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Novel acid-labile and targeted nanoparticles as possible antimalarial drug delivery systems

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Summary

The multistage life cycle of malaria-causing *P. falciparum* is complex, making prevention and treatment difficult. As a result of resistance to many antimalarial drugs, novel compounds with unexplored targets are constantly sought after for the purpose of treating the symptoms of malaria. Here, novel compounds were screened for antiplasmodial activity against the symptom-causing asexual intraerythrocytic malaria-causing parasites. Unfortunately, many novel compounds in the drug discovery pipeline and drugs in clinical use possess underlying pharmacological issues that makes administration challenging. These include low aqueous solubility and short half-life which negatively impact bioavailability resulting in toxicity. This, in turn, increases patient non-compliance and the emergence of drug-resistant strains.

Nanoparticles (NP) have the ability to mask drugs from the external environment while increasing circulation time and often alleviate many issues at once. Furthermore, the selected drugs do not need to be modified. Drug conjugation NPs with a targeting ligand and stimuli-responsive linkers have been extensively researched in many diseases, however, none have been reported for malaria clinically. Here, the first acid-labile targeted NP (tNP) that exploits the biology of infected erythrocytes and the specialised food vacuole (FV) of *P. falciparum* is interrogated for ability to decrease toxicity while retaining antimalarial activity.

This dissertation describes the effect of tNPs on the efficacy and toxicity of selected compounds. *In vitro* haemolysis and cytotoxicity assays revealed that the tNPs are biocompatible to erythrocytes and HepG2 cells. The data also shows that tNPs decrease the toxicity of drugs and the chosen novel compound against human cells. A decrease in antiplasmodial activity was observed *in vitro* for the tNPs when compared to the novel compound and drugs on their own. However, this was due to the biogenesis of the FV and a shortened window of release. Nonetheless, the NP backbone was not active against *P. falciparum* intraerythrocytic parasites whereas tNPs were, showing activity due to released drug. The targeting ligand was also not specific for antiplasmodial activity.

Although a significant loss in activity is observed, the results presented here suggests that these novel acid-labile tNPs serve as an attractive starting point for targeted treatment of malaria with an improved patient tolerance. Furthermore, novel compounds with issues can be selected without having to be modified or completely discarded. Therefore, increasing the chances of finding a variety of compounds that can be used to treat malaria while keeping patients safe.

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List of abbreviations

ACT	Artemisinin based combination therapy
ADMET	Absorption, Distribution, Metabolism, Excretion, Toxicity
AL	Artemether and lumefantrine combination
AP	Aminopyrazine
AR	Artemether
ART	Artemisinin
AST	Astemizole
BI	Benzimidazole
CC_{50}	Half maximal cytotoxic concentration
CQ	Chloroquine
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E8	Elimination 8
EDTA	Ethylene dinitrotetraacetic acid
EM	Emetine
FA	Fusidic acid
FDA	Food and drug administration
FV	Food vacuole
GTS	Global technical strategy
H3D	Drug discovery and development centre
HCQ	Hydroxychloroquine
hpi	hours post invasion
IC_{50}	Half maximal inhibitory concentration
IDC	Intraerythrocytic developmental cycle
IMHB	Intramolecular hydrogen bond
IP	Imidazopyridazines
ITN	Insecticide treated nets
LDH	Lactate dehydrogenase
LF	Lumefantrine
M^2PL	Malaria parasite molecular laboratory
MMV	Medicines for malaria venture

MoA	Mode of action
MR4	Malaria research and reference reagent centre
MSF	Malaria SYBR Green I assay
MW	Molecular weight
NP	Nanoparticle
NPP	New permeability pathway
PB	Pyrido(1,2-a)benzimidazoles
PEG	Polyethylene glycol
PQ	Primaquine
PV	Parasitophorous vacuole
PVL	Polyvalerolactone
PVM	Parasitophorous vacuole membrane
PVP	Polyvinylpyrrolidone
QN	Quinine
RI	Resistance index
RPMI	Roswell Park Memorial Institute
SI	Selectivity index
SU	Stellenbosch University
TCP	Target candidate profile
tNP	Targeted nanoparticle
TPP	Target product profile
UCT	University of Cape Town
WHO	World Health organisation

Chapter 1: Literature review

1.1. Malaria disease burden

Malaria is an infectious disease caused by intracellular parasites belonging to the *Plasmodium* genus. Although preventable, 3.6 billion people are at risk of contracting malaria. An estimated 228 million cases were reported for the 2018 period, 405 000 of which were fatal [1]. Pregnant women and children aged under five years are the most vulnerable, accounting for 67 % of malaria associated mortalities, with a child mortality occurring every two minutes [2, 3]. The disease is endemic to mainly impoverished and resource-limited countries, severely affecting already vulnerable socio-economic structures [1].

Between 2000 and 2015, notable worldwide progress was made towards malaria elimination. During this time, global incidence and mortality rates decreased by 40 % and 60 %, respectively. The World Health organisation (WHO) revealed that no new cases were reported in 27 countries, 8 of which had been malaria free for three or more consecutive years in 2018 (Figure 1.1). However, the majority of African, South American and South-East Asian countries remain malaria endemic. Over 90 % of reported cases and mortalities occur in sub-Saharan Africa with the remainder affecting South-East Asia and South America [3].

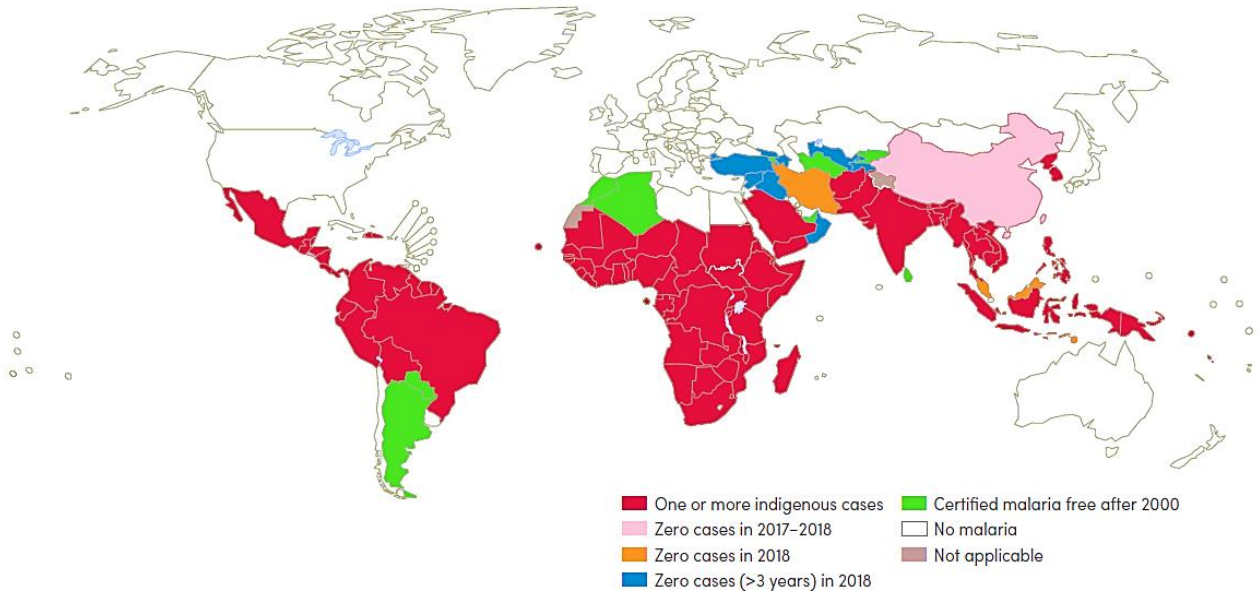


Figure 1.1. The 2018 global malaria case incidence status by country status.
Global malaria case profiles of countries that have either eliminated malaria (white), have no reported malaria cases since 2000 (green), have been malaria free for more than three consecutive years (blue) or no reported malaria cases in 2018 alone (orange). Many countries in Africa, Asia and South America are still burdened with malaria (red). Source: WHO World Malaria Report 2019 (URL: https://apps.who.int/iris/bitstream/handle/10665/275867/9789241565653-eng.pdf?ua=1) [1].

Several regional and global initiatives have been initiated in an effort to help countries reach their malaria elimination goals [4]. An example of this is the E-2020 established by the WHO; 21 countries, including South Africa, that had previously set goals to reach elimination status by 2020. The elimination 8 (E8) is a regional collaboration between 8 southern African countries with aims to achieve malaria elimination by 2020-2030 [3, 5]. However, the malaria mortality reduction rate has slowed down since 2016. South Africa reported 5-fold more cases in 2017 as compared to 2016 and will likely not reach the E2020 and E8 goals [6, 7].

Eliminating malaria has proven to be complicated and exhausting due to the complexity of *Plasmodium* parasites. There are five species of *Plasmodium* parasites that infect humans, namely *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [8]. However, *P. falciparum*, prevalent in sub-Saharan Africa, is responsible for the most severe cases and the highest global mortality [1].

1.2. The life cycle of *P. falciparum*

The complete life cycle of *P. falciparum* is observed through several morphologically distinct stages within the human host and the female *Anopheles* mosquito vector [9]. Over 30 species of *Anopheles* are important malaria vectors with *An. arabiensis* and *An. funestus* responsible for malaria transmission in South Africa [10]. The mosquito vector is host to the sporogonic stages of the *P. falciparum* life cycle whereas the human host houses the exoerythrocytic and intraerythrocytic stages within hepatocytes and erythrocytes, respectively (Figure 1.2).

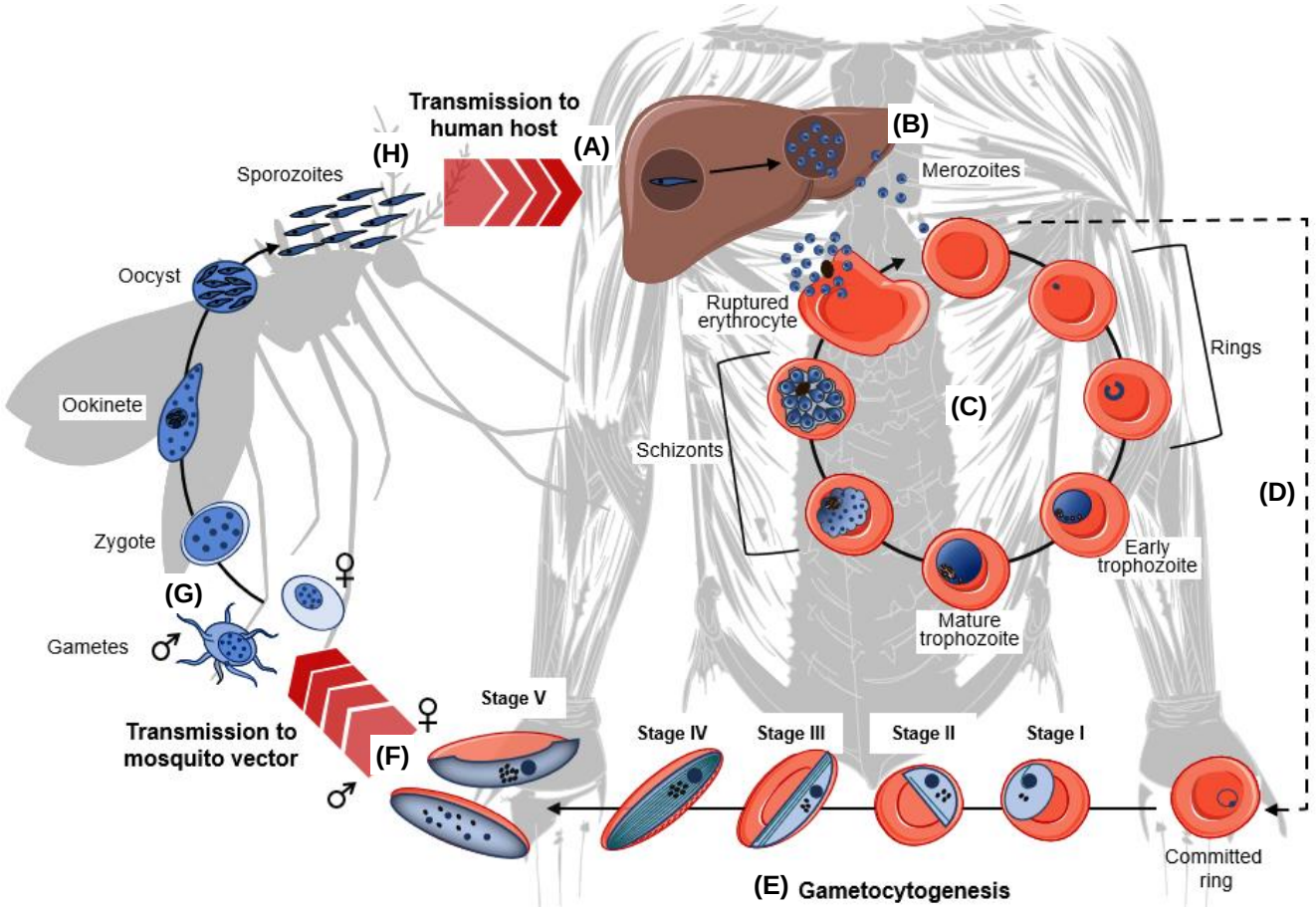


Figure 1.2. The life cycle stages of *P. falciparum* in the human host and mosquito vector.
The life cycle of *P. falciparum* is initiated (A) in the liver where the exoerythrocytic stages occur, beginning when an infected *Anopheles* mosquito transmits sporozoites to the human host during a blood meal. Transmitted sporozoites invade hepatocytes and develop into hepatic schizonts. (B) Hepatocytes rupture, releasing merozoites that can invade erythrocytes, thus initiating the asexual intraerythrocytic stages. (C) Asexual ring-stage parasites mature into trophozoites and subsequently into multinucleated schizonts. Daughter merozoites are released upon erythrocyte rupture and can invade new erythrocytes thereby continuing the asexual intraerythrocytic portion of the life cycle. (D) Some merozoites are sexually committed and will undergo gametocytogenesis, the sexual intraerythrocytic stages. (E) Gametocytogenesis is observed through five morphologically distinct stages. (F) Stage V gametocytes can be taken up by a mosquito vector during a blood meal, initiating the sporogonic stages. (G) In the sporogonic stages male and female gametes fuse to form an ookinete in the midgut of the vector. The ookinete enters the epithelial cells and develops into an oocyst which in turn releases sporozoites to the salivary glands of the mosquito. (H) The complete life cycle of *P. falciparum* is reinitiated when a mosquito takes a blood meal. This image was created using open source software Servier Medical Art and Microsoft PowerPoint.

1.2.1. The asexual life cycle stages

The life cycle of *P. falciparum* begins when an infected female *Anopheles* mosquito takes a blood meal, injecting mature motile sporozoites from its salivary gland into the human host via saliva [11]. Within an hour, transmitted sporozoites migrate through the skin into circulation until they reach the liver and infiltrate hepatocytes, initiating the exoerythrocytic stages (Figure 1.2. A).

Sporozoites then mature into multinucleated hepatic schizonts. Infected hepatocytes rupture, releasing thousands of merozoites into the bloodstream to invade erythrocytes, initiating the asexual intraerythrocytic developmental cycle (IDC) (Figure 1.2. B) [12].

