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Spiroindolone NITD609 is a novel antimalarial drug that targets the P-type ATPase PfATP4

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Spiroindolone NITD609 is a novel antimalarial that targets the P-type ATPase PfATP4

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Abstract:

Malaria is caused by the *Plasmodium* parasite and is a major health problem leading to many deaths worldwide. Lack of a vaccine and increasing drug resistance highlights the need for new antimalarial drugs with novel targets. Antiplasmodial activity of spiroindolones was discovered through whole-cell, phenotypic screening methods. Optimisation of the lead spiroindolone improved both potency and pharmacokinetic properties leading to drug candidate NITD609 which has produced encouraging results in clinical trials. Spiroindolones inhibit PfATP4, a P-type Na^+-ATPase in the plasma membrane of the parasite, causing a fatal disruption of its sodium homeostasis. Other diverse compounds from the Malaria Box appear to target PfATP4 warranting further research into its structure and binding with NITD609 and other potential antimalarial drugs.

Key Terms

- Apicomplexan organisms: a group of parasitic eukaryotic microorganisms that includes *Plasmodium* and *Toxoplasma*.
- Malaria box: an open access collection of 400 structurally diverse antimalarial compounds selected from whole-cell drug screen hits. It consists of 200 drug-like compounds and 200 probe-like compounds, all with unknown mechanism of action.
- NGBS Consortium: public-private collaboration involved in a malaria drug discovery programme to identify new chemotypes for the development of malaria treatments.
- P-type adenosine triphosphatase (P-type ATPase): a family of evolutionary related membrane-bound pumps that self-phosphorylate at a conserved aspartate residue. Most P-type ATPases pump cations enabling the maintenance of electrochemical gradients across cell membranes.
- *Plasmodium bergei*: a murine malaria widely used in an animal model for malaria research.
- Spiroindolones (Spirotetrahydro beta-carbolines): synthetic compound with two stereocentres consisting of a beta-carboline structure connected through a central spiroatom to an indole structure.

Introduction / background

Malaria is an infectious disease caused by the malaria parasite *Plasmodium* and transmitted by female *Anopheles* mosquitoes. The four main species that infect humans are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*. *P. falciparum* and *P. vivax* are the most common with *P. falciparum* causing the most deaths globally [1]. The World Health Organisation estimates there were 207 million cases of malaria and 627,000 malaria deaths in 2012 [2] which is a great improvement since 20 years previous when it caused over 1 million deaths per year [3].
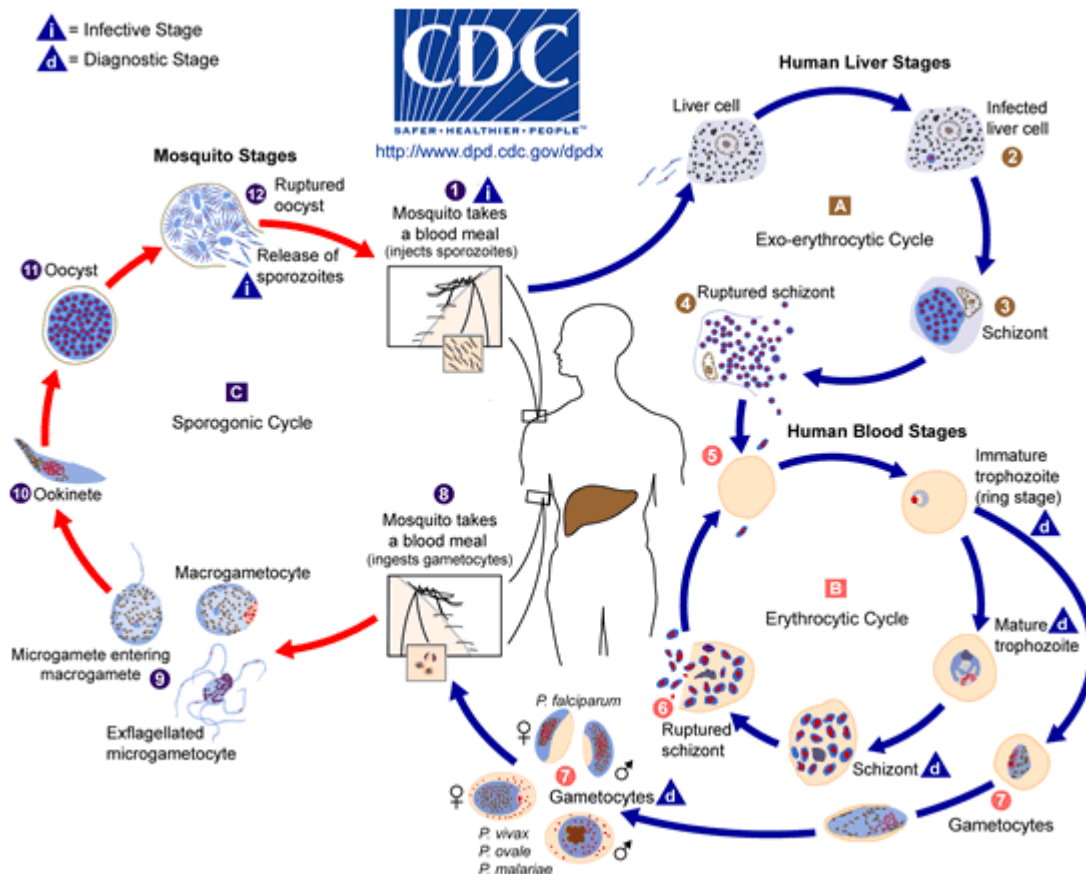

Figure 1. Malaria parasite life cycle. The life cycle is complex involving both human and mosquito hosts. Figure copied from [4]

The life cycle of the malaria parasite is complex with several life stages involving both mosquito and human hosts (Figure 1). When the female *Anopheles* mosquito bites sporozoites are injected with the saliva into the human host (Step 1). The sporozoites travel to the liver and infect the hepatocytes (Step 2) where they develop and multiply asexually to form exo-erythrocytic schizonts (Step 3). These schizonts rupture releasing merozoites (Step 4) into the bloodstream where they enter the erythrocytes (Step 5). They replicate synchronously in the erythrocytes causing the clinically manifested cycle of fever. Ring-stage trophozoites mature into schizonts which rupture releasing merozoites (Step 6). Some of the merozoites develop into male and female gametocytes (Step 7) which are ingested with the blood into the female mosquito gut (Step 8). The male and female gametes fuse to form zygotes (Step 9) in the mosquito's stomach. They then elongate and become motile ookinetes (Step 10) which migrate from the gut and form oocysts (Step 11). The oocysts undergo meiotic division to form sporozoites (Step 12) which migrate to the salivary gland of the mosquito ready to be

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injected into the next human host (Step 1). In *P. vivax* and *P. ovale* some of the liver-stage parasites can remain dormant as hypnozoites for weeks or years. Reactivation of the hypnozoites causes relapses in the disease.

Currently there is no vaccine licensed for use against malaria however it is hoped a vaccination that offers some protection will be introduced in 2015. The RTS,S vaccine is undergoing phase 3 trials and initial results showed it reduced the incidence of malaria by half in children of 12-17 months old for 12 months after vaccination [5]. Most existing antimalarial drugs act against the asexual blood stages of the parasite. Primaquine is the only drug approved that acts against the dormant liver stages of *P. vivax* whose reactivation lead to relapse [6]. For many years chloroquine and sulphadoxine-pyrimethamine were the principal antimalarial treatments however resistance to both drugs led to increased morbidity and mortality from malaria. Artemisinin-based combination therapies (ACTs) , which contain a combination of an artemisinin derivative and another antimalarial drug with a different mechanism of action, are recommended to reduce the risk of drug resistance [1]. ACTs are a major factor in the increased malaria control, consequently it is of great concern that *P. falciparum* has developed some resistance to artemisinin derivatives in South East Asia [7].

Increasing resistance of *Plasmodium* to the existing malaria treatments including artemisinin and its derivatives [7] highlight the need for new antimalarial drug therapies. Key factors in considering the target product profile (TPP) include the vulnerability of many of the patients (many of whom are young children and pregnant women), the lack of medical supervision under which drugs are administered and the need for patient compliance.

