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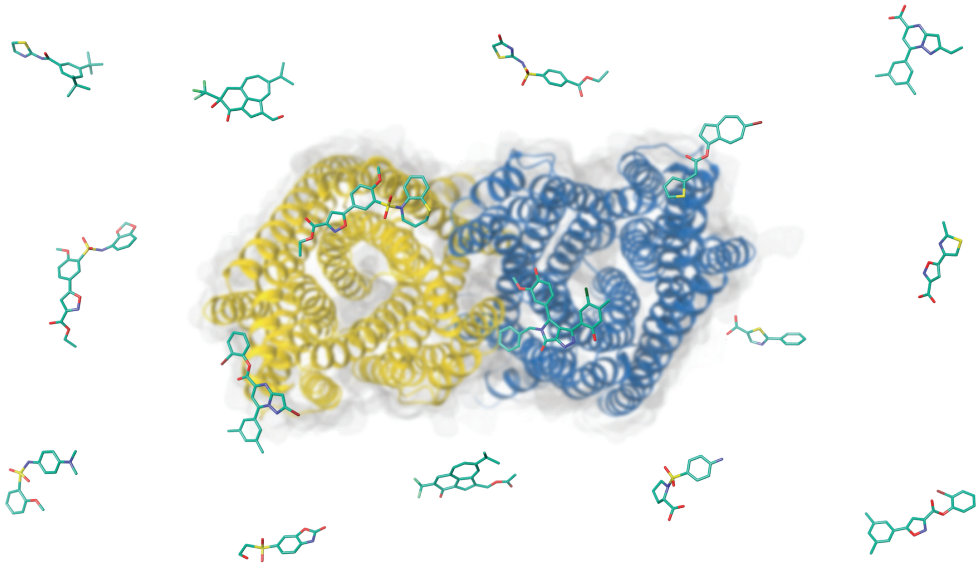
NIKLAS G. JOHANSSON

# DESIGN AND SYNTHESIS OF MEMBRANE-BOUND PYROPHOSPHATASE INHIBITORS AGAINST PATHOGENIC PROTOZOAN PARASITES



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Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

# Design and synthesis of membrane-bound pyrophosphatase inhibitors against pathogenic protozoan parasites

Niklas G. Johansson

ACADEMIC DISSERTATION

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**Supervised by** Gustav Boije af Gennäs, PhD
Drug Research Program
Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

Professor Jari Yli-Kauhaluoma, PhD
Drug Research Program
Division of Pharmaceutical Chemistry and Technology
Faculty of Pharmacy
University of Helsinki
Finland

**Reviewed by** Professor Philippe M. Loiseau, PhD
Antiparasite Chemotherapy
Faculty of Pharmacy
University of Paris-Saclay
France

Professor Marco Mor, PhD
Department of Food and Drug
Faculty of Pharmacy
University of Parma
Italy

**Opponent** R&D Director Félix Calderón, PhD
Tres Cantos Open Lab
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*To all those who inspired it,*
*yet will not read it.*

*”Egentligen gäller det endast detta; att inte tröttna, aldrig bli ointresserad, likgiltig, tappa sin dyrbara nyfikenhet – då tillåter man sig att dö. Så enkelt är det, är det inte?"*

*“It is simply this: Do not tire, never lose interest, never grow indifferent – lose you invaluable curiosity and you let yourself die. It’s as simple as that.”*

*Tove Jansson*

# Abstract

Pathogenic protozoan parasites cause devastating diseases, such as malaria (*Plasmodium* spp.), toxoplasmosis (*Toxoplasma* spp.), leishmaniasis (*Leishmania* spp.) and trypanosomiasis (*Trypanosoma* spp.), which have an enormous health, social and economic impact, particularly in tropical regions of the world. For example, nearly half of the population of the world is at risk of malaria that is responsible for more than 600,000 deaths annually. Interestingly, all of these parasites have in common a homodimeric integral protein, membrane-bound pyrophosphatase (mPPase), consisting of 15 to 17 helices with a molecular weight of 70 to 81 kDa. These mPPase enzymes hydrolyze pyrophosphate, a by-product of many biological processes. Besides taking care of excess pyrophosphate, the energy released through hydrolysis of phosphoanhydride bonds is coupled with the pumping of protons or sodium ions thus creating an ion gradient across the membrane. Consequently, mPPases play an important role in the survival of many organisms under diverse stress situations due to osmotic stress or other energy limitations. Inhibiting the function of mPPases bears promise from a drug discovery perspective, as remarkably these enzymes do not exist in animals or humans. Hitherto, only crystal structures of the hyperthermophilic bacterium *Thermotoga maritima* mPPase (TmPPase) and mung bean *Vigna radiata* (L.) R. Wilczek mPPase have been solved and are amenable for structure-based drug design. Still, most known inhibitors, such as pyrophosphate and bisphosphonate derivatives, are not optimal as drugs due to features including poor stability and extensive hydrophilicity.

The focus of this study was to explore membrane-bound pyrophosphatase as a potential drug target for pathogenic protozoan parasites, mainly *Plasmodium falciparum*. Since most of the currently known inhibitors are phosphorus-based, thus limiting their therapeutic usage, the aim of this thesis was to develop new organic mPPase inhibitors with potential as an alternative approach in the treatment of malaria and other related parasitic diseases. By developing nonphosphorus inhibitors targeting mPPase, the intention was to block the essential ion pump of these parasites. The compounds obtained were first tested in a 96-well plate *in vitro* screening assay using thermostable TmPPase as a model enzyme, resulting in the construction of a small library of drug-like compounds (<500 Da) containing different scaffolds with low micromolar activities. Selected top hits were studied further and evaluated against purified mPPase from *P. falciparum* (PfPPase-VP1) expressed in baculovirus-infected insect cells. In addition, their ability to inhibit the growth of *P. falciparum* in a survival assay in erythrocytes was studied.

In publication I, the discovery and synthesis of the first nonphosphorus, non-substrate analog inhibitor of *Thermotoga maritima* mPPase (TmPPase), with a 6-(aminomethyl)benzo[*d*]thiazol-2-amine core, as well as preliminary structure-activity relationships (SARs) and a X-ray crystal structure of this allosteric inhibitor bound to TmPPase are reported. Unfortunately, the allosteric binding region was not preserved in parasitic mPPase thus indicating that there are alternative ways to inhibit mPPase enzymes. In the subsequent study, publication II, the chemical exploration of phosphate isosteres, which finally led us to 2-bromophenyl 5-arylisoxazole-3-carboxylates, is presented. As some antiparasitic activity was maintained, it is assumed that these inhibitors bind orthosterically in the preserved region of the enzyme even though it has not yet been confirmed by sufficient crystallographic data. Publication III further explores structural analogs of the best isoxazole inhibitor (publication II), which resulted in discovering a new bicyclic scaffold, with a 4,5-dihydropyrrolo[3,4-*c*]pyrazol-6(1*H*)-one core, and its related pyrazolo[1,5-*a*]pyrimidine analogs. These compounds seem promising also against PfPPase and, for that reason further studies thereof are ongoing. Additionally, publication IV describes a cross-project *in vitro* screening that got us to

study azulenes and benzazulenes as potential TmPPase inhibitors. These low micromolar TmPPase inhibitors retained similar activity also in a *P. falciparum* survival assay in erythrocytes. Altogether, these studies offer a different approach for further development of novel drugs against malaria and other diseases caused by pathogenic protozoan parasites.

# Sammandrag

Sjukdomsframkallande urdjursparasiter orsakar förödande sjukdomar, såsom malaria (*Plasmodium* spp.), toxoplasmos (*Toxoplasma* spp.), leishmaniasis (*Leishmania* spp.) och trypanosomiasis (*Trypanosoma* spp.). Dessa sjukdomar leder till enorma hälsomässiga, sociala och ekonomiska konsekvenser, särskilt i tropiska områden i världen. Till exempel malaria leder till mer än 600 000 dödsfall årligen och nästan hälften av världens befolkning befinner sig i riskzonen för att insjukna. Intressant nog har alla dessa parasiter gemensamt ett homodimert integralt protein, membranbundet pyrofosfatas (eng. membrane-bound pyrophosphatase, mPPase), bestående av 15 till 17 helixar med en molekylvikt på 70 till 81 kDa. Dessa mPPase-enzymer hydrolyserar pyrofosfat, en biprodukt i många biologiska processer. Förutom att ta hand om överskottet av pyrofosfat, kopplas energin som frigörs vid hydrolys av fosfoanhydridbindningar till pumpning av protoner eller natriumjoner, vilket skapar en jongradient över membranet. mPPaser har således en viktig roll för överlevnaden av många organismer under diverse stressituationer på grund av osmotisk stress eller andra energibegränsningar. Att hämma funktionen av mPPaser kunde vara ett lovande alternativt för attt upptäckta nya läkemedel, eftersom dessa enzymer inte finns i djur eller människor. Hittills har endast kristallstrukturer av den hypertermofila bakterien *Thermotoga maritima* mPPase (TmPPase) och mungböna *Vigna radiata* (L.) R. Wilczek mPPase lösts och är tillgängliga för strukturbaserad läkemedelsdesign. De flesta kända hämmare, såsom pyrofosfat- och bisfosfonatderivat, är inte optimala som läkemedel på grund av egenskaper inklusive dålig stabilitet och omfattande hydrofilicitet.

Fokus för denna studie var att utforska membranbundet pyrofosfatas som ett potentiellt läkemedelsmål för sjukdomsframkallande urdjursparasiter, främst *Plasmodium falciparum*. De flesta av de för närvarande kända hämmarna är fosforbaserade, vilket begränsar deras terapeutiska användning. Syftet med denna avhandling var således att utveckla nya organiska mPPase-hämmare med utvecklingsmöjligheter som ett alternativt tillvägagångssätt vid behandling av malaria och andra relaterade parasitsjukdomar. Avsikten var att blockera den essentiella jonpumpen hos dessa parasiter genom att utveckla fosforfria hämmare gentemot mPPase. De erhållna föreningarnas biologiska aktivitet utvärderades först *in vitro* med termostabilt TmPPase som modellenzym i 96-brunnsplattor, vilket resulterade i uppbyggnaden av ett litet bibliotek av läkemedelsliknande föreningar (<500 Da) bestående av olika grundstommar (eng. scaffolds) med låga mikromolära aktiviteter. De mest lovande föreningarna studerades vidare och testades mot renat mPPase från *P. falciparum* (PfPPase-VP1) uttryckt i baculovirusinfekterade insektsceller. Dessutom studerades deras förmåga att hämma tillväxten av *P. falciparum* i en överlevnadsanalys i erytrocyter.

I publikation I rapporteras upptäckten och syntesen av den första fosforfria TmPPase-hämmaren, som har en 6-(aminometyl)benso[*d*]tiazol-2-amin -stomme och således inte är en substratanalog, samt preliminära struktur-aktivitetsförhållanden (SAR) och en röntgenkristallstruktur för denna allosteriska hämmare bunden till TmPPase. Tyvärr bevarades inte den allosteriska bindningsregionen i parasitiskt mPPase vilket indikerar att det finns alternativa sätt att hämma mPPase-enzymer. I den efterföljande studien, publikation II, presenteras diverse fosfatisosterer, som slutligen ledde oss till 2-bromfenyl-5-arylisoxazol-3-karboxylater. Eftersom viss antiparasitisk aktivitet bibehölls, antas det att dessa hämmare binder ortosteriskt i den konserverade regionen av enzymet även om detta ännu inte har bekräftats av tillräcklig kristallografisk data. För publikation III studerades ytterligare strukturella analoger av den bästa isoxazolhämmaren (publikation II), vilket resulterade i upptäckten av en ny bicyklisk grundstomme, med en 4,5-dihydropyrrolo[3,4-*c*]pyrazol-6(1*H*)-on -stomme, och dess relaterade pyrazolo[1,5-*a*]pyrimidinanaloger. Dessa föreningar verkar lovande även mot

PfPPase och följaktligen pågår ytterligare studier av dessa. I publikation IV beskrivs en *in vitro* screening av föreningar från anknutna projekt som fick oss att studera azulener och bensazulener som potentiella TmPPase-hämmare. Dessa låga mikromolära TmPPase-hämmare bibehöll liknande aktivitet även i en *P. falciparum*-överlevnadsanalys i erytrocyter. Sammantaget erbjuder dessa studier ett annat tillvägagångssätt för vidareutveckling av nya läkemedel mot malaria och andra sjukdomar orsakade av patogena protozoparasiter.

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*Niklas G. Johansson*

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# List of original publications

This thesis is based on the following publications:

I Vidilaseris, K.; Kiriazis, A., Turku, A.; Khattab, A.; Johansson, N.G.; Leino, T.O.; Kiuru, P.S.; Boije af Gennäs, G.; Meri, S.; Yli-Kauhaluoma, J.; Xhaard, H.; Goldman, A. Asymmetry in Catalysis by *Thermotoga maritima* Membrane-Bound Pyrophosphatase Demonstrated by a Nonphosphorus Allosteric Inhibitor. *Sci. Adv.* **2019**, *5* (5), eaav7574. DOI: 10.1126/sciadv.aav7574

II Johansson, N.G.*; Turku, A.*; Vidilaseris, K.*; Dreano, L.; Khattab, A.; Ayuso Pérez, D.; Wilkinson, A.; Zhang, Y.; Tamminen, M.; Grazhdankin, E.; Kiriazis, A.; Fishwick, C.G.W.; Meri, S.; Yli-Kauhaluoma, J.; Goldman, A.; Boije af Gennäs, G.; Xhaard, H. Discovery of Membrane-Bound Pyrophosphatase Inhibitors Derived from an Isoxazole Fragment. *ACS Med. Chem. Lett.* **2020**, *11* (4), 605–610. DOI: 10.1021/acsmedchemlett.9b00537

III Johansson, N.G.; Dreano, L.; Vidilaseris, K.; Khattab, A.; Liu, J.; Lasbleiz, A.; Ribeiro, O.; Kiriazis, A.; Boije af Gennäs, G.; Meri, S.; Goldman, A.; Yli-Kauhaluoma, J.; Xhaard, H. Exploration of Pyrazolo[1,5-*a*]pyrimidines as Membrane-Bound Pyrophosphatase Inhibitors. *ChemMedChem.* **2021**, *16* (21), 3360–3367. DOI: 10.1002/cmdc.202100392

IV Kiriazis, A.*; Johansson, N.G.*; Vidilaseris, K.; Dreano, L.; Turku, A.; Khattab, A.; Leino, T.O.; Arnaudova, R.; Meri, S.; Boije af Gennäs, G.; Goldman, A.; Yli-Kauhaluoma, J.; Xhaard, H. Exploration of Azulene-Based Compounds as Membrane-Bound Pyrophosphatase Inhibitors. *Manuscript.*

The publications are referred to in the text by their Roman numerals.

Publications related to, but not discussed in this thesis:

Leino, T.O.; Johansson, N.G.; Devisscher, L.; Sipari, N.; Yli-Kauhaluoma, J.; Wallén, E.A.A. Synthesis of 1,3,6-Trisubstituted Azulenes Based on the 1-Acyloxyazulene Scaffold. *Eur. J. Org. Chem.* **2016**, *2016* (33), 5539–5544. DOI: 10.1002/ejoc.201600962

Vidilaseris, K.*; Johansson, N.G.*; Turku, A.; Kiriazis, A.; Boije af Gennäs, G.; Yli-Kauhaluoma, J.; Xhaard, H.; Goldman, A. Screening Protocol for Identification of *Thermotoga maritima* Membrane-Bound Pyrophosphatase Inhibitors. *J. Vis. Exp.* **2019**, (153), e60619. DOI: 10.3791/60619

* Equal contribution

## Author contributions

I NGJ contributed to planning of the study, synthesized and characterized part of the compounds together with AKi and TOL, as well as participated in preparation of the manuscript.

II NGJ contributed to planning of the study, synthesized and characterized part of the compounds together with DAP, who worked under supervision of NGJ. AT and KV carried out the computational and pharmacological parts of the study, respectively. NGJ, AT and KV prepared the manuscript with equal contribution.

III NGJ planned the study, synthesized and characterized part of the compounds together with AL, who worked under supervision of NGJ, as well as prepared the manuscript.

IV NGJ contributed to planning of the study, synthesized and characterized part of the compounds together with AKi, TOL and RA. AKi and NGJ prepared the manuscript with equal contribution.

Publication II is also included in the dissertation of Aaron Wilkinson (University of Leeds, UK).

# Abbreviations

| | |
|---|---|
| Ac | acetyl |
| ACT | artemisin-based combination therapies |
| AP | 3(5)-aminopyrazole derivative |
| ATP | adenosine triphosphate |
| ATPase | adenosine triphosphatase |
| aq | aqueous |
| BCE | before the common era |
| BINAP | 2,2'-bis(diphenylphosphino)-1,1'-binaphthalene |
| Bmim | 1-benzyl-3-methylimidazolium |
| Bn | benzyl |
| bpy | 2,2'-bipyridine |
| Bu | butyl |
| $CI_{95\%}$ | half maximal inhibitory concentration expressed as a 95% confidence interval |
| cod | 1,5-cyclooctadiene |
| DABCO | 1,4-diazabicyclo[2.2.2]octane |
| DCM | dichloromethane |
| DDQ | 2,3-dichloro-5,6-dicyanobenzoquinone |
| DIPEA | *N*,*N*-diisopropylethylamine |
| DMAP | 4-(dimethylamino)pyridine |
| DMF | *N*,*N*-dimethylformamide |
| DMF-DMA | *N*,*N*-dimethylformamide dimethyl acetal |
| dmphen | 4,7-dimethyl-1,10-phenanthroline |
| DNA | deoxyribonucleic acid |
| dppf | 1,1'-bis(diphenylphosphino)ferrocene |
| EDC | 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide |
| Et | ethyl |
| Fmoc | fluorenylmethyloxycarbonyl |
| Fmoc-OSu | *N*-(9-fluorenylmethoxycarbonyloxy)succinimide |
| HATU | 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate |
| HRMS | high-resolution mass spectrometry |
| $IC_{50}$ | half maximal inhibitory concentration |
| IDP | imidodiphosphate |
| $K_i$ | inhibition constant |
| LC-MS | liquid chromatography-mass spectrometry |
| LDA | lithium diisopropylamide |
| Me | methyl |
| mPPase | membrane-bound pyrophosphatase |
| MtPPase | *Mycobacterium tuberculosis* pyrophosphatase |
| mw | microwave |
| MW | molecular weight |
| NIS | *N*-iodosuccinimide |
| NMR | nuclear magnetic resonance |
| PET | positron emission tomography |

| | |
|---|---|
| PfPPase | *Plasmodium falciparum* membrane-bound pyrophosphatase |
| PfPPase-VP1 | *Plasmodium falciparum* vacuolar $H^+$-translocating pyrophosphatase VP1 |
| Ph | phenyl |
| $P_i$ | orthophosphate |
| $pin_2B_2$ | bis(pinacolato)diboron |
| Piv | pivaloyl |
| poly-P | polyphosphate |
| PPase | pyrophosphatase |
| $PP_i$ | pyrophosphate |
| Pr | propyl |
| RNA | ribonucleic acid |
| rt | room temperature |
| SAR | structure-activity relationship |
| sPPase | soluble pyrophosphatase |
| TMH | transmembrane helix |
| TmPPase | *Thermotoga maritima* membrane-bound pyrophosphatase |
| Tf | trifluoromethanesulfonyl |
| TFAA | trifluoroacetic anhydride |
| THF | tetrahydrofuran |
| TMS | trimethylsilyl |
| Ts | toluenesulfonyl |
| *p*-TSA | *para*-toluenesulfonic acid |
| V-$H^+$-ATPase | vacuolar-type adenosine triphosphatase |
| VrPPase | *Vigna radiata* membrane-bound pyrophosphatase |
| WHO | World Health Organization |
| Xantphos | (9,9-dimethyl-9*H*-xanthene-4,5-diyl)bis(diphenylphosphane) |

# 1 Introduction

If you would ask someone to name the most dangerous animal in the world – snakes, spiders or even humans are rather recurring replies. Of course, there are some variations depending on the definition of dangerous. For instance, if the definition were the number of people killed annually, mosquitoes would undoubtedly be at the top of the list, as various species cover most regions of the world. In fact, it is not the mosquito itself, but rather the result of it acting as a vector for devastating diseases such as malaria (*Anopheles* mosquitoes), as well as dengue fever, yellow fever, chikungunya, and Zika virus disease (*Aedes* mosquitoes) that is the real reason behind the resulting mortality and morbidity. Malaria is a vector- and blood-borne protozoan infection that has plagued humanity for countless generations and probably accounted for more deaths than any other disease.[1] The name of the disease originates from Italian for bad (*mal*) and air (*aria*). Malaria was formerly known as ague or marsh fever and was commonly associated with humid areas such as swamps. While malaria nowadays is associated with predominantly tropical areas and significantly affects young children in Africa, the disease has formerly existed in colder areas even in Finland.[2,3] Malaria parasites are transmitted to humans by mosquitoes having a blood meal. First the parasites migrate to the liver and change forms, and then they head for the red blood cells (erythrocytes) to reproduce. When another mosquito bites an infected human, the mosquito becomes a carrier of the disease and can pass it on. The typical symptoms range from fever, chills and anemia to even coma or death. Although malaria is easily transferable and potentially a deadly disease which is commonly linked to poverty and economic hindrance, it is both preventable and treatable.