The IDC begins when a merozoite comes into contact with the membrane of an erythrocyte causing the erythrocyte to deform. This allows merozoites to penetrate the membrane forcing its way into the erythrocyte [13]. Merozoites then develop into metabolically nondescript ring stages observed 0-24 h post invasion (hpi) [14], which subsequently mature into metabolically active trophozoites (22-36 hpi) [15]. This is followed by multiple rounds of nuclear division resulting in a multinucleated schizont 36-48 hpi from which 24-36 daughter merozoites are released upon erythrocyte rupture (Figure 1.2. C) [16].

Released merozoites invade new erythrocytes perpetuating the asexual intraerythrocytic portion of the life cycle. This perpetuation leads to the exponential increase in parasitaemia associated with the asexual proliferating stages [17]. The clinical symptoms of malaria (headache, fever, nausea, anaemia, muscle aches, digestive problems and shaking) which are characteristic of uncomplicated malaria, are observed during the multiple erythrocytic ruptures that occur every 48 h [18]. If left untreated, uncomplicated malaria can become severe, which is commonly the result of microvascular obstruction of capillaries, leading to organ damage, placental infection and ultimately death [19].

1.2.2. The sexual life cycle stages

A small proportion of schizonts commit to sexual differentiation and will release merozoites of the same sex [20]. Merozoites from a sexually committed schizont develop into sexually committed ring stages after erythrocyte invasion (Figure 1.2. D) [21]. Sexually committed ring-stage parasites then undergo gametocytogenesis: sexual differentiation into quiescent, non-dividing male and female gametocytes [22] in a biased 4:1 ratio, respectively [23]. Gametocytogenesis, accomplished within 10-14 days following erythrocyte invasion in *P. falciparum* [24, 25] is observed through five morphologically distinct stages classified as either early (stages I/II/III) or late (stage IV/V) stage gametocytes (Figure 1.2. E) [26]. Stage I-IV gametocytes are sequestered in the bone marrow and are absent from blood circulation to avoid clearance by the spleen [27, 28]. Maturation causes the erythrocytes to elongate, becoming a crescent-shaped thin layer around the parasite [22, 26]. Finally, stage V gametocytes are released into the circulatory system

[27] in preparation for transmission to a female *Anopheles* mosquito during a blood meal (Figure 1.2. F) where the remainder of the life cycle of *P. falciparum* occurs.

A change in environment between the human host and the mosquito vector, following a blood meal, stimulates the induction of gametogenesis. These environmental stimuli include a decrease in temperature, the presence of xanthurenic acid and an increase in pH [29-31]. Male gametocytes exflagellate into eight microgametes, whereas female gametocytes roundup to form macrogametes ready for fertilisation. Fertilisation produces a diploid zygote which matures into a motile ookinete (Figure 1.2. G) [32, 33] that then enters the epithelial cells of the midgut wall. The ookinete develops into an oocyst that produces haploid sporozoites after multiple rounds of replication [34, 35]. Oocysts then rupture releasing sporozoites into the body cavity of the mosquito, completing the life cycle of *P. falciparum* [36, 37]. Sporozoites migrate from the body cavity into the salivary glands of the mosquito, wherefrom transmission to a human host can occur via saliva during another blood meal (Figure 1.2. H) [32].

The differential multistage development of *P. falciparum* is imperative for parasite survival and transmission. The existence of organelles such as the food vacuole (FV) which is generated during the life cycle of *P. falciparum* parasites, is imperative for growth and survival.

1.3 Biogenesis of the food vacuole

Biogenesis of the FV, an acidic organelle that evolved for specialised function within *P. falciparum* parasites, occurs during the IDC and is one of the most important biological processes governing parasite biology (Figure 1.3) [38, 39]. The FV plays a role in the detoxification of oxygen radicals [40], storage of non-degradable biomolecules and regulation of intracellular osmolarity of the infected erythrocyte [41-43], all of which are essential for parasite survival. Since the parasite increases in surface area, almost expanding to the entire erythrocyte volume [44], it has been suggested that the degradation of erythrocyte cytoplasm might be necessary to make room for the parasite to grow. However, the main function of the FV is the degradation of haemoglobin which is the primary constituent of erythrocytic cytosol [45, 46]. Haemoglobin degradation supplies intraerythrocytic parasites with amino acids for the synthesis of new proteins since only a limited capacity of *de novo* amino acid synthesis is possible [46]. The degradation of haemoglobin, unfortunately, also results in the release of toxic haem which is neutralized by parasite primarily

by the formation of haemozoin crystals [47]. Therefore, in order for parasites to survive, haemoglobin degradation and haem detoxification are essential [48].

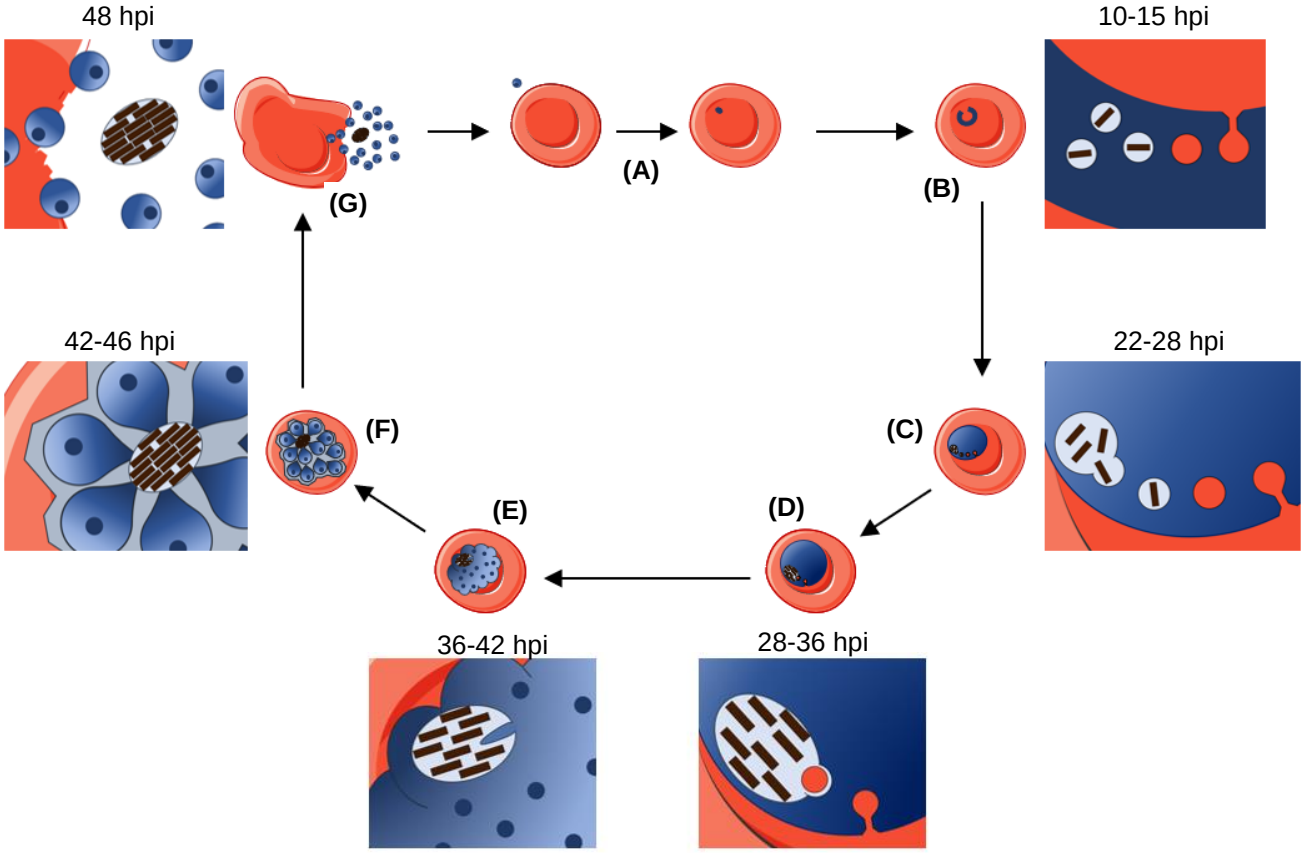


Figure 1.3. Biogenesis of the specialised food vacuole of *P. falciparum* parasites.
The food vacuole (FV) is formed *de novo* following (A) erythrocyte invasion by a merozoite. (B) After 10-15 h post invasion (hpi) where the parasite progresses into late ring stages, erythrocyte cytoplasm is taken up through micropinocytosis forming small vesicles, where some haemoglobin digestion occurs. (C) Endocytosis of the host's cytoplasm continues until the early trophozoite stages where the small vesicles start to fuse to form a larger FV 22-28 hpi. (D) A complete, intact FV is observed in the mid to late trophozoite stages (28-36 hpi) where the majority of haemoglobin digestion and haem degradation occurs. (E) This continues until early schizont-stage parasites (36-42 hpi) where the FV membrane fold as the FV shrinks, slowing down the feeding process. (F) The condensed FV in late schizonts (42-46 hpi) is now separated from the individual merozoites ready to be (G) released once the erythrocyte ruptures 48 hpi. This image was made using open source software Servier Medical Art and Microsoft PowerPoint. Adapted from [49].

The FV forms *de novo* following erythrocyte invasion by a merozoite (Figure 1.3. A) [50]. Intracellular merozoites are enclosed within a newly formed parasitophorous vacuole (PV) and the PV membrane (PVM), separating it from the cytoplasm of the surrounding erythrocyte [48]. The inner membrane, which develops from the PVM, rapidly disappears during the early ring stages, allowing more efficient nutrient diffusion into the parasite cytoplasm [51]. During the mid to late ring stages (10-15 hpi), host erythrocyte cytoplasm is taken up through micropinocytosis and

thereafter concentrated into small food vesicles (Figure 1.3. B) [52]. As the parasite matures to early trophozoite stage parasites (22-28 hpi), the small vesicles coalesce to form a single, large FV (Figure 1.3. C) [49]. Portions of haemoglobin-rich host cytoplasm continue to be pinched off and transported to the FV within small vesicles [49, 53]. The majority of erythrocyte cytoplasm consumption and subsequent haemoglobin degradation is observed in the now large, consolidate FV of mid to late trophozoite stages (Figure 1.3. D) [48]. Here, the very dense haemozoin crystals can also easily be observed with a light microscope [54]. Feeding continues until the early schizont stages where the FV shrinks due to feeding slowing down (Figure 1.3. E). The FV, which is now separated from the individual merozoites (late schizont) becomes condensed (figure 1.3. F). Once the erythrocyte ruptures, the FV is released along with merozoites (Figure 1.3. G).

The adaptability and plasticity of *P. falciparum* has made efforts towards prevention, control and elimination of malaria more complicated. Therefore, to achieve malaria elimination goals an integrative strategy that targets the vector and various stages of the parasite is required.

1.4. Malaria prevention and control

1.4.1. Vector control

The aim of vector control is to prevent parasite transmission by hindering any contact between infected mosquitoes and humans, through the use of insecticides. Long-lasting insecticides (carbamates, organophosphates, organochlorides and pyrethroids) [55] are commonly used for insecticide-treated mosquito nets (ITN) and indoor residual spraying [7, 19]. The most efficient vector control method is the use of ITNs with more than half of the global population having been protected [56]. Unfortunately, the incorrect use of ITNs e.g. for fishing or agriculture, further limits their efficacy rendering humans unprotected [4]. Furthermore, mosquitoes continuously evolve, resulting in resistance to many insecticide classes as well as changes in behavioural and feeding patterns [57]. As a result, other methods of control recently explored include mosquito larvae control through larvicides, the destruction of mosquito breeding areas and the search for endectosides, molecules that are ingested by the host to target the mosquito vector during a blood meal [10, 58, 59].

1.4.2. Parasite control

Parasite control strategies target the various life cycle stages of *P. falciparum* and in so doing, disrupt development of the symptom-causing asexual intraerythrocytic stages and the sexual

gametocyte stages. Current explored parasite control strategies include vaccine development and antimalarial drugs.

1.4.2.1. Vaccine development

There are three main strategies for vaccine development. One strategy is exoerythrocytic vaccines that target the hepatic stages to prevent progression to intraerythrocytic stages. Another strategy is to develop vaccines that target the intraerythrocytic stages to prevent disease. Lastly, the development of vaccines that target gametocytes to prevent transmission of parasites from the human host to the mosquito vector. [60].

Vaccine development for malaria has been challenging due to the large, complex genome of *P. falciparum* parasites which also have highly effective host immune evasion strategies [61]. The most promising vaccine candidate RTS,S/AS01 (developed by GlaxoSmithKline), effective against the exoerythrocytic stages of parasite development, has completed phase III clinical evaluation [62]. Unfortunately, RTS,S/AS01 fails to provide long-term prevention, and a highly variable patient response was reported [63] with a maximum efficacy of 56 % in children under the age of 5 [64]. A fully effective candidate that provides long-term protection is ideal [62, 65]. Therefore, in the absence of an ideal vaccine, parasite control mainly relies on the use of antimalarial drugs.

1.4.2.2. The use of antimalarial drugs for chemotherapy and chemoprevention

The most successful parasite control strategy is the use of antimalarial drugs [2]. However, before antimalarial drugs can be administered, suspected patients need to be diagnosed correctly and very quickly. Therefore, diagnosing methods such as rapid diagnostic tools and microscopy are important for correct treatment and control. There are many antimalarial drugs used to treat malaria and these are classified either by structure or mode of action (MoA).

Quinine (QN) was the first known antimalarial compound and is the parent compound from which many antimalarials are derived [66]. Quinine derivatives can be classified as either an amino alcohol and quinoline; chloroquine (CQ) is a 4-aminoquinoline, primaquine (PQ) a 8-aminoquinoline, and lumefantrine (LF) an amino alcohol. Quinine and derivatives interfere with the haem degradation process in the FV as the MoA [19]. PQ is effective against the intrahepatic stages and is also gametocytocidal, showing potential as a transmission-blocking drug. However, PQ causes haemolysis in erythrocytes that are deficient of glucose-6-phosphate dehydrogenase

which resulting in anaemia in the said individuals [67, 68]. CQ is one of the most potent and cheap antimalarial drugs to date, however, widespread resistance was reported during the 1970's [69].

Antifolates target either dihydrofolate reductase or dihydropteroate synthase, enzymes involved in the folate pathway [2]. Folate metabolism produces a cofactor essential for the synthesis of thymine and purine nucleotides used in DNA replication [70]. Although DNA replication is an essential process in humans and malaria-causing parasites, humans are able to obtain essential folic acids through the diet whereas *Plasmodium* rely on the synthesising process. Therefore, antifolates target parasites with a high selectivity [2]. Drugs in this class include pyrimethamine and sulfadoxine which were used after CQ was no longer administered. However, parasites developed rapid resistance to this class as well [71, 72].

Artemisinin (ART) is an endoperoxide extracted from *Artemisia annua*. ART and derivatives, such as artemether (AR) and artesunate, are highly potent against malaria parasites but have a very short plasma half-life [73]. Artemisinins are most effective when used in combination with at least one partner drug that has a longer half-life, usually an amino alcohol or 4-aminoquinoline. This specific partnering of drugs with an ART derivative is termed artemisinin-based combination therapy (ACT) and is currently the WHO recommended first-line standard of treatment for malaria infections [74]. Other advantages of combination therapy over monotherapy include decreased adverse effects and a lowered chance for parasites to form resistance [75]. Unfortunately, resistance to ACTs has been reported in various parts of the world, including Africa [76, 77].