Ideally new antimalarial drugs should [1,8]:

- be effective against the blood stages of the key species of parasite that cause malaria in humans, including those with drug-resistance,
- kill the hypnozoites of *P. vivax* and *P. ovale* therefore preventing relapse,
- kill the gametocytes to prevent transmission,
- kill sporozoites and exo-erythrocytic schizonts therefore preventing the initial infection when used prophylactically,
- have a novel mechanism of action,
- have good oral availability,
- be safe,
- cure by a single dose,
- be cheap to produce.

Discovery of spiroindolones as antimalarial agents

At a time when there was optimism that the ration drug design approach, based on the molecular understanding of the biology of the malaria parasite, would produce new antimalarial drugs the spiroindolones were identified using older, whole-cell, phenotypic screening methods based on the growth inhibition of *P. falciparum* [9]. A library of 12,000 pure natural and structurally similar synthetic molecules was screened using whole-cell proliferation assays with cultured cells from intra-erythrocytic *Plasmodium* parasites [10,11] (Figure 2). From the 275 compounds that had submicromolar activity against *P. falciparum* those that weren't active against multi-drug resistant forms of the parasite and those that were cytotoxic to mammalian cells were excluded leaving 17 compounds. Since it is important that a new antimalarial drug can be administered in tablet form the oral bioavailability of the remaining compounds was assessed. Based on its activity against *Plasmodium* and favourable pharmacokinetic profile a synthetic compound (**1**) related to the spiroazepineindole

class was selected for medicinal chemistry lead optimisation. Compound **1** had good oral bioavailability in mice with F = 59% and an oral half-life of nearly 4 hours [6].
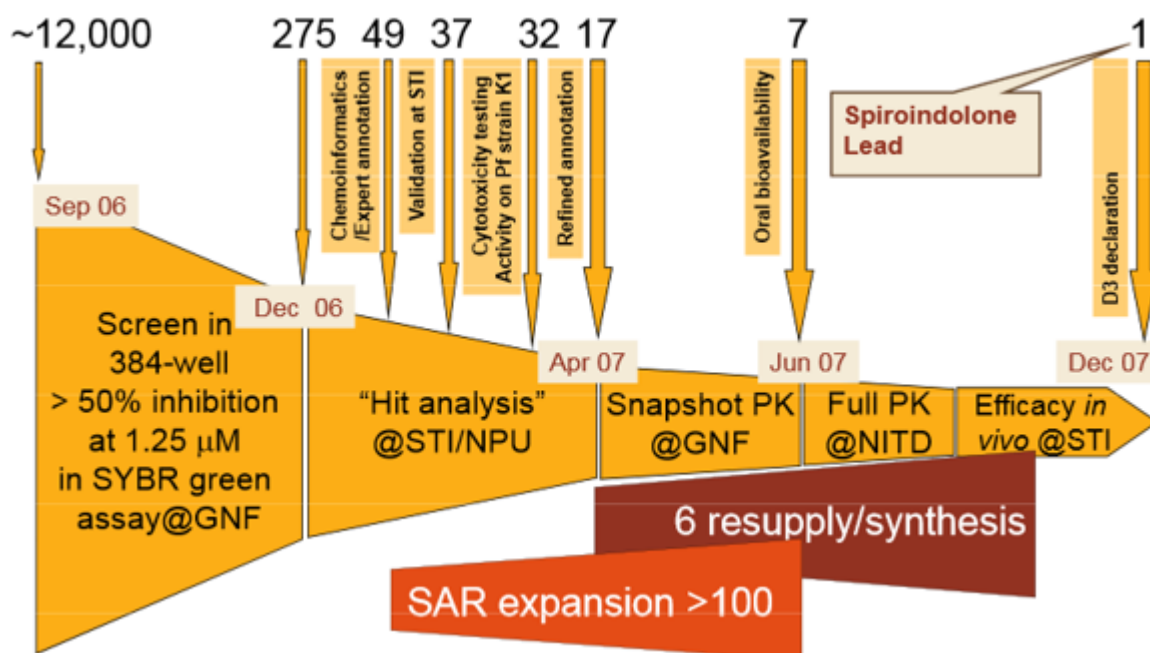

Figure 2. Overview of the screening process that identified the spiroindolone lead compound. Figure copied from [12].

The initial compound identified (**1**) was a racemic mixture consisting of 1R,3S & 1S,3R and 1S,3S & 1R,3R pairs of enantiomers that had moderate potency against wild-type (NF54) and chloroquine-resistant (K1) strains of *Plasmodium* (Figure 3). A single 100mg/kg dose of racemic compound **1** led to a 96% decrease in parasitaemia in the *P. berghei*-infected mouse model.

The reaction to create compound **1** yielded an unequal mixture such that there was a 9:1 excess of the 1R,3S & 1S,3R pair of enantiomers [11]. Structure-activity relationship (SAR) analysis showed that the 1R,3S stereoisomer (**1a**) was over 250 times more potent against *P. falciparum* than the 1S,3R stereoisomer (**1b**) (Figure 3). SAR analysis of further spiroindolone compounds consistently indicated that the 1R,3S stereoisomer was essential to be active against *P. falciparum* [11]. Only one enantiomer being active suggests the existence of a discreet target.
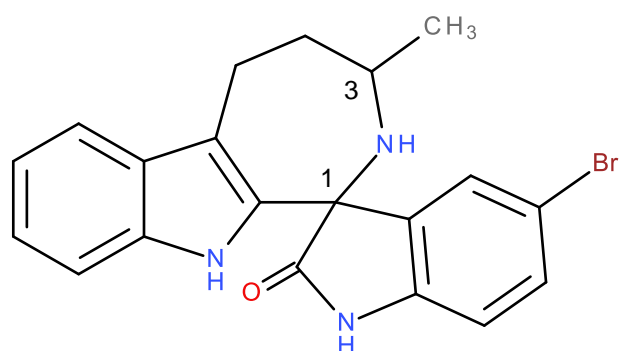
Compound 1 – racemic
NF54 EC_{50} = 90 nM
K1 EC_{50} = 80nM
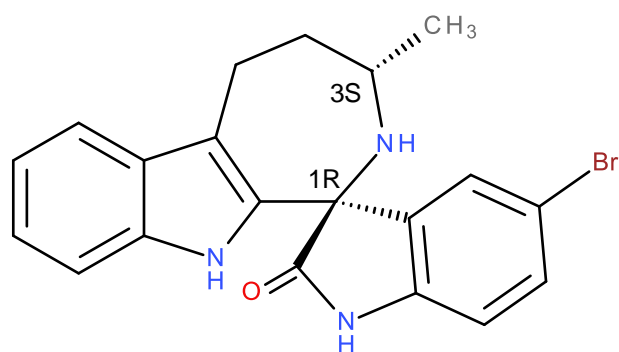
Compound 1a – 1R,3S active enantiomer
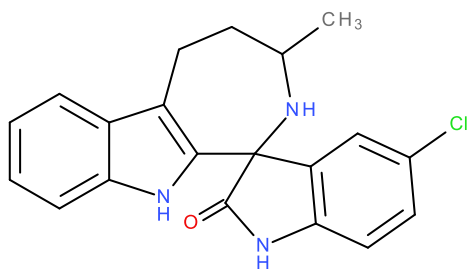NF54 EC_{50} = 20 nM
K1 EC_{50} = 30 nM
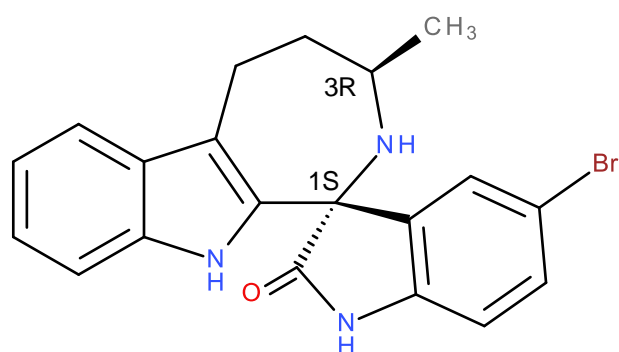
Compound 1b – 1S,3R inactive enantiomer
NF54 EC_{50} = >5000 nM
K1 EC_{50} = > 5000 nM

Figure 3. Molecular structure of the lead compound. The racemic **1,** active enantiomer **1a** (1R,3S) and inactive enantiomer **1b** (1S,3R). Data from [11].