Complex life cycles of many pathogenic protozoan parasites offer both challenges and opportunities. For example, when treating malaria there are many chemotherapeutic targets to choose from. However, the difficulty is to find a target that affects all necessary parasite forms. In general, the drug development process itself is rather complex, time-consuming and expensive, requiring experts from various fields and multiple challenges to overcome before a potent and safe drug is approved. Sometimes nature suggests which direction to go, once the active ingredients in medicinal plants used in the treatment of various diseases have been deciphered. In the case of malaria, bark from the cinchona tree (*Cinchona* L. spp.) was used in South America long before quinine, its active ingredient, was known.[1] Similarly, the currently well-known antimalarial drug artemisinin is originating from traditional Chinese medicine and extracts of sweet wormwood (*Artemisia annua* L.) have been used long before the reason for the disease was first decrypted in the late 19$^{th}$ century. In 1880, the French scientist Charles Laveran discovered *Plasmodium* parasites in malaria patients and the British military doctor Ronald Ross found them in mosquitoes seventeen years later. Figure 1 presents a short overview of central milestones in the research of malaria as well as how fast resistance has evolved against existing antimalarial drugs.

Even though the number of malaria cases has been decreasing during the last century, increased temperatures allow insect vectors to spread to new areas.[4,5] In addition, rising resistant strains against drugs and pesticides require continuous improvements even of the most potent drugs and pesticides in the market.[6] Synthesis of drugs from natural sources in laboratories, as well as optimization of their structures to improve their pharmacodynamics and pharmacokinetic properties, are a necessity to meet the need from the market. However, it is demanding to follow the biosynthesis routes of Mother Nature. Even the most skilled synthetic chemist with years of training frequently needs to consider alternative routes or simplify target structures whenever plausible. These approaches are still indispensable by offering different, complementary treatment options such as vaccines or new drug targets.

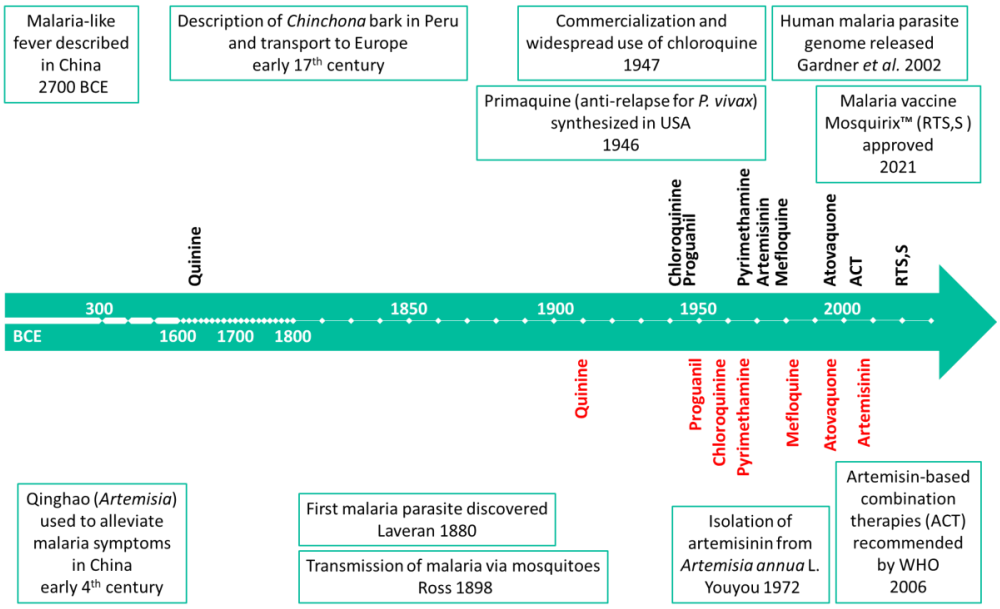



**Figure 1** *Timeline of important occurrences in malaria research including key antimalarial drugs (marked in black when they were introduced and in red when resistance was found).*

## 1.1 Protist diseases

Protozoa are eukaryotic organisms that pose a major health concern to this day. Protist diseases, including malaria (*Plasmodium* spp.), leishmaniasis (*Leishmania* spp.), toxoplasmosis (*Toxoplasma* spp.), and trypanosomiasis (*Trypanosoma* spp.), cause mortality and morbidity globally. *Plasmodium* and *Toxoplasma* are examples of apicomplexan parasites, which use an important apicoplast organelle for transition between hosts. On the other hand, *Leishmania* and *Trypanosoma* are flagellated protists known as kinetoplastids due to their DNA-containing, kinetoplast organelles. These parasites often have complex life cycles and rely on vectors as carriers of the disease from one host to another. Even though several of these diseases are curable, the increasing number of drug-resistant strains is desperately urging for new alternative treatment options to cope with these diseases. Moreover, current remedies might rely on expensive drugs suffering from side effects or limited availability.

### 1.1.1 Malaria (*Plasmodium* spp.)

Even though malaria is both preventable and treatable, the World Health Organization (WHO) reported around 241 million cases of malaria infections and more than 600,000 deaths worldwide in 2020.[7] Approximately ⅔ were children under the age of five and 94% of this burden were in Africa. Malaria is spread by female *Anopheles* mosquitoes acting as a vector for several different species of *Plasmodium* parasites.[1] Five species have been reported to infect human: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. The dominating species in Africa is *P. falciparum*, which also is considered to be the most harmful one. This is due to the fact that a *P. falciparum* infection can progress to severe illness even with lethal outcome if not treated in time. In countries outside Africa,

*P. vivax* is the most prevalent species of the *Plasmodium* parasites. Besides blood-stage parasites, *P. vivax* also produce latent (dormant) liver-stage parasites that can reactivate months after the infective mosquito bite and cause relapse. Initial symptoms of malaria – chills, fever and headache – may be challenging to recognize if mild, but usually they appear 10–15 days after infection. Vector control such as insecticide-treated nets and indoor residual spraying, as well as early diagnosis and rapid treatment are crucial to reduce the prevalence of malaria and prevent deaths.

The different species of *Plasmodium* parasites have similar lifecycles.[8–10] At the start, an infected mosquito injects several hundred sporozoites into the cutaneous tissue of the human host (Figure 2). Within the next minutes to hours, the sporozoites leave the injection site and enter the blood vessels until some reach and invade the liver hepatocytes. In the liver-stage, which takes almost a week, they go through a first phase of asexual multiplication (exoerythrocytic schizogony) producing many uninucleate merozoites (about 10,000 in *P. vivax*/*P. ovale* and up to 30,000 in *P. falciparum*) that flood out into the blood. These merozoites invade red blood cells and undergo a second phase of asexual multiplication (erythrocytic schizogony) producing more merozoites that invade other red blood cells and continue to multiplicate. This intraerythrocytic cycle lasts only about two days, whereas the maturing into gametocytes can take around one week. As the infection progresses, some merozoites develop into female (macrogametocytes) and male (microgametocytes) gametocytes that circulate in the peripheral blood until another mosquito takes a blood meal. These gametocytes generate zygotes (ookinetes) in the mosquito's stomach. These zygotes invade the gut wall and transform into oocysts, which eventually grow until rupturing to release sporozoites. After nearly two weeks, these sporozoites make their way to the salivary glands of the mosquito and are ready to be injected as soon as the mosquito feeds on another human host to continue the malaria cycle.

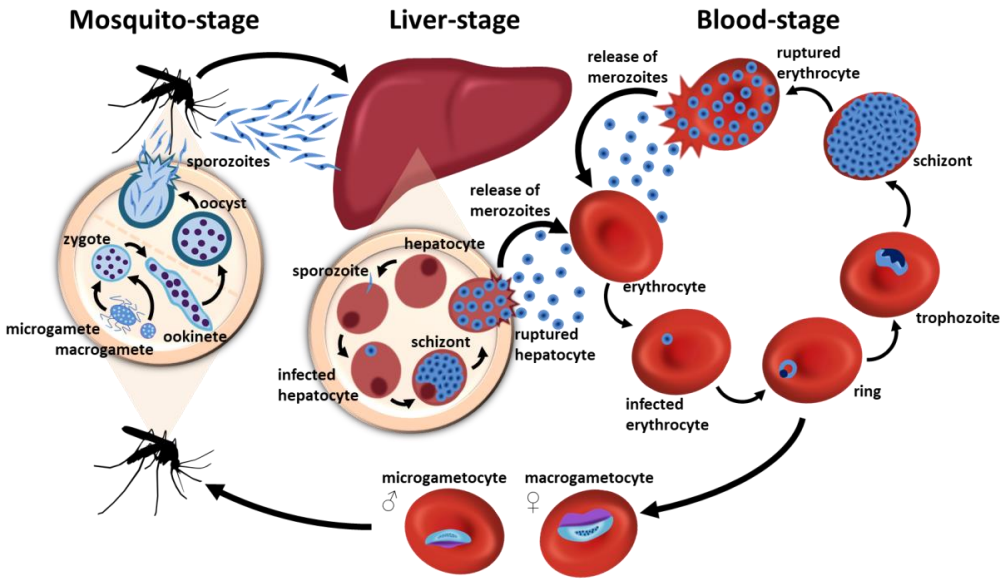



**Figure 2** *Simplified, graphical lifecycle of the malaria parasite in the mosquito vector and human host. First, a malaria-infected mosquito bites a human and the parasites undergo several phases both in the liver and later in red blood cells. When another mosquito takes a blood meal from the infected human, the parasites go through several phases in the gut before they finally reach the salivary glands of the mosquito, ready to be transmitted to the next human host.*

In general, antimalarial drugs (Figure 3) are classified based on their antiplasmodial activity in the parasitic life cycle or by their chemical structure. From a biological perspective, there are five classes of antimalarials: prophylaxis, gametocytocides, blood schizonticides, tissue schizonticides, and sporontocides.[11] Prophylactic drugs, used for the prevention of malaria especially for travelers, include primaquine, proguanil and pyrimethamine. Gametocytocides, as artemisinin and chloroquine, destroy blood-stage, female, and male gametocytes. As a result, these antimalarials inhibit the transfer of malaria parasites from an infected person to an uninfected mosquito. Sulfadoxine, mefloquine and quinine are examples of blood schizonticides that stop early symptoms of malaria by disrupting the asexual erythrocyte forms of the parasites. Tissue schizonticides, such as primaquine and pyrimethamine, can prevent relapse of dormant hypnozoites in the liver-stage of *P. ovale* and *P. vivax* infections. Primaquine and pyrimethamine are also sporontocidal i.e., they inhibit the development of oocytes in the mosquito-stage and thereby inhibit the transmission of the disease back to humans. The corresponding chemical classification of the aforementioned antimalarials comprises six classes: 8-aminoquinolines (primaquine), biguanides (proguanil), diaminopyrimidines (pyrimethamine), artemisinin-based (artemisinin and derivatives thereof e.g., artemether and artesunate), 4-aminoquinoline (chloroquine), and quinine-based (quinine, mefloquine).

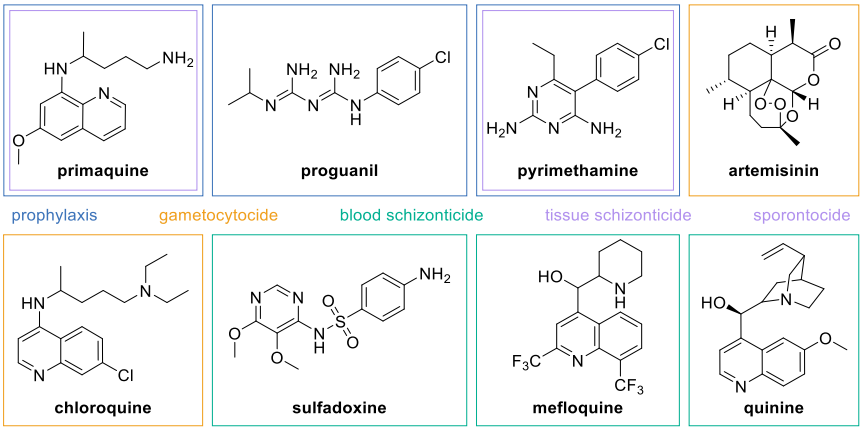



**Figure 3** *Various antimalarial drugs currently used in mono and/or combination therapies.*

The discovery of artemisinin, including its analogs and combination therapies thereof, was a significant achievement indeed. However, continuous drug development of novel antimalarials is a prerequisite to tackle malaria that has been around and evolved together with humans for ages. Innovative approaches, including development of diverse drug candidates (Figure 4),[12–15] malaria vaccine trials[16,17] and open-source drug discovery projects[18–20] are needed to complement current treatments. Particularly, as global warming allows *Plasmodium* parasites to spread to new areas that previously were too cold for the vectors to thrive. To cope with the complex lifecycle and rising resistant strains of the parasites against drugs and insecticides, multiple options exist. Possible approaches range from modifying existing antimalarials and combination therapies, to finding new drug targets or more drastic approaches as eradicating the vectors.

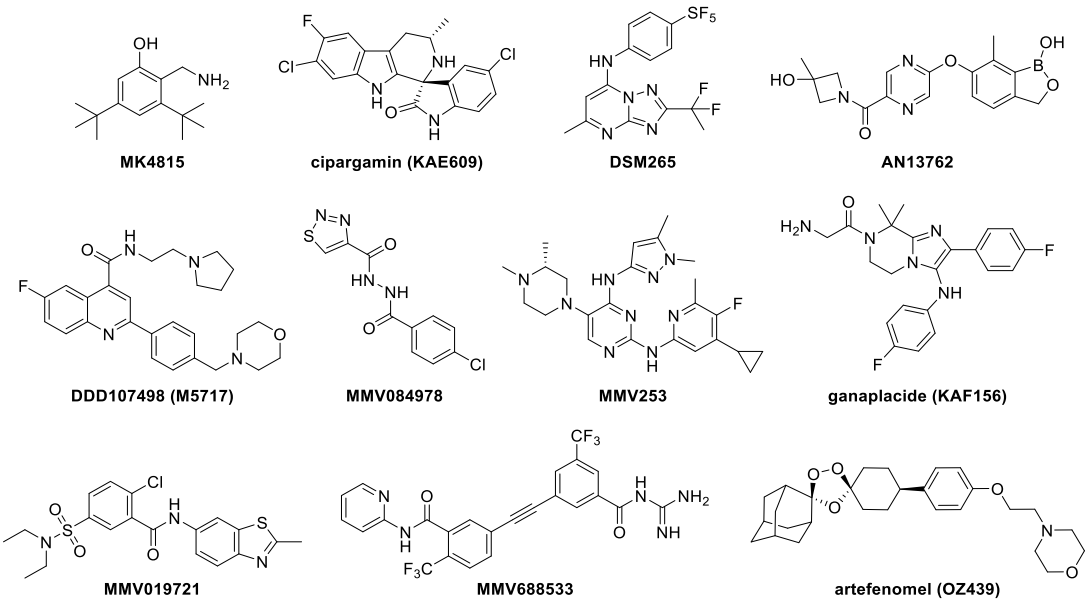



**Figure 4** *A variety of potential antimalarial drug candidates at different stages of the drug discovery and development pipeline.*

### 1.1.2 Toxoplasmosis (*Toxoplasma* spp.)

Besides malaria, other infectious diseases are also due to related apicomplexan parasites. For instance, toxoplasmosis (*Toxoplasma* spp.) is usually spread to humans as a result of contact with infected cat feces or through consumption of infected water or food.[21] It is considered one of the most prevalent human infections. The disease has a complex life cycle with an acute and a latent stage. At the acute stage, the parasite actively multiplicates causing tissue and organ damage, muscle ache and fever, but even encephalitis, myocarditis or pneumonitis for persons with weak immune system. Normally initial symptoms are quite mild and flu-like for some weeks to months. However, the disease can be life-threatening for a weak immune system and the parasites will stay dormant in the body even though the symptoms disappear. Elsewise healthy persons have mild to no apparent symptoms and do not generally need treatment. In more severe cases drugs are available, including pyrimethamine (Figure 5), sulfadiazine and folinic acid drug combination, but alternative treatment options are still needed.

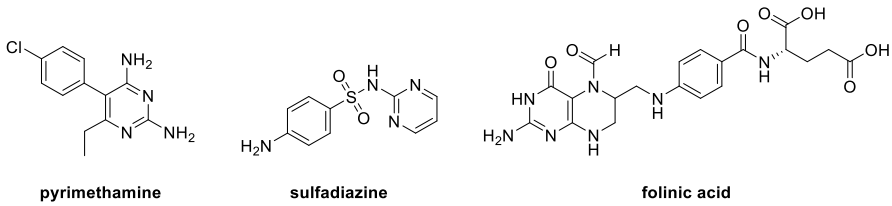



**Figure 5** *Different drugs used in the treatment of toxoplasmosis*

The main life cycle of toxoplasmosis (Figure 6) is between cats or other feline (definite host) and birds, rodents or other mammals (intermediate host).[22] However, human serves as an unfortunate end host in the elongated path. When a felid eats an infected tissue cyst, the bradyzoites invade epithelial cells, start dividing, and differentiate into female and male gametocytes. Some gametocytes form zygotes and differentiate into oocysts that are excreted in feces, which directly or indirectly get into contact with the intermediate host or human due to contact with contaminated water, soil or plants. As the intermediate host consumes contaminated foodstuff, sporozoites are released in the intestine. The sporozoites form tachyzoites and undergo asexual reproduction in cells until they rupture and release more tachyzoites into other tissue cells that eventually form cysts containing bradyzoites in various organs. The tissue cyst loaded with bradyzoites are then ready to be passed on to the next carnivore (feline or human). If a human gets infected, either by ingesting infective oocysts from cat feces or raw meat containing bradyzoites, the parasites penetrate the intestine and eventually form cysts particularly in the brain, eye, heart, lung, and muscle tissue. Additionally, the infection might be passed down to the fetus during pregnancy.

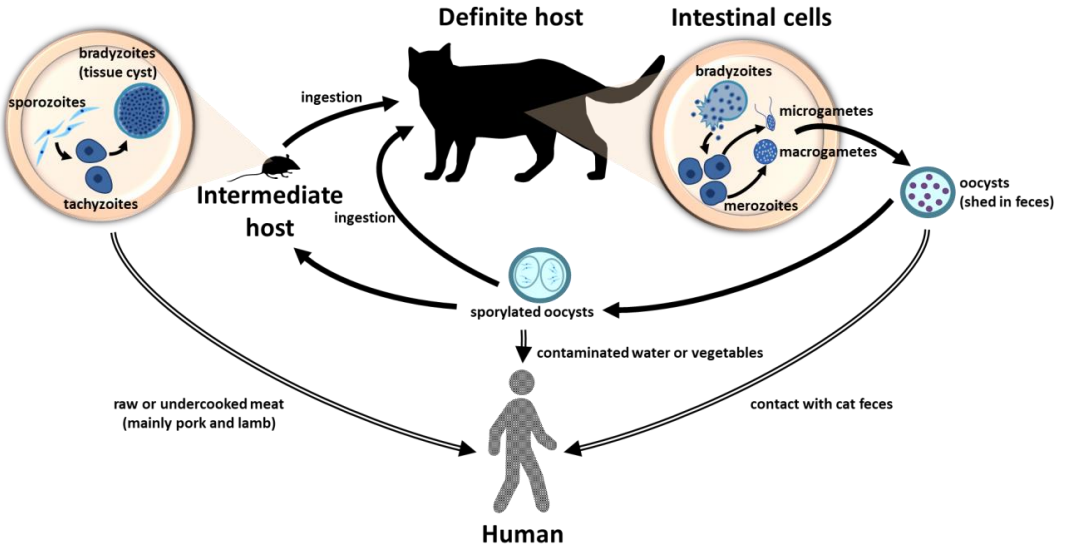



**Figure 6** *Shortened lifecycle of the Toxoplasma gondii parasite comprising of a sexual phase in the definite host (feline) and an asexual phase in the intermediate host (mammals, rodents, birds), as well as transmission to human.*

### 1.1.3 Leishmaniasis (*Leishmania* spp.)

Another source of infectious diseases is kinetoplastid protists.[23,24] Female, blood-feeding sandflies (*Phlebotomine* or *Lutzomyia*) act as a vector for several species of the parasite (*Leishmania* spp.) causing leishmaniasis.[25,26] Even though most people infected with the parasite do not develop any symptoms, the disease might be devastating especially when associated with malnutrition and weak immune system. The main forms of the disease are cutaneous, mucocutaneous, visceral (kala-azar) and post-kala-azar dermal leishmaniasis. Cutaneous leishmaniasis is the most common form, causing

skin ulcers on exposed body parts including the face, arms, and legs, as well as leaving permanent scars. Mucocutaneous leishmaniasis is even worse, affecting mucosal tissue such as mouth, nose and throat cavities that are partially or totally destroyed by non-healing wounds. Visceral leishmaniasis is the worst form with symptoms including fever, weight loss, anemia and swollen spleen with a potentially fatal outcome if not treated in time. However, there is still an additional risk of developing non-fatal post-kala-azar dermal leishmaniasis, causing skin rashes typically in the face that might spread to other parts of the body.[27] Current drugs (Figure 7) such as sodium stibogluconate, meglumine antimoniate, amphotericin B, paromomycin, pentamidine isethionate, miltefosine, fluconazole, itraconazole and ketoconazole suffer from side-effects and pharmacokinetic issues. In addition, the complex lifecycle ranging from flagellated promastigotes in the vector to amastigotes in the human host, allows the parasite to remain in the body post-treatment and can cause relapse for example during immunosuppression.