Continuous drug resistance to various antimalarials threatens the main strategy of parasite control and the progress made towards the decrease in malaria infections and fatalities. Therefore, novel compounds and strategies are required.

1.5. Strategies for identification and development of novel compounds

The continual generation of new molecules is required as drug-resistant *Plasmodium* parasites will always be a concern. Therefore, antimalarial drug discovery and development focuses on introducing novel compounds with unexplored MoA. Additionally, drugs need to be affordable, especially for the poor communities most affected by malaria, and potent against *Plasmodium* parasites yet safe enough to be given to pregnant women and children [78]. Furthermore, a single dose in an orally bioavailable format is ideal.

The Medicines for malaria venture (MMV), a not-for-profit organisation, was created to support the WHO mandate of developing affordable medicines and has a proposed minimum standard for medicine required for elimination [58]. Clear goals have been proposed by the MMV for the types of medicines needed, which are defined as target product profiles (TPPs). TPPs comprise of one or more active molecules (target candidate profiles (TCPs)) targeting at least one of the life cycle stages of malaria-causing parasites (Figure 1.4).

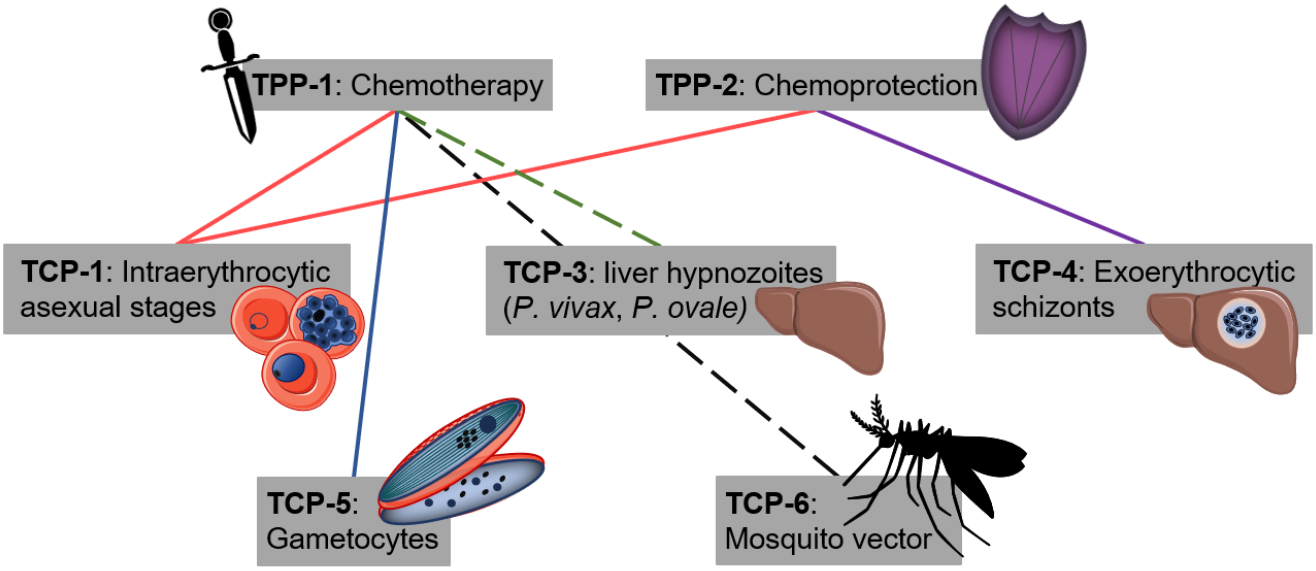


Figure 1.4. Overview of the Medicines for malaria's strategy for ideal antimalarial medicines.
The relationship between the individual target candidate profiles (TCPs) required to form the two target product profiles (TPPs) with solid lines used for relevance to *P. falciparum* in the human host. TPP-1 focusses on treating infected individuals whereas TPP-2 aims to protect individuals from infection. A combination of TCP-1 (asexual intraerythrocytic stages) and TCP-5 (gametocytes) activity defines TPP-1 against *P. falciparum* in the human host whereas TPP-2 requires a partnering of TCP-1 and TCP-4 (hepatic schizonts) activity. TCP-3 (liver hypnozoites) is relevant only to *P. vivax* and *P. ovale* infections whereas TCP-6 targets the mosquito vector. This image was adapted from [58] using images from Microsoft word and open source software Servier Medical Art.

There are five TCPs: TCP-1, TCP-3, TCP-4, TCP-5 and TCP-6, categorised based on the life cycle stage that is affected. TCP-3 are molecules that target dormant hypnozoites in the liver, which are only present in *P. vivax* and *P. ovale* infections. Therefore, TCP-3 molecules are not relevant to *P. falciparum.* Chemoprotection (TPP-2) involves seasonal chemoprevention in endemic areas and for individuals who plan to visit malaria endemic areas. TPP-2 medicines strategise the combination of TCP-1 and TCP-4 molecules. TCP-4 focuses on the clearance of liver schizonts whereas TCP-1 clears the asexual intraerythrocytic stages in an effort to treat the symptoms of malaria [58]. The quinine derivatives, antifolates and ART derivatives are all

examples of TCP-1 molecules [79]. TPP-1, the envisioned medicine for treatment of *P. falciparum* infections, is a combination of TCP-1 and TCP-5 molecules. TCP-5 target the gametocyte stages to prevent human to vector transmission [58]. Transmission-blocking aims to either eliminate stage V gametocytes or render them unable to progress to gametogenesis. Since PQ has many side effects, there is no universally used TCP-5 antimalarial.

The MMV has a comprehensive list of new compounds at different phases of clinical development. Among others are kinase inhibitors developed on the African continent such as MMV390048 and UCT943, both of which possess TCP-1, TCP-4, TCP-5 and TCP-6 activity [80-82]. Several other compounds in clinical development target the gametocyte stages including tafenoquine as well as the endoperoxides OZ439 and OZ277, all of which show potential as TCP-5 molecules towards TPP-1 medicines [83, 84].

Whilst multistage activity is a preferred characteristic of any new potential antimalarial, the search for TCP-1 candidates remains the first priority, to treat symptomatic patients and save lives.

1.6. Drawbacks in antimalarial drug treatment

Although resistance is one of the greatest concerns regarding antimalarial drugs, other factors render therapy difficult. To maximise patient cooperation and simplicity of treatment even in remote areas, drugs are administered in an orally bioavailable format. However, some drugs have poor oral bioavailability due underlying pharmacological issues including toxicity, low aqueous solubility, as well as low physical and metabolic stability [85, 86].

The ACT, Coartem, is a combination treatment of AR and LF where AR is potent against *P. falciparum* parasites and LF is the slow acting partner drug that removes residual parasites. However, LF is highly lipophilic which translate to low aqueous solubility, and a high lipid diet is required when taking the medicine [87]. A low aqueous solubility can result in variable bioavailability thereby affecting clinical response. As much as 40 % of candidates in the drug discovery and development pipeline as well as approved candidates possess low aqueous solubility [88].

Rapid renal clearance or degradation of therapeutic agents is a concern as sufficient doses may not reach the intended target tissue. For example, most ART derivatives including AR have a short plasma half-life and a poor oral bioavailability. Less than 35 % of the ingested drug reaches the

blood stream and even less the target tissue, therefore, multiple doses are required [89]. Drugs with insufficient solubility, stability and bioavailability are required in higher doses which can lead to side-effects clinically [90]. This coupled with multiple doses and a change in diet increases the possibility of patient non-compliance which in turn increases the chances of treatment failure and the formation of resistant strains.

In order to improve the efficacy and therapeutic index of novel compounds, new strategies need to be developed. The most used strategy to improve various shortcomings of antimalarial drugs is the development of synthetic derivatives that possess improved pharmacological properties. It is crucial that drug candidates have their pharmacokinetic issues improved before clinical trials, therefore, chemical modifications need to occur early on at the drug discovery stage [91]. In addition to cost implications, chemical modifications may also result in the improvement of one shortfall at the expense of another. Artemisone and artelinic acid are derivatives of ART with improved toxicity and half-life, respectively [92, 93]. However, the ART derivatives have only recently made it into clinical trials, revealing how much time is required. Furthermore, the development of synthetic derivatives can be costly.

In recent years, nanocarriers have been explored as an approach to overcome these challenges through use drug delivery systems [94].

1.7. Nanoparticles as drug delivery systems

Nanocarriers consist of nanoparticles (NP) that can be used as drug delivery systems with the main goal being to protect the drug from extracellular degradation or renal clearance. NPs can also improve target specificity and the pharmacokinetic profile as well as reduce the administration dose and dose frequency required, thereby reducing possible dose related toxicities i.e. side effects [94].

1.7.1. Encapsulation *vs*. polymer conjugation

Drugs are partnered to NPs either through encapsulation or conjugation. Encapsulation is described as the physical entrapment of a therapeutic agent within the NP, which usually has an amphiphilic backbone. Polymer conjugation is described as chemically conjugating the drug to the polymeric backbone using a linker (Figure 1.4) [95].

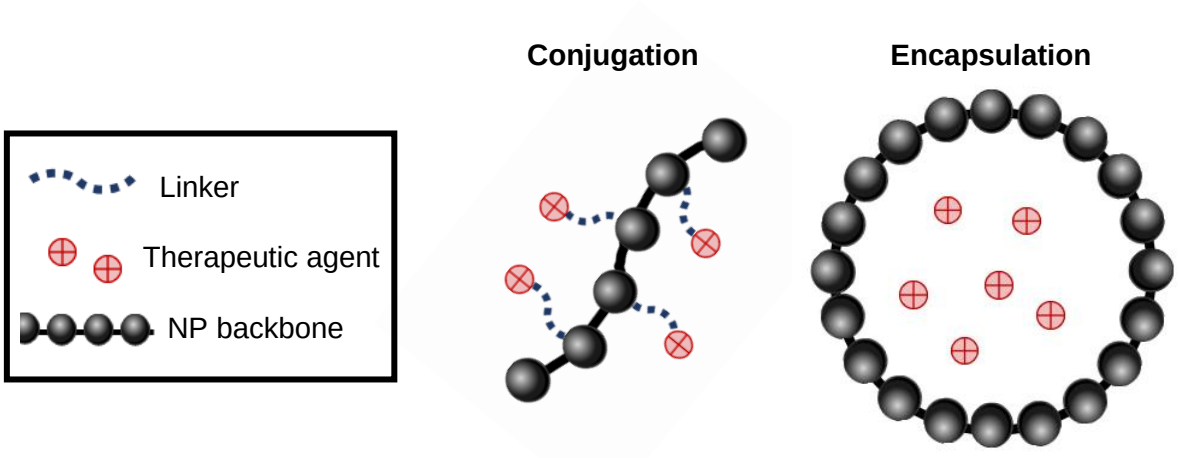


Figure 1.5. The difference between encapsulation and conjugation of therapeutic agents.
Medicines are either physically encapsulated into an amphiphilic backbone where the therapeutic agent is free within the nanoparticle (NP) or chemically conjugated to a polymeric NP backbone where the therapeutic agent is attached by a linker. Adapted from [95].

The encapsulation of a therapeutic agent into a NP backbone requires a simpler method of synthesis and less time. Furthermore, a larger variety of drug classes can be incorporated into the core of the NP. In other words, if the core of the NP is hydrophobic then any hydrophobic drug can be encapsulated and vice-versa. Unfortunately, many encapsulations have been found to release drugs early, before reaching the target site [95]. Nonetheless, encapsulation has been highly successful in several instances with examples such as the first Food and drug administration (FDA) approved anticancer nanomedicine Doxil, which consists of a highly toxic antibacterial agent encapsulated in a liposomal NP [96]. This resulted in decreased toxicity and increased the duration of drug circulation.

Chemical conjugation is a more complicated process [95] but reduces the chances of premature drug release because of the addition of a linker, which can be designed to respond only to a specific environmental stimulus. This is based on several theories with the most prominent strategy being that of Helmut Ringsdorf where the NP includes a linker to attach the therapeutic agent to a biocompatible and water soluble backbone [97]. Polymer conjugations are consequently more stable against non-specific environmental changes resulting in a higher circulation time [95, 98]. Stimuli-responsive linkers mimic biological systems by exploiting an external stimulus to allow a change in properties resulting in the release of the drug from the NP backbone [99, 100]. Commonly used stimuli are temperature, pH, light, electric field and even concentration e.g. glucose or salts [101]. Unfortunately, the attachment to a linker limits drug groups that can be conjugated to the NP.

NPs in the form of encapsulations and conjugations for antimalarial use is an emerging field which can be used to alleviate issues associated with drugs. Furthermore, NPs can be modified to be targeted to a specific tissue or cell type.

1.7.2. Active and passive delivery of NPs

Normal drug administration results in unselective distribution throughout various tissues in the body [95, 102, 103]. Therefore, only a small portion reaches the desired target, and a higher dose is required to achieve a therapeutic effect. To improve tissue specificity, NPs take advantage of two main methods: passive and active NP delivery both of which rely on differences between the diseased and healthy tissue. Very few active methods have been investigated with the majority of research based on only exploiting passive delivery.

Passive transport relies on structural differences caused by the disease. Uptake of drugs and also NPs, by implication, mostly rely on structural changes such as increased permeability, that is typically observed in diseased or infected cells [104]. This means that NPs can be passively taken up into the diseased cells at a higher concentration than into healthy cells, due to enhanced permeability features. For instance, cancerous cells typically have enhanced permeability and additionally have the ability to retain small molecules or NPs at a higher concentration than normal tissue [104, 105]. Comparatively, in *P. falciparum*-infected erythrocytes new structural changes include the deposit of nonspecific porous-like structures called ‘new permeability pathways’ (NPP), making the membrane of an infected erythrocyte more permeable as intracellular parasites mature to the trophozoite and schizont stages [14]. Unfortunately, passive NP delivery has some shortcomings. NPs may still distribute towards healthy cells and a small concentration may make it across the membrane. Therefore, there is a need to develop NPs that lower the distribution to healthy cells, enhancing uptake into only diseased or infected cells.

Active NP delivery enhances the effects of passive delivery by making the NP more specific to diseased or infected cells through the addition of a ligand. These specific ligands (termed targeting ligands) can be carbohydrates, antibodies, peptides or proteins [106] that are then conjugated to the NP for delivery to the diseased or infected cells such as *P. falciparum*-infected erythrocytes in the case of malaria [98]. Targeting ligands are usually selected based on the knowledge that diseased cells have different receptors on their surfaces as compared to healthy cells [107]. If a specific receptor is known then a ligand can be designed or synthesised to target the receptor, if not, then other methods such as panning of a library of ligands can be done to find those that bind

to the diseased or infected cells. The addition of such a targeting ligand is meant to allow the NP to have a higher affinity towards the diseased or infected cells resulting in a higher chance of uptake into the cell even through NPP. In the case of *P. falciparum* infections, erythrocytes undergo several membrane changes in lipid composition, proteins and cell surface knobs, to which ligands can be attracted [102].

1.8. Work leading up to this project

This study was part of a collaboration with Prof Bert Klumperman as principal investigator of the Advanced Macromolecular Structures Group at Stellenbosch University (SU). This group designed and developed the NPs as described below. The main aim was to generate a polymer-based NP drug delivery system to which the therapeutic agent was conjugated through an acid-labile linker for controlled release.