Optimisation of spiroindolone lead led to drug candidate NITD609

Most antimalarial drugs act on the parasite in the blood stages therefore it is desirable for the drug to stay in the plasma compartment and not to enter the body fat. Substitutions to the bromine atom were made to make the molecule less lipophilic since lipophilic molecules are more likely to enter the adipose tissue from the plasma. Replacing the 5'-bromide with a 5'-chloro produced a compound with improved balance of potency, pharmacokinetics and synthetic accessibility [11] (Figure 4 – Compound **2**). Mono- and di-substitutions with other halogens produced unfavourable results.

Compound **2** (racemate) – 5'-bromo replaced with 5'-chloro
NF54 EC$_{50}$ = 84 nM
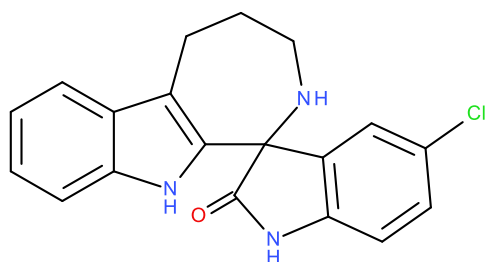
Compound **3** (racemate)
NF54 EC$_{50}$ = 86 nM
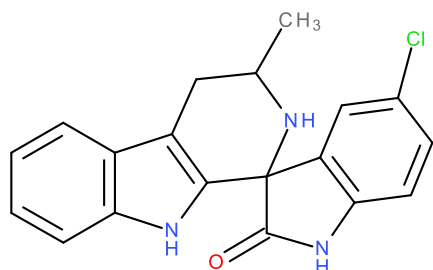
Compound **4** (racemate)
NF54 EC$_{50}$ = 27 nM
K1 EC$_5$ = 21 nM
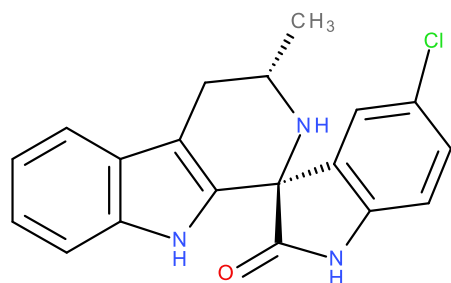
Compound **4a** (1R,3S)
NF54 EC$_{50}$ = 9.2 nM
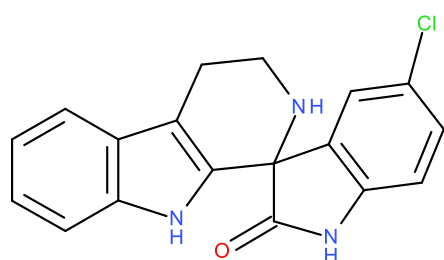
Compound **5** (racemate)
NF54 EC$_{50}$ = 126 nM
K1 EC$_5$ = 104 nM

Figure 4. Compounds **2**, **3**, **4**, **4a** & **4**. 5'-bromo replaced with 5-'chloro (**2**). Alterations to the azepine ring to give compound **4**. Removal of the methyl from the 7-membered ring does affect potency (**2** & **3**) however decreases potency 5-fold when removed from the 6-membered ring (**4** & **5**).

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There was some scope for alterations to the central seven-membered azepine ring [6] (Figure 4). Adding another carbon atom to the azepine ring of compound **2** (NF54 IC$_{50}$ = 84nM) produced an inactive compound (structure not shown: NF54 IC$_{50}$ > 5000nM) however potency was increased 3-fold when removing a carbon atom to make a 6-membered ring tetrahydro-β-carboline derivative (**4**) (Figure 4). Removal of the C3 methyl from the tetra-hydro-β-carboline structure resulted in nearly a 5-fold loss of potency (**5)** however its removal did not affect the potency for the azepineindole structure (**3**). The C3 methyl could only be substituted with trifluoromethyl.

Compound	4 (racemate)	4a (1R,3S)	4b (1S,3R)
NF54 IC$_{50}$ (nM)	27	9.2	>5000
***In vitro* (liver microsomes) Clearance CL$_{int}$**			
Mouse	Medium	High	Low
Human	Medium	High	Low
***In vitro* (liver microsomes) Microsomal Half-life t$_{1/2}$ (min)**			
Mouse	26.5	1.8	103
Human	9.9	1.2	95
CYP2C9 inhibition (µM)	n/a	1.51	>10.00
***In vivo* (mice) Clearance CL (mL/min/kg)**	n/a	49.66	n/a

Table 1. Pharmacokinetic profile of spiroindolone 4. *In vitro* metabolic stability measured using liver microsomes. *In vivo* clearance measured in mice at a single 5mg/kg iv dose. CL = clearance; CL$_{int}$ = Intrinsic clearance; n/a = not available. Data from [6,11]

The spiroindolones represented good drug candidates as they generally had good *in vitro* solubility and permeability, were not cytotoxic to human cell lines, had low cardiotoxicity potential, low genotoxicity and did not significantly bind to human receptors, kinases or ion channels [11]. There was a significant difference in the metabolic stability and CYP450 inhibition between the pairs of enantiomers with the inactive 1S,3R having more favourable properties [11]. A microsomal clearance assay was used to predict the metabolic stability and it was found that the active 1R,3S enantiomers had a high clearance, poor metabolic stability with liver microsomes and inhibited CYP2C9 preferentially (Table 1).

The benzene ring of the indole moiety was identified as being susceptible to metabolism by oxidation and therefore leading to high clearance. A systematic approach was taken to modifying the indole ring of compound **4a** to improve its half-life in the presence of liver microsomes (Figure 5). Aryl halides are usually too stable to be metabolised so halides were added to the benzene ring to protect it from oxidation. Substituting a fluorine atom at C7 position of the indoline was the most effective in increasing the half-life (**7a**) [6]. Substitutions

on C5, C6 and C8 did not improve the metabolic stability however they did increase the potency with substitutions at C6 and C7 being the most effective (**9a** & **10a**).
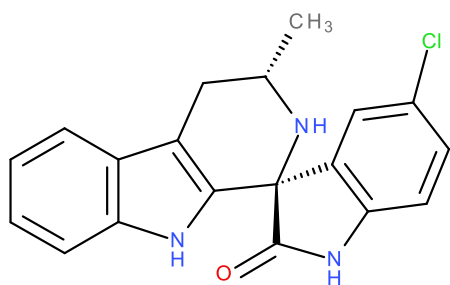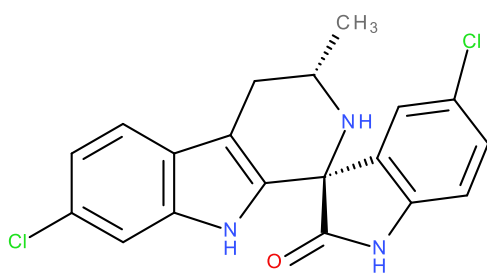
Compound **4a** (1R,3S)
NF54 EC_{50} = 9 nM
Hepatic CL_{int} mouse = high
Hepatic CL_{int} human = high
CYP2C9 inhibition (µM) = 1.51
In vivo CL (mL/min/kg) = 49.66
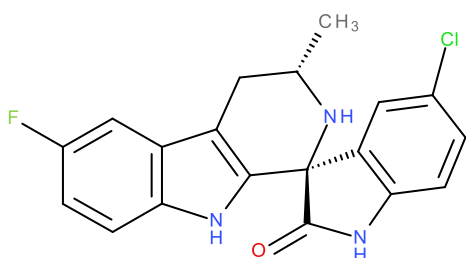
Compound **6a** (1R,3S)
NF54 EC_{50} = 3 nM
Hepatic CL_{int} mouse = high
Hepatic CL_{int} human = medium
Half-life $t_{1/2}$ mouse (min) = 4.2
Half-life $t_{1/2}$ human (min) = 10
CYP2C9 inhibition (µM) = 1.72
In vivo CL (mL/min/kg) = 24.01
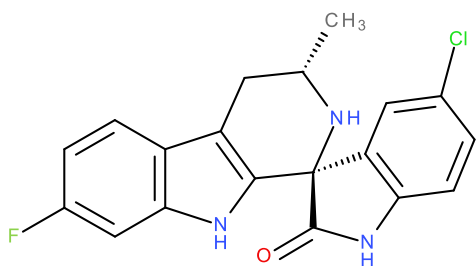
Compound **7a** (1R,3S)
NF54 EC_{50} = 3.5 nM
Hepatic CL_{int} mouse = low
Hepatic CL_{int} human = low
Half-life $t_{1/2}$ mouse (min) = 53
Half-life $t_{1/2}$ human (min) = 53