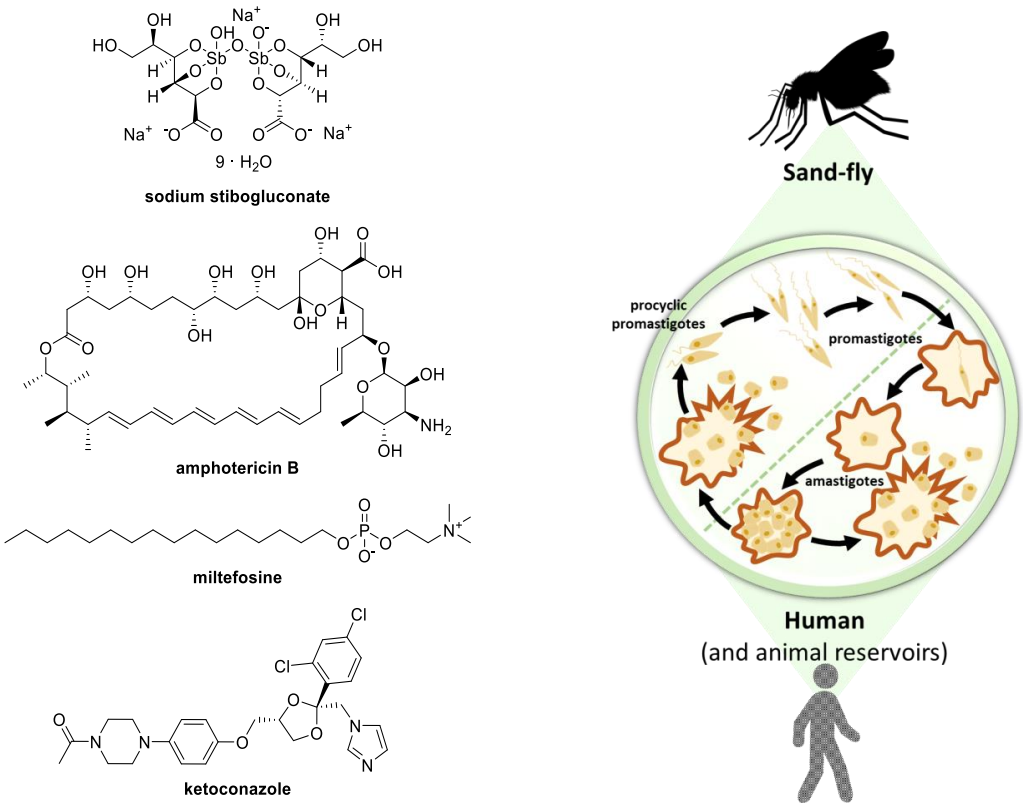



**Figure 7** *Different drugs used in the treatment of leishmaniasis (left), and simplified lifecycle of the Leishmania parasite in the vector and host (right).*

As an infected sand-fly takes a blood meal, promastigotes are injected into the human host (Figure 7).[23,24] First the promastigotes are phagocytized by macrophages, and are then transformed into amastigotes inside macrophages. The amastigotes multiply in cells (including macrophages) of various tissues e.g., spleen, liver or bone marrow. As the next sand-fly takes a blood meal, infected macrophages are ingested releasing amastigotes into the vector. Finally, the amastigotes transform

into promastigotes in the midgut, where they divide, migrate to the proboscis and are ready to be injected to the next host.

### 1.1.4 Trypanosomiasis (*Trypanosoma* spp.)

Several species of kinetoplastid parasites (*Trypanosoma* spp.) are responsible for the potentially life-threatening illnesses known as American trypanosomiasis (*T. cruzi*) or Chagas disease, and African trypanosomiasis (*T. brucei*) or African sleeping sickness.[23,24] Chagas disease occur in South America/Latin America, and is primarily spread by hematophagous triatomine bugs also known as kissing bugs. In fact, it is not the bite itself spreading the disease. The factual issue is the bugs' tendency to leave feces or urine next by, which is then accidentally smeared into wound, other skin lesion, eyes or mouth by the host. Likewise, the *T. cruzi* infection can be developed by consumption of food contaminated with the parasitic excrements of the vector. The initial acute phase having the parasite in the blood, usually have vague or no symptoms and lasts two to three months. In the chronic stage, the parasite causes progressive tissue damage by hiding in the heart or digestive muscles. Chagas disease is curable if treated rapidly enough after being infected, but current treatments are based on merely two drugs: nifurtimox and benznidazole (Figure 8).

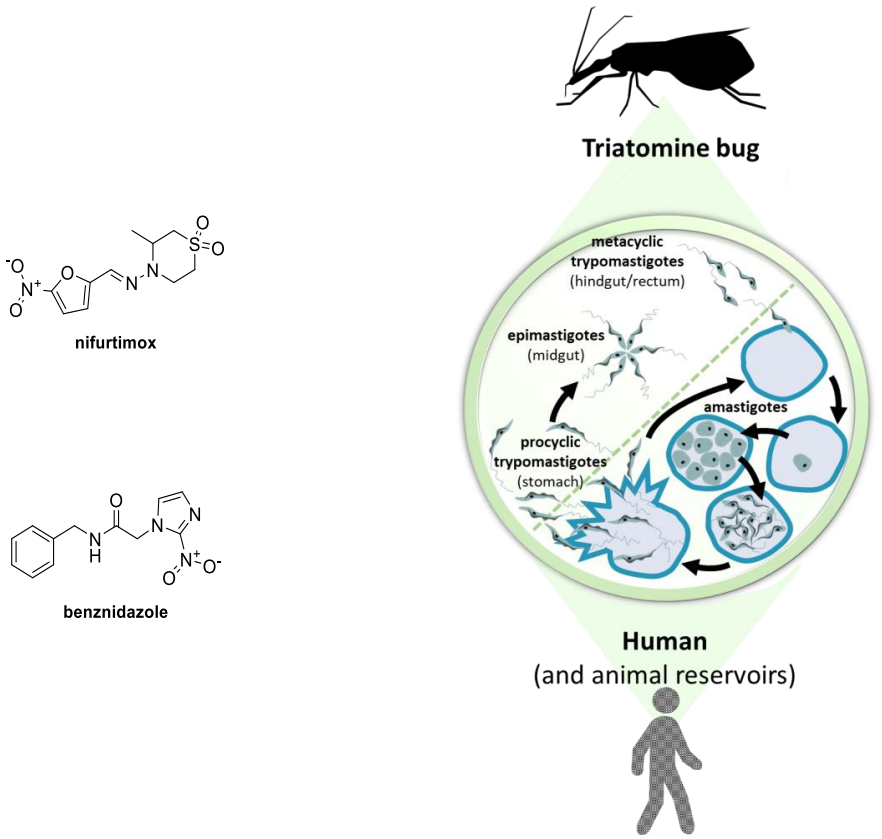



**Figure 8** *Current drugs used in the treatment of Chagas disease (left), and shortened lifecycle of the Trypanosoma cruzi parasite in the vector and host (right).*

The lifecycle of Chagas diseases commences, when an infected triatomine bug takes a blood bite (Figure 8). [23,24] Inopportunely, it also leaves feces containing metacyclic trypomastigotes nearby the bite wound, allowing the *T. cruzi* parasites to enter the human host. The metacyclic trypomastigotes transform into amastigotes, multiply, and infect other cells by transforming to intracellular amastigotes forming new sites of infection. The intracellular amastigotes transform into bloodstream trypomastigotes, burst out of the cell and enter the bloodstream to be taken up by the next bite of another triatomine bug. In the vector, the bloodstream trypomastigotes transform into epimastigotes, multiply in the midgut and transform into metacyclic trypomastigotes in the hindgut, ready to be passed on to another host.

African sleeping sickness is spread by *Glossina* tsetse flies in sub-Saharan Africa.[23,24] The disease can be further divided into West (*T. brucei gambiense*) and East (*T. brucei rhodesiense*) African trypanosomiasis, of which *T. b. gambiense* is responsible for about 95% of cases. *T. b. gambiense* develops first as a chronic infection, emerging after some months or years, before eventually affecting the central nervous system in the advanced stage of the disease. In contrast, *T. b. rhodesiense* causes an acute infection already within a few weeks or months involving crossing the blood-brain barrier and affecting the central nervous system. Both forms are fatal if not treated in time. In the first stage, suramin or pentamidine is used as a treatment, whereas eflornithine and/or nifurtimox, as well as fexinidazole or melarsoprol are used in the second stage (Figure 9).

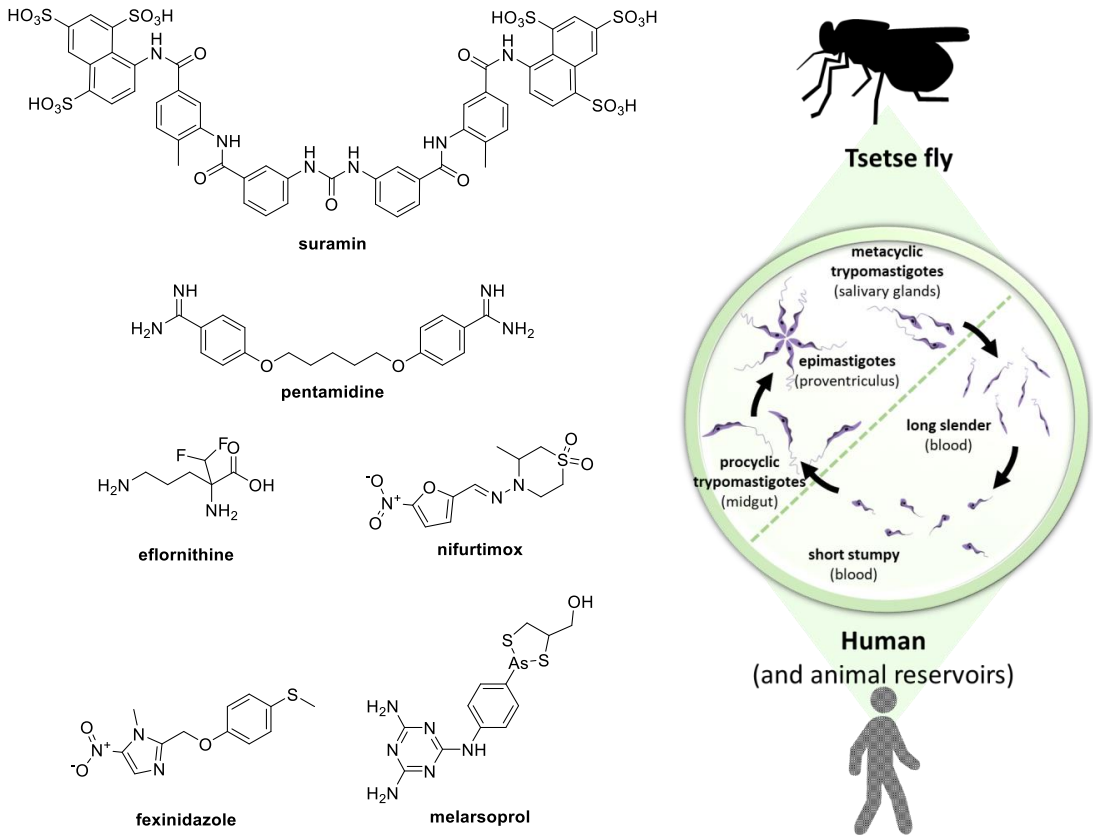



**Figure 9** *Different drugs used in the treatment African sleeping sickness (left), and simplified lifecycle of the Trypanosoma brucei parasite in the vector and host (right).*

The lifecycle of African sleeping sickness contains the bite of an infected tsetse fly (Figure 9).[23,24] During the blood meal, metacyclic trypomastigotes are injected into a human. The *Trypanosoma brucei* parasites differentiate into bloodstream trypomastigotes, which multiply in various human body fluids e.g., blood, lymph or spinal fluid. Another tsetse fly takes a blood meal ingesting bloodstream trypomastigotes, which transform into procyclic trypomastigote and reproduce in the midgut. Then they migrate towards the salivary glands where they transform into epimastigotes, multiply and transform into metacyclic trypomastigotes ready to be delivered to a new host.

## 1.2 Membrane-bound pyrophosphatases

Pyrophosphatases (PPases) are enzymes commonly found in many living species and take care of pyrophosphate, formed as a byproduct in different biosynthetic reactions such as DNA, RNA and protein synthesis.[28] There are both soluble and integral membrane PPases. Membrane-bound pyrophosphatase (mPPase), the primary enzyme of this study, is a homodimeric integral protein that hydrolyzes pyrophosphate into orthophosphate. As a result, mPPases act as ion pumps producing an ion gradient over the membrane. In 1966, Baltscheffsky *et al.*[29] reported the first mPPase of the photosynthetic bacterium *Rhodospirillum rubrum*, in their studies of light-induced formation of pyrophosphate ($PP_i$). Later studies further showed that these enzymes in fact are proton pumping mPPases.[30,31] In addition to some bacteria,[29,32] mPPases have also been found in several different species, including plants,[33,34] protozoan parasites,[35] and archaea.[36] Furthermore, some mPPases improve stress resistance in plants and are essential for the survival of protozoan parasites making these enzymes potential drug targets.[37]

### 1.2.1 Structure and function

Membrane-bound pyrophosphatases consist of 15 to 17 transmembrane helices per monomer with a molecular weight of 70 to 81 kDa.[38] These enzymes differ in several aspects compared to the other PPase protein families. PPases are generally divided into two major categories: membrane-bound PPases and ubiquitous soluble PPases (sPPases). According to their distinct structures, they are divided into four families: soluble cytoplasmic PPases (Families I–III) managing $PP_i/P_i$ homeostasis in the cell accompanied by integral membrane PPases (Family IV) that also pump protons.[39] For example, Family I and Family II PPases are evolutionarily unrelated soluble proteins, found in and vital for most living organisms.[38] mPPases have a unique structure and do not resemble sPPases apart from their common enzymatic activity. However, mPPases are only capable of hydrolyzing about ten $PP_i$ molecules per second, approximately 20- or 200-fold slower than sPPases in Family I and Family II, respectively. Whereas sPPases are found in all known living organisms, mPPases exist only in protozoan parasites, plants and some species of archaea and bacteria.

Currently the crystal structures from merely two mPPases have been solved – *Thermotoga maritima* (TmPPase) and *Vigna radiata* (VrPPase).[40–42] These homodimeric integral membrane proteins are symmetrical. Each monomer consists of 16 transmembrane helices (TMHs) forming two concentric layers with six (TMH5–6, TMH11–12, and TMH15–16) and ten (TMH1–4, TMH7–10, and TMH13–14) helices in the inner and outer layer, respectively. The regions can further be divided into a hydrolytic center, a coupling funnel, an ion gate, and an exit channel (Figure 10).[43] From a drug discovery perspective, the focus has been on the inner layer forming both the hydrolytic center and

the coupling funnel. This active site is closed upon substrate binding by a long loop (TMH5–6), and is re-opened after completed hydrolysis and subsequent ion pumping.[40–42]

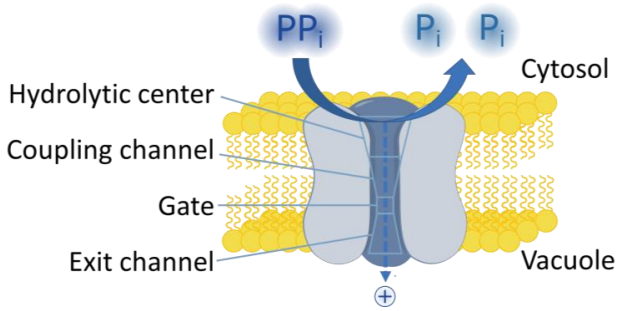



**Figure 10** *Simplistic illustration of pyrophosphate ($PP_i$) hydrolysis into orthophosphates ($P_i$) by mPPase, and subsequent ion pumping across the membrane.*

This process of hydrolyzing pyrophosphate into orthophosphate is coupled to the generation of an electrochemical gradient by translocation of ions $H^+$ and/or $Na^+$ across membranes and can be regulated by monovalent cations (dependence of $K^+$).[43] Also the reverse reaction ($2\ P_i \rightarrow PP_i$) has been observed, but the pyrophosphate synthesis merely creates an equilibrium rather than active transport and usually at a slower rate.[32] mPPases are thus primary ion pumps, fueled by $PP_i$ hydrolysis in the active site channel, having all required machinery in the same subunit enabling enhanced enzyme function and energy control.[44] In addition to the protein family, these ion pumps can be classified based on their cation specificity, as well as the requirement of potassium ions for activity.[45] Both $Na^+$ and $Na^+/H^+$-PPases, as well as some $H^+$-PPases, are $K^+$-dependent.[34] However, other $H^+$-PPases ($K^+$-independent $H^+$-PPases) are showing full activity even in the absence of $K^+$.[36] Moreover, some $Na^+/H^+$-PPases can only pump $H^+$ in low $Na^+$ concentrations ($Na^+$-dependent $Na^+/H^+$-PPases), whereas others can pump both $Na^+$ and $H^+$ regardless of $Na^+$ concentration (True $Na^+/H^+$-PPases).[46] Noteworthy, $Na^+$-PPases are only found in prokaryotes such as the bacteria *T. maritima* but neither in protozoan parasites nor plants. On the contrary, both $K^+$-dependent and $K^+$-independent $H^+$-PPases have been found in protozoan parasites, plants, and prokaryotes.

### 1.2.2 Relevance for prokaryotes, plants, and pathogens

Pyrophosphate is a side product of metabolic processes including nucleotide, lipid and protein synthesis.[47,48] Therefore, hydrolysis of $PP_i$ is significant to prevent toxic buildup that would otherwise inhibit essential biosynthetic pathways. Reduced $PP_i$ levels in the cytoplasm hence act as a thermodynamic driving force for metabolic processes. This fundamental pyrophosphate metabolism by specific hydrolases, i.e., some type of pyrophosphatases is essential for all organisms known to date. The focus herein has been ion pumping mPPases that convert pyrophosphate hydrolysis to a $Na^+$ or $H^+$ gradient. These enzymes belong to a protein family originally discovered in photosynthetic bacteria and plants[29,34,49] later to be found in parasitic protists, archaea, and certain species of bacteria, albeit they do not occur in animals and humans.[50,51] In archaea and bacteria, mPPases are found in the cell membrane,[50] while in algae, plants and protists, they can be located in acidocalcisomes, the vacuole, and/or Golgi apparatus as well.[52,53] Although no homologous proteins exist in animals and

humans, mPPases play an essential role in the survival of many organisms during diverse stress situations due to osmotic stress or other energy limitations such as anoxia, intense light, low temperature and mineral deficiency.[54]

Usually mPPases are located in the cell membrane of prokaryotes but they also exist in the membranes of acidocalcisome-like organelles.[55] Upon substrate binding on the cytoplasmic side, these proteins pump $Na^+$ or $H^+$ into either the periplasm or into organelles. Moreover, as for example, the hyperthermophilic marine bacterium *Thermotoga maritima* is found near undersea volcanic vents, a low energy environment, it might utilize $PP_i$ (a waste product of ATP-hydrolysis) as a secondary source of energy. TmPPase was initially thought to pump protons when characterized in 2001,[56] but later studies revealed it to be a $K^+$-dependent sodium pump.[32] Due to its high thermal stability[57] allowing increased stability during room temperature assays, TmPPase has been used for biochemical and structural studies.[40,43] Additionally, both $K^+$-dependent and $K^+$-independent $H^+$-PPases have been found in the vacuole of plants. Similarly, these enzymes are highly significant for their survival during periods of abiotic stress ranging from drought[58,59] and high soil salinity[60] to coldness and anoxia.[61] Overexpression of mPPases, in comparison to normal or low levels, has indeed been shown to be beneficial for the viability and survival of plants.[62]

Characteristically both $K^+$-dependent and $K^+$-independent $H^+$-PPases can be found within the same species of protozoan parasites. This might be an evolutionary residue or perhaps to provide better control under different cellular and environmental conditions. Many parasitic protists, including *Plasmodium falciparum*, *Toxoplasma gondii*, *Trypanosoma brucei*, and *Leishmania donovani*, possess these enzymes.[37] In contrast to many of the recently mentioned protists having only $K^+$-dependent mPPases,[63–65] *Plasmodium* also has a $K^+$-independent type.[66] Although mPPases are predominantly found in the acidocalcisome organelle,[35] some also exist in the Golgi apparatus and parasite cell membrane.[67] Acidocalcisomes are important for many parasites e.g., when it comes to essential regulation of pH and osmotic pressure during the parasitic lifecycle. This organelle, which is common for many parasites, is known to accumulate positively charged compounds including diamines,[68] metal ions such as $Ca^{2+}$ and $Mg^{2+}$, along with phosphorus derivatives such as bisphosphonates and polyphosphates.[53,69] Furthermore, acidocalcisomes have been associated with functions including short-chain polyphosphate (poly-P) metabolism, storage of cations, calcium homeostasis, intracellular pH homeostasis, as well as osmoregulation. For instance, acidocalcisomes and poly-P play a central role in the response of trypanosomatids to various sorts of stress, particularly at low-energy conditions.[53,69–73] The ionic gradient across the acidocalcisome, maintained by mPPases and vacuolar-type adenosine triphosphatase (V-$H^+$-ATPase), is important for ordinary function of the acidocalcisome such as in osmotic homeostasis when the parasite is passed on from an insect vector into the mammalian bloodstream.[53] Knocking out mPPase leads to a severe drop in poly-P levels and loss of the acidocalcisome acidity, thereby obstructing the ability of the parasites to stabilize their intracellular pH when they are exposed to external alkaline environments.[72] In *Toxoplasma gondii*, mPPase knockouts increased sensitivity to extracellular conditions and compromised virulence in mice.[71] Additionally, in parasitic protozoa, such as *P. falciparum*, *T. gondii*, *L. donovani*, *T. brucei*, and *T. cruzi*, pyrophosphate (and bisphosphonate) derivatives are able to inhibit growth.[66,74] However, this is thought to result from the action of both soluble and membrane-bound pyrophosphatases,[74,75] in particular by inhibition of the prenyl diphosphate synthase (isoprenoid pathway).[76]

### 1.2.3 Known inhibitors

Inorganic pyrophosphatases are inhibited by diverse compounds, including substrate analogs,[77–80] calcium[81–83] or fluoride[84–86] ions, arsenate[87] or tetranitromethane,[88] along with covalent inhibitors such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide[89] or fluorescein 5-isothiocyanate.[90] Despite the fact that sPPases and mPPases perform the same task – hydrolysis of pyrophosphate – both the active site and mechanism differ, which hypothetically allows specific inhibition.[54] Naturally, the mPPase inhibition is affected by several factors including conditions related to pH,[91] temperature,[92–94] time as well as concentration of the inhibitor.[90] In general, most documented mPPase inhibitors are phosphorous and structurally similar to orthophosphate (**1**, Figure 11) but lack the phosphoanhydride bond.[54,65,95] For instance, imidodiphosphate (**2**, $K_{i,app}$ = 12 µM) and methylenediphosphonate (**3**, $K_{i,app}$ = 68 µM) showed inhibitory activity against VrPPase.[96] However, dichloromethylenediphosphonate (**4**) was completely inactive. Other variations led to the most potent inhibitors aminomethylenediphosphonate (**5**, $K_{i,app}$ = 1.8 µM), hydroxymethylenediphosphonate (**6**, $K_{i,app}$ = 5.7 µM), and ethane-1-hydroxy-1,1-diphosphonate (**7**, $K_{i,app}$ = 6.5 µM).