1.8.1. The structure and rationale of the NPs

The are many options for polymers to use in the synthesis of NP backbones. Polyethylene glycol (PEG) is the “golden standard” for drug therapy polymers because it is non-toxic, water-soluble and FDA approved [108]. Additionally, PEG increases the molecular weight (MW) and hydrophilicity high enough to avoid renal clearance, increasing the duration of therapeutic agents in blood circulation. Polyvinylpyrrolidone (PVP), known as a plasma expander for trauma patients, possesses the same advantages as PEG [109]. Furthermore, PVP has a longer plasma half-life and lower tissue distribution than PEG, making it attractive for tissue-specific drug delivery [110]. For this study, novel amphiphilic NPs were made from a polyvinylpyrrolidone-block-polyvalerolactone backbone (PVP-b-PVL) where PVP and PVL compose the hydrophilic and hydrophobic components of the amphiphilic NP backbone, respectively.

As previously discussed, stimuli-responsive linkers can be used for conjugation of selected therapeutic agents to the NP backbone. Here, a pH responsive, acid-labile linker was used. The linker is stable at physiological pH (pH 7.4) and becomes unstable at a pH of 4.5 to 5.5 (acidic environment) releasing the drug from the polymeric NP backbone. The purpose of a pH responsive linker was to exploit the acidic environment of the FV of *P. falciparum* parasites, ensuring release only once the NP is taken up into an infected erythrocyte (Figure 1.6).

Since the best method is to take advantage of both active and passive NP delivery, a targeting ligand was conjugated to the NP backbone to enhance targeting to infected erythrocytes. With the

knowledge that erythrocytes change when infected, Eda, Eda and Sherman (2004) screened a phage display library of various peptides on both infected (50-90 % parasitaemia) and non-infected erythrocytes [102]. After the sixth panning, the remaining peptides were sequenced and only 8 peptides had attached to only infected erythrocytes. Although the receptors of these peptides is not yet known, they had a high affinity towards infected erythrocytes. Only GSRSKGT was hydrophilic and highly water soluble *in vitro* and would be beneficial for the exterior space to accommodate the self-assembly of the NP [111]. The peptidic targeting ligand, GSRSKGT, was then conjugated to the NP and is henceforth described as the targeted NP (tNP).

The tNP is expected to remain intact at physiological pH, target *P. falciparum*-infected erythrocytes as a result of the targeting ligand and once taken up by the erythrocyte make its way to the FV where the acid-labile linker would release the therapeutic agent (Figure 1.6. B). This is contrary to the NP that would only take advantage of passive delivery into both infected and non-infected erythrocytes (Figure 1.6. B). Once inside infected erythrocytes, it is proposed that the nanocarriers will distribute throughout the *P. falciparum* parasite and eventually enter the food vacuole (FV). The FV has an approximate pH of 5.0-5.5 whereas *P. falciparum* parasites have a pH between 7.0 and 7.4 [112] and erythrocytes have a pH of 7.1 [112, 113] (Figure 1.6). Therefore, once inside the FV the pH responsive linker will degrade, releasing the drug from the rest of the NP component. This target-specific drug release should ensure maximum antimalarial efficacy.

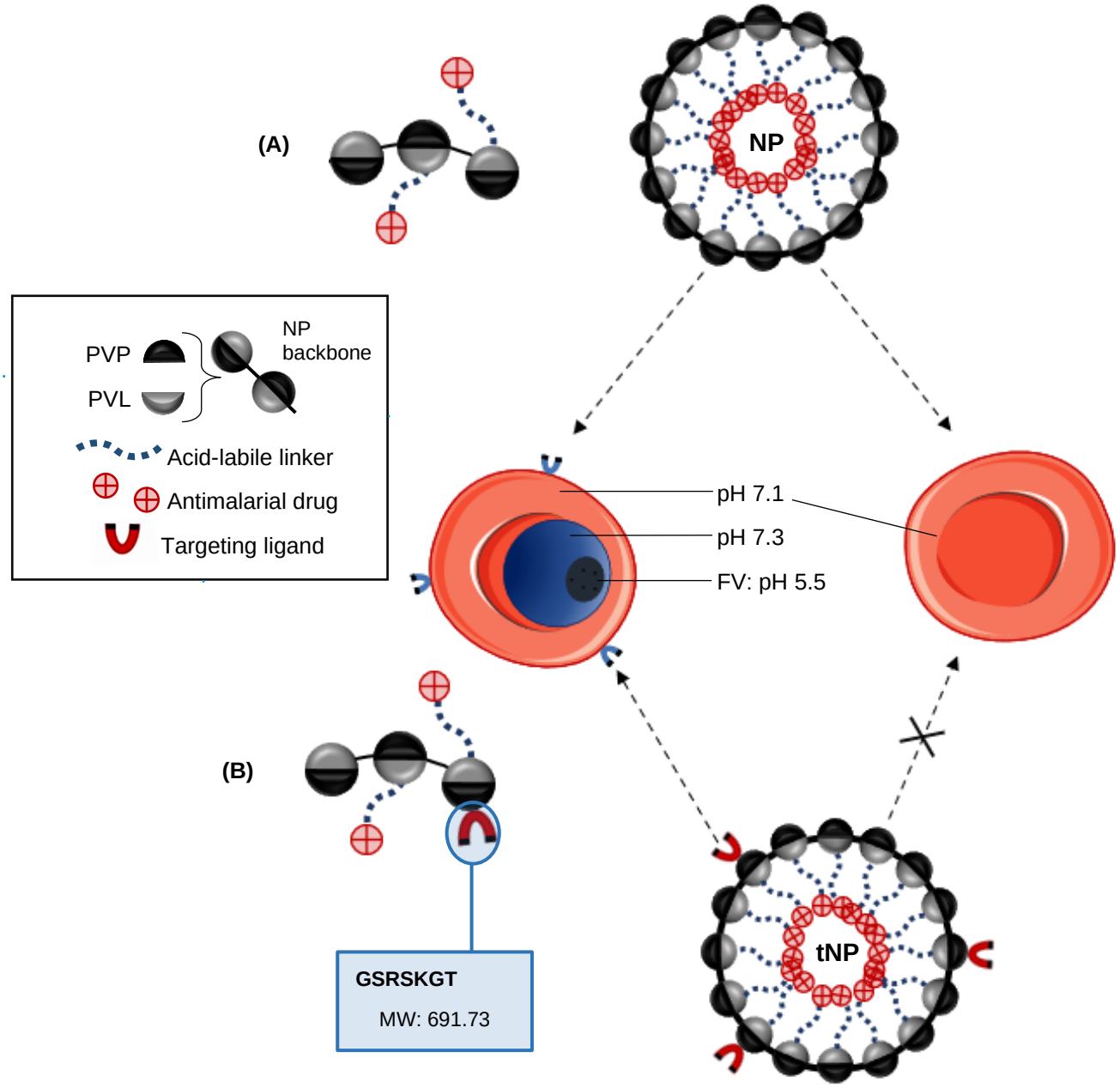


Figure 1.6. The design and action of acid-labile novel nanoparticles (NPs) and targeted nanoparticles (tNPs) for treatment against *P. falciparum* parasites.

The (A) nanoparticles (NP) were synthesised with an amphiphilic backbone consisting of hydrophobic polyvalerolactone (PVL) and hydrophilic polyvinylpyrrolidone (PVP). This would assemble into a micelle structure to further protect the antimalarial drug. The drug would only be released under the acidic environment of the FV (pH 5.0-5.5). However, (B) targeted NP (tNP) containing the peptide ligand GSRSKGT, with a higher affinity for infected erythrocytes, would be taken up in the same way and released in the FV. Further ensuring only targeted release. This image was adapted from Burrows *et al*., 2017 using images from Microsoft word and open source software Servier Medical Art using information provided by Dr Lisa Fortuin from Stellenbosch University.

Both the NP and tNP systems were used in this study, in the first instance as a proof-of-principle by conjugating with known antimalarial drugs that have known solubility, toxicity and instability

issues, to evaluate the performance of the system. Secondly, the NPs containing a novel active compound identified though a whole cell screening approach was used to explore its potential to improve performance of the new compound as potential antimalarial candidates.

1.9. Aim

To determine whether acid-labile targeted NPs (tNPs) would improve antiplasmodial activity and toxicity of antimalarial drugs and be useful to improve performance of antimalarial candidates.

1.10. Hypothesis

Novel targeted, acid-labile tNPs that mask drugs will improve antiplasmodial activity and toxicity of antimalarial drugs.

1.11. Objectives

The following individual objectives were completed to achieve the aim of the project.

1. Evaluate the safety of the novel acid-labile NP systems on mammalian and *Plasmodium* cell lines.
2. Determine the effect of incorporating known antimalarial drugs into the acid-labile NP on antimalarial activity and toxicity.
3. Identify hit antimalarial compounds through whole cell phenotypic screening.
4. Determine the effect of incorporating the new antimalarial hit compounds into the acid-labile NP system.

1.12. Research Outputs

1.12.1. Conferences and Symposia

Leshabane M., Fortuin L., Mbizana S., Klumperman B., Coertzen D. and Birkholtz L. Exploring novel targeted nanocarriers as possible antimalarial drug delivery systems. 4th South African Malaria Research Conference. Poster presentation. Pretoria, South Africa, July 2019.

Leshabane M., Fortuin L., Mbizana S., Klumperman B., Coertzen D. and Birkholtz L. Exploring novel targeted nanocarriers as possible antimalarial drug delivery systems. NRF Communities of Practice: Discovering drugs to eliminate malaria symposium. Poster presentation. Pretoria, South Africa, Nov 2019.

1.12.2. Publications

Leshabane M., Dziwornu G., Reader J., Coertzen D., Moyo P., van der Watt M., Erlank E., Chisanga K., Nsanzubuhoro C., Ferger R., Venter N., Koekemoer L., Chibale K., Birkholtz L. Benzimidazole derivatives are potent against multiple life cycle stages of *Plasmodium falciparum* malaria parasites. Submitted to ACS Infectious Diseases (ID: 2020-00910v).

Fortuin L., Leshabane M., Pfukwa R., Coertzen D., Birkholtz L. and Klumperman B. (2020) Facile route to targeted, biodegradable polymeric prodrugs for the delivery of combination therapy for malaria. ACS Biomaterials Science and Engineering 6 (11), 6217-6227. doi: 10.1021/acsbiomaterials.0c01234.

Jokonya S., Langlais M., Leshabane M., Reader P. W., Vosloo J., Pfukwa R., Coertzen D., Birkholtz L., Rautenbach M. and Klumperman B. (2020) Poly(N-vinylpyrrolidone) anti-malaria conjugates of membrane disruptive peptides. ACS Biomacromolecules 21 (12), 5053-5066. doi: 10.1021/acs.biomac.0c01202.

Coertzen D., Reader J., van der Watt M., Leshabane M., Langeveld H., Cheuka P.M., Dziwornu G.A., Chibale K., Birkholtz L. Exploring the transmission-blocking activity of antiplasmodial 3,6-diarylated Imidazopyridazines. Submitted to Transactions of the Royal Society of South Africa (ID: TTRS-2020-0065).

Chapter 2: Materials and methods

2.1. Statement of ethical clearance

All *in vitro* experiments were carried out at the Malaria Parasite Molecular Laboratory (M^2PL) which has a certified biosafety level P2 facility. The M^2PL holds ethical clearance from the University of Pretoria's Faculty of Natural and Agricultural Sciences (reference number 180000094) for the cultivation of parasites and from the Faculty of Health Sciences (reference number 506/2018) for the use of human erythrocytes to this end.

2.2. Novel compounds

A total of 277 compounds from various classes were synthesised at and obtained from the University of Cape Town's (UCT) Drug Discovery and Development Centre (H3D) as part of a collaborative effort. The compounds, CQ and emetine (EM) were then dissolved in 100 % dimethyl sulfoxide (DMSO, Sigma, USA) to a 10 mM concentration and stored at either -20 °C for long-term storage or 4 °C when used regularly.

2.3. Collection of blood and storage of erythrocytes

Human whole blood (blood type A^+ or O^+) was collected in anticoagulated blood bags (Adcock Ingram, South Africa) from consenting donors. Packed erythrocytes resuspended to a final haematocrit of 50 % in incomplete Roswell Park Memorial Institute (RPMI) 1640 culture media containing 2 % (w/v) D-glucose (Sigma-Aldrich, USA), supplemented with additional 2 % (w/v) D-glucose, 25 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonate (HEPES), 0.2 mM hypoxanthine, 23.8 mM sodium bicarbonate (Sigma-Aldrich, USA) and 0.024 mg/ml gentamycin (Fresenius, Germany) and stored at 4 °C for no more than 2 weeks [114].

2.4 Cultivation and synchronisation of *in vitro* intraerythrocytic asexual *P. falciparum* parasites

Cryopreserved drug-sensitive (NF54 strain) and multidrug-resistant (K1 strain) *P. falciparum* parasites were obtained from BEI resources under the MR4 repository (Malaria research and reference reagent centre, Manassas, USA). The parasites were thawed and, thereafter, cultivated *in vitro* using an adaptation of the Trager and Jensen method [115] as previously described [114]. Parasite cultures were maintained at 5 % haematocrit in complete RPMI-1640 culture media [incomplete culture media supplemented with 5 g/L AlbuMAX II (Life Technologies, USA)]. AlbuMAX II is a bovine serum albumin source used to replace serum and results in decreased

variability between samples while maintaining optimum parasite health. Cultures were gassed with a hypoxic tri-gas (90 % N_2, 5 % O_2 and 5 % CO_2) following daily replenishment of media and kept at 37 °C rotating at ~60 rpm. The rotary platform is necessary to encourage merozoite invasion into erythrocytes while minimising multiple invasions of a single erythrocyte [116-118]. Parasite viability and parasitaemia was monitored daily with a light microscopy (1000x magnification, oil immersion) of methanol-fixed, Giemsa-stained thin blood smears. For optimal parasite growth conditions, parasitaemia was adjusted to 4-6 % and 2-3 % for ring and mature asexual stages, respectively.

Although *in vivo* parasites maintain synchrony throughout intraerythrocytic development, *in vitro* parasite synchrony decreases over several life cycles [115]. Therefore, synchronisation of *in vitro P. falciparum* parasites is essential for stage-specific studies of malaria parasites. For this purpose, 5 % (w/v) D-sorbitol (Sigma-Aldrich) was used to ensure >95 % synchrony. Non-infected human erythrocytes are impermeable to sorbitol, however, as *P. falciparum* parasites mature membrane fluidity increases permitting sorbitol uptake into erythrocytes. Mature asexual parasites are, therefore, selectively eliminated through osmolysis resulting in a culture containing mainly non-infected and ring-stage infected erythrocytes. Parasites were synchronised thrice (once every 48 h cycle when ring-stage parasites were observed) and thereafter used for subsequent experiments.

2.5. Evaluation of antiplasmodial activity of compounds against the intraerythrocytic asexual stages

A >95 % ring-stage synchronised intraerythrocytic asexual *P. falciparum* parasite culture was used to determine the antiplasmodial activity using the Malaria SYBR Green I fluorescence-based (MSF) assay platform [114]. The assay is based on the intercalation of SYBR Green I into double-stranded DNA (dsDNA). Since human erythrocytes lack nuclei and are, consequently, devoid of DNA [119], any intercalation will be into the DNA of the parasite. Therefore, a higher fluorescence signal is detected where a higher proportion of dsDNA is present, a direct measure of parasite proliferation.