Compound **8a** (1R,3S)
NF54 EC_{50} = 4 nM
Hepatic CL_{int} mouse = low
Hepatic CL_{int} human = low
CYP2C9 inhibition (µM) = 4.11
In vivo CL (mL/min/kg) = 60.08
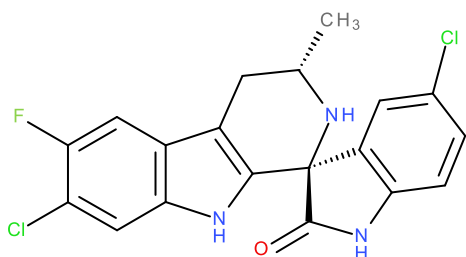
Compound **9a** (1R,3S) (NITD609)
NF54 EC_{50} = 0.9 nM
Hepatic CL_{int} mouse = low
Hepatic CL_{int} human = low
Half-life $t_{1/2}$ mouse (min) = 49
Half-life $t_{1/2}$ human (min) = 76
CYP2C9 inhibition (µM) = 5.42
In vivo CL (mL/min/kg) = 9.75
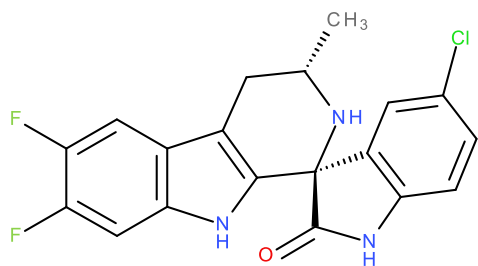
Compound **10a** (1R,3S)
NF54 EC_{50} = 0.2 nM
Hepatic CL_{int} mouse = low
Hepatic CL_{int} human = low
Half-life $t_{1/2}$ mouse (min) = 49
Half-life $t_{1/2}$ human (min) = 56
CYP2C9 inhibition (µM) = 7.35
In vivo CL (mL/min/kg) = 8.53

Figure 5. Substitutions on the indole ring system improved the metabolic stability and potency of the spiroindolones. Data from [6,11].

Figure 6. Top: spiroindolone pharmacophore; Middle: molecular structure of NITD609 showing key features; Bottom 3-dimensional structure of NITD609. R1 = CH_3 or CF_3; R2 = Cl or Fl; R3 = H, F or CL; R4 = H, F or Cl.

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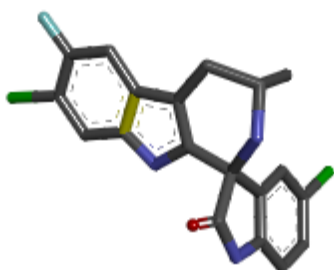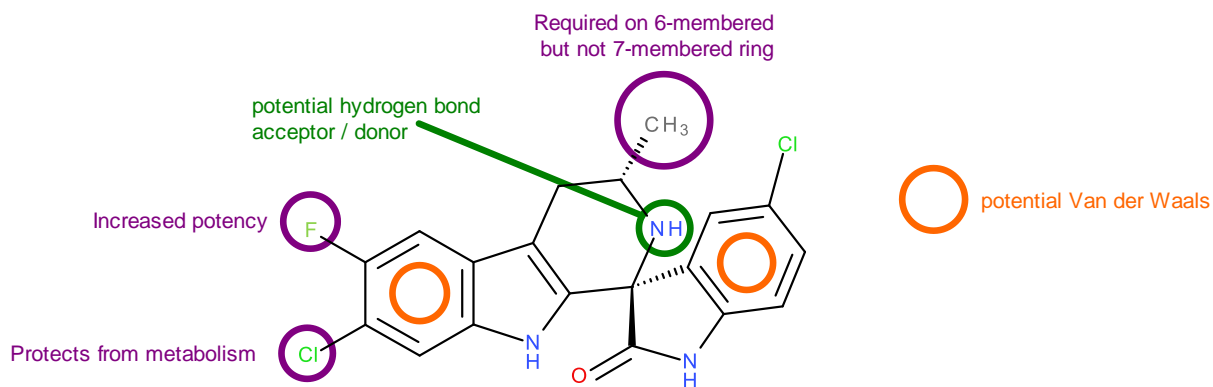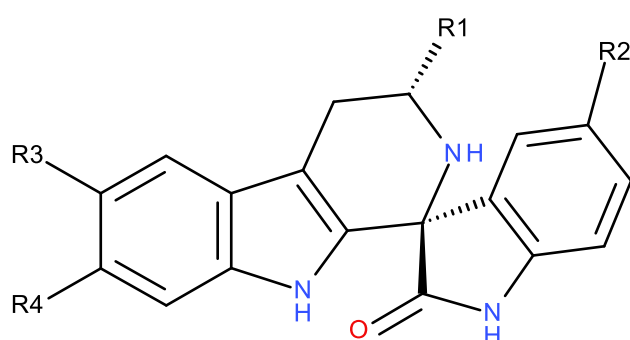The spiroindolones had been identified as potential new antimalarial drugs however their mechanism of action was unknown. Compound **9a** (NITD609, Figure 6) showed the best combination of potency and oral exposure and went forward for further preclinical evaluation.

NITD609 displays antimalarial activity against the asexual blood stage, gametocyte and oocyte stages of *Plasmodium*.

The optimisations from *in vitro* studies using microsomal clearance assays proved to correspond well to antimalarial activity in the *P. berghei*-infected mouse model with **4a**, **9a** & **10a** demonstrating better efficacy in reducing parasitaemia than chloroquine and artesunate [6]. Each of the spiroindolones prolonged survival of the mice more than chloroquine and artesunate when a single oral dose of 30mg/kg was administered (Table 2). Even better results were achieved when dosed orally once daily for 3 days with 60-90% of mice being cured.

Compound	1 x 30 mg/kg			3 x 30 mg/kg		
	Activity (%)	Survival (days)	Cure rate (%)	Activity (%)	Survival (days)	Cure rate (%)
Chloroquine	99.7	8.7	0	99.9	14.0	0
Artesunate	92.2	7.3	0	99.0	11.8	0
4a	99.9	10.7	0	99.9	18.8	60
9a (NITD609)	99.6	13.3	0	99.8	29.1	90
10a	99.6	12.0	0	99.8	23.8	80

Table 2. *In vivo* antimalarial activity in *P. berghei*-infected mouse model. Data from [6]**.**

NITD609 achieved *in vitro* inhibitory concentration (IC$_{50}$) values of 0.5 to 1.4nM against a panel of wild-type and drug-resistant *P. falciparum* with no significant decrease in activity against the drug-resistant strains [10]. Resistant strains included those with resistance against chloroquine, pyrimethamine, and mefloquine however artemisinin-resistant strains were not included in the panel.

Ex vivo assays were performed using fresh isolates of *P. falciparum* and *P. vivax* from malaria patients on the Thai-Burmese border where chloroquine resistance is known to occur [10]. NITD609 and artesunate were equally effective with IC$_{50}$ <10nM against both *P. falciparum* and *P. vivax*. The asexual blood stages are associated with the morbidity and mortality of malaria. *In vitro* sensitivity assays using *P. falciparum* at ring, trophozoite and schizont phases of the human blood stages determined that NITD609 was most effective against schizonts [10].

As well as being effective against the asexual blood stages, another study found that NITD609 acted in a dose-dependent manner against the sexual gametocytes which are responsible for

transmission of the parasite [13] (Figures 7 & 8). Most existing antimalarial drugs do not effect gametocytes therefore patients treated with these drugs can still transmit malaria. NITD609 was more effective in clearing gametocytes than lumefantrine, primaquine and artemether. In this *in vitro* study the efficacy of NITD609 in clearing gametocytes could not be compared to that of primaquine which is used in human patients to prevent transmission *in vivo* as it does not have antimalarial activity *in vitro.* The lack of *in vitro* activity of primaquine was expected as it is a prodrug and it is necessary for it to be metabolised to induce its gametocytocidal properties. NITD609 also decreased the oocyte count when added to the blood meal of mosquitos in a standard membrane feeding assay (SMFA) [13].

These results showed that NITD609 not only acted on the asexual blood stages of the parasite but also on the sexual stages that are involved in transmission of malaria however higher concentrations were required.

