of these compounds against *Tb*PTR1 and *Tb*DHFR, as well as against human DHFR, has been extensively investigated to assess their selectivity for the protozoan enzymes. Furthermore, their cytotoxicity and antiparasitic effects were evaluated in cell-based assays. Additionally, crystal structures of both enzymes in complex with selected compounds have been successfully obtained. These findings will help elucidating the structural-activity relationship (SAR) of these compounds series and guide the design and synthesis of innovative folate inhibitors as antiprotozoal agents.

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# Activity-based Protein Profiling to investigate the interactome of the antimalarial early lead Plasmodione

## Subject area: Omics technologies

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In 2021, malaria is estimated to cause 247 million clinical episodes and 619 000 deaths, it is caused by parasites of the genus *Plasmodium*. Several antimalarial drugs have been developed over the years, but the parasite quickly develops resistance to all of them. Plasmodione (PD) is a novel early lead antimalarial drug that is highly effective in limiting the proliferation of malaria parasites in vitro at the nM level with very moderate toxicity to host cells. Literature data suggest that this early lead drug may have multiple modes of action *via* its main metabolite, by interacting with distinct protein targets expressed in the various parasite stages. The aim of the STSM project is to identify putative plasmodione targets in the P. falciparum proteome through the affinity-based protein profiling (ABPP) strategy. The approach aims to photolabel Plasmodium protein targets by using photoreactive and 'clickable' PD-derived probes and identify them by mass spectrometry methods. The general ABPP strategy is based on several steps: 1) UV irradiation of the ABPP probe with the cell lysate, 2) conjugation of biotin by a click chemistry-based reaction (CuAAC), 3) enrichment of the biotinylated proteins by pull-down from streptavidin beads, 4) digestion of the enriched proteins and analysis by liquid chromatography-mass spectrometry (LC-MS/MS). Activity-based probes were first tested on a model protein, recombinant glutathione reductase.[1] The optimization involved different screening conditions: from UV-irradiation under oxygen-free conditions to the click reaction under oxygen-free conditions with a biotin azide and pull-down of the adducts through biotin-streptavidin beads. The whole procedure was applied to *S. cerevisiae* cell systems to validate the experimental workflow. After validation, the method was applied to *P. falciparum* cell extracts. [1]

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# Preclinical investigation of H80 action vs miltefosine through imaging and mass spectrometry proteomics for a sustainable lead development and clinical translation

## Subject area: Drug design & structural biology

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The primary objective of therapeutic research for Leishmaniasis is to replace the currently few existing drugs, that are fairly toxic and require prolonged parenteral administration, with an orally-available option that is less toxic and involves short treatment cycles [1]. In this context, an important advance has been the recent demonstration that an oral treatment with miltefosine (MIL, figure 1a) can bring to complete recovery in over 90% of patients with anthroponotic Leishmania visceralis (LV), but with severe side effects [2].