Preliminary screens against drug-sensitive NF54 parasites were performed at 1 µM and 5 µM pressure (dilutions made in complete media resulting in <0.1 % DMSO). Only compounds with good (>50 % inhibition at 1 µM and >75 % inhibition at 5 µM) were evaluated further for dose-response on both drug-sensitive (NF54) and multidrug-resistant (K1) parasites, whereby

compounds were serially diluted. Compounds with poor (<50 % inhibition at 1 µM and <75 % inhibition at 5 µM) and moderate (<50 % inhibition at 1 µM and >75 % inhibition at 5 µM or >50 % inhibition at 1 µM and <75 % inhibition at 5 µM) preliminary activity were not taken further. Untreated parasites were used as negative control for inhibition of proliferation and the positive control was CQ (0.5 µM) for the preliminary and dose-response screens.

The drug treated parasite suspensions (1 % parasitaemia and 1 % haematocrit) in 96 well plates were incubated stationary at 37 °C under hypoxic conditions for 96 h. Following incubation, equal volumes of parasite suspension and SYBR Green I lysis buffer [0.0002 % (v/v) SYBR Green I dye (Invitrogen, USA), 20 mM Tris pH7.5, 0.08 % (v/v) Triton X-100 (Sigma-Aldrich, USA), 5 mM ethylene dinitrotetraacetic acid (EDTA) and 0.0008 % (w/v) saponin (Merck, South Africa)] were added to new, clear 96 well microplates and thereafter incubated in the dark at room temperature for 1 h. SYBR Green I fluorescence was measured at an excitation wavelength of 490 nm and emission wavelength of 520 nm using a GloMax® Explorer Detection System with Instinct® (Promega, USA). Fluorescence from treated parasites was normalised to that obtained from the positive control for inhibition (background fluorescence) and expressed as a percentage of the untreated control. Data were analysed in Excel and GraphPad Prism v 6.0 (GraphPad Software Inc., La Jolla, CA, USA) was used to generate non-linear regression curves from which half-maximal inhibitory concentrations (IC_{50}) were obtained. IC_{50} is the concentration at which 50 % inhibition of parasite proliferation is observed.

2.6. *In vitro* cultivation of HepG2 liver carcinoma mammalian cells

Human hepatocellular liver carcinoma cells (adherent HepG2 line) were thawed at 37 °C followed by the addition of pre-warmed (37 °C) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat inactivated 10 % (v/v) foetal bovine serum (GE Healthcare, USA). Antibiotic agent 1 % (v/v) penicillin/streptomycin (Sigma-Aldrich, USA) was added to prevent bacterial contamination. HepG2 cells were maintained at 37 °C under 5 % CO_2 and 90 % humidity (Heracell 150i, Thermofisher Scientific, USA) as previously described in literature [114] with media replaced every 2-3 days as needed. Cell confluency was monitored at 400x magnification (10x field magnification and 40x objective lens) using a phase contrast microscope until 70-80 % monolayer confluency was observed. Once sufficiently confluent, the media was aspirated, and the adherent cells washed thrice with 1x PBS. Cells were detached by incubating in trypsin-EDTA (Sigma-Aldrich, USA) at 37 °C for 5 min and thereafter pelleted through 1000 rpm centrifugation

for 2 min followed by a media wash step. Passaged cells were stained with trypan blue as an indicator of cell viability, to facilitate the counting of cells under a light microscope (400x magnification) with a haemocytometer. Cells were either split and maintained further or prepared for subsequent experiments.

2.7. *In vitro* cytotoxicity evaluation of novel compounds

Cytotoxicity was determined using the lactate dehydrogenase (LDH) leakage assay as previously described [114]. LDH is an intracellular enzyme that catalyses the interconversion of lactate and pyruvate. When the cell membrane integrity is compromised, or the cell dies, intracellular components such as LDH are released to the extracellular space. The LDH leakage assay is based on the extracellular existence of LDH due to loss of cell membrane integrity [120].

A cell culture with >97 % viable cells was seeded into 96-well plates ($2x10^4$ cells per well) and incubated overnight at 37 °C under CO_2 rich conditions (5 % CO_2 and 90 % humidity). Following completion of the 24 h incubation, cells were treated at 2 µM compound concentrations and incubated for an additional 48 h under the same conditions. DMEM complete culture media was used as background control, untreated viable cells as negative control and cells treated with triton-x 100 [1 % (v/v)] solution as the positive control for cytotoxicity while EM (10 µM) and CQ (2 µM) as reference controls.

After the 48 h incubation, the plates were centrifugation at 600 x *g* for 10 min after which the supernatant was transferred with LDH cytotoxicity assay reagent (Cytoselect LDH kit, Biocom Africa) to new 96 well plates in a 9:1 ratio as per kit recommendations. A further incubation at 37 °C under 5 % CO_2 and 90 % humidity for 1 h then followed. The kit contains a water soluble tetrazolium dye (WST-1), which is converted to formazan by LDH. Formazan was detected at an absorbance wavelength of 450 nm [120], using the SpectraMax Paradigm microplate reader (Molecular devices, USA). The background absorbance was subtracted from treated HepG2 cells and thereafter normalised to the positive control for cytotoxicity and represented as a percentage value. Data were analysed in Microsoft Excel and GraphPad Prism v 6.0.

2.8. NPs and interrogative antimalarial drugs

Antimalarial drug HCQ and drug combination of AR and LF, henceforth referred to as AL, were dissolved in 100 % dimethyl sulfoxide (DMSO, Sigma, USA) to a 10 mM concentration and stored at 4 °C (-20 °C for long-term storage). The NPs, NP backbone and tNPs were dissolved to 1 mM

in DMSO followed by water in a 1:9 ratio. The NPs were then stored at -20 °C. The mass of each NP and tNP was 20 % drug and 80 % NP backbone.

2.9. *In vitro* haemolytic toxicity

Erythrocytes (2 % final haematocrit) were exposed to NPs as well as antimalarial drugs and incubated for 48 h at 37 °C. Untreated erythrocytes were the negative control for haemolysis and erythrocytes treated with triton-x 100 [1 % (v/v)] were the positive control for 100 % haemolysis. Once the incubation time had lapsed, erythrocyte suspensions were centrifuged at 3000 x *g* for 3 min. Thereafter, the supernatant was transferred to new 96 well plates at a 3:7 dilution in water [121]. The haemoglobin content in the suspensions was measured at an absorbance of 540 nm using a SpectraMax Paradigm microplate reader (Molecular devices, USA) [98]. The amount of released haemoglobin in the supernatant is indicative of cell lysis and can used as an inverse measure of cell viability. Treated erythrocytes were normalised to the positive control for haemolysis and represented as a percentage. Data were analysed in Microsoft Excel and, where necessary, GraphPad Prism v 6.0 was also used.

2.10. Dose-response evaluation of NPs against intraerythrocytic asexual parasites

A >95 % ring-stage synchronised NF54 *P. falciparum* parasite culture was then used to determine antiplasmodial activity using the MSF assay [114]. Untreated parasites and 0.5 µM CQ were used as the negative and positive control for inhibition, respectively. Parasites were exposed to various concentrations of NPs, tNPs and associated drugs. Dose-response curves generated in GraphPad Prism v 6.0 were used to determine IC_{50} values for three independent biological replicates each consisting of technical triplicates.

2.11. *In vitro* cytotoxicity assays

A cell culture with >97 % viable HepG2 cells was used for the LDH leakage assay. Cytotoxic drug EM (10 µM) was used as reference control. HCQ and AL were all treated at 10 µM whereas the NPs and tNPs were treated at concentration equivalent to 10 µM of drug component. A minimum selectivity index (SI) of 10 is recommended by the MMV [122, 123], therefore, NPs were also treated at more than 10X antiproliferative IC_{50} in order to confirm their potency against malaria parasites. Data were analysed in Microsoft Excel and GraphPad Prism v 6.0.

2.12. *In silico* aqueous solubility and lipophilicity predictions

Several physicochemical parameters were chosen as predictor variables to describe the best candidate for conjugation to the NP backbone. These physicochemical predictor variables were logS and logP for predictions of solubility and hydrophilicity at pH 7.4, respectively, as well as MW. Osiris DataWarrior v 5.0.0 (www.openmolecules.org), MolSoft LLC v 1.5.1 (www.molsoft.com) and ALOGPS v 2.1 (www.vcclab.org/lab/alogps) were all used to predict the selected variables *in silico* using SMILES provided by UCT. Acceptable values described below were based on literature.

Insufficient aqueous solubility (low logS) and a high MW results in low absorption [88, 124]. A high partition coefficient (octanol/water), logP, which translate to low hydrophilicity also results in poor absorption or permeation [124]. Compounds with a logS>-5.7 were classified as soluble according to Jorgensen's rule of three [125]. A logP between 1.35 and 1.8 is optimal for oral and intestinal absorption [126], however, Lipinski's rule of 5 accepts logP<5 and MW<500 Da [124].

Chapter 3: Results

PART I: Evaluation of NP with known antimalarials as interrogative drugs

3.1. Evaluation of the NP backbone

The NP backbone used in this study is made from an amphiphilic polymer consisting of PVP which is not toxic to human erythrocytes [110]. Two independent samples of the same NP backbone were evaluated, as each was later used independently for the conjugation of the different interrogative drugs.

3.1.1. The NP backbone does not cause haemolysis

The NP system was intended to target *P. falciparum*-infected erythrocytes, therefore minimal toxicity against non-infected erythrocytes was expected. Significant haemolysis of erythrocytes would cloud analysis of antiplasmodial activity caused by the NP backbone as burst erythrocytes could lead to false positive readouts on parasite viability, since parasite viability is directly dependent on intact erythrocytes. Erythrocyte lysis was evaluated for erythrocytes treated with the NP backbone using the haemolysis assay (Figure 3.1).

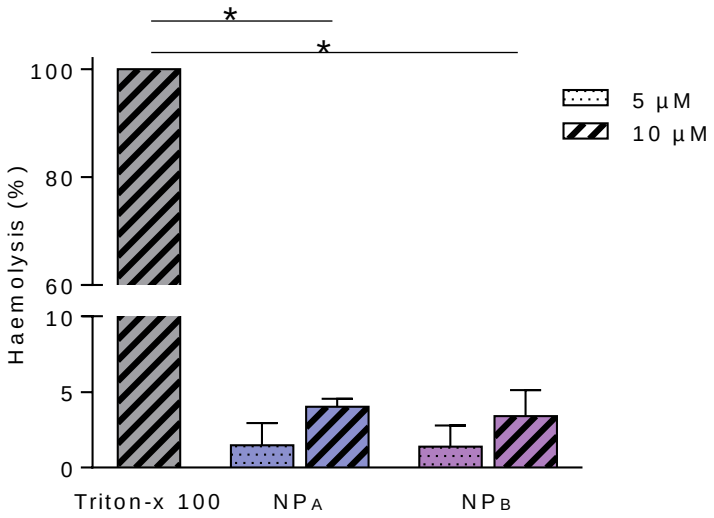


Figure 3.1. Haemolytic effect of nanoparticle (NP) backbone on human erythrocytes.
Erythrocyte lysis caused by 5 µM (dotted bars) and 10 µM (striped bars) of two samples of the same NP backbone, samples NP_A (blue) and NP_B (purple), determined using the haemolysis assay. The positive control for erythrocyte lysis was Triton-x 100 (grey bar). Data are from three independent biological experiments performed in technical triplicates, ± SEM. Significance was determined using the paired Student's *t*-test (*P<0.05).

As NP_A and NP_B are different samples of the same NP backbone, it was expected that a similar haemolysis would result from both. A 1.1-fold and 1.2-fold difference in haemolysis was observed between NP_A and NP_B at 5 µM and 10 µM of the NP backbone. Haemolysis caused by the NP backbone at 5 µM and 10 µM was significantly lower (P<0.05, n=3, paired Student's t-test) than that caused by haemolytic detergent triton-x 100 (Figure 3.1) with even the highest haemolysis observed lower than 5 %. Furthermore, there was no significant difference between 5 µM and 10 µM for either NP_A or NP_B. The results also inferred that any possible antiplasmodial activity by the NP backbone would not be due to haemolysis of infected erythrocytes.

3.1.2. The NP backbone is not cytotoxic

It is known that PVP is not toxic to erythrocytes, however, little information exist on its effects on other cell types [110]. To confirm that the PVP backbone would not cause toxicity to other mammalian cells types, the LDH release assay was used to determine cytotoxicity of the NP backbone on HepG2 liver cells. Triton-x 100 was used as the positive control with cytotoxic drug EM as reference control (Figure 3.2).

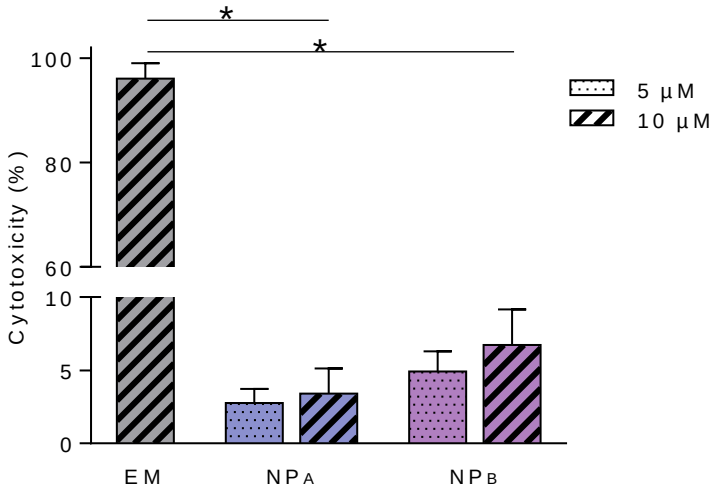


Figure 3.2. Cytotoxic effect of the nanoparticle (NP) backbone on HepG2 mammalian hepatocytes. Inhibitory effect of 5 µM (dotted bars) and 10 µM (striped bars) of two samples of the same NP backbone, samples NP_A (blue bars) and NP_B (purple), on HepG2 cells as determined using the lactate dehydrogenase (LDH) cytotoxicity assay. The positive control for HepG2 cell cytotoxicity was emetine (EM). Data are from three independent biological experiments performed in technical triplicates ± SEM. Significant differences were determined using the paired Student's t-test (*P<0.05).

The NP backbone showed significantly less (P<0.05, n=3, paired Student's t-test) cytotoxicity towards HepG2 cells than the reference control EM, with a 20-fold difference and 17-fold difference for the NP_A backbone and NP_B backbone at 10 µM. Although less cytotoxicity was

observed for NP_A than NP_B (1.7-fold at 5 µM and 2.0-fold at 10 µM), this was not significant. Furthermore, there was no significant difference observed between 5 µM and 10 µM NP backbone treatment for either of the samples (Figure 3.2). The results indicate that the NP backbone is not toxic to mammalian liver cells.

3.1.3. Cultivation and synchronisation of intraerythrocytic asexual parasites

To investigate antiplasmodial activity against intraerythrocytic *P. falciparum* parasites, it was first necessary to culture healthy, viable and highly synchronised *P. falciparum* parasites *in vitro*. The life-cycle progression of *in vitro P. falciparum* cultures was monitored and parasitaemia determined as a measure of viability (Figure 3.3).