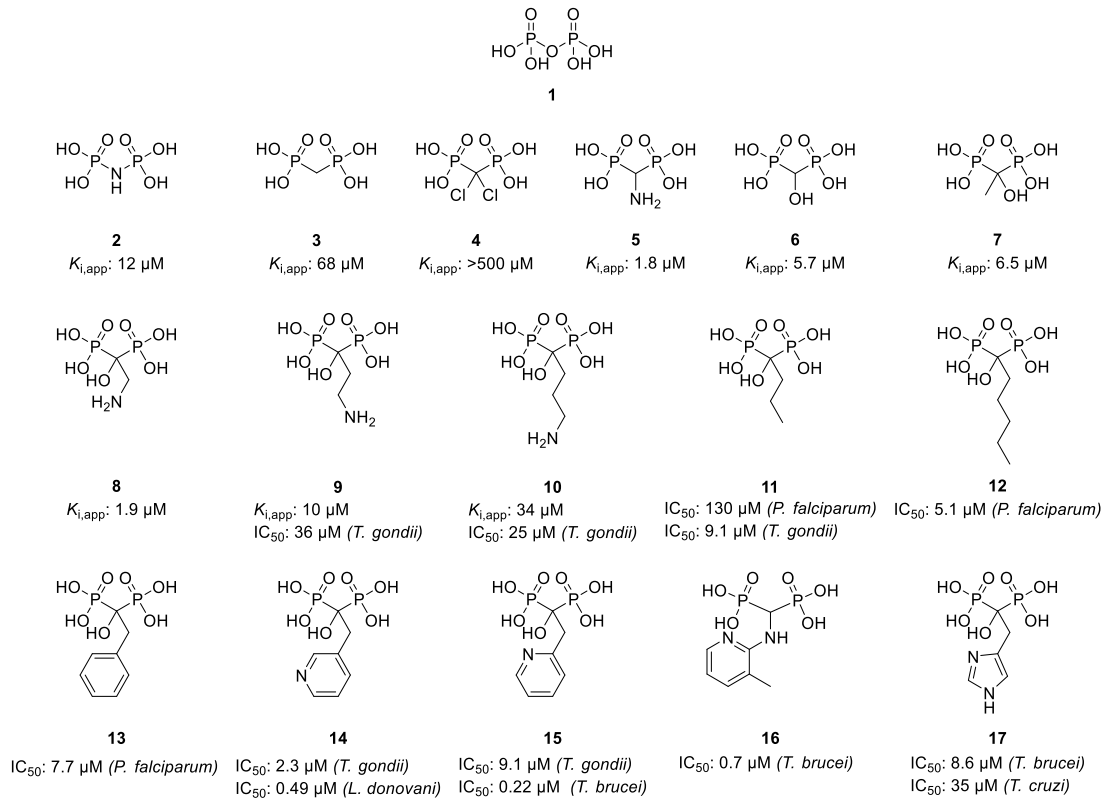



**Figure 11** *Structure of orthophosphate (**1**), a variety of known phosphorus VrPPase inhibitors, as well as selected antiparasitic bisphosphonates against T. gondii, P. falciparum, L. donovani, T. brucei and T. cruzi.*

Non-hydrolyzable bisphosphonates, where the central pyrophosphate oxygen has been replaced with a carbon substituted with various side chains, have been studied as potential antiparasitic[74,97] as well

as farnesyl diphosphate inhibiting[98,99] agents. Furthermore, some bisphosphonates have been reported to competitively inhibit sPPases and mPPases.[75] Besides hydrophilic groups, such as α-$NH_2$ or α-OH that might increase metal ion chelating capacity, additional nitrogen-rich side chains have been particularly favorable for VrPPase inhibition. The shorter the side chain of the primary amine substituent, the more the activity was favored, for instance when comparing compounds **8–10** ($K_{i,app}$ = 1.9–24 μM). Analogous antiparasitic bisphosphonates showed promising results, too.[74] For example, the *Plasmodium* parasites seemed to favor non-nitrogen containing bisphosphonates (**12**, **13**). *Toxoplasma* parasites tolerated aliphatic amino substituents (**9**, **10**), but alkyl (**11**) or heteroaryl (**14**, **15**) substituents were preferred showing similar low micromolar activity as the *Plasmodium* parasites. Even though *T. cruzi* parasites merely showed moderate inhibition (**17**), the pyridinyl-substituted bisphosphonates **14–16** had submicromolar activity against *L. donovani* and *T. brucei* parasites.

So far, nonphosphorus mPPase inhibitors have not been as extensively studied as phosphorus containing ones. Nevertheless, some acylspermidine derivatives found in soft coral *Sinularia* sp. did inhibit VrPPase, even though spermidine itself did not inhibit nor prevent the inhibition of this enzyme.[100] Structurally, compounds **18**–**22** (Figure 12) were rather similar to *N′*,*N″*,*N‴*-trimethylspermidines acylated with unsaturated fatty acids, mainly differing by the orientation of the double bond(s) and length of the methylene chain. All these compounds showed mPPase inhibition but did not inhibit yeast cytosolic PPase. The most effective compounds for inhibition of $PP_i$ hydrolysis were **21** and **22**, both with $IC_{50}$ values below 20 μM. Neither of these compounds did inhibit soluble cytosolic PPase, V-ATPase, plasma membrane $H^+$-ATPase, nor mitochondrial ATP synthase. Although the long lipophilic tail is likely to interact at the interface of helices and the membrane lipids, the methylated spermidine moiety possibly binds to regions other than the catalytic site.

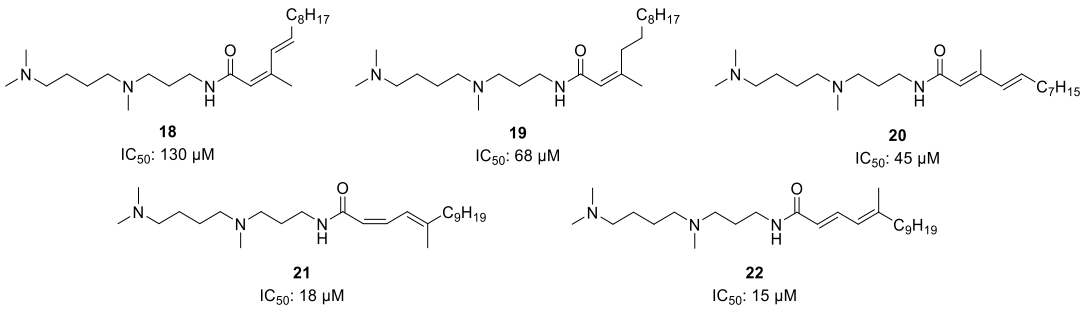



**Figure 12** *Acylspermidine mPPase inhibitors.*

Other non-substrate analogs are for instance 1,2,4-triazines, studied for their antibacterial activity but also reported to inhibit inorganic pyrophosphatases.[101] The fortuitous discovery of **23** (Figure 13) occurred due to the PPase-coupled assay used in the high-throughput screening, originally intended to detect *o*-succinyl benzoic acid coenzyme A synthetase inhibitors. However, this initial hit is merely a weak ($IC_{50}$ = 150 μM) inhibitor of *Mycobacterium tuberculosis* PPase (MtPPase). To optimize the antimicrobial activity and MtPPase activity, a series of additional 3-(3-aryl-pyrrolidin-1-yl)-5-aryl-1,2,4-triazine analogs was synthesized. Mainly substitutions or expansions of the furanyl moiety were done with phenyl rings bearing electron donating or withdrawing groups, which led to a 2–4-fold gain in activity for the top MtPPase inhibitors **25–29** ($IC_{50}$ = 33–63 μM). Small changes, as replacing the

chlorine atom of **23** with a hydroxyl group as in **24**, caused all inhibitory activity to be lost. Benzyl protection of **24** restored the abolished activity, as compound **25** demonstrated a 2-fold gain in activity. Further substitution of the furan ring seemed benefical too. In fact, replacing the furan moiety with a mono- or disubstituted phenyl ring (**27**–**29**) showed comparable activities. Although compound **28** ($IC_{50}$ = 33 µM) indicated both enzyme and antibacterial inhibition, MtPPase inhibiting **26** ($IC_{50}$ = 38 µM) lacked antibacterial activity. On the other hand, compound **24** had antibiotic activity without showing any detectable inhibition of MtPPase. Consequently, there appears to be a lack of general correlation between the enzyme inhibition and antibacterial activity versus *M. tuberculosis*.

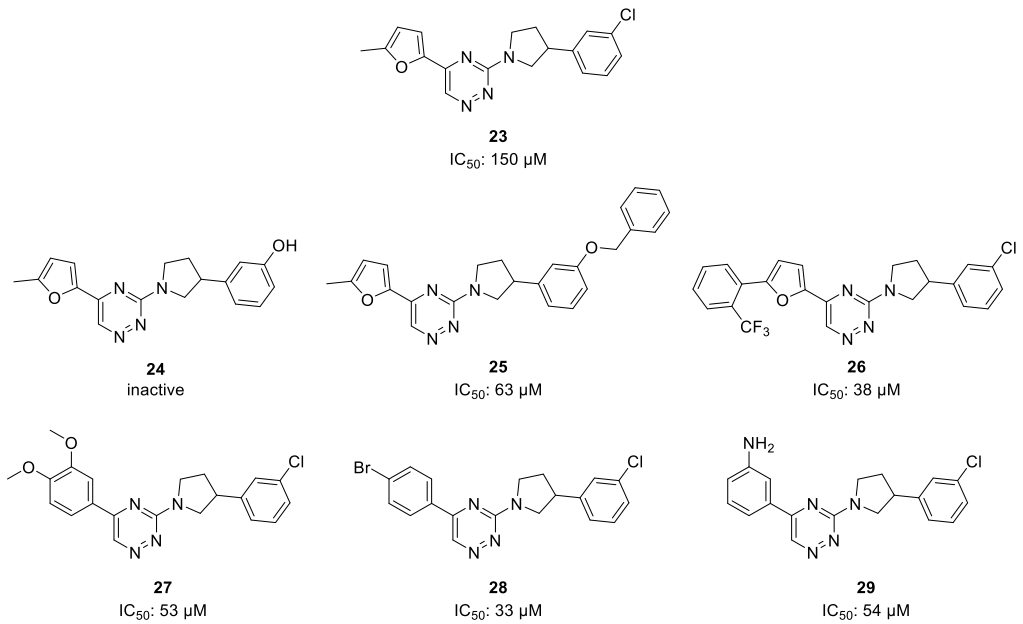



**Figure 13** *Selection of 1,2,4-triazine MtPPase inhibitors.*

### 1.2.4 Potential drug target

Membrane-bound pyrophosphatases have been suggested to be valuable targets for chemotherapeutic action.[53,66,102,103] Nevertheless, only a few crystal structures have been solved, limiting structure-based drug design. Some of these structures have been obtained from co-crystallization experiments with the substrate analog imidodiphosphate (IDP), yet only very few nonphosphorus mPPase inhibitors have been reported.[91,100] Most known inhibitors, such as pyrophosphate and bisphosphonate derivatives, are not optimal as drugs due to their physicochemical properties, for example poor stability and extensive hydrophilicity. As a chemotherapeutic target, mPPases offer significant advantages. Firstly, they would allow for targeting not only the digestive vacuole in the asexual blood-stage parasites but also the gametocyte-stage.[103–105] Secondly, since no human homologues exist, inhibitors are highly likely to be specific, provided that phosphorus compounds are avoided due to their tendency to recognize unrelated structural motifs.[106,107] In addition, targeting nonconserved binding sites of PPases could allow species-selective inhibitors to be developed.[39]

To develop new inhibitors against mPPases it is almost fundamental to identify the ligand-binding sites of known inhibitors for structure-based drug design. Obviously, there are more than one possible sites of action. The catalytic pocket on the cytoplasmic side of the protein, where competitive inhibitors such as IDP and bisphosphonates bind, is however rational as a starting point. Crystal structures with these compounds can be used as initial indications of important amino acid residues that could be targeted in further compound design of specific mPPase inhibitors. As previously mentioned, the protein has two rings of helices, one inner and one outer (Figure 14). The inner ring (TMH5–6, TMH11–12, and TMH15–16) is more appealing from a drug discovery perspective since it is critical for the substrate hydrolysis and ion pumping. In addition to a hydrolytic center located circa 20 Å above the membrane surface, the inner ring also forms a coupling channel, a gate below the membrane surface, and an exit channel.[40] This funnel-shaped pocket of the hydrolytic center is highly conserved between mPPases and contains sixteen conserved polar residues important for substrate binding as for example seen in the crystal structure of VrPPase in complex with IDP.[40,42] Furthermore, the coupling funnel contains eight conserved polar residues – forming an ionic network – and mutation of these residues affect the normal function of the enzyme.[108] Hence, these sites could serve as prospective target sites in early drug design. Beneath the coupling funnel, there are the ionic and hydrophobic gates, of which the latter prevents ion back-flow from the periplasm to the cytoplasm. Finally, the exit channel allows release of ions to the periplasmic side. Notably, the exit channel is not conserved among mPPases, indicating that it does not play a central role in the ion pumping mechanism.

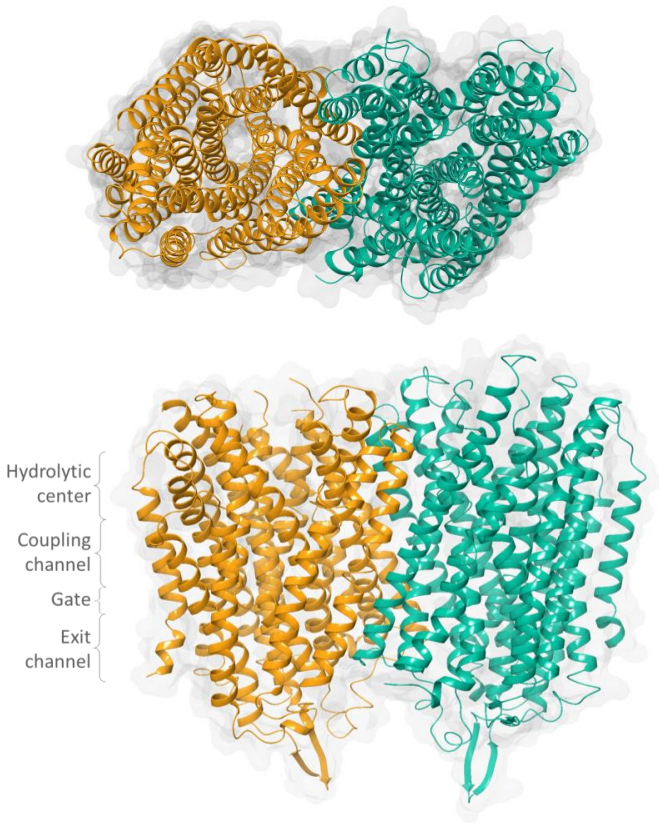



**Figure 14** *Top and side view of the dimeric mPPase structure.*

## 1.3 A selection of cyclic core structures

Heterocycles bearing nitrogen, oxygen and/or sulphur, which are common in various five- or six-membered rings, are biologically interesting compounds. They offer a useful tool for the tuning of physicochemical properties such as solubility, polarity, lipophilicity, and hydrogen bonding capacity, besides acting as versatile molecular framework from where ligands can be grown. Some of these cores, including quinolone, chromone, and benzothiophene, are so common in biologically active agents that they are considered to be privileged structures.[109,110] Thus, a major part of all drugs known to date can be described by these kind of molecular features. Furthermore, fundamental reactions, such as amidations, ester hydrolysis, and Suzuki–Miyaura coupling, are vital in medicinal chemistry and regularly used in the synthesis and modifications of drug-like scaffolds.[111] Various heterocycles are frequently found in natural products too.

### 1.3.1 Benzothiazoles

The benzothiazole ring system consists of a benzene ring fused to a thiazole at its 4- and 5-position. This scaffold has been reviewed[112–114] for its diverse biological activities, including anticancer,[115–117] anticonvulsant,[118–120] anti-inflammatory,[121–123] antimicrobial,[124–126] antiparasitic (e.g. antileishmanial,[127,128] antimalarial[129–131] and antitrypanosomal[132,133]), antitubercular[134] or antiviral[135–137] effects. Besides occurring in marine and terrestrial natural compounds, there are also several drugs with benzothiazole-based scaffolds currently in use (Figure 15).

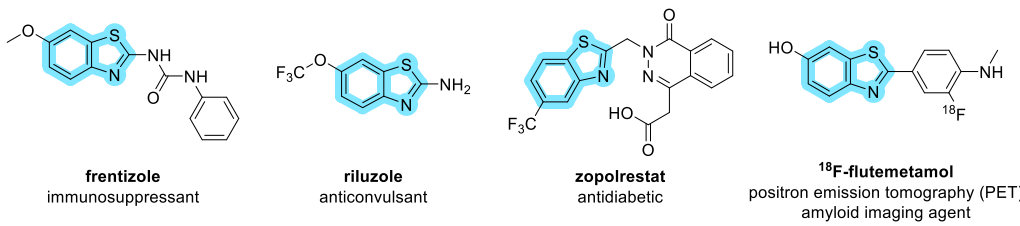



**Figure 15** *Examples of benzothiazole containing bioactive molecules.*

From a synthetic viewpoint, the desired substitution pattern and/or available starting materials offer several alternatives to make benzothiazole analogs. It might be favored to form the core or further functionalize available substituents of a pre-made core. Electrophilic reactions, such as nitration, are limited to the benzo part of the molecule, whereas the 2-position is more prone to react with nucleophilic reagents. In the simplest case, no substituents are present. Alternatively, useful synthetic handles including halogens or amino groups are preferred. More relevant to this study are 2-aminobenzothiazole derivatives that generally can be made in various ways ranging from early pioneer work[138–140] to more modern approaches. The main methods include cyclocondensation or intramolecular oxidative cyclization to form the bicyclic ring system, from aryl amines,[141,142] aryl isothiocyanates,[143,144] aryl thioureas,[145,146] *o*-haloanilines,[147] *o*-aminothiophenols[148] or *o*-halothioureas,[149] substitution of the 2-position in benzothiazole[150,151] or 2-halobenzothiazole,[152,153] as well as further functionalization of 2-aminobenzothiazole[154,155] (Figure 16).

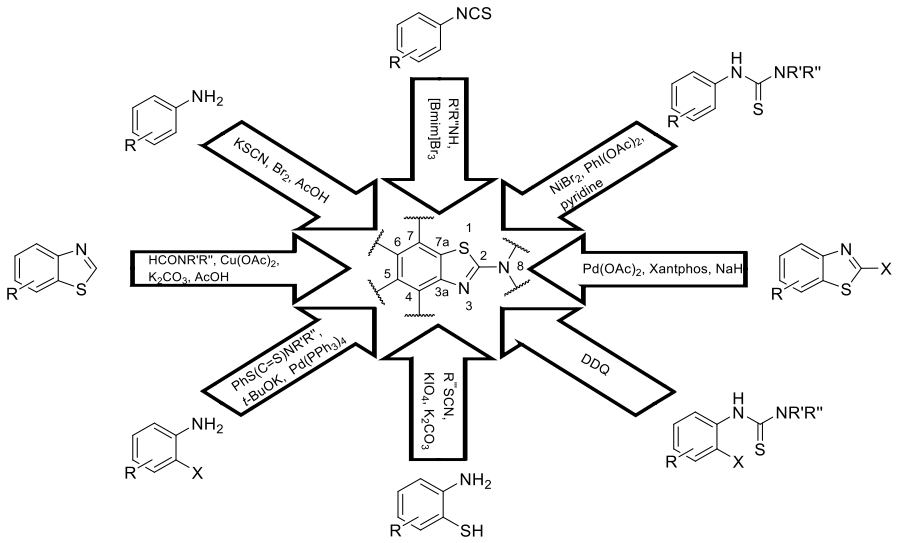



**Figure 16** *A few alternative synthetic methods to form the benzo[d]thiazole core.*

### 1.3.2 Isoxazoles

Isoxazoles are aromatic, five-membered heterocycles with one oxygen and one nitrogen atom at adjacent positions. This scaffold has a wide variety of biological activities reviewed,[156–159] and for instance anticancer,[160–162] anti-inflammatory,[163,164] antimicrobial,[165–168] antiparasitic (e.g. antileishmanial,[166,169,170] antimalarial,[171–173] antitrypanosomal[169,173–175]), antitubercular,[176,177] or antiviral[178–180] effects have been reported. Isoxazoles can also be found in drugs (Figure 17) and bioactive natural products, such as leflunomide and muscimol, respectively.