Thus, the aim of our drug discovery study is to identify new leads, which are more active than MIL, and allow an oral administration. During our studies on trypanosomatidic diseases, we have identified a new lead, named H80 (figure 1b), showing low micromolar EC50 in macrophages infected with Leishmania infantum, L. donovani and L. major. It also showed low propensity to develop drug resistance when compared to MIL [3]. Therefore, H80 is considered an interesting antileishmanial agent active against both visceral and cutaneous Leishmaniasis in vitro and displays an activity like that of MIL (Figure 1c) [3]. The objective of the present work is to study the mechanism of action and the target of H80, that are so far unknown, making it difficult to improve the compound activity by rational design. To achieve this goal, we decided to combine a fluorescence analysis with a mass spectrometry proteomic approach. We decided to replicate a previous proteomic experiment where L. Donovani strain was treated with different concentration of H80 and miltefosine. [Leda Severi, PhD thesis, 2018]. Samples were fractionised to underlined different protein's expression in different subcellular localization (cytoplasmatic, intermediate and mitochondrial fraction). [4] We replicated the experiment with L. infantum strain treated with concentration of H80 and miltefosine corresponding to EC10, EC50 and EC90. The comparison with the same proteomic experiments on L. Donovani revealed over 50% mutual DEPs with the same direction of expression, in particular proteins involved in the membrane transports fatty acid biosynthesis and ribosomal assembling resulted significantly upregulated in the parasites treated with miltefosine and H80. We also have developed imaging studies based on the intrinsic fluorescence of H80, to follow its internalization behaviour inside promastigote cells. Fluorescence studies with two different sources of lights (DAPI, and CFP as control) revealed that H80 is up-taken through vesicles with an endocytosis mechanism. The distribution of the compound in the cells is predominantly cytoplasmic, lateral to the nucleus, which remains vesicle-free. These results were confirmed by a second fluorescence-based immunoassays with Rabit-α-luciferase and A21244 Alexa647 probe (Figure 1d). In conclusion, by exploiting different fluorescent imaging studies we have demonstrated that our compound H80 is internalized by the amastigotes through vesicles, via endocytosis, and localizes in the cytoplasm.

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# Single cell transcriptomics reveals altered myeloid cell profile despite the administration of an early miltefosine cure in *Leishmania infantum*-infected rhesus macaques.

## Subject area: Parasitology and Omics

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Visceral leishmaniasis (VL) is a chronic and frequently lethal disease. VL occurs in 88 countries around the world. In the context of One Health VL is a typical disease in which environmental changes, vectors, and domestic dogs are critical for humans. The human disease is lethal if not treated. Current drug regimens to treat visceral leishmaniasis are associated with a significant frequency of infection relapses, particularly in immunosuppressed patients. Detailed knowledge of the cells and tissues that harbor parasites after completion of a drug treatment regimen is required. Here, using a rhesus macaque (RM) model of visceral leishmaniasis (1, 2), a miltefosine (HePC) cure of 21 days at the dose of 5 mg/kg was administrated. We show that *Leishmania infantum* can still be detected after HePC therapy in different tissues including, spleen, bone marrow, peripheral and mesenteric lymph nodes (LNs) despite this early therapy. A lower drug penetration was observed in LNs that may explain the persistence of parasites, culminating in MLN relapse 3 months after completion of treatment. Sorting of splenic neutrophils, monocytes/macrophages and DCs demonstrate the presence of parasites after HePC treatment. Finally, using single-cell transcriptomic analysis (scRNA) that may provide insights about myeloid cell heterogeneity based on gene expression (cellular atlas), we show that the absence of parasite eradication in the spleen is associated with the absence of full immune cell restoration. The identification of parasite reservoirs post-HePC treatment, associated with immune cell profile alteration, is of crucial importance for opening new horizons for a cure for visceral leishmaniasis.

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# Synthesis of chemical probes based on the early lead redox-active antiplasmodialagent, plasmodione, for metabolic and imaging studies

## Subject area: Medicinal Chemistry

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One of the main research topic of the CBM team is focused on the development of redox-active antiparasitic drug-candidates based on the 3-benzylmenadione core, in particular the antimalarial plasmodione (PD) [1]. Experimental data indicate that the early lead drug PD could exhibit several modes of action (MoA) depending on parasitic stages, and protein targets. PD is proposed to be activated in the parasite through a cascade of redox reactions generating several metabolites, in particular its key metabolite (PDO). However, metabolites generated from redox-cyclers are produced in trace amounts requiring effective analytic methods to characterize their locus of action. To identify the generated PD metabolites and fully characterize PD MoA we designed and synthetized various chemical probes for metabolic and imaging studies. An activity-based protein profiling (ABPP) strategy in *Plasmodium* parasites was investigated to fish PD protein targets by crosslinking upon photoirradiation [2] (Vittoria Monaco's STSM project). Besides, we synthesized the heavy enriched $^{13}C$*18*-PD to track the metabolites from *P. falciparum* protein reaction mixtures, e.g. with the *P. falciparum* $NADP^+$ ferredoxin reductase [3], and 'clickable' PD analogues to perform localisationexperiments by fluorescence microscopy. Some data will be presented.