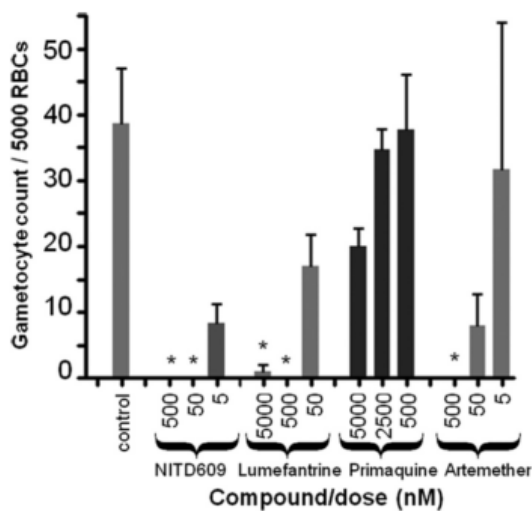**Figure 7. Effect of antimalarial drugs on early gametocytes *in vitro*. Copied from** [13]**.**
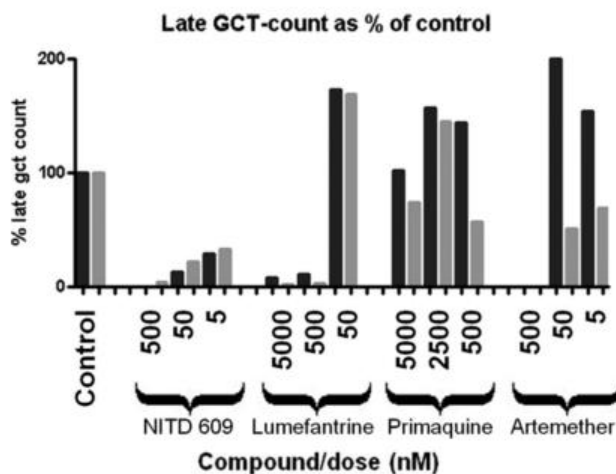

Figure 8. Effect of antimalarial drugs on late gametocytes *in vitro*. Copied from [13]**.**

NITD609 did not prevent infection of *P. berghei* mice when given prophylactically before administration of a sporozoite injection [14]. This indicated that NITD609 was ineffective against the liver stages of *Plasmodium*.

Spiroindolones target PfATP4

Using an incorporation assay with radiolabelled methionine and cysteine it was demonstrated that NITD609 blocked protein synthesis in P. falciparum within 1 hour [8,10] (Figure 9). This effect was similar to that of the known protein translator inhibitors anisomycin and cycloheximide. The effect of NITD609 was in contrast to the effect of artemisinin and mefloquine suggesting a different mechanism of action.
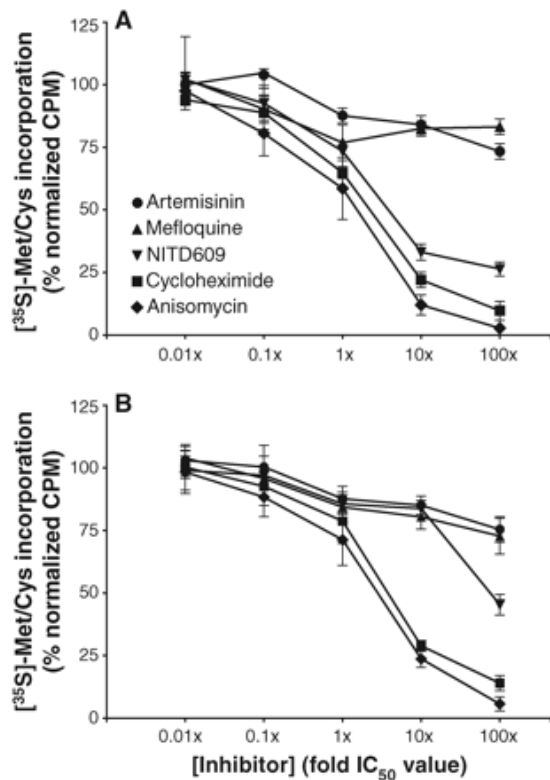
Figure 9. Effect of NITD609 on protein synthesis in *P.* falciparum. Incorporation of radiolabelled methionine and cysteine after 1 hour of exposure was monitored to evaluated rate of parasite protein synthesis. A: wild-type clones; B: NITD609-R drug-resistant clones. Copied from [10].

In vitro genetic methods were used to determine the likely target of NITD609 [10,15]. Malaria parasites were grown in the presence of a test compound until resistant strains developed. For NITD609 it took 3-4 months of *in vitro* selection to increase the IC_{50} 7 to 10-fold [10,15] indicating that NITD609 does not readily select for high-level resistance *in vitro*. Genomic analysis was used to compare the parent and resistant lines which enabled identification of the genes involved with the resistance. Most of the differences in the NITD609-resistant line were found in the gene for the P-type cation-transporter ATPase4 (PfATP4). This was supported by the reverse genetic approach of creating transgenic parasites with mutations on the PfATP4 and showing that these were resistant to the spiroindolones NITD609 and NITD678 but not artemisinin and mefloquine (Figure 10).

Figure 10. NITD609 (A) and NITD678 (B) are less effective against parasites with pfatp4 mutations. Artemisinin (C) and mefloquine (D) did were still effective against parasites with pfatp4 mutations. Dd2attB: strain of parasite; PbEF1α: *P. beghei* promoter;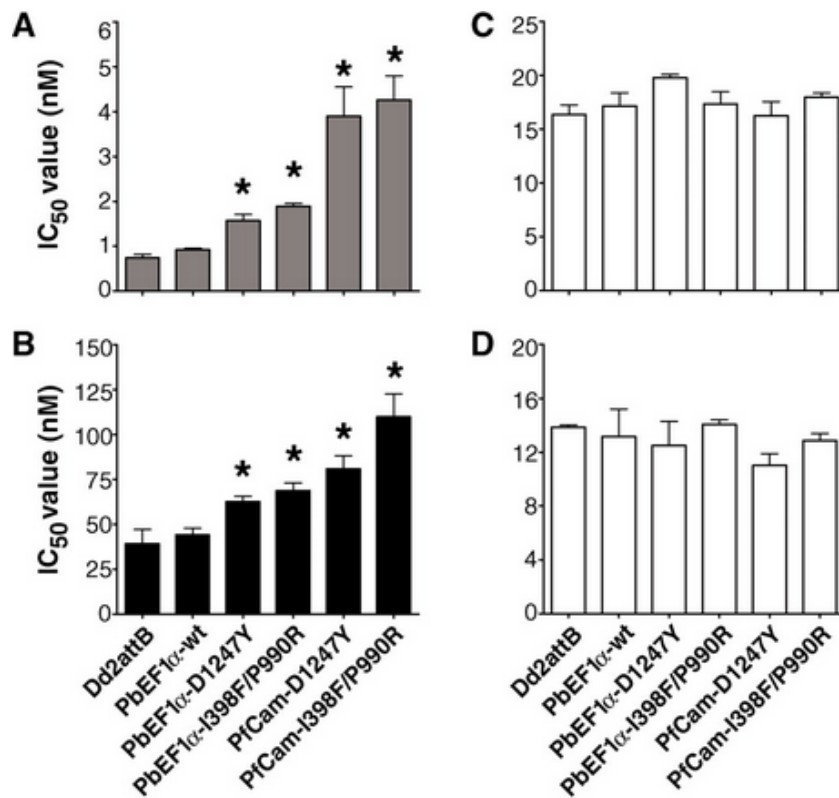 PfCam: *p. falciparum* calmodulin promoter; wt: wildtype; D1247Y: resistant clone; P990R: resistant clone. * P<0.0001 compared to DD2aatB strain. Copied from [10].

The role of membrane transport proteins in the malaria parasite

A malaria parasite that has infected a human erythrocyte needs to acquire key nutrients from the extracellular environment and export metabolites to support its high level of metabolic and biosynthetic activity whilst maintaining its own cytosolic composition. The normal rate of uptake across the erythrocyte plasma membrane of some of these nutrients does not meet the requirements of the parasite and it may also be in competition with the host cell for these nutrients [16].

Human blood plasma is a high-$[Na^+]$/low-$[K^+]$ environment and uninfected human erythrocytes are a low-$[Na^+]$/high-$[K^+]$ environment [17]. Around 12-14 hours after entering a human erythrocyte *P. falciparum* creates new permeability pathways in the plasma membrane of the host erythrocyte increasing its permeability. This enables the uptake of nutrients into the infected cell however also allows the flow of Na^+ and K^+ across their respective concentration gradients so they reach levels close to those in the extra-erythrocytic plasma [17]. Even though this causes increased $[Na^+]$ in its extracellular environment the parasite is able to maintain a low cytosolic $[Na^+]$ [17,18].