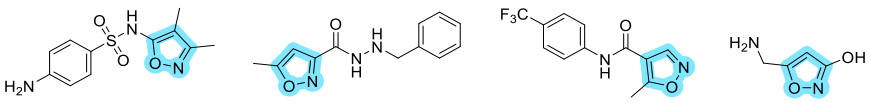



**Figure 17** *A selection of isoxazoles with biological activities.*

Sometimes direct functionalization of the isoxazole core is performed, whereas sometimes modification of substituted isoxazoles is explored. The choice is generally based on the required substitution pattern, such as 5-arylisoxazole-3-carboxylates in this study, as well as existing starting materials. The fact that nucleophiles are more prone to attack the 3-position, whereas electrophilic substitution tends to take place at the 4-position needs to be considered as well. Since early studies[181–183], isoxazoles have been frequently synthesized by condensation of a 1,3-dicarbonyl compound with hydroxylamine via an oxime intermediate undergoing subsequent cyclization and dehydration. More current synthetic methods also include 1,3-dipolar cycloaddition of an alkyne with a nitrile oxide formed *in situ* from e.g., chlorooximes in the presence of a base. The former route might be slightly longer than the latter, but it also offers greater regioselectivity. Furthermore, the intermediate 1,3-

dicarbonyl compound can be used in the synthesis of other heterocycles, which might be favorable from a synthetic medicinal chemistry perspective. Direct functionalization[184] of isoxazoles or isoxazoles decorated with suitable coupling handles is often desired, as this could allow easy modifications without having to start the synthesis from scratch for each substitution pattern. Depending on available starting materials different approaches to construct the isoxazole core from a range of substrates including aldehydes,[185–187] alkynes,[187–189] enones,[190–192] diynes,[193] nitroacetates,[194,195] ketones,[179,196,197] oximes,[198] and ynones,[199] has also been reported (Figure 18).

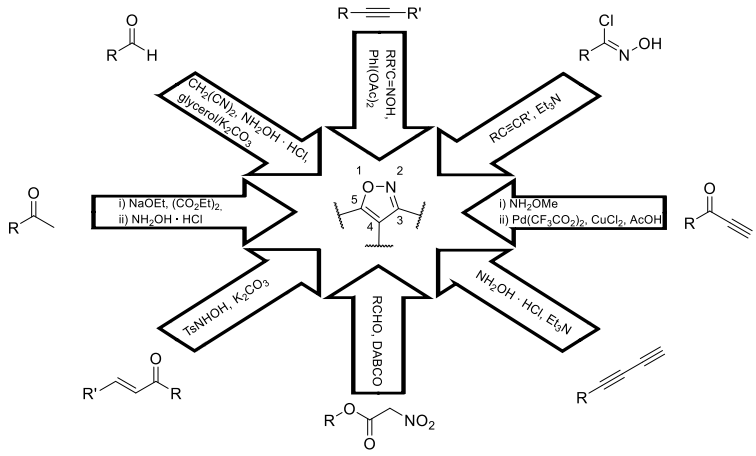



**Figure 18** *A variety of synthetic methods to make the isoxazole core.*

### 1.3.3 Pyrazolo[1,5-*a*]pyrimidines

Pyrazolopyrimidines show structural resemblance to prevalent purine analogs. Depending on how the pyrazole is fused to the pyrimidine moiety, the heterocyclic ring system have either three or four nitrogen atoms. Hence, in the case of pyrazolo[1,5-*a*]pyrimidine there are only three nitrogen atoms, as one nitrogen is shared by both the pyrazole and the pyrimidine ring. This scaffold has been reviewed[200,201] for its biological activities, such as antimicrobial,[126,202–204] anticancer,[205–207] anti-inflammatory,[208,209] antiparasitic (e.g. antileishmanial,[210] antimalarial[211,212] and antitrypanosomal[210,211]), or antiviral[213,214] effects, and occurs in a number of drugs (Figure 19).

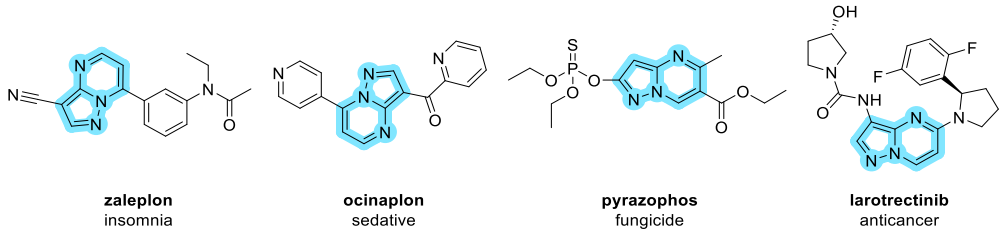



**Figure 19** *Examples of pyrazolo[1,5-a]pyrimidines with biological activities.*

Functionalization of the pyrazolo[1,5-*a*]pyrimidine core might be favored during or after the ring construction. Alternative convenient ways of introducing substituents are to use customized precursors decorated with the desired substituents or suitable coupling handles for further functionalization. Also, electrophilic (formylation, halogenation, nitration and nucleophilic aromatic substitution reactions can take place on the pyrazole and pyrimidine side, respectively. Since initial experiments,[215–218] typical ring forming reactions include an appropriate 3(5)-aminopyrazole (AP) together with a 1,3-bis-electrophile such as unsaturated nitriles,[219–221] unsaturated carbonyl compounds,[207,222–226] 1,3-diketones,[214,227–229] ketoesters,[226,230,231] ketonitriles,[232] enaminones,[206,233–235] enaminonitriles,[236,237] halovinyl aldehydes,[238,239] acetals,[240] enones,[241] ketenes[242] or ketones[243,244] (Figure 20).

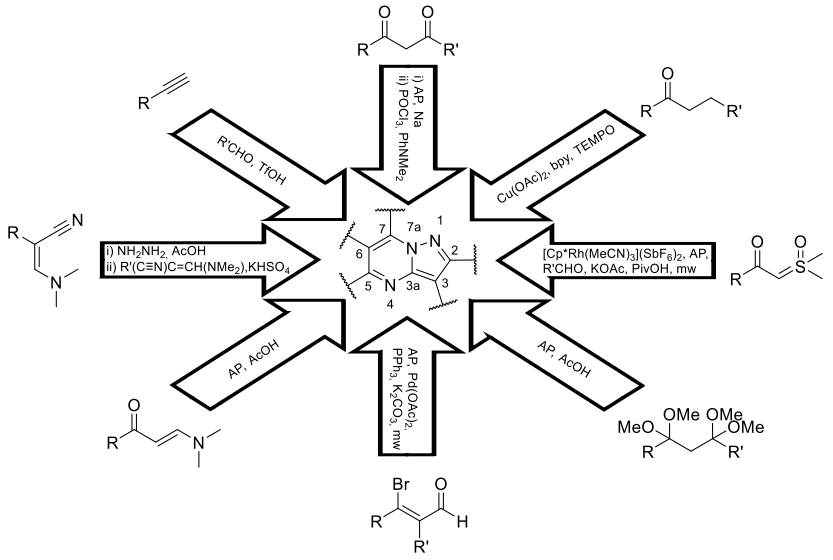



**Figure 20** *An assortment of synthetic methods to construct the pyrazolo[1,5-a]pyrimidine core.*

### 1.3.4 Azulenes

Azulene i.e. bicyclo[5.3.0]decapentaene and guaiazulene i.e. 1,4-dimethyl-7-(propan-2-yl)azulene, are aromatic hydrocarbons made up of a fused five- and seven-membered ring. The nature of the azulene ring system differs in several aspects to that of its structural isomer naphthalene. The most noticeable is the characteristic blue color of azulene (*azul* is Spanish for blue) in comparison to naphthalene that is white. In addition, the distribution of electron density giving azulene a dipole moment with an electron-rich and electron-poor five and seven-membered ring, respectively. Although azulene derivatives are found in nature, they have not been extensively studied within medicinal chemistry. Consequently, the current amount of azulene-derived drugs is rather low and azulenes have merely been studied as bioisosteric replacements of some unsaturated ring systems.[245,246] This could be an interesting prospect for potential applications, as azulenes have been reviewed[247–249] e.g., for their anticancer,[250–253] anti-inflammatory,[253,254] antidiabetic,[255] antiviral,[256] and antimicrobial[257] activity. Despite the fact that azulenes show a range of diverse biological activities (Figure 21), the amount of approved drugs is rather scarce at present.[258]

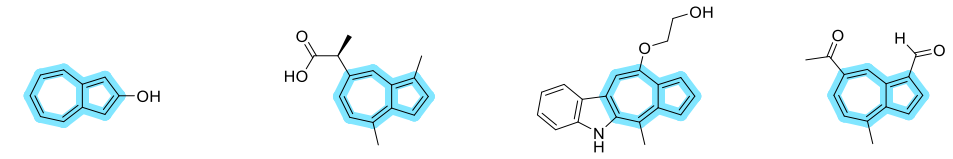



**Figure 21** *Examples of azulenes with biological activities.*

Due to the distinctive electron distribution of azulene, the five- and seven-membered ring is typically more prone to react with electrophiles and nucleophiles, respectively. The electron-rich five-membered ring, especially the 1- and 3- position, reacts readily through electrophilic substitution such as halogenation, Vilsmeier–Haack formylation, and Friedel–Crafts acylation.[259] On the other hand, this reactivity might lead to undesired metabolic consequences, which could be decreased by suitable substitution such as a deactivating or steric moiety. Substitution of the electron-deficient seven-membered ring with nucleophiles, mainly at the 4-, 6- and 8-position, is yet rather restricted. Complementary functionalization can be done in some cases by various cross coupling reactions as well.[260,261] Therefore it is often useful to introduce convenient substituents already in the formation of the azulene core as an alternative to direct functionalization of the azulene or guaiazulene structure. Initial work on the synthesis of azulenes used rather harsh reaction conditions,[262–265] and were later replaced by alternative methods.[266–269] In one of the early dehydrogenation free methods, the azulene ring is formed from pyridine through thermal cyclization of an intermediate pentafulvene derivative. Modified versions thereof, including alkyl pyridinium salts, are still regularly used.[270,271] Complementary approaches include the use of tropones,[272,273] 2*H*-cyclohepta[*b*]furan-2-ones,[274–276] cycloheptatrienes,[277,278] tropylium salts,[279] β'-bromo-α-diazo ketones[280,281], or thiophene *S*,*S*-dioxides,[282,283] as well as direct functionalization of the azulene core[284] (Figure 22).

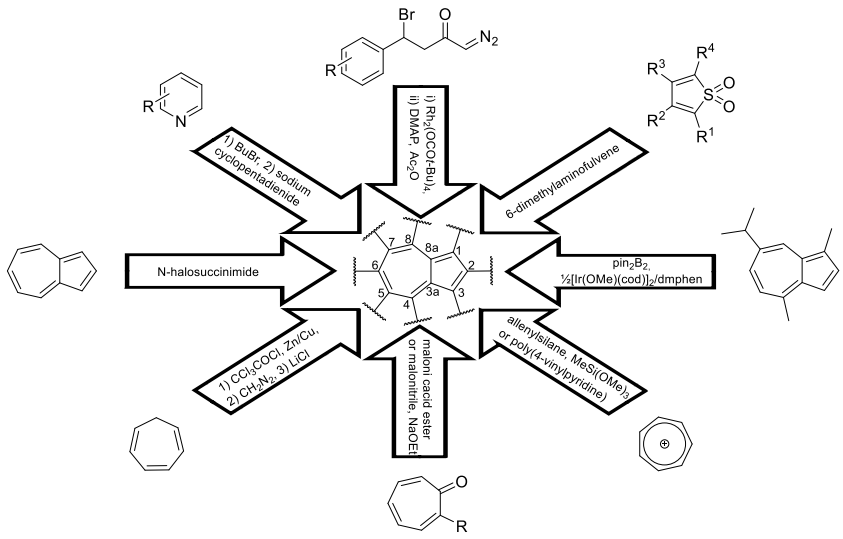



**Figure 22** *A choice of synthetic methods to construct azulene derivatives.*

# 2 Aims of the study

The focus of this study was on membrane-bound pyrophosphatases (mPPases) and their potential as a drug target against pathogenic protozoan parasites by affecting the activity of this essential ion pump. More precisely, we focused on the development of nonphosphorus mPPase inhibitors with prospective as an alternative approach in the treatment of parasitic diseases including malaria (*Plasmodium* spp.). As a starting point, we used a virtual screening of phosphate mimicking fragments for finding new inhibitors and subsequent biological testing of commercially available analogs. In addition, selected compounds from a proprietary library were tested. An initial molecule collection of potential mPPase inhibitors was constructed to cover enough chemical space to study their preliminary structure-activity relationship (SAR). Mainly heterocyclic scaffolds, such as benzo[*d*]thiazole-, isoxazole-, pyrazolo[1,5-*a*]pyrimidine and azulene-derived compounds, were synthesized and tested against our model assay using mPPase from *Thermotoga maritima* (TmPPase). The best hits were further tested using mPPase from *Plasmodium falciparum* (PfPPase) and in a *P. falciparum* survival assay in erythrocytes.

The more specific aims were:

- To design and synthesize benzo[*d*]thiazole-based inhibitors with the potential for specific inhibition of TmPPase and PfPPase, as well as activity in a *P. falciparum* survival assay, and gain deeper understanding of the mPPase binding site based on X-ray crystallographic studies (Publication I).

- To design and synthesize isoxazole-based inhibitors with the potential for specific inhibition of TmPPase and PfPPase, as well as activity in a *P. falciparum* survival assay, and study their SAR (Publication II & III).

- To design and synthesize pyrazolo[1,5-*a*]pyrimidine-based inhibitors with the potential for specific inhibition of TmPPase and PfPPase, as well as activity in a *P. falciparum* survival assay (Publication III).

- To design and synthesize azulene-based inhibitors with the potential for specific inhibition of TmPPase and PfPPase, as well as activity in a *P. falciparum* survival assay, and study their SAR (Publication IV).

# 3 Materials and methods

All synthesized compounds were >95% pure as determined by liquid chromatography-mass spectrometry (LC-MS), and characterized by high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) spectroscopy, whereas commercially obtained compounds had purities >90% as specified by the vendors. The preliminary biological evaluation of compounds against TmPPase, as well as for selected compounds against PfPPase, was done by the Goldman group at the Department of Biosciences, Division of Biochemistry, University of Helsinki. Selected compounds were further studied in a *P. falciparum* survival assay survival assay in erythrocytes by the Meri group at the Malaria Research Laboratory, Translational Immunology Research Program, Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki. More specific information regarding materials and equipment, as well as synthetic, analytical, biological and computational methods used in this study is described in the original publications I–IV, in the related publications[285,286] and in the related supporting information thereof. The material can be found from the website of the respective journal or can be requested from the author.

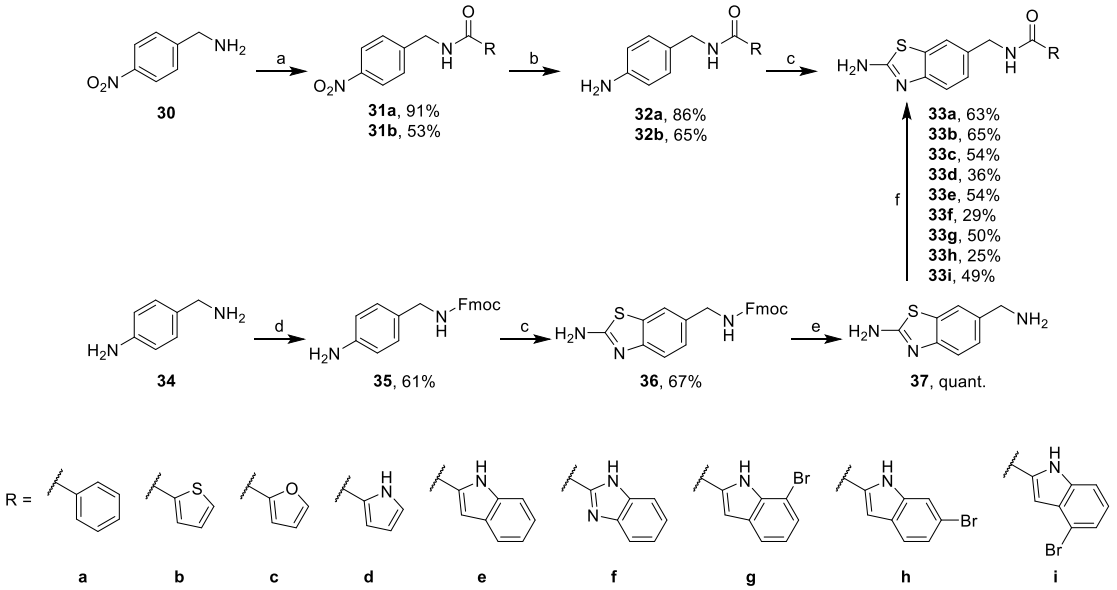
# 4 Results and discussion

Herein, the main findings in publications I–IV as well as unpublished data are presented. This study is a screening process started from a combination of *in silico* mining of phosphate isosteres[107] complemented by commercial analogs and synthesized compounds. In addition to this approach, the starting point was further complemented by a cross-project screening approach of some previously synthesized in-house compounds from our research group. As the primary goal was to synthesize the required derivatives for biological testing, no further optimization of the reaction conditions were attempted at this stage of the study. Predominantly, four chemically diverse inhibitor classes were synthesized. These derivatives were initially tested against our recently developed 96-well plate TmPPase model, which led to the discovery of novel nonphosphorus TmPPase inhibitors showing activities in the low micromolar range. From an evolutionary perspective, VrPPase resembles parasitic mPPases more than TmPPase. However, the distant relationship of TmPPase is acceptable as the active site is highly conserved between different organisms. In addition, these proteins can be produced in high quantity and purity. Interestingly, preliminary studies of selected antimalarials showed that they did not inhibit TmPPase, thereby implying the potential of mPPases as unexploited, potential drug targets. The most potent hits were further evaluated in a PfPPase inhibition assay, as well as for their ability to inhibit the growth of *P. falciparum*.

## 4.1 Design, synthesis and evaluation of 2-aminobenzo[*d*]thiazoles

Two different synthetic routes for 2-aminobenzo[*d*]thiazoles had previously been reported by our research group.[137] In short, a three-step synthetic route was used to obtain amides **33a** and **33b** (Scheme 1). The amidation of 4-nitrobenzylamine (**30**) was achieved using either benzoic acid together with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 4-dimethylaminopyridine (DMAP) in anhydrous *N*,*N*-dimethylformamide (DMF), or 2-thiophenecarbonyl chloride and triethylamine in tetrahydrofuran (THF). Subsequent Pd/C-hydrogenation of the nitro group in **31a** and **31b** gave the corresponding amines. Using ammonium thiocyanate in glacial acetic and bromine, as well as subsequent treatment with aqueous ammonia formed the 2-aminobenzo[*d*]thiazole core in **33a** and **33b**. As the yields were rather low for the other substrates, an alternative four-step route was used for most of the compounds. The key intermediate **37** was obtained in a moderate yield from diamine **34**. 4-Aminobenzylamine (**34**) was protected using *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) to give **35**, followed by 2-aminobenzo[*d*]thiazole formation. Fmoc deprotection of **36** to amine **37**, was followed by amidation and gave reasonable yields of the previously low-yielding pyrrole **33d** and indole **33e** derivatives. The 2-aminobenzo[*d*]thiazole in **33a–33i** have an important amide moiety, which works as a synthetically favorable connection point for straightforward amidations using the key intermediate **37** and various carboxylic acids or acyl chlorides. Generally, amine **37** was used with an equimolar amount of the desired carboxylic acid derivative, EDC·HCl and DMAP in anhydrous DMF. The amide couplings were carried out at room temperature for one to two days to give reasonable yields of compounds **33c** and **33e–33i**. However, in the case of **33d** no coupling agent was needed as (trichloroacetyl)pyrrole and triethylamine in DMF were used as a complementary method. However, none of these compounds contained a bromine atom that could have been beneficial to facilitate future structural studies. For instance, the anomalous signal of bromine atoms can aid in detecting the

presence of the molecule as well as recognising its orientation in low resolution (<3.5 Å) diffraction data. For that reason, some new analogs (**33g–33i**) were synthesized in the usual manner to develop a deeper understanding of the binding site.




**Scheme 1** *Synthesis of 2-aminobenzo[d]thiazoles from 4-nitrobenzylamine (**30**) or 4-aminobenzylamine (**34**). Reagents and conditions: (a) Benzoic acid, EDC·HCl, DMAP, DMF, 17 h, (**31a**), or 2-thiophenecarbonylchloride, $Et_3N$, THF, 27 h, (**31b**); (b) $H_2$, 10% Pd/C, EtOH-EtOAc, 4 h; (c) (i) $NH_4SCN$, $Br_2$, AcOH, 10–15 °C→rt, overnight (ii) 25% $NH_3$ (aq) (**33a**, **33b**); (d) Fmoc-OSu, $Et_3N$, MeCN, DMF, 1 h; (e) piperidine, DMF, 1.5 h; (f) furan-2-carboxylic acid, indole-2-carboxylic acid, benzimidazole-2-carboxylic acid, 7-bromoindole-2-carboxylic acid, 6-bromoindole-2-carboxylic acid, or 4-bromoindole-2-carboxylic acid, and EDC·HCl, DMAP, DMF, rt, 1–2 d (**33c**, **33e**–**33i**), or 2-(trichloroacetyl)pyrrole, $Et_3N$, DMF, 23 h (**33d**).*

At the starting point of this project, there were predominantly phosphorus-containing mPPase inhibitors reported such as pyrophosphate (and bisphosphonate) derivatives. These compounds are, however, not suitable as drugs due to their poor stability and extensive hydrophilicity. In order to circumvent these issues we took a starting point from the recently solved crystal structures[40,41] and the developed *Thermotoga maritima* mPPase (TmPPase) model system.[286,287] One of the earliest hits in the in-house screening process led to the revivification of a series of 2-aminobenzo[*d*]thiazoles, initially developed for anticancer, antimicrobial and antiviral activities.[137] Initially, several 2-aminobenzo[*d*]thiazole derivatives were studied (Publication I). The unsubstituted benzamide **33a** (Figure 23) was not active. Replacing the phenyl ring with five-membered heteroaromatic substituents (as in to **33b**–**33d**) gave varying results. Although a thiophene-2-carboxamide **33b** did not gain any activity, the corresponding furan, pyrrole and indole derivatives **33c**–**33e** had $IC_{50}$ values less than 26 µM. Interestingly, the most potent inhibitor **33e** ($IC_{50}$ = 1.7 µM) lost all activity if the indole moiety was replaced with a benzimidazole ring (as in **33f**). The brominated derivatives **33g–33i** unfortunately led to reduced activity in comparison to the first set of analogs, even if a bromine atom at the 7-position (**33g**) was somewhat tolerated. Structural studies of **33e** together with imidodiphosphate revealed it to be the first nonphosphorus allosteric inhibitor of *Thermotoga*

*maritima* membrane-bound pyrophosphatase. This substrate binds next to the exit channel contrary to the targeted site. The hydrophobic clamp formed can lock the exit channel in the closed formation, which prevents the full motions required for the monomer to undergo a full catalytic cycle. The transferability to pathogenic mPPases were nevertheless ineffective since this binding site turned out to be in a region not too conserved between different species.