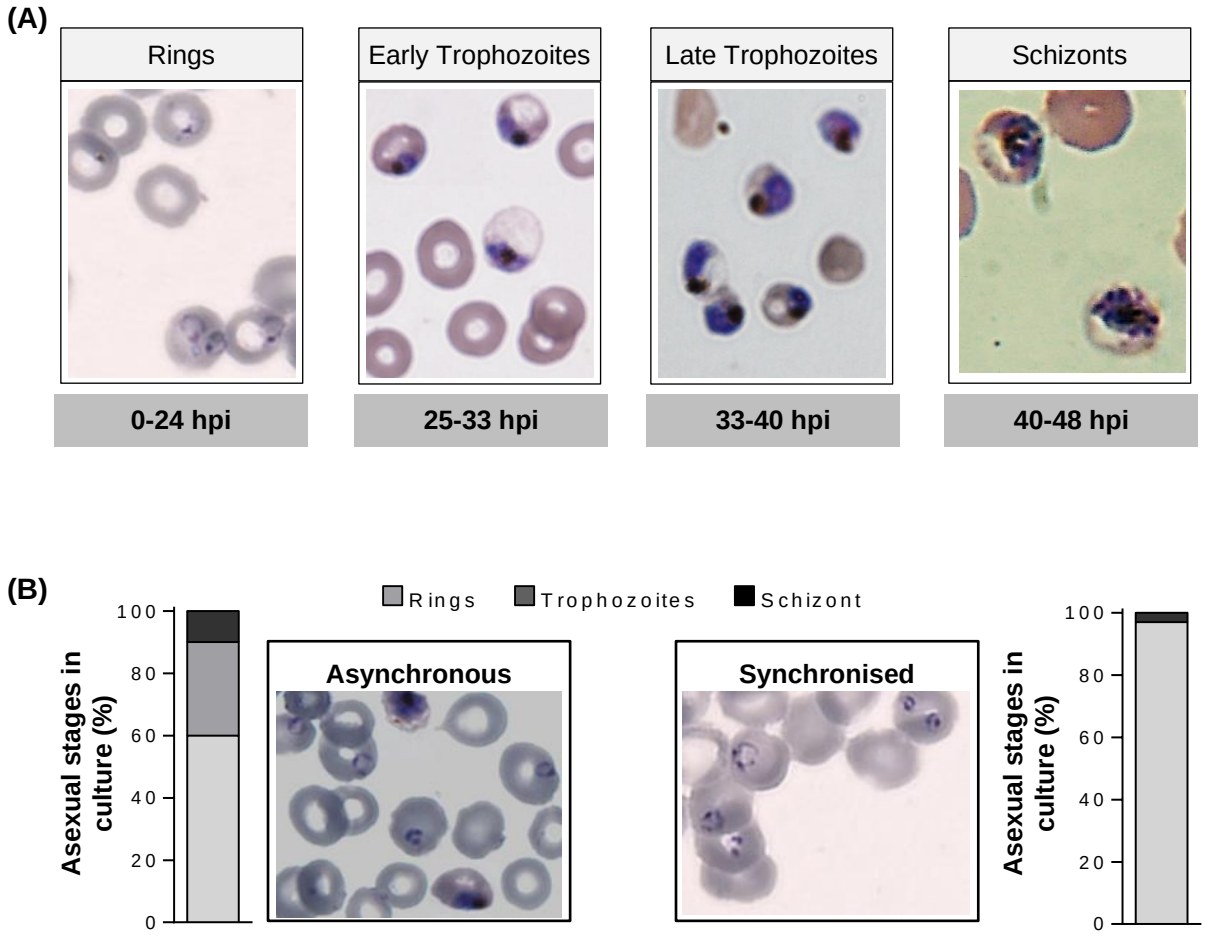


Figure 3.3. Microscopic evaluation of *P. falciparum* intraerythrocytic asexual parasites *in vitro*. Giemsa-stained thin blood smears were viewed under a light microscope at 1000x magnification. Parasitaemia was monitored daily and used as a measure of viability along with (A) the observance of the different morphological stages within the full 48 h life cycle of *P. falciparum* parasites *in vitro* and classified based on the hours post invasion (hpi). (B) Asynchronous cultures were synchronised with D-Sorbitol to obtain a culture with a population of >95 % ring-stage parasites before cultures were used for any other experiments.

The IDC of *P. falciparum* was observed over a ~48 period *in vitro* (Figure 3.3. A). Ring-stage parasites were observed up to 24 hpi while early trophozoites were observed 24-33 hpi. Although a haemozoin crystal is seen for all trophozoites, late trophozoites have more nucleic content (stained blue) which was easily identified by the parasite covering a larger area of the host erythrocyte, leading to a decreased visibility of the erythrocyte membrane. Schizonts were identified by the presence of multiple nuclei (>2) rather than a consistent blue nucleic mass (trophozoite). Late trophozoites and schizonts were observed 33-40 hpi and 40-48 hpi, respectively. Each schizont releases 24-36 daughter merozoites, therefore a >2x higher parasitaemia was expected following erythrocyte re-invasion. A 3-4x conversion rate was observed for trophozoite- to ring-stage parasites. Asynchronous cultures with a mixed stage population were synchronised using D-sorbitol to ensure a culture of >95 % ring stages (Figure 3.3. B). Overall, healthy parasites were observed consistently and were then used for subsequent experiments.

3.1.4. The NP backbone has poor activity against the asexual *P. falciparum* stages

Since it was concluded that the that the NP backbone neither causes overt cellular toxicity nor haemolysis, the antiplasmodial activity of the NP backbone was also determined with the use of the MSF assay to ascertain whether the NP backbone would result in parasite death (Figure 3.4). Parasites were exposed to serially diluted concentrations of the polymer with the highest concentration of 10 µM.

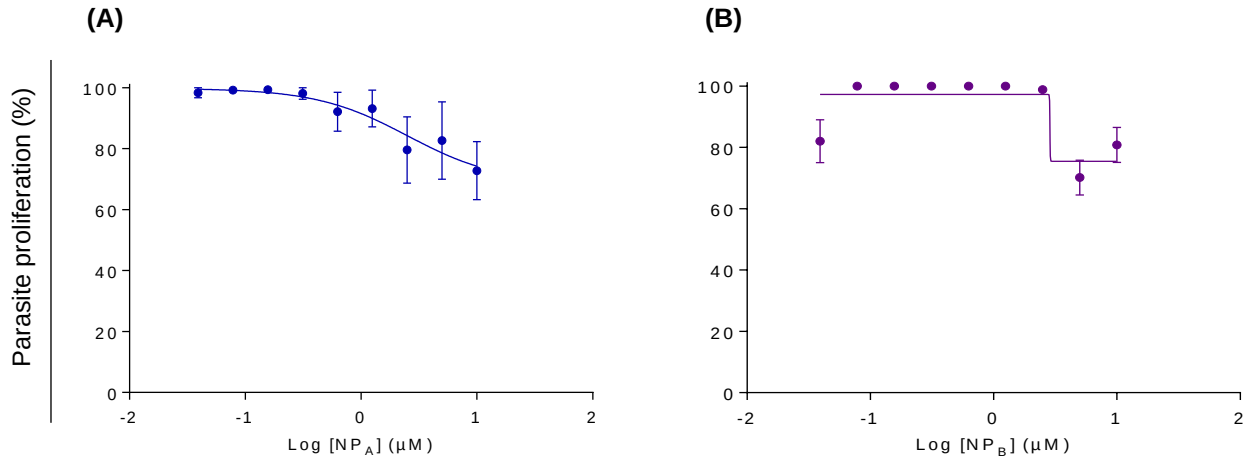


Figure 3.4. Dose-response of the nanoparticle (NP) backbone against *P. falciparum* asexual stages. Dose-response evaluation of *P. falciparum* parasites exposed to (A) NP_A and (B) NP_B, two samples of the same NP backbone, for 96 h. Data are from three independent biological experiments performed in technical triplicates, ± SEM.

Neither NP_A nor NP_B abolished parasite proliferation dramatically, with 70 % parasite proliferation still present, even at the highest tested concentration of 10 µM (Figure 3.4. A and B). As a result of this observation the NP backbone was classified as minimally active against *P. falciparum* parasites.

Any activity observed for the NP would be due to the release of the conjugated therapeutic agent alone and not due to any haemolytic or antiplasmodial activities of the backbone itself.

3.2. Validation of antiplasmodial activity of interrogative antimalarial drugs on asexual intraerythrocytic stages

Well-known antimalarial drugs were chosen, as the therapeutic agent, to test the NP system. In order to be conjugated to the NP backbone, drugs needed to have a hydroxyl (OH) group. CQ was a commonly used drug and is still used for malaria research. However, CQ does not have an OH group. The hydroxylated derivative of CQ, HCQ, was chosen instead as it is also potent against *P. falciparum* parasites. Currently, only drug combinations are used for malaria treatment, therefore AL, a well-established drug combination of AR and LF was chosen. LF contains the desired OH group while AR is hydrophobic and could be incorporated into the hydrophobic core. The antiplasmodial activity of the interrogative drugs was determined on drug-sensitive (NF54) intraerythrocytic asexual *P. falciparum* parasites *in vitro* using the MSF assay with CQ used as the positive control for inhibition of parasite proliferation (Figure 3.5).

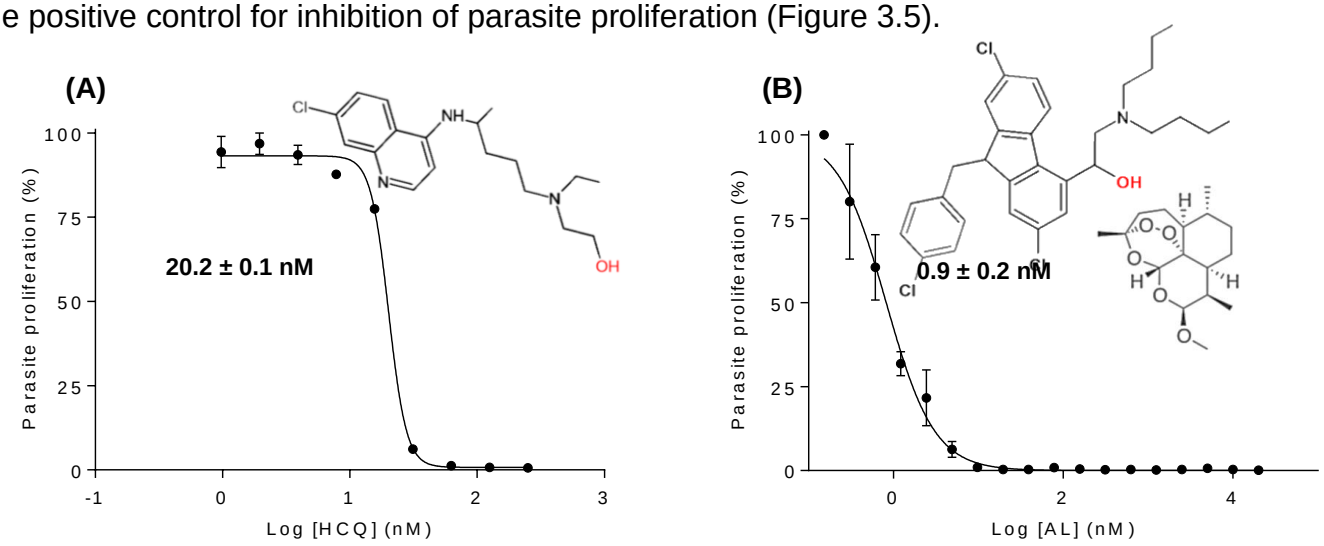


Figure 3.5. Dose-response curves of antimalarial drugs against *P. falciparum* asexual stages.
Dose-response evaluation of interrogative antimalarial drugs (A) hydroxychloroquine (HCQ) and (B) combination of artemether and lumefantrine (AL) against intraerythrocytic asexual stages of *P. falciparum* parasites treated over 96 h. Non-linear regression curves were drawn in GraphPad Prism v 6.0 from which IC_{50} values (bold text) were obtained. Data are from three independent biological experiments performed in technical triplicates, ± SEM. Where not visible, error bars fall within the symbols.

The activity of both the AL combination and HCQ were confirmed as potent with nanomolar IC_{50}<50 nM (Figure 3.5). The determined IC_{50} for HCQ was 20.2 ± 0.1 nM and this was similar to published data [127] (Figure 3.5. A). An IC_{50} of 0.9 ± 0.2 nM for AL was observed as seen in figure 3.5.B. Synergism has been previously reported for the AL drug combination, therefore a very low IC_{50} was expected [128].

With this baseline activity of the known antimalarial drugs confirmed, as well as the non-toxic nature of the NP established *in vitro*, the antimalarial drugs were subsequently conjugated to the NP backbone to make NPs and tNPs.

3.3. Evaluation of NP with interrogative antimalarial drugs

The interrogative drugs were conjugated to the NP backbone via the acid-labile linker and named NP-AL and NP-HCQ for conjugated AL and HCQ, respectively.

3.3.1. NPs with interrogative drugs are active against asexual *P. falciparum* parasites

The MSF assay was used to determine whether the NP conjugates with interrogative drugs, NP-AL and NP-HCQ, would be active against drug-sensitive (NF54) intraerythrocytic asexual *P. falciparum* parasites (Figure 3.6).

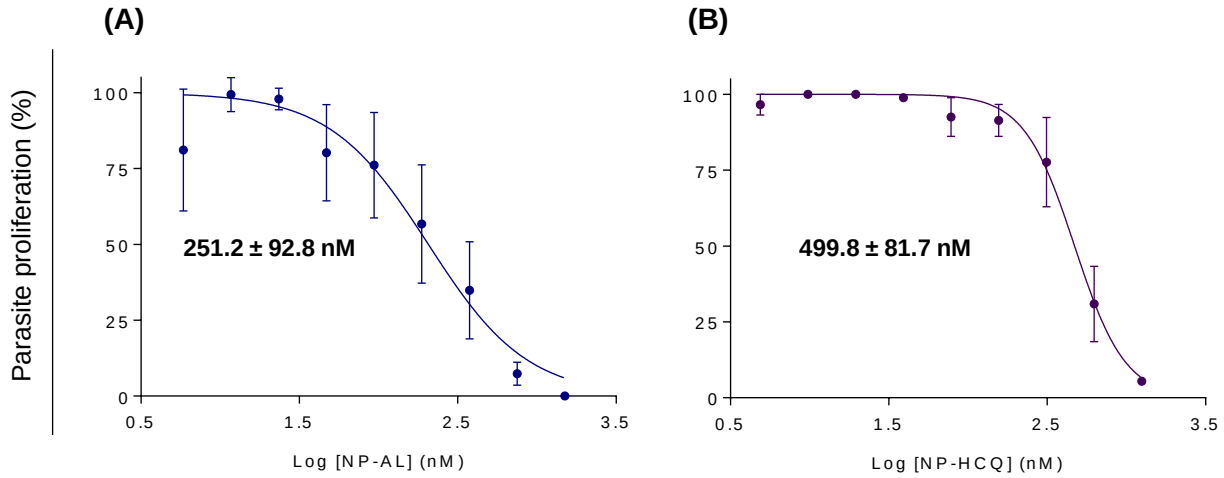


Figure 3.6. Dose-response of nanoparticles (NPs) with interrogative antimalarial drugs.
The antiplasmodial activity of nanoparticles with interrogative drug (A) artemether: lumefantrine combination (AL), NP-AL and (B) hydroxychloroquine (HCQ), NP-HCQ against drug-sensitive (NF54) intraerythrocytic asexual parasites. Non-linear regression curves were drawn in GraphPad Prism v 6.0 from which IC_{50} values (bold text) were obtained. Data are from three independent biological experiments performed in technical triplicates, ± SEM. Where not visible, error bars fall within the symbols.