Some protozoa and lower plants expel Na^+ via an *exitus natrus (ENA)* P-type Na^+-ATPase which is closely related to the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) and plasma membrane Ca^{2+}-ATPases (PMCA) [17]. It was known that the *P. falciparum* genome codes for 13 P-type ATPases, two of which are known to be Ca^{2+}-ATPases but none had been identified as Na^+-ATPase.

PfATP4 is a P-type Na⁺-ATPase

PfATP4 belongs to a subclass (Type 4) of P-type ATPases that are unique to apicomplexan organisms [19]. Its absence in mammals makes it a good drug target. Until recently it PfATP4 was believed to be a P-type Ca^{2+}-ATPase due to its similarity with the Ca^{2+}-ATPases of the endoplasmic reticulum [19,20] however recent studies by indicated it is a P-type Na+-ATPase [17,21] (discussed in more detail later in this section).

PfTP4 is a 190kDa protein found in the parasite plasma membrane [22]. Genetic sequencing showed it contained the features common to P-type ATPases including sequence motifs of a highly conserved phosphorylation site and nucleotide-binding site [19]. Modelling techniques indicated ten transmembrane helices and that mutations associated with spiroindolone resistance were localised in these transmembrane helices (Figure 11). PfATP4 has an extended hydrophilic N-terminal region and lacks the long hydrophilic C-terminal seen in plasma-membrane-type ATPases. The extended M7/M8 extracellular loop which is has a high proportion of acidic residues is predicted to be involved in ion transport [19].
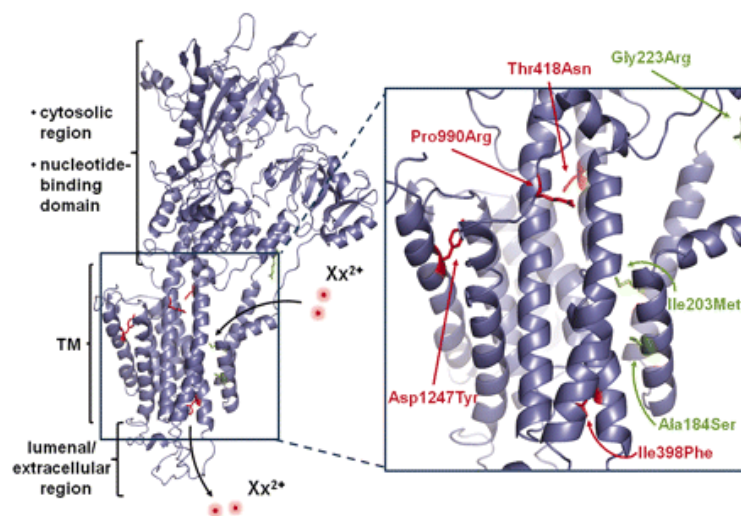

Figure 11. Model of predicted structure of PfATP4. Residues associated with mutations causing resistance are shown in red for NITD609-R and green for NITD678-R. These mutations are located in the transmembrane helices. Copied from [10]

Spillman et al investigated the effect of a variety of ionophores and ion transport inhibitors on Na⁺ regulation in *P. falciparum* [17]. The known P-type ATPase inhibitor sodium orthovanadate impaired Na⁺ regulation as did the known ENA Na⁺-ATPase inhibitor furosemide suggesting the involvement of ENA P-type Na⁺-ATPase in maintaining the low cytosolic [Na⁺] despite the high extracellular levels. Ouabain which inhibits all known Na⁺/K⁺-ATPases at the concentrations used had no significant effect on [Na⁺]$_i$ indicating that Na⁺/K⁺-ATPases were not involved. When the parasites were suspended in a glucose-free extracellular environment which causes depletion of ATP, there was an increase in [Na⁺]$_i$ which also supports the involvement of an ATPase in maintaining the low [Na⁺]$_i$. Analysis of the amino acid sequence showed that PfATP4 share an amino acid sequence that is known to be highly conserved in ENA Na⁺-ATPases and important for Na⁺ transport. This sequence is not present in SERCA, PMCA or Na⁺/K⁺-ATPases.

It is proposed that PfATP4 acts as an ENA Na⁺-ATPase and actively pumps Na⁺ out of the intra-erythrocytic parasite and that the efflux of Na⁺ is associated with an influx of H⁺ into the parasite (Figure 12) [17]. A further study demonstrated that the acid load caused by the influx of H⁺ is likely to be countered by the extrusion of H⁺ by a V-type H⁺-ATPase [21].
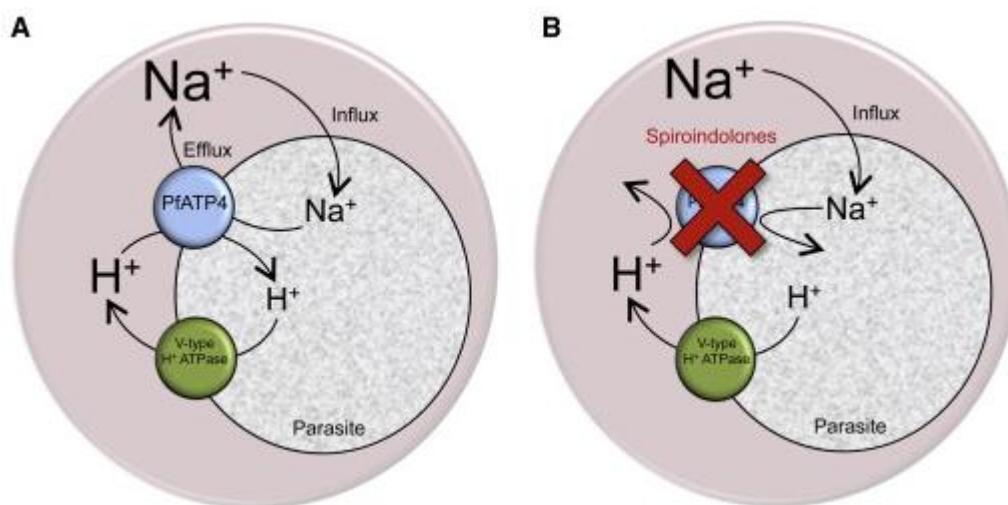
Figure 12. Proposed mechanism of action of spiroindolones targeting PfATP4 in the plasma membrane of *P. falciparum* [17]. Inhibition of PfATP4 by spiroindolones leads to a rise in intracellular Na^+ and increase in intracellular pH.

Spiroindolones inhibit PfATP4
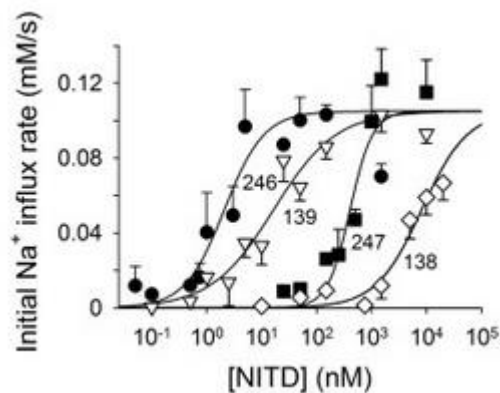
Spironindonlone	IC$_{50}$ inhibition of parasite proliferation (nM)	IC$_{50}$ disruption of Na+ regulation (nM)
● NIDT246	0.12 ± 0.02	2.1 ± 0.8
▽ NITD139	4.0 ± 5.8	16.6 ± 5.7
■ NITD247	41.6 ± 5.8	407 ± 117
◇ NITD138	2300 ±200	820000 ± 1700

Figure 13. The effect of four spiroindolones on the rate of influx of Na$^+$. The order of potency for parasite proliferation inhibition corresponds with the order for Na$^+$ disruption. Copied from [17].

Four different spiroindolones were shown to cause an increase in [Na$^+$] in a dose-dependent manner and the order of potency in increasing [Na$^+$] corresponded their ranking inhibiting parasite proliferation [17] (Figure 13). Spiroindolone NITD246 was shown to reduce membrane-associated ATPase activity in membrane preparations from infected erythrocytes suspended in a high concentration of Na$^+$. It was also shown that PfATP4 mutations corresponding with spiroindolone resistance reduced sensitivity to Na$^+$ disruption and ATPase activity by spiroindolones. These results supported the hypothesis that spiroindolones inhibit PfATP4 preventing sodium ions being pumped out of the parasite and leading to a fatal disruption of parasite sodium homeostasis (Figure 12).