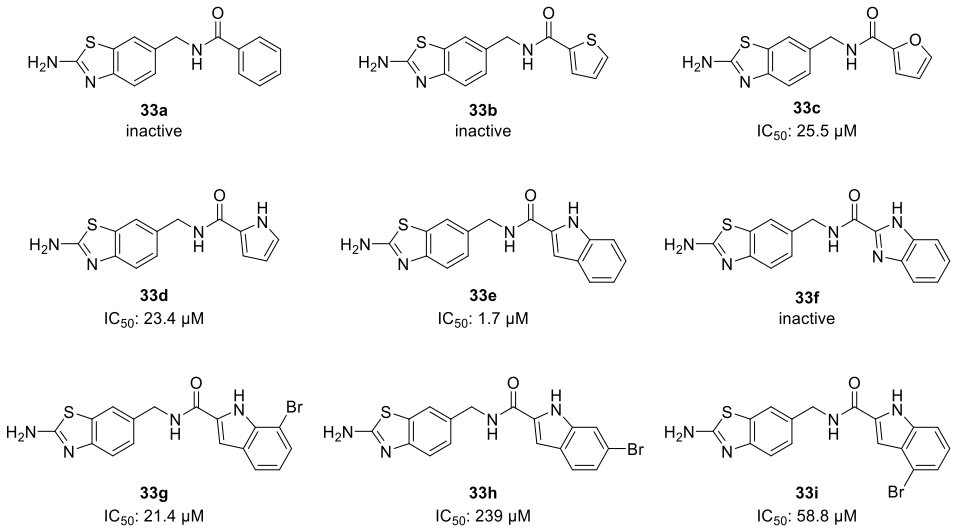
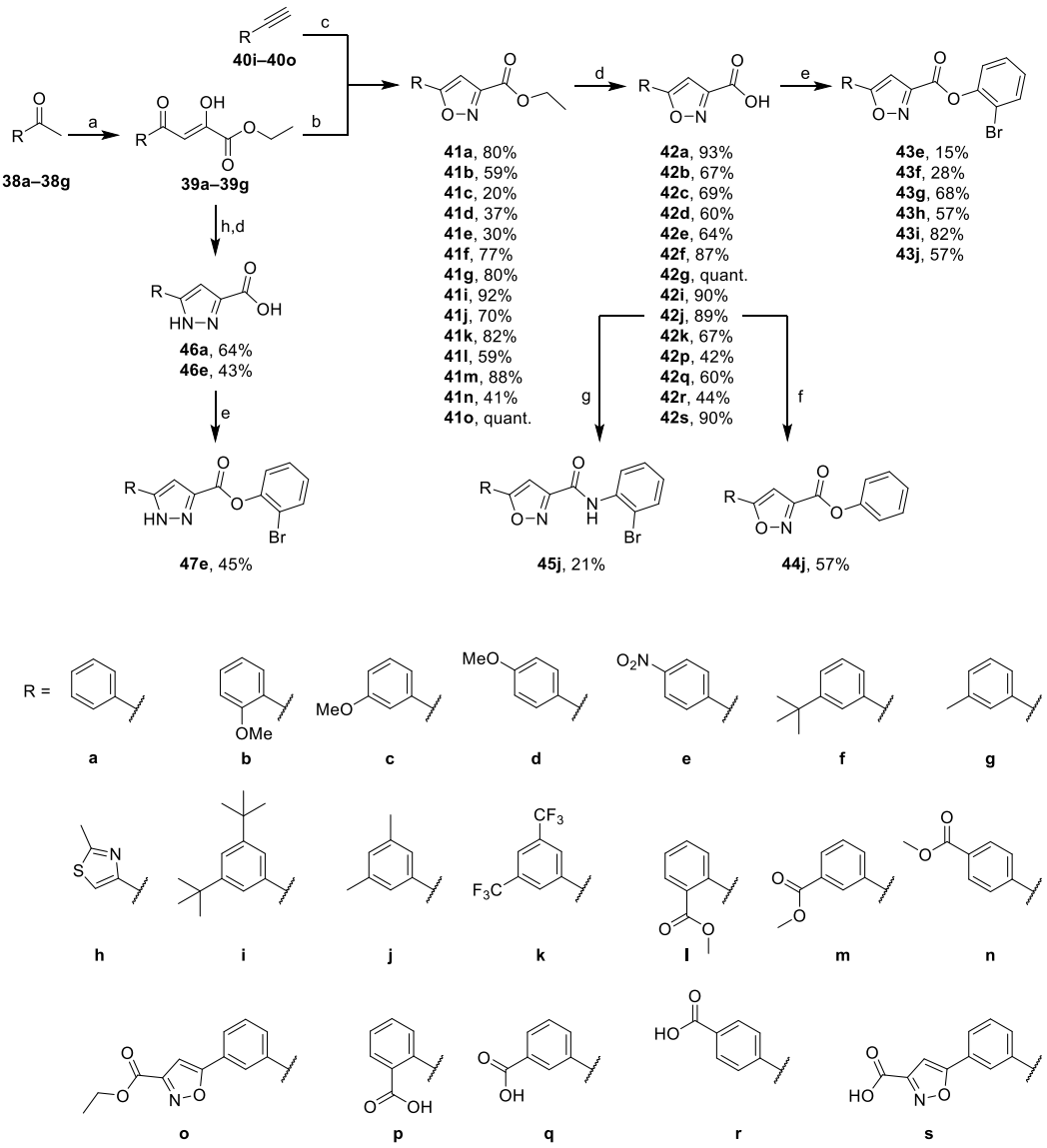



**Figure 23** *Selected screening hits of the in-house screen, including the best hit **33e** and structural analogs thereof, as well as their half maximal inhibitory concentration ($IC_{50}$) against TmPPase.*

## 4.2 Design, synthesis and evaluation of isoxazoles

Although this project is based on multiple scaffolds, one of the main branches of this study was focused on the 3,5-disubstistuted isoxazole core (Publication II). The main synthesis routes consist of condensation, cycloaddition, cycloisomerization and functionalization. Initially we were attracted to use lattermost one, as it would allow easy variation of the substituents using suitable synthetic handles, for example, boronates and Pd-catalyzed coupling reactions. This attempt was nonetheless abandoned due to limited success, except for the cycloaddition reaction that was later used for some analogs. However, as merely a limited selection of starting materials including alkyne moieties were affordably available, we also relied largely on robust enolate chemistry (Scheme 2). This opened up a possibility to use an assortment of acetophenone analogs decorated with various electron withdrawing (**e**, **k**–**n**, **p**–**r**) and donating (**b**–**d**, **f**, **g**, **i**, **j**) groups. The ethyl 5-arylisoxazole-3-carboxylates **41** were either formed from aryl alkynes **40i**–**40o** and ethyl 2-chloro-2-(hydroxyimino)acetate (forming nitrile oxide *in situ*) in a one-step [3 + 2] cycloaddition reaction, or from aryl ketones **38a**–**38g** and diethyl oxalate in a crossed condensation reaction to give **39a**–**39g**, followed by a ring condensation with hydroxylamine. Similarly, corresponding pyrazoles could conveniently be made by replacing the hydroxylamine with hydrazine. After that, the ethyl carboxylates were, in the presence of lithium hydroxide, hydrolyzed to their corresponding carboxylic acids **42** and **46**, some of which were further activated as acyl chlorides and converted to other esters as e.g., 2-bromophenyl 5-arylisoxazole-3-carboxylates (**43e**–**43j**, **47e**).




**Scheme 2** *Synthesis of isoxazoles from ketones or alkynes, as well as pyrazoles from ketones. Reagents and conditions: (a) NaOEt, diethyl oxalate, EtOH, reflux, 2 h (**39a**) or NaOEt, diethyl oxalate, EtOH/Et₂O, rt, overnight (**39b–39g**); (b) H₂NOH·HCl, EtOH, reflux, 3 h (**41a–41g**); (c) Ethyl 2-chloro-2-(hydroxyimino)acetate, NaHCO₃, MeCN, rt, 3–5 d (**41i–41o**); (d) LiOH, EtOH/H₂O, 1–48 h (**42a–42g**); (e) 2-Bromophenol, EDC·HCl, DMAP, DIPEA, DCM, rt, overnight (**43e**, **47e**), or (i) (COCl)₂, DMF, DCM, rt, 0.5–1 h, (ii) 2-bromophenol or phenol, Et₃N, 0 °C → rt, 1–5 h (**43f–j**, **44j**); (f) N₂H₄·H₂O, AcOH, EtOH, rt, 5 h; (g) 2-Bromoaniline, KOtBu, THF, rt, 1 h; (h) N₂H₄·H₂O, AcOH, EtOH, rt, 5 h.*

Following the previous findings, 16 phosphate-mimicking fragments were purchased as a pilot screen and tested in the TmPPase assay at 100 µM concentration. The compounds of this fragment library had a size range of 143–338 Da and consisted of commonly known phosphate isosteres such as boronate, isoxazole, sulfone and sulfonamide moieties.[107] Four of the compounds (**48–51**, Figure 24)

showed inhibition greater than 50%. The fragments were all rather heteroatom-rich bicycles of which the isoxazole fragment was chosen for further exploitation.

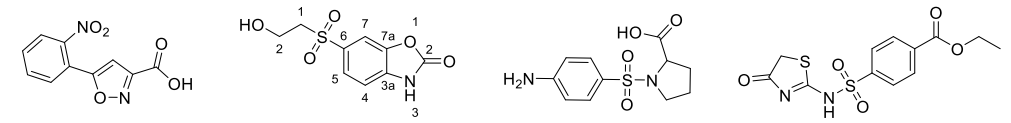



**Figure 24** *The best hits, including their molecular weight (MW) and their $IC_{50}$ expressed as a 95% confidence (given in square brackets) against TmPPase, of the pilot screen.*

From these initial hits, we decided to move forward with the smallest and synthetically appealing isoxazole fragment (Publication II and III). Since the other fragments contained either sulfone or sulfonamide linkers, a sulfonamide moiety exploration of commercially available 5-arylisoxazole-3-carboxylates was performed. A small sulfonamide series of carboxylic acid and alkyl ester analogs, where the *ortho*-nitro substituent predominantly had been replaced with a *para*-methoxy group, were tested. Although the inhibition of the best sulfonamide (**52**, Figure 25) achieved a 2-fold $IC_{50}$ improvement, the screen did not promote any further advancements. Introducing sulfonamides to the 5-arylisoxazole-3-carboxylate core showed overall sensitivity to minor alterations and were hence rounded off. However, a simple arylsulfonamide lacking the isoxazole moiety indicated promising activity and might be followed up. Replacements of the aryl moiety with heteroaryl analogs showed that some changes were well tolerated, as in the 2-methylthiazol-4-yl analog (**42h**), whereas others were not, as in the corresponding 1,5-dimethyl-1*H*-pyrazol-4-yl analog (**53**). Compound (**42h**) was, in addition to IDP, *de facto* used as a positive control in the TmPPase assay due to its low micromolar activity ($IC_{50}$ = 17 µM).

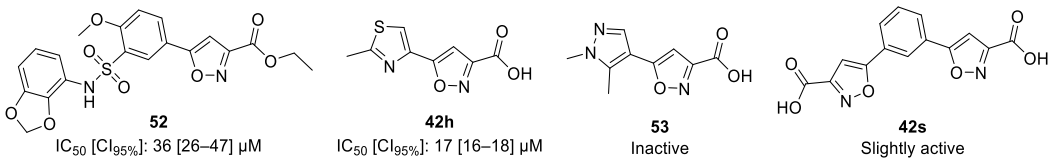



**Figure 25** *The most potent sulfonamide hit, selected 5-heteroarylisoxazole-3-carboxylates and the dicarboxylic isoxazole analog, as well as their $IC_{50}$ against TmPPase.*

After this early exploration of analogs to isoxazole **48**, we decided to study how various aryl substituents would affect the activity. The simplest unsubstituted phenyl analog **42a**, as well as the corresponding substituted analogs decorated with either electron withdrawing or donating substituents, did not show any improvement in the activity. A similar outcome was obtained from the molecular modeling-supported approach of bridging the positively charged interaction sites in the substrate site with, for example, dicarboxylic isoxazole analog **42s** (Figure 25).

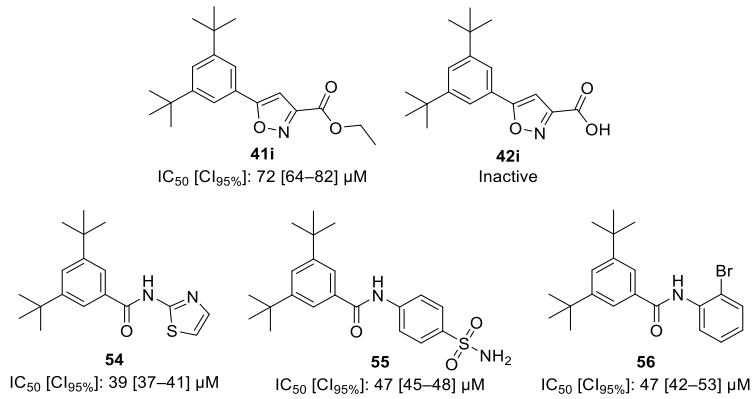



**Figure 26** *5-(3,5-Di-tert-butylphenyl)isoxazoles and selected 3,5-di-tert-butylphenyl analogs, including their $IC_{50}$ against TmPPase.*

Through the synthesis route developed, the corresponding ethyl carboxylates were also available for testing. However, most of the esters were not showing any noteworthy activities apart from ethyl 5-(3,5-di-*tert*-butylphenyl)isoxazole-3-carboxylate (**41i**, Figure 26) that was more active than its corresponding acid analog (**42i**). This finding led us to further explore the importance of the 3,5-di-*tert*-butylphenyl moiety. Three of these analogs (**54–56**), containing a thiazole, *para*-phenylsulfonamide or *ortho*-bromophenyl fragment, showed a 2-fold gain in comparison to the starting point (**48**). Coincident modifications of the isoxazole moiety with other heterocycles showed that both pyrazole (as in **46a**, Figure 27) or thiazole (as in **57**) were more active than their corresponding isoxazole analog (**42a**). Altogether, these observations demonstrate the significance of the thiazole ring (as in **42h** and **57**) and the 3,5-di-*tert*-butylphenyl moiety (as in **41i** and **54–56**).

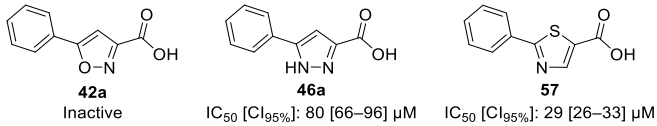



**Figure 27** *Heterocyclic modifications of the central core, as well as their $IC_{50}$ against TmPPase.*

To better support further structural information via X-ray crystallographic studies, compounds containing bromine atoms were desired. As some of the compounds tested indicated the importance of an *ortho*-bromophenyl fragment (e.g. **56**) this fragment was introduced as *ortho*-bromophenyl carboxylates of our hitherto most favorable compounds (**41i**, **42h**, **57**). Unfortunately, this modification led to a 27-fold and 1.6-fold loss in activity for esters **43h** and **58** (Figure 28) compared to their corresponding carboxylic acids (**42h** and **57**), respectively. However, the alteration of compound **41i** to 2-bromophenyl carboxylate **43i** was favorable. Low micromolar activities were similarly seen for other alkylated analogs (**43f**, **43g**, **43j**) in an attempt to gain higher ligand efficiency. Alterations to the alkyl groups were allowed but removing the bromine (as in **44j**) was only somewhat tolerated with a 3.6-fold loss of inhibition. To enhance the chemical stability, and to explore different interactions, the ester bond was replaced with the corresponding amide (**45j**), which triggered a complete loss of activity. However, alternative bioisosteric replacements could possibly have been more favorable.

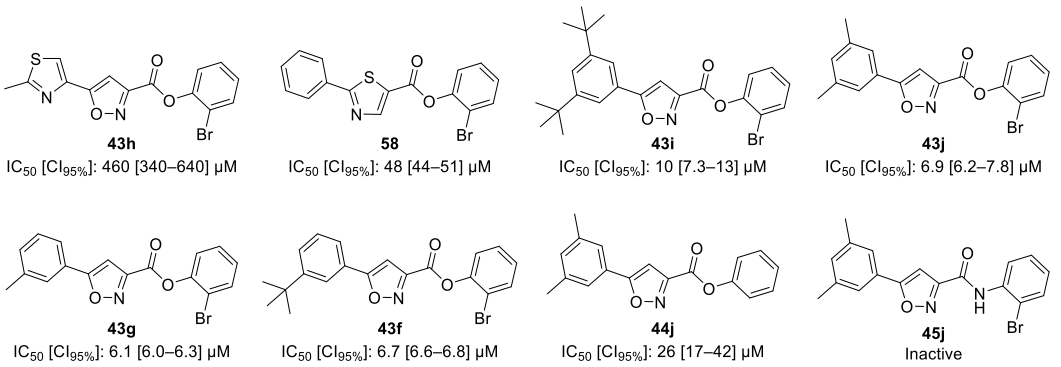
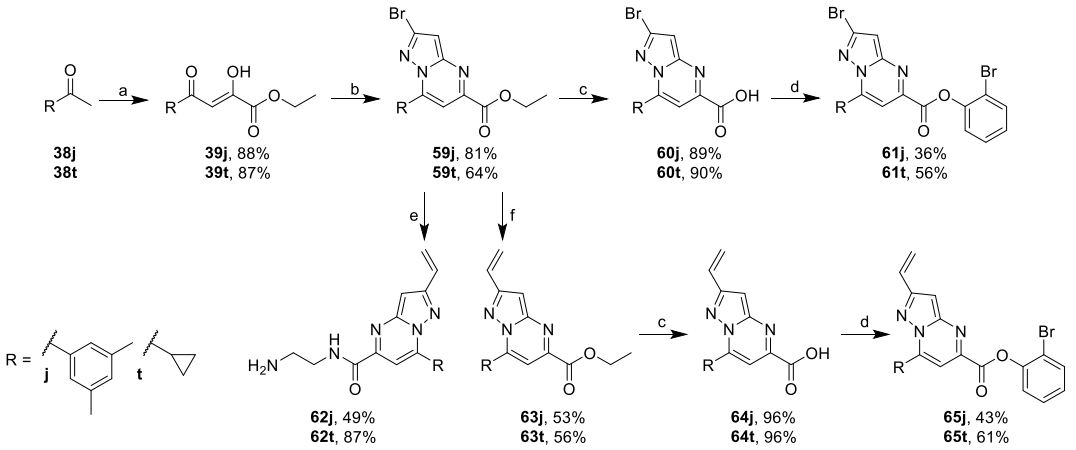



**Figure 28** *An assortment of alterations, including their $IC_{50}$ against TmPPase.*

Consequently, the potential of carboxylates **43i** and **43j** (Figure 28) to inhibit also pathogenic membrane proteins was studied using expressed PfPPase-VP1 gene (PF3D7_1456800) in baculovirus-infected insect cells. The inhibition assay, performed on the PfPPase-VP1 in the membrane, showed that only compound **43j** ($IC_{50}$ = 55 μM) inhibited the PfPPase-VP1 activity at the tested concentrations. Thus, carboxylate **43j** was further tested in a *P. falciparum* survival assay in erythrocytes indicating a somewhat modest inhibition ($IC_{50}$ = 99 μM). These findings might not prove a direct mechanism of action, as inhibition may occur via soluble pyrophosphatases or some other mechanism. However, similar isoxazole compounds have also been found to inhibit *T. cruzi* parasites,[174] underlining the relevance of these hits for anti-parasitic drug discovery.

## 4.3 Design, synthesis and evaluation of pyrazolo[1,5-*a*]pyrimidines

Compound **39j** (Scheme 2), formerly used as a key intermediate in the ring condensation of isoxazoles and pyrazoles (Publication II), has also been used in the synthesis of pyrazolo[1,5-*a*]pyrimidines.[226] Crossed condensation of diethyl oxalate and 3,5-dimethylacetophenone (**38j**) or acetylcyclopropane (**38t**), followed by ring condensation of **39j** and **39t** using 3-bromo-1*H*-pyrazol-5-amine formed the pyrazolo[1,5-*a*]pyrimidine core (Scheme 3, Publication III). Ethyl carboxylates **59j** and **59t** were hydrolyzed into the corresponding acids **60j** and **60t** using lithium hydroxide and esterified with 2-bromophenol to give the corresponding aryl esters **61j** and **61t**. The new analogs seemed to tolerate modification of the isoxazole to a larger central core. However, as the molecular weight started to exceed 500 Da, we consequently sought to shrink the aryl moiety (**j**) to a smaller cyclopropyl ring (**t**). This was, however, not beneficial for the activity in most cases. The introduction of the 2-bromo substituent in the pyrazole ring also opened for further functionalization possibilities using Suzuki or some other palladium-mediated coupling reactions. For that reason, we made the corresponding vinyl analogs **63j**–**65j** and **63t**–**65t**, which led us to the unanticipated simultaneous amidation and Suzuki coupling giving **62j** and **62t**.