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# Fish-borne zoonotic diseases with special emphasis on parasites and risks for public health
## Subject area: parasitology

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Aquaculture as the controlled process of cultivating aquatic organisms, especially fish for human consumption, is one of the fastest growing food production sectors in the world and is an important contributor to global food supply and economic growth. Fish is a good source of proteins, fats (poly-unsaturated fatty acids), vitamins (including, vitamin A, vitamin B2, vitamin B6, and others), and minerals (iron, calcium, iodine, potassium, and other minerals) and many parts of the world are used as the main component in the human diet (Longwe & Fannuel, 2016). But, despite that, fish-derived zoonotic diseases have caused considerable problems in the aquaculture industry and fishery worldwide. With the world's growing population and potential global trade of aquaculture and fish, the risk of aquatic-derived zoonoses in humans is increasing (Ziarati et al. 2022).

Zoonoses are infectious diseases that are transmitted between animal species to humans (Han et al. 2016). Several causative agents of infectious diseases, including bacteria, viruses, parasites, and fungi, can be transmitted from animals to people through different routes (Rahman et al. 2020), but Shamsi (2019) considered that there are few important zoonotic diseases within aquatic organisms, from a public health point of view. Certain parasite species from fish have been reported in humans, but only a few cause serious diseases. The fish-derived parasitic cestodes (e.g. *Diphyllobothrium* spp.), nematodes (e.g. *Anisakis* spp.), and trematodes (e.g. *Opisthorchis* spp.) are mainly transmitted to humans through the consumption of improperly cooked or raw fish.

Therefore, Ziarati et al. (2022) concluded that the incidence of zoonotic diseases can be reduced by proper fish processing, mainly by thermal treatment. The prevalence of zoonotic agents in fishes should be regularly monitored to evaluate the prevalence of pathogens in both wild and cultured fish populations.

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# Changing paradigm to confront zoonotic Leishmaniasis: One Health perspective from Portugal
## Subject area: Parasitology & One Health

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Then genus *Leishmania* comprises protozoan parasites with potential to infect both animals and humans. Infected individuals may develop disease, largely described as leishmaniasis (or as the leishmaniases). This is a neglected tropical disease for which natural transmission is mostly dependent on phlebotomine sand fly insects. Depending on whether a mammalian host, other than man, is necessary for the parasite to complete its life cycle, zoonotic and anthroponotic leishmaniasis may occur. The most severe infection phenotype is visceral leishmaniasis (VL), fatal if left untreated and associated to infection by species of the *Leishmania donovani* complex. *Leishmania infantum* is one of these species, being responsible for human zoonotic VL (ZVL) and canine leishmaniasis (CanL) in different regions and countries of both the New and Old Worlds, including Europe. Southern European countries are highly endemic for CanL, but human ZVL has largely been under reported. Due to climate change, globalization, migration, human and animal movements, the risk of establishment and transmission of ZVL is increasing as permissive phlebotomine sand flies spread and occupy of new ecosystems in northern European regions. Portugal harbors competent vectors for the successful zoonotic transmission of *L. infantum* and has a high prevalence of its infection among canine reservoirs. Notwithstanding, the occurrence of human and feline leishmaniasis in Portugal has not been accurately estimated, nor has the risk of introduction of different Old World or naive New World *Leishmania* spp. within our ecosystem. This project aims to update the current epidemiology of canine and feline leishmaniasis in mainland Portugal, identify potential sylvatic reservoirs, describe phylogeny of the circulating *Leishmania* strains and their drug sensitivity profiles against to the available and licensed leishmanicidal therapies.

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