Determination of three dimensional structure required

The three-dimensional structure of PfATP4 has been predicted using modelling techniques however it has not been determined by X-ray crystallography. The structure of PfATP4 and its binding with ligands, including the spiroindolones and other drugs that target it should be ascertained. Understanding the active sites of PfATP4 and the binding interactions that occur will allow more effective drugs to be created.

Pharmacokinetics

An important factor in a successful antimalarial treatment is ensuring patient compliance which can be improved by reducing the pill burden and treatment duration. Existing antimalarial drugs have to be taken from one- to four-times daily for up to seven days [23] which is often difficult to achieve in areas where there is a lack of medical supervision.

The pharmacokinetic properties observed when NITD609 was administered to mice and rats indicated that a once-daily dosing regimen might be appropriate (Table 3). It had excellent oral bioavailability, a good half-life, moderate volume of distribution and low systemic clearance.

Species	Actual dose (mg/kg)	Route	C_{max} (μM)	T_{max} (h)	AUC_{inf} (μM*h)	F (%)	V_{ss} (L/kg)	CL (mL/min/kg)	Elim. $T_{1/2}$(hour)
Mice	5.4	i.v.	—	—	23.88	—	2.11	9.75	3.44
	24.6	p.o.	9.17	1	138.65	100	—	—	10.02
Rats	5	i.v.	—	—	61.32	—	3.04	3.48	10.69
	23.7	p.o.	11.68	8	524.15	100	—	—	27.73

Table 3. Pharmacokinetic data for NITD609 in female CD-1 mice and Wistar rats. i.v. = intravenous; p.o. = oral; C_{max} = maximum drug serum concentration; T_{max} = Time at which C_{max} observed; AUC = area under curve; F = oral bioavailability; V_{ss} = volume of distribution; CL – clearance; $T_{1/2}$ Elim = Half-life. Copied from [10].

Safety and selectivity of NITD609 *in vitro*

The safety of a new antimalarial drugs is essential, particularly due to the vulnerability of the patient population, which includes many young children, pregnant women and those with comorbidities, living in areas that often have limited medical resources. The malaria parasite within the human body is the intended target for NITD609 therefore it is important that it is not cytotoxic to human cells at the concentrations required for parasite cytotoxicity. NITD609 showed no significant cytotoxicity against a panel of *in vitro* mammalian cell lines [10]. For each of the cell lines tested the concentration of NITD609 that lead to 50% cell death (CC_{50}) was at least 10µM. Since the IC_{50} was < 1nM the selectivity index (CC_{50}/IC_{50}) > 10,000. Some antimalarial drugs can be cardiotoxic as they inhibit hERG channels however NITD609 showed low binding affinity to hERG as well as other human ion channels, G-protein coupled receptors and enzymes. When administered to rats for 14 days at 10-20 times the calculated effective dose required to reduce parasitaemia by 99% (ED_{99}) there were no adverse events observed.

Clinical trials

NITD609 (which is now known as KAE609 or cipargamin) underwent a phase 2 clinical trial in 3 locations in Thailand to assess its antimalarial efficacy, safety and adverse events (ClinicalTrials.gov number NCT01524341) [24]. KAE609 was administered to adults with uncomplicated *P. vivax* or *P. falciparum* malaria at 30mg per day for 3 days. This was a small study with only 21 patients however the results were very encouraging. KAE609 cleared the parasites rapidly from the blood of the patients with median complete parasite clearance time of 12 hours. The median parasite half-life clearance was 0.95 hours for *P. vivax* and 0.90 hours for *P. falciparum* which is excellent when considered compared to artensunate for which less than 1% of patients infected with *P. falciparum* have half-life clearance of under 1 hour.

The safety, tolerability and pharmacokinetics of KAE609 in healthy male adult patients after single and multiple oral dosing was assessed in a randomised, double-blind, placebo-controlled study [25]. KAE609 was generally well tolerated however some mild to moderate adverse events were recorded which were mostly gastrointestinal and genitourinary and increased with rising doses.

Two further clinical trials are scheduled [26]. One to find the minimum inhibitory concentration for a single dose of KAE609 to reduce parasitaemia to zero in adult male patients with *P. falciparum* monoinfection (ClinicalTrials.gov Identifier: NCT01836458), this will help to identify the optimal dose of KAE609. The other to assess the efficacy and safety of KAE609 in adults with acute malaria mono-infection (ClinicalTrials.gov Identifier: NCT01860989).

PfATP4 pathway targeted by diverse structures

In a recent *in vitro* study 28 out of 400 (7%) compounds in the 'Malaria Box' affected Na^+ and pH regulation in a manner consistent with PfATP4 inhibition [27]. Six of these, which had chemically diverse structures, were analysed further and were all found to have reduced efficacy against spiroindolone-resistant parasites with pfatp4 mutations indicating that they all interact with PfATP4. These results suggest that PfATP4 is important for parasite survival, can be inhibited by a variety of compounds and is readily accesses by compounds in the extracellular medium. Researchers at GlaxoSmithKline have identified at least four further scaffolds unrelated to NITD609 that may act via the same PfATP4 pathway [28]. These results indicate that PfATP4 may be an important target in the fight against malaria and certainly warrants further research to understand its action, molecular structure and binding sites.

Repurposing of NITD609 for use against other apicomplexan parasites

As well as its activity against *Plasmodium,* NITD609 is also active against the related apicomplexan parasite *Toxoplasma gondii* which causes toxoplasmosis [29]. *T. gondii* ATPase4 (TgATP4), which corresponds to PfATP4, was identified as the likely target. Modelling techniques showed that PfATP4 and TgATP4 share a large degree of sequence identity particularly around the transmembrane region of the ATPase4 structure where the spiroindolone binding site is proposed to be and the residues involved in drug resistance (Figure 14).
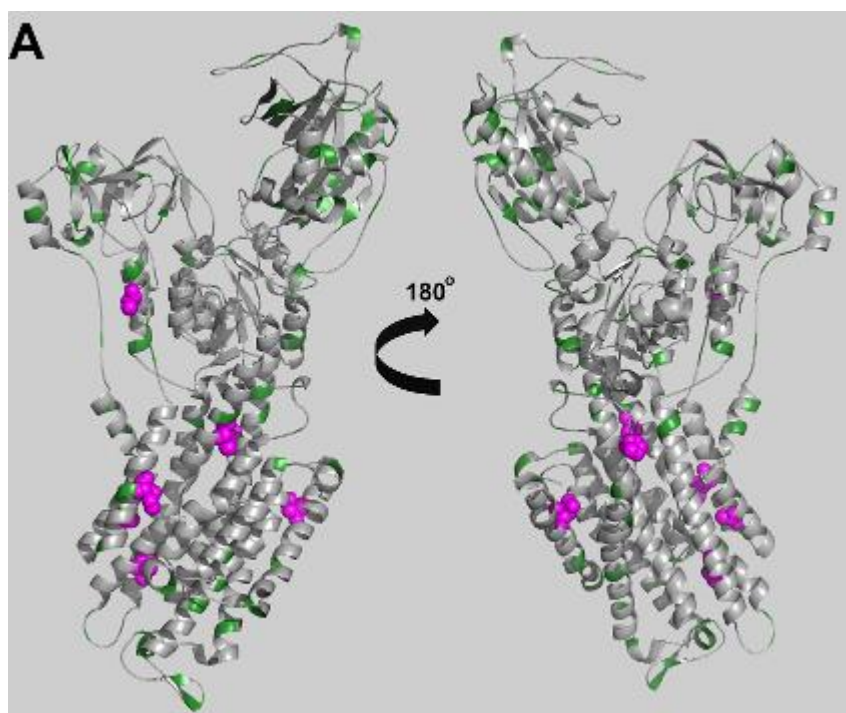

Figure 14. Modelled structure of TgATP4. Green indicates residues that differ significantly between *T. gondii*, *P. falciparum* and *P. vivax*. Magenta spheres indicates residues altered in spiroindolones resistance. Copied from [29]

Future perspective

NITD609 is currently in Phase II clinical trials and is the first antimalarial drug with a novel action to reach this stage since atovaquone/proguanil in 1996 [8]. To reduce the risk of resistance developing antimalarial drugs should always be administered in combination therefore if it is successful in clinical trials a suitable partner drug will need to be identified which could delay its introduction into clinical use.