**Scheme 3** *Synthesis of pyrazolo[1,5-a]pyrimidines from 3,5-dimethylacetophenone (**38j**) or acetylcyclopropane (**38t**). Reagents and conditions: (a) KOtBu, diethyl oxalate, THF, rt,1 h; (b) 5-Bromo-1H-pyrazol-3-amine, HCl (aq), EtOH, mw, reflux, 30 min; (c) LiOH, EtOH/$H_2O$, 16–72 h; (d) 2-Bromophenol, HATU, DIPEA, DMF, rt, overnight; (e) Potassium trifluoro(vinyl)borate, Pd(dppf)$Cl_2$, ethylenediamine, n-PrOH, mw, 125 °C, 15 min; (f) Potassium trifluoro(vinyl)borate, Pd(dppf)$Cl_2$, $Et_3N$, EtOH, mw, 125 °C, 15 min.*

Taking inspiration from the previously designed isoxazole scaffold, we were curious about incorporating more heteroatoms by using a bicyclic central core decorated with familiar substitution pattern and/or substituents. Furthermore, screening of commercial analogs of the hitherto best inhibitors revealed a promising bicyclic scaffold with a 4,5-dihydropyrrolo[3,4-*c*]pyrazol-6(1*H*)-one core (Publication III). These hybrid approaches led us to focus on pyrazolo[1,5-*a*]pyrimidines as a potential scaffold. As the direct scaffold hopping strategy resulted in similar activity, we decided to move on continuing to improve the overall physicochemical properties of the compound as the molecular weight of the direct analog **61j** (Figure 29) was around 500 Da. The quick fix was to change the 3,5-dimethylphenyl ring to some smaller carbocycle, such as a cyclopropyl ring. However, these analogs were not active at all in most cases. Alternatively, replacing the bromine in the 2-position with various coupling handles e.g., vinyl as in **65j** kept the activity intact. Interestingly, we managed to remove the 2-bromophenyl group without any loss of activity in the case of **65j**, whereas neither of the corresponding carboxylic acids **64j** nor **64t** showed any activity against TmPPase.

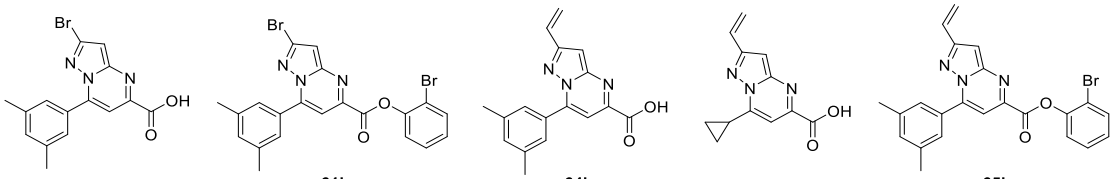
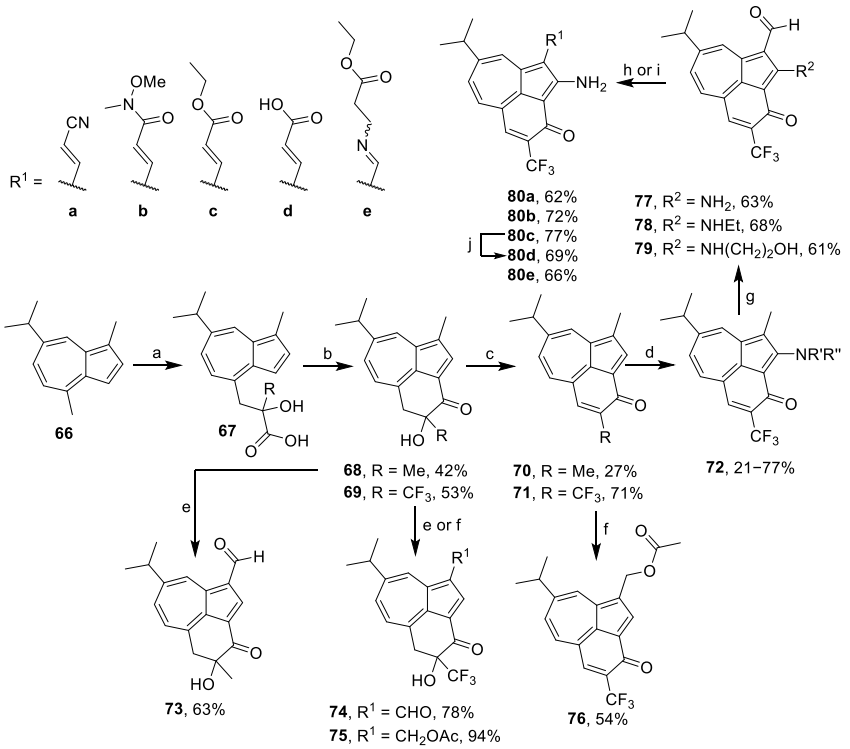



**Figure 29** *Selected 2,5,7-trisubstitued pyrazolo[1,5-a]pyrimidines as well as their $IC_{50}$ against TmPPase.*

The three most potent compounds were chosen for follow-up studies and tested against purified mPPase from *P. falciparum* (PfPPase-VP1) expressed in baculovirus-infected insect cells. As in the case of the isoxazole series, there was a severalfold loss in activity from the primary assay. The $IC_{50}$ values of vinyl derivative **64j** and **65j** (Figure 29) were 130 µM and 74 µM, respectively. Compound **61j** ($IC_{50}$ = 58 µM) appeared to be the most promising one and it was further evaluated in a parasite survival assay in erythrocytes. The growth of *P. falciparum* was inhibited with an $IC_{50}$ value of 31 µM showing a 2-fold improvement to the PfPPase assay. However, this might also be linked to inhibition of some other protein in the parasite such as insoluble pyrophosphatases, or due to colloidal aggregation.

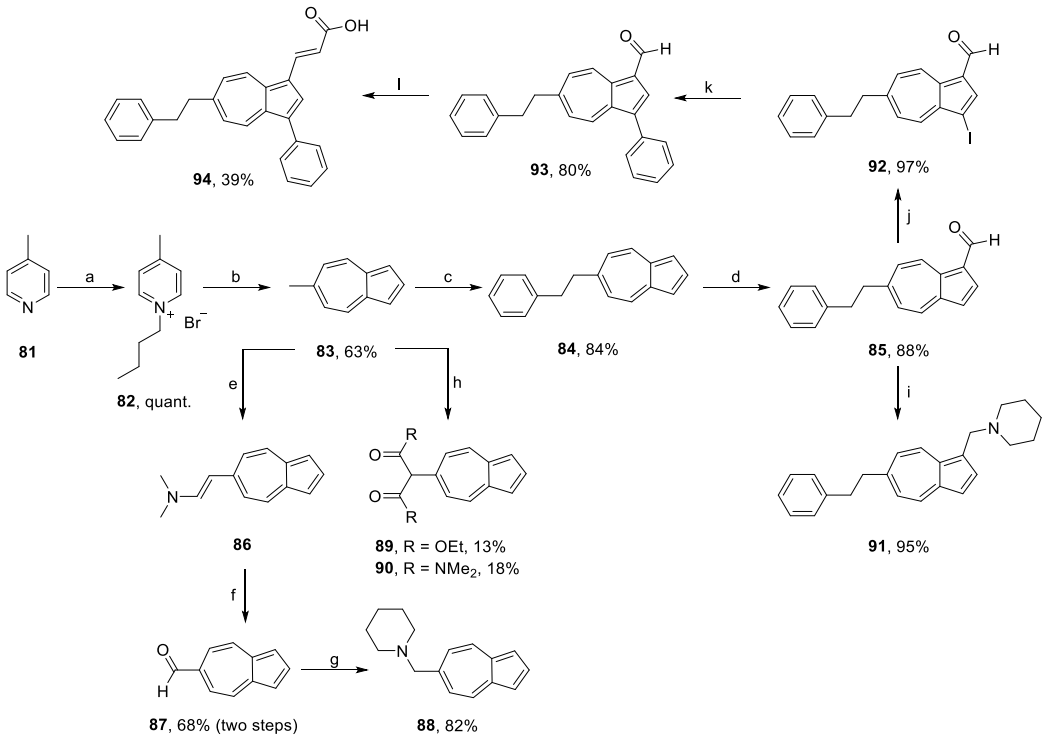
## 4.4 Design, synthesis and evaluation of azulenes

Previous findings of our research group had led us into the multicolored world of azulenes.[271,285,288,289] Depending on the desired substitution pattern, this chemistry has been developed either starting from naturally occurring guaiazulene (Scheme 4), or by construction of the azulene core itself from commercially available starting materials such as 4-picoline (Scheme 5) or halogenated cinnamic acid derivatives (Scheme 6). In the first route (Scheme 4), the 4-methyl group in guaiazulene (**66**) is first deprotonated with LDA, followed by alkylation using trifluoromethyl or ethyl pyruvate.[288,289] Subsequent ester hydrolysis and intramolecular Friedel-Crafts acylation promote ring annulation and formation of the tricyclic dihydrobenzo[*cd*]azulen-3-one core. This dihydrobenzazulene scaffolds, **68** and **69**, were used as such for DDQ oxidation of the 1-methyl group to give the corresponding 1-acetoxy (**75**) or 1-formyl (**73**, **74**) analogs (Publication IV). Removal of the chiral center in **68** and **69** by TFAA-mediated dehydration led to structurally more rigid benzo[*cd*]azulen-3-ones that also could be subjected to DDQ oxidation. However, the conversion of 1-acetoxy derivative **76** was about half of its unsaturated analog **75**. On the other hand, the three initial synthetic steps were more favorable for the key intermediate **71** (R = $CF_3$) than for the corresponding methyl analog **70**. Besides 1-methyl oxidation using DDQ, having a quinone methide moiety merged in the benzazulene core also opened up for oxidative amination/thiolation at the 2-position.[289] Diversely substituted amines were used to give primary, secondary and tertiary 2-aminobenzazulenes (**72**) with a range of functional groups and heterocycles (Publication IV). DDQ oxidation of these compounds gave the corresponding aldehydes (e.g., **77–79**). Additionally, the introduced formyl moiety of **77** could further be functionalized using various phosphine ylides in a Wittig reaction (**80a–80c**) or by reductive amination with amino acids (**80e**) for example. Subsequent hydrolysis of ethyl ester **80c** gave the corresponding carboxylic acid **80d**.



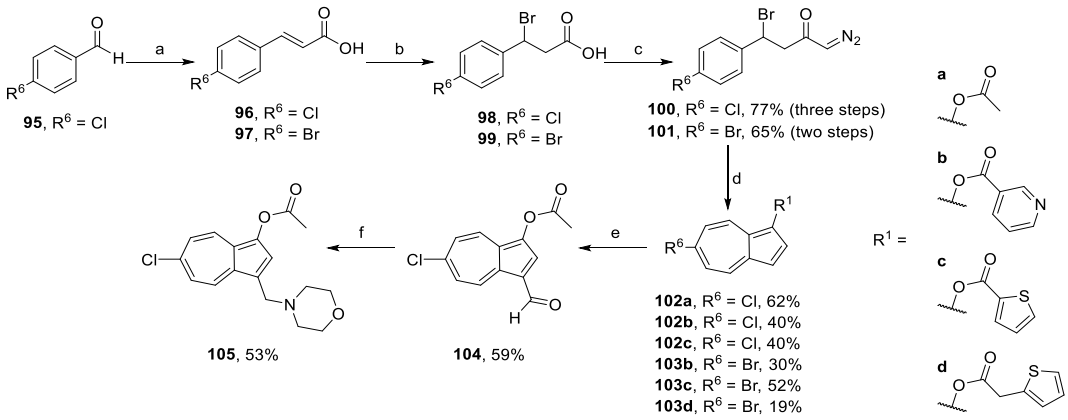

**Scheme 4** *Synthesis of benzazulenes from guaiazulene. Reagents and conditions: (a) 1) LDA, $Et_2O$, −16 °C, 25 min, then trifluoromethyl pyruvate or ethyl pyruvate, −16 °C to rt, 45 min, 2) NaOH, MeOH-THF-$H_2O$, 0 °C, 2 h, then rt, 6 h; (b) p-TSA, $CHCl_3$, rt, 6 d; (c) TFAA, pyridine, 0 °C, 2 h, then 65 °C, 4 h (**70**), or TFAA, pyridine, 0 °C, 3.5 h (**71**); (d) Prim. or sec. amine, 1,4-benzoquinone, THF, or amine hydrochloride, $Et_3N$, DMF/MeOH, 0 °C to rt, 2–48 h; (e) DDQ, MeOH, rt, 1 h (**73**, **74**); (f) DDQ (1 equiv.), AcOH, rt, 4.5–5.5 h (**75**, **76**); (g) DDQ, THF-$H_2O$, −16 °C, 30 min; (h) Wittig reagent, THF, microwave (mw), 75–90 °C 2–5 h (**80a–80c**); (i) β-Alanine-OEt·HCl, $NaBH(OAc)_3$, $Et_3N$, THF, rt, 2 d; (j) THF-MeOH-$H_2O$, rt, 25 h.*

In the second route, 4-picoline (**81**, Scheme 5) is *N*-alkylated using 4-bromobutane and the resulting pyridinium salt **82** was treated with sodium cyclopentadienide to give 6-methylazulene (**83**).[271] Deprotonation of this intermediate with LDA in the presence of electrophiles gave various 6-substituted azulene derivatives. For instance, **83** and benzylbromide, *N*,*N*-dimethylformamide dimethyl acetal (DMF-DMA), ethyl chloroformate or dimethylcarbamoyl chloride was used to give compound **84**, enamine **86**, diethyl carboxylate **89** or dimethylamine **90**, respectively. Subsequent oxidation of the double bond in **86** resulted in the corresponding 6-formylazulene (**87**) that was used in reductive amination with piperidine to give **88**. Vilsmeier–Haack reaction of **84** led to monoformylation and the corresponding aldehyde **85** could be used in reductive amination to give the tertiary amine **91**. Halogenation of the 3-position in **85** using *N*-iodosuccinimide (NIS) was a convenient way to introduce a coupling handle in **92**. The iodine substituent was used in Suzuki cross coupling to give **93**, and subsequent Knoevenagel condensation resulted in carboxylic acid **94**.




**Scheme 5** *Synthesis of azulenes from 4-picoline. Reagents and conditions: (a) BuBr, EtOH, reflux, 21 h; (b) NaH, cyclopentadiene, DMF, rt, 5 min, then microwave (mw), 200 °C 15 min; (c) 1) LDA, THF, 0 °C to rt, 30 min, 2) BnBr, −78 °C to rt, 40 min; (d) Vilsmeier reagent, DCM, 0 °C to rt, 30 min; (e) DMF-DMA, DMF, 140 °C, 7 h; (f) $NaIO_4$, THF-$H_2O$, rt, 1 h; (g) piperidine, $NaBH(OAc)_3$, DCM, rt, 5 h; (h) 1) LDA, THF, 0 °C to rt, 30 min, 2) ethyl chloroformate or dimethylcarbamoyl chloride, −78 °C to rt, 1 h; (i) piperidine, $NaBH(OAc)_3$, DCM, rt, 5 h; (j) NIS, DCM, 0 °C, 1 h; (k) $PhB(OH)_2$, $Pd(dppf)Cl_2$, BINAP, $Cs_2CO_3$, PhMe, mw, 110 °C, 1 h; (l) malonic acid, piperidine, pyridine, MW, 130 °C, 30 min.*

In the third route, cinnamic acid **96** (Scheme 6) was made from the corresponding *para*-chlorobenzaldehyde through Knoevenagel condensation with malonic acid.[285] The double bond of the cinnamic acid derivatives **96** and **97** was hydrobrominated with HBr in acetic acid. Subsequent activation of the carboxylic acids **98** and **99** using oxalyl chloride led to the corresponding acyl chlorides that were treated with (trimethylsilyl)diazomethane. The formed diazoketones **100** and **101** were then treated with rhodium(II) pivalate in order to form the azulene ring system, followed by instantaneous esterification using various electrophiles to give 1-acyloxy azulenes **102a–102c** and **103b–103d**. Further functionalization could also be obtained, for example, by using the halogen at the 6-position in various cross coupling reactions. Formylation using phosphoryl chloride gave **104** that was subsequently converted to **105** through reductive amination with morpholine.




**Scheme 6** *Synthesis of azulenes from p-chlorobenzaldehyde. Reagents and conditions: (a) malonic acid, piperidine, pyridine, 105 °C, 21 h; (b) 33% HBr in AcOH, rt, 4–9 d; (c)1) $(COCl)_2$, PhH, 65 °C, 18 h, 2) $TMSCHN_2$, MeCN, 0–4 °C, 24 h; (d) 1) $Rh_2(OPiv)_4$, DCM, rt, 1.5 h, 2) acetic anhydride, nicotinoyl chloride hydrochloride, thiophene-2-carbonyl chloride, or 2-thiopheneacetyl chloride, DMAP, rt, 5–15 min; (e) $POCl_3$, DMF, rt, 45 min; (f) morpholine, $NaBH(OAc)_3$, DCM, rt, 2.5 h.*

Screening of the proprietary in-house compound library led, besides the aforementioned 2-aminobenzo[*d*]thiazoles (Publication I), to the exploration of azulene-based derivatives (Publication IV). The initial hits, **68** and **69** (Figure 30), were racemic dihydrobenzazulenes with $IC_{50}$ values in the 75–90 μM range. Oxidation of the 1-methyl group in **69** to the corresponding 1-formyl (**74**, $IC_{50}$ = 5.2 μM) or 1-acetoxy (**75**, $IC_{50}$ = 29 μM) derivative improved the activity 17- and 3-fold, respectively. Removal of the chirality through dehydration of the dihydrobenzazulenes consequently gave the structurally more rigid benzazulene core. Compounds **70** and **71** were both more active than the initial hits. Similarly, rigidification of **75** led the most potent benzazulene **76** with an $IC_{50}$ value of 1.5 μM.

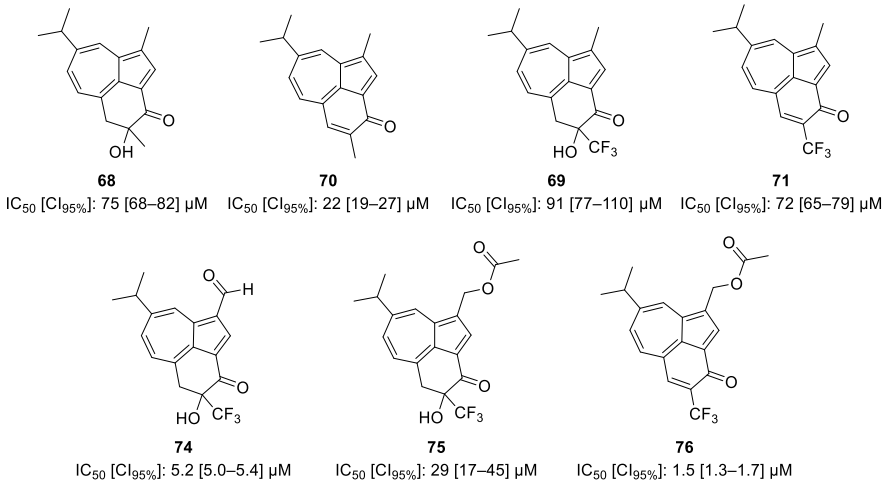



**Figure 30** *Initial hits and early modifications of the dihydrobenzazulene and benzazulene scaffold, including their $IC_{50}$ against TmPPase.*

Oxidative amination at the 2-position of the benzazulene core led in some cases to low micromolar activities, for example, bearing carboxylic acids, whereas other attempts were completely inactive (Figure 31). However, this position was also sensitive to modifications. Adding an amino group to **71** led to 2-fold gain in activity for the primary 2-aminobenzazulene **106**. On the contrary, the corresponding methylamine **107** showed no activity, even if ethylamine **108** had an $IC_{50}$ of 6.0 µM. Further elongation of the ethyl chain was moderately tolerated for the carboxylic acid **109** ($IC_{50}$ = 38 µM) but not at all for the corresponding alcohols **110** and **111**. Similar lack of activity was noticed for methyl carboxylate **80c**, although low micromolar activity could be recovered replacing the ester group with a carboxylic acid (**80d**, $IC_{50}$ = 15 µM). The aromatic carboxylic acid **112** showed activity in the same range. Even though the corresponding aromatic alcohol **113** was merely moderately tolerated, it was noticeably better than the aliphatic alcohols. In addition, subsequent oxidation of the methyl group at the 1-position indicated it not to be beneficial for the 2-aminobenzazulenes, as shown by loss in activity for both **77** and **78**. The most potent benzazulene **76** was indeed oxidized at the 1-position but unsubstituted at the 2-position. Consequently, these azulene-based scaffolds showed susceptibility towards functional group modifications and gave rise to ambiguous SAR. Moreover, these scaffolds have a large aromatic core that make them potentially promiscuous.