As expected, the NPs with interrogative drugs were active against *P. falciparum* parasites. A dose-response curve was obtained for each NP for which NP-AL resulted in an IC_{50} of 251.2 ± 92.8 nM (Figure 3.6. A) and NP-HCQ an IC_{50} of 499.8 ± 81.7 nM (Figure 3.6. B). The results showed that NP-AL and NP-HCQ resulted in sub-micromolar antiplasmodial activity.

3.3.2. The antiplasmodial activity of the NPs is due to the interrogative antimalarial drugs

Since it was established that the NP backbone is minimally active against *P. falciparum*, it was important to determine how much more active NP-AL and NP-HCQ was than NP_A and NP_B. This was achieved by comparing the activities of the NPs to the NP backbone. This would not only determine the change in activity of NP with and without therapeutic agent (interrogative drugs) but would also be a first measure to confirm release of the therapeutic agent from the NP backbone as is expected once inside infected erythrocytes. Since the NP backbone did not decrease parasite proliferation by 50 %, even at 10 µM treatment, the IC_{50} for the NP backbone was assumed to be >10 µM. Furthermore, the activated NP were calculated by the entire NP.

Table 3.1. Evaluation of antiplasmodial activity of nanoparticles (NPs) with interrogative antimalarial drugs.

Comparison of observed activity of the NP backbone and the NPs with interrogative drugs. Data are from three independent biological experiments performed in triplicate, ± SEM.

	IC_{50} (nM)	Fold change[a]
NP_A (backbone)	> 10 000[b]	1
NP-AL	251.2 ± 92.8	>40**
NP_B (backbone)	> 10 000[b]	1
NP-HCQ	499.8 ± 81.7	>20**

[a] Fold change is defined as the IC_{50} ratio of empty NP to activated NP with significant values (paired Student *t*-test, ***P*< 0.01).

[b] Highest NP concentration used. Maximum activity resulted in <30 % inhibition of parasite proliferation.

NP-AL and NP-HCQ were significantly (*P*<0.01, n=3, paired Student *t*-test) more active than the NP backbone alone as observed with respective 40-fold and 20-fold differences (Table 3.1). The results substantiate that the potency of the NPs was due to the conjugated drug and not as a result of the NP backbone.

3.3.3. Conjugation to nanoparticle decreases antiplasmodial activity of interrogative drugs

Once it was established that the NPs possessed antiplasmodial activity that was due to the conjugated interrogative drugs, it was also important to determine whether NPs would result in improved activity of the interrogative drugs as proposed. This was achieved by comparing the activities of the interrogative drugs, AL and HCQ, to NP-AL and NP-HCQ. The IC_{50} of the NP-AL and NP-HCQ was adjusted to represent only the amount of the drug in the NP for comparability.

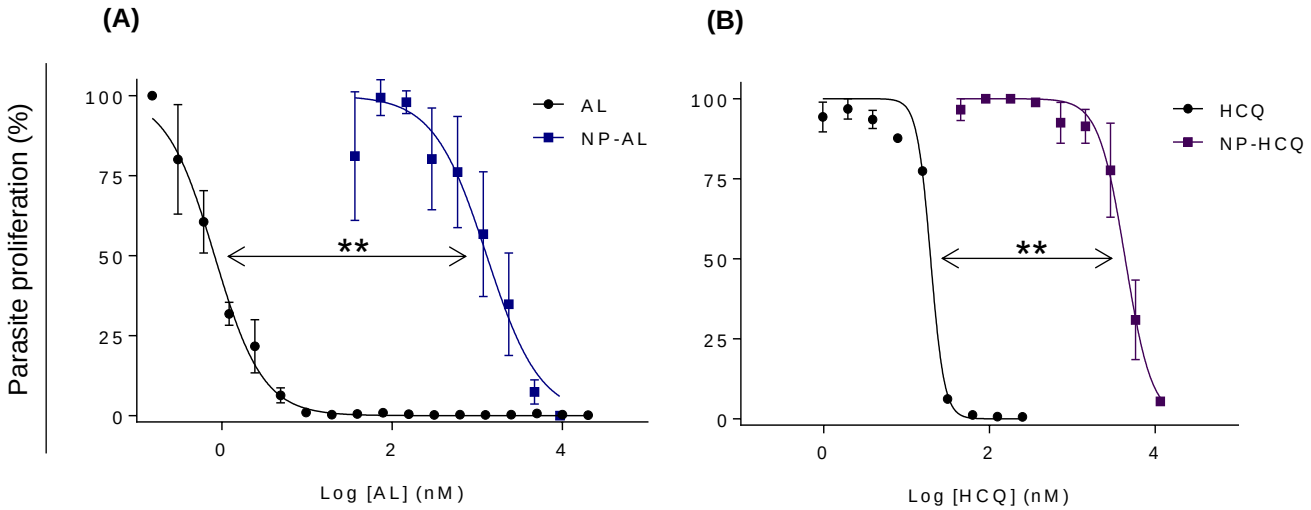


Figure 3.7. The effect of nanoparticles (NPs) on the antiplasmodial activity of interrogative drugs. The difference in activity (IC_{50} shift) between interrogative antimalarial drugs (●) (A) combination of artemether and lumefantrine (AL) or (B) hydroxychloroquine (HCQ) and the respective nanoparticles (NPs), NP-AL (■) and NP-HCQ (■). Non-linear regression curves were drawn in GraphPad Prism v 6.0 from which IC_{50} values (bold text) were obtained. The NPs were represented by the proportion of antimalarial drug equivalent Data are from three independent biological experiments performed in technical triplicates, ± SEM. Where not visible, error bars fall within the symbols. Significant differences were determined using the paired Student's *t*-test (**P<0.01).

As seen in figure 3.7. A, NP-AL was 1700-fold less active than the free drug AL, a significant difference (P<0.01, n=3, paired Student's *t*-test). Similarly, a significant 220-fold decrease (P<0.01, n=3, paired Student's *t*-test) in activity was observed for NP-HCQ when compared to HCQ (Figure 3.7 B). It was evident that the conjugation of AL or HCQ to the NP backbone lowered activity significantly. Therefore, the NPs did not result in improved activity.

Since the NP with interrogative drugs did not improve activity on their own, it was then important to determine whether the addition of the peptidic targeting ligand (GSRSKGT) would result in improved activity as hypothesised.

3.3.4. The addition of a targeting ligand to NPs does not improve antiplasmodial activity

The NP backbone was sparsely decorated with a peptide (GSRSKGT) that was previously thought to have a higher affinity towards *P. falciparum*-infected erythrocytes [102]. Targeted NPs (tNPs), tNP-AL and tNP-HCQ, were also evaluated against the asexual intraerythrocytic stages of *P. falciparum* parasites *in vitro*. This activity was then compared to the non-targeted NP to determine essentiality of the targeting ligand for improving antiplasmodial activity (Figure 3.8).

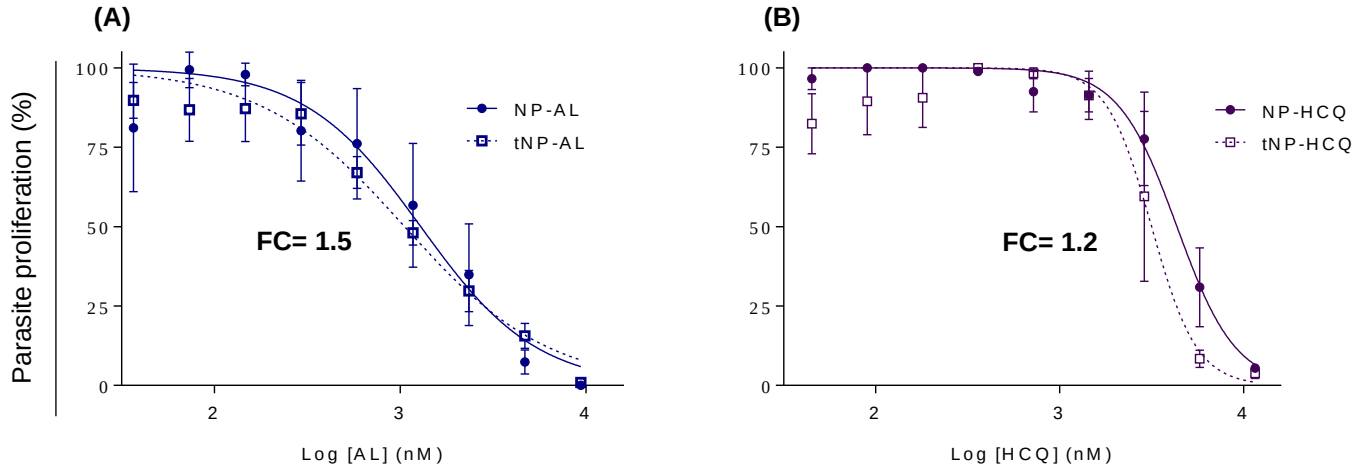


Figure 3.8. The impact on antiplasmodial activity of adding peptidic (GSRSKGT) targeting ligand to nanoparticles (NPs).

The difference in activity (IC_{50} shift) between (A) NP and targeted NP with interrogative antimalarial drug combination of artemether and lumefantrine (AL), tNP-AL (□, dotted curve) and NP-AL (●, solid curve). (B) IC_{50} shift between NP and targeted NP with hydroxychloroquine (HCQ), tNP-HCQ (□, dotted curve) and NP-HCQ (●, solid curve). Data are from three independent biological experiments performed in technical triplicates, n=3 ± SEM. Non-linear regression curves were drawn in GraphPad Prism v 6.0 from which IC_{50} values were obtained. Where not visible, error bars fall within the symbols. Significant fold change (FC) in IC_{50} (bold numbers) were determined using the paired Student's *t*-test (*P<0.05).

The addition of the targeting ligand was expected to improve antiplasmodial activity of the NPs with interrogative drugs as only the target tissue would be reached. Even though a 1.5-fold (Figure 3.8. A) and 1.2-fold (Figure 3.8. B) improvement was observed for tNP-AL and tNP-HCQ compared to the non-targeted counterpart, NP-AL and NP-HCQ, respectively, this difference was not significant. Although most intra-assay and inter-assay coefficient of variance (CV) values were <20 % for NP-HCQ and NP-AL, tNP-HCQ yielded an intra-assay and inter-assay %CV >20 % for a single concentration for one biological replicate. Furthermore, inter-assay %CV >20 % was observed for some of the concentrations due to one biological replicate being different to the other two for both tNP-AL and NP-AL.

Since neither the NPs nor tNPs improved activity when compared to the interrogative drugs, it was beneficial to determine whether the NPs and tNPs were advantageous for other factors.

3.3.5. NP and tNPs decreases haemolysis of interrogative antimalarial drugs

The haemolysis of the NPs, tNPs and interrogative drugs on erythrocytes was tested at 5 µM and 10 µM of the interrogative drug proportion. This was done to determine whether conjugation to the NP backbone would decrease any haemolysis caused by the interrogative drugs (Figure 3.9).

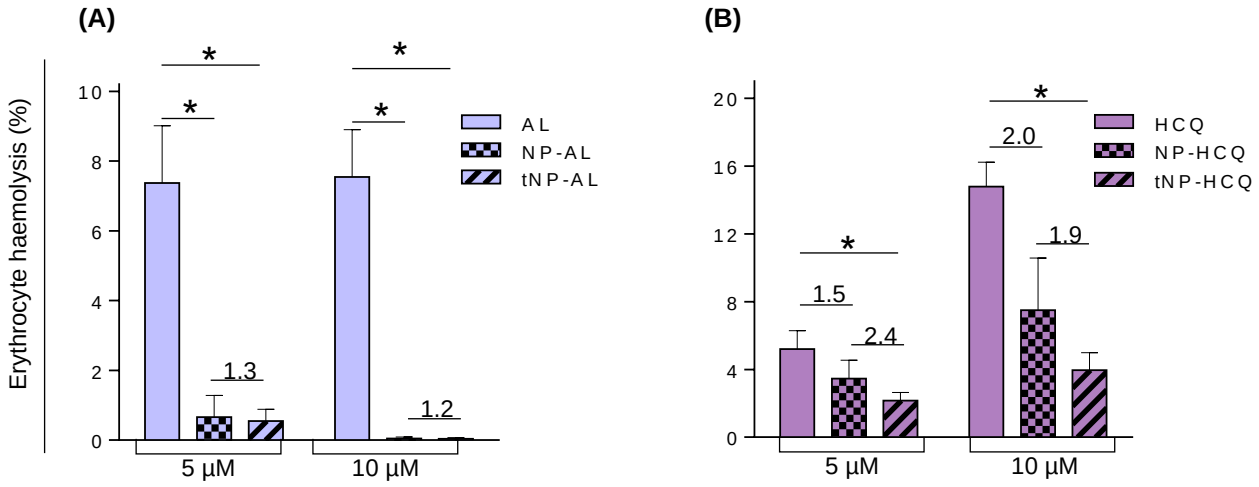


Figure 3.9. Effect of nanoparticles (NP) and targeted nanoparticles (tNPs) on the haemolysis of interrogative antimalarial drugs.
The change in haemolysis caused by conjugation of interrogative antimalarial drugs (A) artemether and lumefantrine combination (AL) and (B) hydroxychloroquine (HCQ) to NPs (chequered bars), NP-AL and NP-HCQ, and tNPs (striped bars), tNP-AL and tNP-HCQ. Erythrocyte haemolysis was determined at 5 µM and 10 µM of drug equivalent using the haemolysis assay. Data are obtained from three independent biological replicates of technical triplicates, n=3 ± SEM with significant differences calculated using the paired Student's *t*-test (**P*<0.05) with data.

Haemolysis caused by NP-AL and tNP-AL was consistently less than 2 %, significantly less (*P*<0.05, n=3) than that caused by AL, with a 3.7-fold and 3.8-fold decrease for tNP-AL at 5 µM and 10 µM, respectively (Figure 3.9. A). Therefore, at both concentrations, NPs and tNPs significantly improved safety towards erythrocytes *in vitro*. HCQ caused 5.2 % and 14.8 % haemolysis at 5 µM and 10 µM, respectively. NP-HCQ did not significantly improve the haemolysis of HCQ at either 5 µM or 10 µM concentrations (Figure 3.9. B). Contrary to NP-HCQ, tNP-HCQ was 4.0-fold less haemolytic than HCQ at 10 µM and 3.6-fold less haemolytic at 5 µM, indicating a significant decrease (*P*<0.05, n=3, paired Student's *t*-test). There was no significant difference between the NPs and tNPs for either of the drugs at any concentration, indicating that the targeting ligand was not significant to decrease haemolysis. The results indicate that the NP are biocompatible to healthy non-infected erythrocytes.

3.3.6. NPs and tNPs decrease the cytotoxicity of interrogative antimalarial drugs

It was then essential to determine whether the NP and tNP would decrease toxicity in other mammalian cell types. The cytotoxicity on HepG2 cells was also determined for the NPs and tNPs at 10 μM interrogative drug equivalents (Figure 3.10).