PfATP4 is emerging as an important target in the fight against malaria and further research is needed to understand its structure, function and how drugs may target it in the fight against malaria. The three-dimensional, crystal structure of the PfATP4 protein should be determined as well as its binding interactions with the spiroindolones and some of the diverse compounds from the Malaria Box. With this knowledge a rational approach can be utilised to create further

drugs for the treatment of malaria and possibly other apicomplexan parasites such as *Toxoplasma gondii*.

Executive Summary:

- Resistance of *Plasmodium* to artemisinin emphasises the need for new antimalarial drugs with a novel mode of action.
- Spiroindolones were discovered to have antimalarial activity using whole-cell screening methods however their target and mode of action were unknown.
- It was determined that the 1R,3S configuration was required for activity against *Plasmodium*.
- Optimisation of the spiroindolone using SAR analysis lead improved both potency and pharmacokinetics and led to drug candidate NIT609.
- Spiroindolones inhibit PfATP4 of the *Plasmodium* parasite resulting in a fatal increase in sodium ions in the parasite.
- PfATP4 is a P-type Na^+-ATPase located in the parasite plasma membrane that pumps sodium ions out of the parasite.
- The spiroindolones have a distinct mechanism of action from the existing antimalarial drugs.
- *In vitro s*piroindolones do not readily select for resistance and do not show cross-resistance to existing antimalarial drugs.
- Pharmacokinetic studies indicate NITD609 may be compatible with once-daily oral dosing.
- NITD609 is currently in Phase 2 clinical trials where so far it has shown encouraging results.
- The crystal structure of PfATP4 and its binding interactions with the spiroindolones or other drugs has not been determined.
- Other compounds in the Malaria Box, with diverse molecular structures, also appear to target PfATP4. This suggest that PfATP4 is an important target that should be investigated further.

Acknowledgement

References

1. Wells TNC, Alonso PL, Gutteridge WE. New medicines to improve control and contribute to the eradication of malaria. *Nat. Rev. Drug Discov.* 8(11), 879–91 (2009).

2. World Health Organisation. World Malaria Report 2013 [Internet]. Available from: http://www.who.int/malaria/publications/world_malaria_report_2013/report/en/ (Accessed on 30 September 2014).

3. Greenwood B. Treatment of Malaria — A Continuing Challenge. *N. Engl. J. Med.* [Internet]. 371(5), 474–475 (2014).

4. Centers for Disease Control and Prevention. Malaria Biology [Internet]. (2012). Available from: http://www.cdc.gov/malaria/about/biology/ (Accessed on 30 September 2014).

5. Karunamoorthi K. Malaria Vaccine : A Future Hope to Curtail the Global Malaria Burden. *Int. J. Prev. Med.* 5(5), 529–538 (2014).

6. Smith PW, Diagana TT, Yeung BKS. Progressing the global antimalarial portfolio: finding drugs which target multiple Plasmodium life stages. *Parasitology.* 141(1), 66–76 (2014).

7. Wongsrichanalai C, Sibley CH. Fighting drug-resistant Plasmodium falciparum : the challenge of artemisinin resistance The Early Indications of Diminishing Efficacy. *Clin. Microbiol. Infect.* 19(10), 908–916 (2013).

8. Mäser P, Wittlin S, Rottmann M, Wenzler T, Kaiser M, Brun R. Antiparasitic agents: new drugs on the horizon. *Curr. Opin. Pharmacol.* 12(5), 562–6 (2012).

9. Wells TNC. Is the Tide Turning for New Malaria Medicines? *Sci.* 329 (5996), 1153–1154 (2010).

10. Rottmann M, McNamara C, Yeung BKS, *et al.* Spiroindolones, a potent compound class for the treatment of malaria. *Science* 329(5996), 1175–80 (2010).

11. Yeung BKS, Zou B, Rottmann M, *et al.* Spirotetrahydro beta-carbolines (spiroindolones): a new class of potent and orally efficacious compounds for the treatment of malaria. *J. Med. Chem.* 53(14), 5155–64 (2010).

12. Diagana T. Spiroindolones : a new chemotype for the treatment of malaria The NGBS consortium [Internet]. Available from: http://www.mmv.org/sites/default/files/uploads/docs/news/Spiroindolones_MMV_proje ct_of_the_year_FINAL.pdf (Accessed 30 September 2014).

13. Van Pelt-Koops JC, Pett HE, Graumans W, *et al.* The spiroindolone drug candidate NITD609 potently inhibits gametocytogenesis and blocks Plasmodium falciparum transmission to anopheles mosquito vector. *Antimicrob. Agents Chemother.* 56(7), 3544–8 (2012).

14. Meister S, Plouffe DM, Kuhen KL, *et al.* Imaging of Plasmodium liver stages to drive next-generation antimalarial drug discovery. *Science.* 334(6061), 1372–7 (2011).

15. Flannery EL, Fidock DA, Winzeler EA. Using genetic methods to define the targets of compounds with antimalarial activity. *J. Med. Chem.* 56(20), 7761–71 (2013).

16. Kirk K, Lehane AM. Membrane transport in the malaria parasite and its host erythrocyte. *Biochem. J.* 457(1), 1–18 (2014).

17. Spillman NJ, Allen RJW, McNamara CW, *et al.* Na(+) regulation in the malaria parasite Plasmodium falciparum involves the cation ATPase PfATP4 and is a target of the spiroindolone antimalarials. *Cell Host Microbe* 13(2), 227–37 (2013).

18.   Mauritz JMA, Seear R, Esposito A, *et al.* X-ray microanalysis investigation of the changes in Na, K, and hemoglobin concentration in plasmodium falciparum-infected red blood cells. *Biophys. J.* 100(6), 1438–45 (2011).

19.   Krishna S, Woodrow C, Webb R, *et al.* Expression and functional characterization of a Plasmodium falciparum Ca2+-ATPase (PfATP4) belonging to a subclass unique to apicomplexan organisms. *J. Biol. Chem.* 276(14), 10782–7 (2001).

20.   Dyer M, Jackson M, McWhinney C, Zhao G, Mikkelsen R. Analysis of a cation-transporting ATPase of Plasmodium falciparum. *Mol. Biochem. Parasitol.* 78(1-2), 1–12 (1996).

21.   Spillman NJ, Allen RJW, Kirk K. Na+ extrusion imposes an acid load on the intraerythrocytic malaria parasite. *Mol. Biochem. Parasitol.* 189(1-2), 1–4 (2013).

22.   Krishna S, Webb R, Woodrow C. Transport proteins of Plasmodium falciparum: defining the limits of metabolism. *Int. J. Parasitol.* 31(12), 1331–42 (2001).

23.   Dandapani S, Comer E, Duvall JR, Munoz B. Hits, leads and drugs against malaria through diversity-oriented synthesis. *Future Med. Chem.* 4(18), 2279–2294 (2012).

24.   White NJ, Pukrittayakamee S, Phyo AP, *et al.* Spiroindolone KAE609 for Falciparum and Vivax Malaria. *N. Engl. J. Med.* 371(5), 403–410 (2014).

25.   Leong FJ, Li R, Jain JP, *et al.* A First-in-Human Randomized, Double-Blind, Placebo-Controlled, Single- and Multiple-Ascending Oral Dose Study of Novel Antimalarial Spiroindolone KAE609 (Cipargamin) To Assess Its Safety, Tolerability, and Pharmacokinetics in Healthy Adult Volunteers. *Antimicrob. Agents Chemother.* 58(10), 6209–14 (2014).

26.   ClinicalTrials.gov. KAE609 [Internet]. (2014). Available from: https://clinicaltrials.gov/ct2/results?term=KAE609&Search=Search (Accessed 30 September 2014).

27.   Lehane AM, Ridgway MC, Baker E, Kirk K. Diverse chemotypes disrupt ion homeostasis in the malaria parasite. *Mol. Microbiol.* (2014).

28.   Gamo F-J. Antimalarial drug resistance: new treatments options for Plasmodium. *Drug Discov. Today. Technol.* 11, 81–88 (2014).

29.   Zhou Y, Fomovska A, Muench S, Lai B-S, Mui E, McLeod R. Spiroindolone that inhibits PfATPase4 is a potent, cidal inhibitor of Toxoplasma gondii tachyzoites in vitro and in vivo. *Antimicrob. Agents Chemother.* 58(3), 1789–92 (2014).