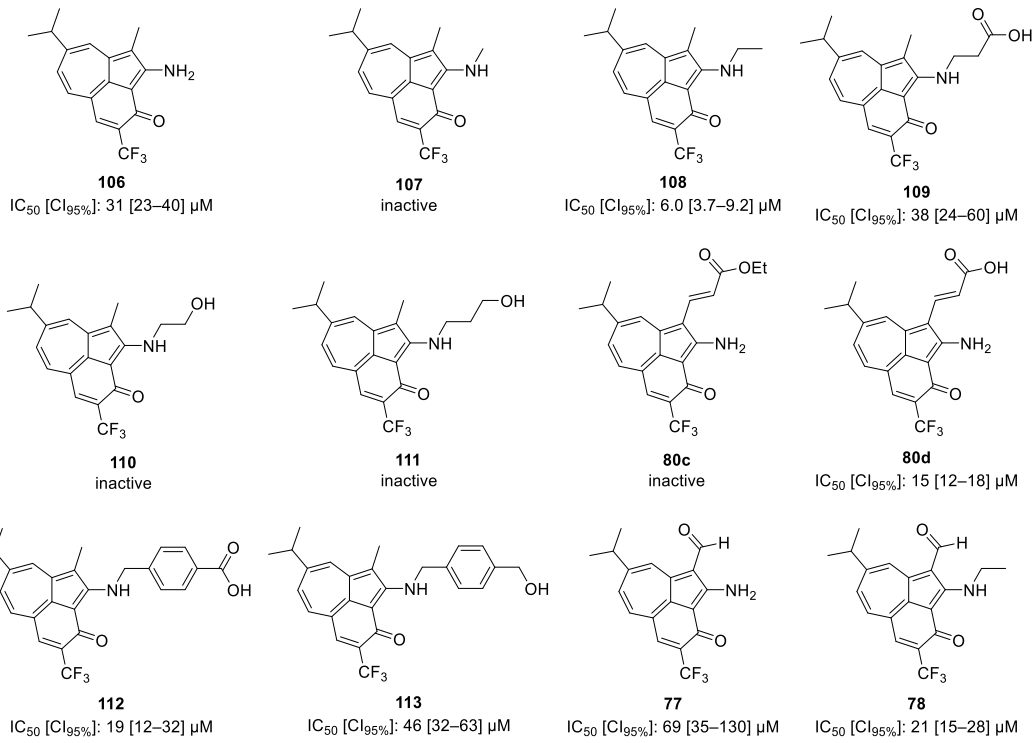



**Figure 31** *Selected 2-aminobenzazulenes and modifications thereof, including their $IC_{50}$ against TmPPase.*

Rational simplification by reducing the overall size of the dihydrobenzazulene and benzazulene derivatives left us merely with the azulene core. Functionalization of the 2-position in azulene was not as straightforward as for the benzazulenes, though. Azulene **94** ($IC_{50}$ = 36 µM, Figure 32) bearing

the same substituent at the 1-position as benzazulene **80d** (Figure 31) showed a small loss in activity, which was comparable to the $IC_{50}$ value of the structurally related carboxylic acid **109**. The modification of the five-membered appears to be important as none of the merely 6-substituted azulenes (**88**–**90**, **114**) showed any inhibition. Introduction of halogens such as bromine not only opened possibilities for further functionalization of the seven-membered ring but may also be helpful for forthcoming X-ray crystallography studies. Altering the position of the halogen substituent, as in the 5- and 6-halogenated compounds **115** and **102a** bearing a 1-acetoxy group, showed activities in the 14–30 µM range. Further exploration of the 3-position, as in compounds **104** and **105**, was well tolerated with a small gain in activity compared to **102a**. Furthermore, alternative 6-halogenared carboxylates were explored, most of them showing comparable inhibition for various substituents at the 1-position, including compounds **116** ($IC_{50}$ = 35 µM) and **102b** ($IC_{50}$ = 27 µM). Generally these brominated and chlorinated derivatives showed moderate to good activity, the best azulene **103d** having an $IC_{50}$ value of 5.2 µM.

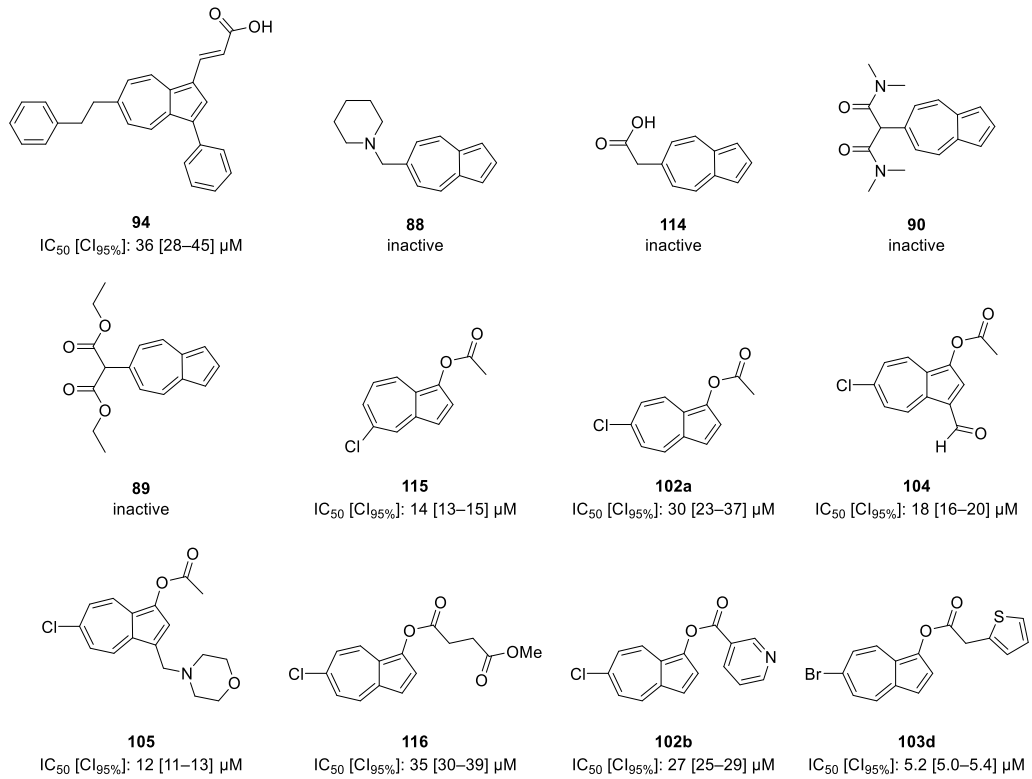



**Figure 32** *An assortment of azulene alterations and their $IC_{50}$ against TmPPase, and their $IC_{50}$ against TmPPase.*

A selection of azulene derivatives were further evaluated for their potential to inhibit the growth of *P. falciparum* in a parasite survival assay in erythrocytes. The $IC_{50}$ values of the benzazulene derivatives were inconclusive and occasionally contradictory to the results from the primary TmPPase assay. Benzazulene **110** (Figure 31) was inactive in the primary assay but showed a severalfold improvement in activity the survival assay ($IC_{50}$ = 26 µM). Yet again, this could be a consequence

of, for example, inhibition of some other protein in the parasite via some other mechanism, or because of colloidal aggregation. Azulenes **102b** and **103d** (Figure 32) showed a 5-fold and 2-fold improvement in activity in parasites compared to the TmPPase assay, respectively. On the contrary, dihydrobenzazulene **74** (Figure 30) showed a 2-fold loss in activity in the survival assay. In addition to low micromolar TmPPase inhibition, azulene **103d** ($IC_{50}$ = 2.6 µM) showed in fact the hitherto best translation also against *P. falciparum* growth in erythrocytes.

# 5 Summary, conclusions and outlook

Pyrophosphate hydrolyzing and ion pumping membrane-bound pyrophosphatases do not exist in humans but are essential for many pathogenic protozoan parasites. Especially for *Plasmodium* spp. causing malaria but also other protist diseases having complex life cycles where protozoan cell types occur in transitions between environment, for example, from host to vector and vice versa. These dimeric, integral proteins have a unique crystal structure, which was solved for the first time a decade ago for *Thermotoga maritima* and *Vigna radiata* mPPase, respectively. Although VrPPase is closer in terms of evolutionary relationship to parasitic mPPases, TmPPase was used as the primary model assay in this study. TmPPase has only a distant relationship with parasitic mPPase, but the active site of the drug target is in fact well conserved across organisms. In this way, the protein can be produced in high quantity and purity. The structure of TmPPase in different conformations has been solved as well. Subsequent evaluation of the antiparasitic activity of the best inhibitors is verified using PfPPase-VP1 or in a parasite survival assay of *P. falciparum* in erythrocytes. Furthermore, targeting enzymes absent in animals lowers the probability of harming human cells. For that reason, mPPases are interesting, potential drug targets for development of new treatments for devastating protist diseases as malaria and other neglected tropical diseases.

The design, synthesis, and biological evaluation of nonphosphorus mPPase inhibitors presented herein may offer an alternative approach against protist diseases, especially in the continuous combat concerning drug-resistant parasites. To evaluate selectivity against mPPase from human pathogens and on-target potency, both synthesized and commercial compounds were screened against TmPPase and PfPPase. Initial hits, both from structure-based design approaches as well as available in-house compounds from other projects, were identified through our TmPPase-based model assay. Of the top inhibitors, the most synthetically appealing one with an isoxazole core was chosen for further refinement. The serendipitous in-house screening resulted in two alternative scaffolds as well. These inhibitors were synthesized using various synthetic strategies and attempts to co-crystallize the best scaffolds with mPPase were performed. This structure determination of the substrate-enzyme complex would allow studies of the binding site/mode, which could provide valuable information for development of selective inhibitors. If these nonphosphorus inhibitors were to bind at the same target site as the bisphosphonates, it appears that an amino or a hydroxyl substituent at the α-carbon might interact with charged amino acid residues. Additionally, the size and hydrophobicity suggest the catalytic site might be located in a small hydrophobic pocket in the protein. On the other hand, this remains to be confirmed by further crystallographic studies of the best nonphosphorus inhibitors.

In the beginning, the crystal structure was solved for the 2-aminobenzo[*d*]thiazole-based inhibitors, originating from in-house cross-project screening. Different aromatic heterocycles were evaluated but none were superior to indole **33e** (Figure 33). This demonstrates the importance of hydrogen bonding and aromaticity, as well as the tolerance of both hydrogen bond donating and accepting ring structures. However, removal or substitution of the fused phenyl ring led to a loss in activity. Similarly, a benzimidazole ring (**33f**) caused all activity to be lost. At physiological pH, both the 2-aminobenzo[*d*]thiazole and the benzimidazole would be protonated and thus repel each other leading to loss of the π–π head-to-tail stacking. As the TmPPase:IDP:**33e** structure showed two protein homodimers binding a single inhibitor dimer each, a potential continuation could be to try to link the compounds together for increased stability. Unfortunately, the low micromolar activity of **33e** in TmPPase was 40-fold lower in PfPPase with no anti-plasmodial activity in the *P. falciparum* survival assay. This is because the inhibitor binds to the enzyme in an allosteric manner near to the exit channel, in a nonconserved region. Aside from the catalytic region, this observation further

demonstrates the potential of alternative binding sites that significantly affect substrate binding. Furthermore, this indicates the unexploited potential of species-specific regions in the development of selective mPPase inhibitors.

The isoxazole series led to the discovery of several low micromolar TmPPase inhibitors. Although the initial hit **48** was active, the expansion of various carboxylic acid analogs were not advantageous. Apart from compound **41i**, most of the corresponding ethyl carboxylates showed little to no activity. On the other hand, several 2-bromophenyl carboxylates (**43f**, **43g**, **43j**) showed low micromolar activity. This might be a result of the esters acting as a prodrug, assisting the inhibitor through some hydrophobic part of the enzyme where the acids were unsuccessful. Supporting this assumption is that the more stabile amide derivative **45j** lost all activity. However, an amide bond is also less flexible than the ester bond, which could restrict the inhibitor from altering conformation as required to affect TmPPase. Alternative bioisosteres could perhaps offer increased chemical stability without loss of inhibitory activity. One of the early hits in this exploration, compound **42h**, was on the contrary more active as the carboxylic acid than its corresponding 2-bromophenyl carboxylate. As most 2-bromophenyl 5-arylisoxazole-3-carboxylates showed low micromolar activity, this highlight the importance of predominantly alkyl-substituted aryl moieties over heteroaryl substituents. Hence, these compounds might have alternative binding modes or sites. One weakness of especially the *tert*-butyl phenyl carboxylates is reduced water solubility at concentrations greater than their $IC_{50}$ values, which could result in colloidal aggregation. This could partially explain the varying activity of isoxazoles bearing sulfonamide functional groups. Minor changes to one of the most promising hits, such as adding a methyl group or decreasing the ring size caused the activity to be lost. Further exploration of structural analogs are needed before more comprehensive conclusions can be made as the inhibitory activity could be merely an artifact due to poor solubility, for example. Out of these, the most potent isoxazole (**43j**, Figure 33) showed moderate inhibition of PfPPase-VP1. In the *P. falciparum* survival assay in erythrocytes **43j** inhibited the growth of *P. falciparum* with an $IC_{50}$ of 99 μM. However, this is severalfold above the limit of no detectable colloidal aggregation. Alternatively, the inhibitor affects the parasite via some other mechanism or target. Unfortunately, the attempts of obtaining crystallographic data to guide us further in the design of new inhibitors were not successful. A reasonable continuation could be try to incorporate bromine atoms to aid crystallographic studies also of other promising inhibitors that perhaps would not be washed out in the crystallization experiments. Another approach could be to make covalent analogs of the inhibitors to bind them irreversibly to the protein for structural studies and subsequent molecular modelling of desirable interactions to target next. Bioisosteric replacements of susceptible moieties could be helpful too. As there was a noticeable difference between aryl and heteroaryl substituents at the 5-position of the isoxazole, a similar replacement of the carboxylate could have drastic effects on the activity.

The isoxazole series was followed by further exploration of the most prominent hits. In addition to the synthesized pyrazolo[1,5-*a*]pyrimidine series, several commercial congeners were evaluated. For instance, a 4,5-dihydropyrrolo[3,4-*c*]pyrazol-6(1*H*)-one scaffold and simple sulfonamide derivatives showed the most promising results of the commercial compounds but were not yet followed-up. However, in comparison to the isoxazole series, even the most promising pyrazolo[1,5-*a*]pyrimidines (**61j**, **64j**, **65j**) had slightly higher $IC_{50}$ values in the TmPPase based assay. Similarly to the isoxazole series, both the 2-bromophenyl and 3,5-dimethyl phenyl group were important for the activity of the pyrazolo[1,5-*a*]pyrimidines. The 3,5-dimethyl phenyl group was crucial for most of these inhibitors. However, the 2-bromophenyl group could be replaced with the corresponding carboxylic acid if also the bromine atom at the 2-position was swapped to a vinyl moiety as in **64j**.

Interestingly, both **64j** and its corresponding 2-bromophenylcarboxylate (**65j**) showed similar activity, whereas there have previously been no correlation between the activity of carboxylic acid and carboxylate analogs. This could be an indication of the carboxylate being hydrolyzed in the assay conditions, which is beneficial for the water solubility. On the other hand, the same trend is not noticed for analogs of **61j** (Figure 33). Therefore, it appears as the bromo or vinyl substituent at the 2-position affect the binding site or mechanism. Pyrazolo[1,5-*a*]pyrimidine **61j** resembles the most promising isoxazoles and showed similar activity against the PfPPase-VP1. Furthermore, **61j** inhibited the growth of *P. falciparum* in the survival assay with an $IC_{50}$ of 31 μM. As the variation of the central core from isoxazole to pyrazolo[1,5-*a*]pyrimidine was well tolerated, an exploration of alternative heterocycles or more substitution of the previous cores could perhaps result in additional interactions and better enzyme inhibition. Both **42h** and **64j** showed no sign of colloidal aggregation even at high concentrations and could be suitable candidates for follow-up studies.

Following these previous explorations of nonphosphorus inhibitors, the in-house cross-project screening revealed yet another scaffold. These azulene-derivatives were further studied but led to somewhat conflicted SAR in the primary TmPPase based assay. Some even showed low micromolar values but were sensitive to modifications. Although the two initial dihydrobenzazulene hits **68** and **69** were comparably active, their corresponding 1-formyl analogs varied considerably. Compound **73** was inactive, whereas **74** (Figure 33) showed low micromolar activity. The formyl group could plausibly react with lysine or other appropriate amino acid residues in the protein. These compounds were evaluated as racemic mixtures. Removal of the chiral center through dehydration gave the more rigid benzazulenes. These derivatives have a built-in *o*-methide moiety that was used in oxidative amination or thiolation of the 2-position. However, the 2-amino-substituted benzazulenes showed vast diversity in activity and were susceptible to alterations. The best improvement in activity was for ethylamine **108** ($IC_{50}$ = 6.0 μM). The corresponding methylamine **107** was completely inactive, although both the isopropylamine analog and primary 2-aminobenzazulene showed moderate activity. Modifying the ethyl chain to a primary alcohol of similar length (**110**, **111**) did not show any activity either. The oxidation of **108** to the corresponding 1-formyl derivative was tolerated but not beneficial. Conflicting activities were also for many heterocyclic and aliphatic derivatives. However, a terminal carboxylic acid moiety appear to be generally important for the activity, perhaps due to interaction with positively charged amino acid residues or cations.

The potentially promiscuous benzazulenes were further simplified to only the azulene core, a substantial common moiety of both the previous scaffolds. In accordance with the benzazulenes, also oxygen-rich substituents seemed to be favored. Several 1,6-disubstituted azulenes were evaluated and demonstrated tolerance both to modification of the 6-halogen substituent from chlorine to bromine, and various ester moieties. Although additional substitution at the 3-position led to a small improvement in activity, none of the 6-monosubstituted derivatives showed any activity. The best brominated analogs might be helpful in crystallographic studies to guide the design of improved inhibitors. Assorted azulenes were evaluated in the *P. falciparum* survival assay in erythrocytes. However, the benzazulenes showed diverged results, which might be due to inhibition of some other protein or perhaps through colloidal aggregation. The best benzazulene was **76** (Figure 33), with an $IC_{50}$ of 1.5 μM against TmPPase and low micromolar activity in the survival assay of *P. falciparum* in erythrocytes. Also the most potent azulene, **103d**, showed low micromolar inhibition of TmPPase along with an $IC_{50}$ of 2.6 μM in the *P. falciparum* survival assay. Especially the lipophilic benzazulenes were suffering from poor solubility, as well as decorated with groups labile for hydrolysis or attack of nucleophilic amino acid residues. On the other hand, the azulenes were more

soluble and demonstrated more tolerance to functional group modifications. Expansion of the 6-halogen substituent or 3-position could be appealing sites to continue this exploration.

As it is challenging to get a comprehensive SAR of these diverse scaffolds due to diverged data, it is likely that a major part of them interact differently with the enzyme. However, at this stage of the study a practical feature to proceed with would be compounds having good solubility and activity. In addition to further alterations of the most promising PfPPase and *P. falciparum* growth inhibitors (Figure 33), it would be interesting to follow-up some of the side-trails that arose during this study. For instance, increased flexibility by introducing suitable linkers could improve the activity or added chirality to enhance the selectivity. Additionally, screening against other pathogenic protozoan mPPases could point out species-selective variations similarly as noticed for the allosteric TmPPase inhibitor **33e**.

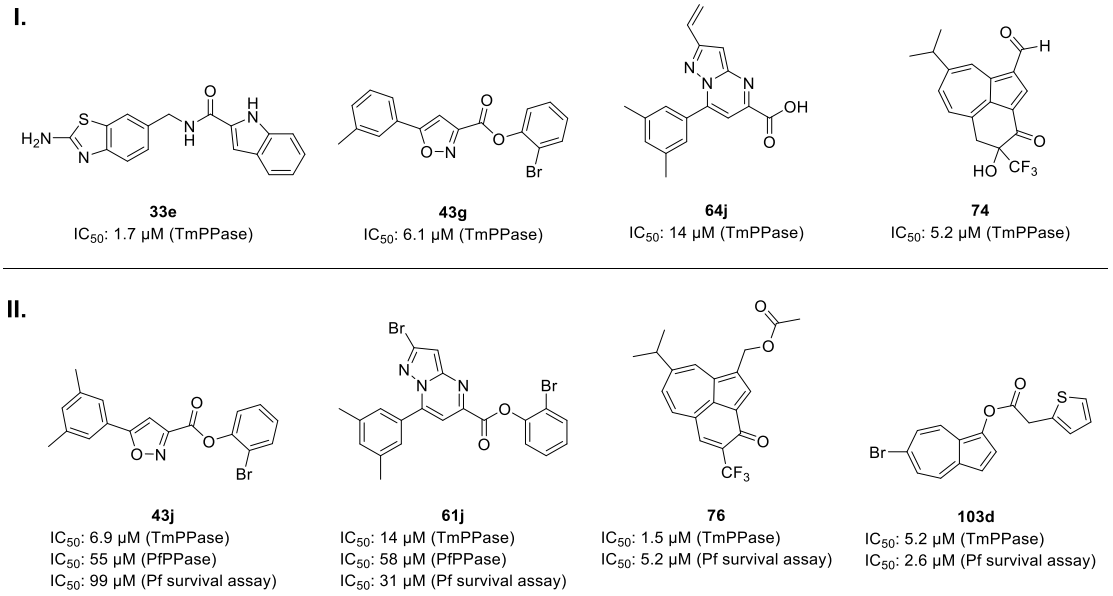



**Figure 33** *Some of the most promising (I) TmPPase, (II) PfPPase and P. falciparum growth inhibitors found in this study, as well as their $IC_{50}$ values.*

At the start of this study no nonphosphorus inhibitors targeting TmPPase were known. Throughout this project six diverse scaffolds (2-aminobenzo[*d*]thiazoles, isoxazoles, pyrazolo[1,5-*a*]pyrimidines, dihydrobenzazulenes, benzazulenes, and azulenes) have been shown to inhibit TmPPase with $IC_{50}$ values in the 2–15 μM range. Some of these inhibitors moderately inhibited PfPPase-VP1 ($IC_{50}$ values 55–130 μM). Furthermore, in a parasite survival assay in erythrocytes the studied scaffolds inhibited *P. falciparum* growth ($IC_{50}$ values 3–99 μM), the best one with an $IC_{50}$ value of 2.6 μM. Although several issues remain to be solved, including an increased on-target potency and selectivity against species-specific mPPases, identifying new inhibitors that bind selectively to the active site of mPPase may lead to novel treatment options against several human pathogens. In order to gain a better understanding of SAR, more and divergent compounds need to be explored. The continuation of this project will attempt to make covalent inhibitors to aid crystallographic studies, thereby verifying the binding site and supporting future design of new inhibitors.

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