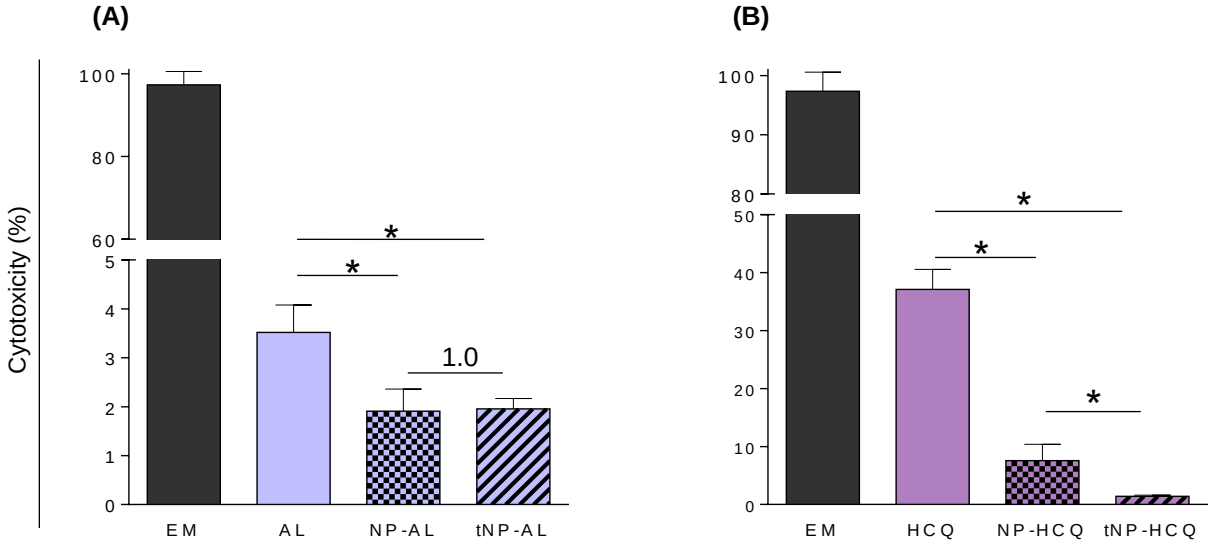


Figure 3.10. Effect of nanoparticles (NPs) and targeted nanoparticles (tNPs) on the cytotoxicity of interrogative antimalarial drugs.

The determination of whether NPs (chequered bars) and tNPs (striped bars) decreases the cytotoxicity of interrogative antimalarial drugs (A) artemether and lumefantrine combination (AL) and (B) hydroxychloroquine (HCQ) on HepG2 hepatocellular cells. Cytotoxicity was determined at 10 μM of drug equivalent using the LDH assay. Significant differences were determined using the paired Student's *t*-test (**P*<0.05) with data from three independent biological replicates of technical triplicates, n=3 ± SEM.

The NPs and tNPs decreased cytotoxicity significantly (n=3, *P*<0.05*,* paired Student *t*-test) for both AL (Figure 3.10. A) and HCQ (Figure 3.10. B). Therefore, the NP system reduces the cytotoxicity of drugs to HepG2 cells. HCQ resulted in ~37 % cytotoxicity at 10 μM, which was expected as the non-hydroxylated CQ has been shown to be active against hepatocyte carcinoma cells at similar concentrations [129]. A significant (*P*<0.05, n=3) 7.8-fold decrease was observed between NP-HCQ and tNP-HCQ indicating a significance for the targeting ligand to decreasing the cytotoxicity of HCQ, (Figure 3.10. B). However, the significance of the targeting ligand for cytotoxicity was not observed for tNP-AL (Figure 3.10. A).

Once the experiments using NP and tNP with interrogative drugs were completed, a novel compound that would benefit from this conjugation had to be selected.

PART II: Screening of novel compounds

Here, novel chemical classes were investigated that are either kinase inhibitors or inhibit β-hematin formation or β-hematin formation. A total of 277 novel compounds from 6 different classes; 14 aminopyrazines (AP), 56 astemizoles (AST), 24 benzimidazoles (BI), 26 Fusidic acid (FA) derivatives, 32 imidazopyrazines (IP), and 125 pyrido(1,2-α)benzimidazoles (PB). These novel compounds were synthesised at UCT with Prof Kelly Chibale as the principal investigator H3D.

3.4. *In vitro* antiplasmodial activity of novel compounds

Preliminary screens of the novel compounds were performed against drug-sensitive (NF54) asexual intraerythrocytic *P. falciparum* parasites using the MSF assay platform. A stringent selection threshold of >50 % inhibition of parasite proliferation at 1 µM and >70 % inhibition at 5 µM was used to ensure that only highly active compounds would be selected for dose response evaluation (Figure 3.11). Acceptable assay quality control parameters Z'-factor >0.6 and CV (%) consistently <20 % were observed. CQ was used as the positive control drug.

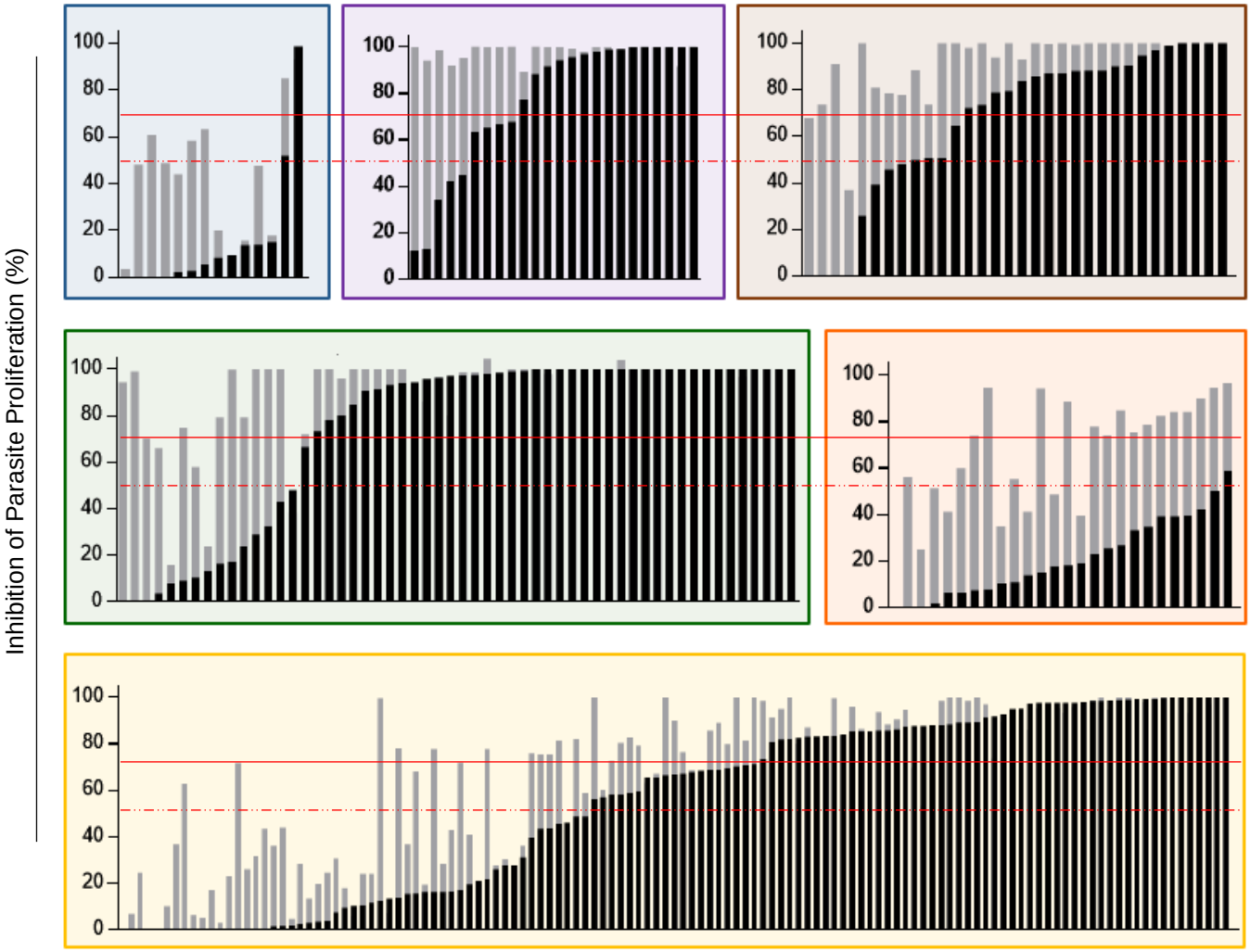


Figure 3.11. Preliminary screens of novel compounds with antiplasmodial activity against asexual intraerythrocytic parasites.

Inhibition of drug-sensitive (NF54) asexual intraerythrocytic *P. falciparum* parasites exposed to 96 h of 1 µM (black bars) and 5 µM (grey bars) of novel compounds from the aminopyrazines (blue), fusidic acid derivatives (orange), benzimidazoles (purple), imidazopyrazines (brown), astemizoles (green), pyrido(1,2-α)benzimidazoles (mustard) classes. Data bars are organised by increasing activity at 1 µM exposure. The selection threshold of 50 % (red dashed line) and 70 % (red solid line) were used to identify potentially highly active compounds. Data are from a single biological experiment performed in technical triplicates ± SD.

From the 277 compounds screened, 152 met the selection criteria of >50 % inhibition of parasite proliferation at 1 µM and >70 % inhibition at 5 µM (Figure 3.11). The most active compounds were from the BI and IP classes with a >70 % hit rate whereas a hit rate <10 % was observed for the FA and AP classes.

The selected hits were further investigated for dose-response against asexual intraerythrocytic stages to determine IC_{50} (Figure 3.12).

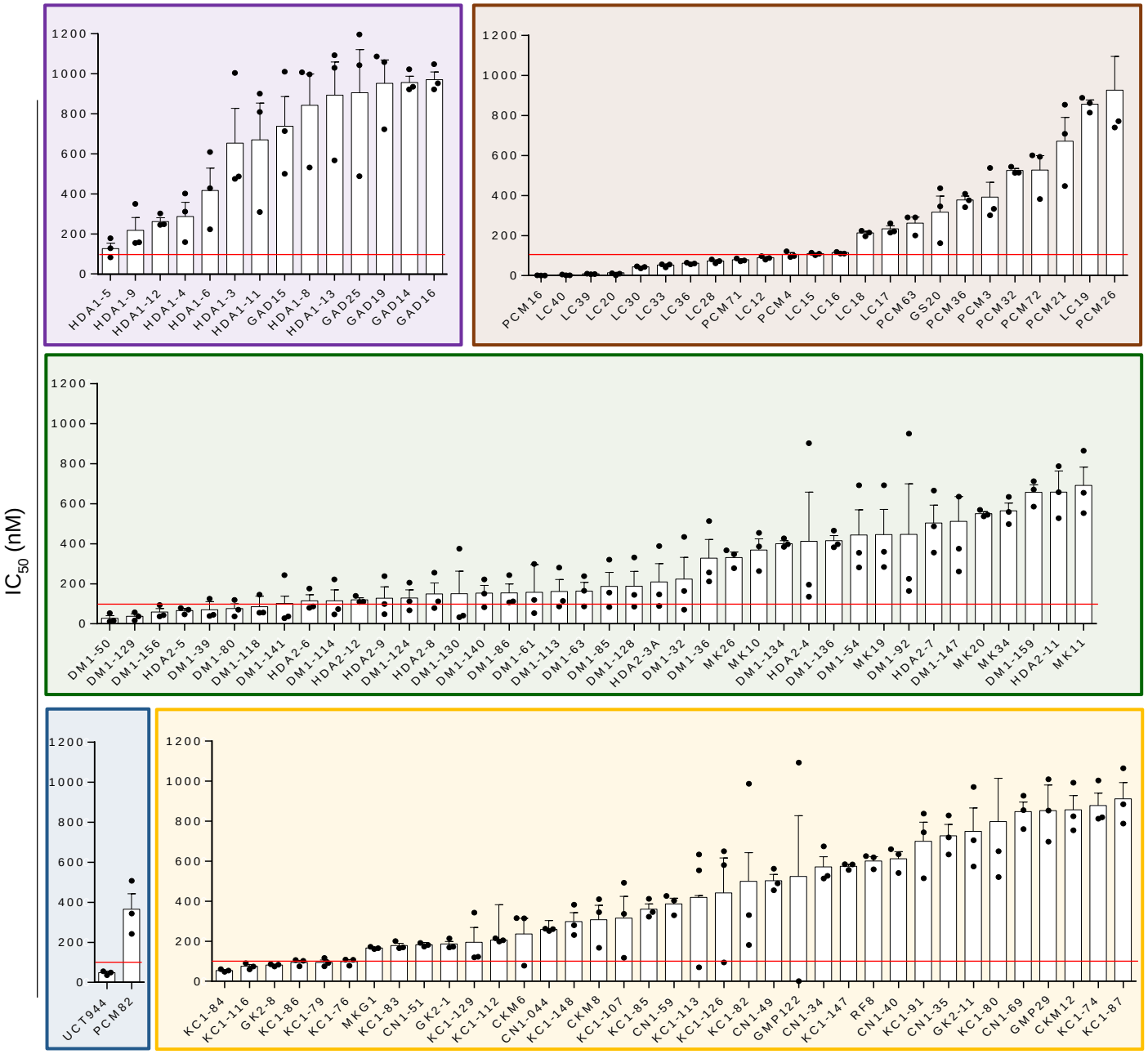


Figure 3.12. Antiplasmodial IC_{50} values of hits with good activity against *P. falciparum* parasites.
In vitro IC_{50} values of selected hit compounds with good antiplasmodial activity (IC_{50}<1 µM) against asexual intraerythrocytic drug-sensitive (NF54) parasites. Compounds are grouped according to classes; aminopyrazines (blue), astemizoles (green), benzimidazoles (purple), imidazopyrazines (brown), pyrido(1,2-α)benzimidazoles (mustard) for average IC_{50} (bar) with individual experiments (•). Compounds with IC_{50}<100 nM fall below the red dotted line. Data are for three independent biological experiments of technical triplicates, n=3 ± SEM.

Only 117 of the 152 selected compounds were highly active (IC_{50} value<1 µM). The most active classes were the ASTs and IPs with average IC_{50}s<200 nM observed for majority of the compounds; 24 compounds and 16 compounds, respectively. The BI were the least active class

with most of the compounds showing IC_{50}s>600 nM (Figure 3.12). From this data, 24 compounds with IC_{50}<100 nM (potent compounds): 1 AP, 7 AST, 10 IP, 6 BP, were selected for further investigation.

3.5. Cross-reactivity of potent hit compounds

Resistance continues to be a concern; therefore, novel compounds need to also be active against parasite strains that have developed resistance to antimalarial drugs. To prove antiplasmodial efficacy across strains that possess different drug susceptibilities, cross-reactivity studies were done. The selected potent hits were exposed to drug-resistant (K1) asexual intraerythrocytic *P. falciparum* parasites to determine resistance indices (RI) (Figure 3.13).

CQ resulted in a resistance index (RI) of 13.2 depicting that a 13.2-fold loss in efficacy was observed against drug-resistant strains. This was expected as K1 parasites are resistant to CQ. Although potent against both drug-sensitive and -resistant strains with IC_{50} of 0.7 nM and 50.4 nM, respectively, PCM16 was 72-fold less effective against the multidrug-resistant K1 strain, indicating possible failure of treatment if used in future. However, PCM16 is still one of the most potent active hits and could still be considered as a lead compound, with the focus to overt resistance. Overall, little to no loss in efficacy was observed between the strains for the rest of the potent compounds as RI<10 were observed.

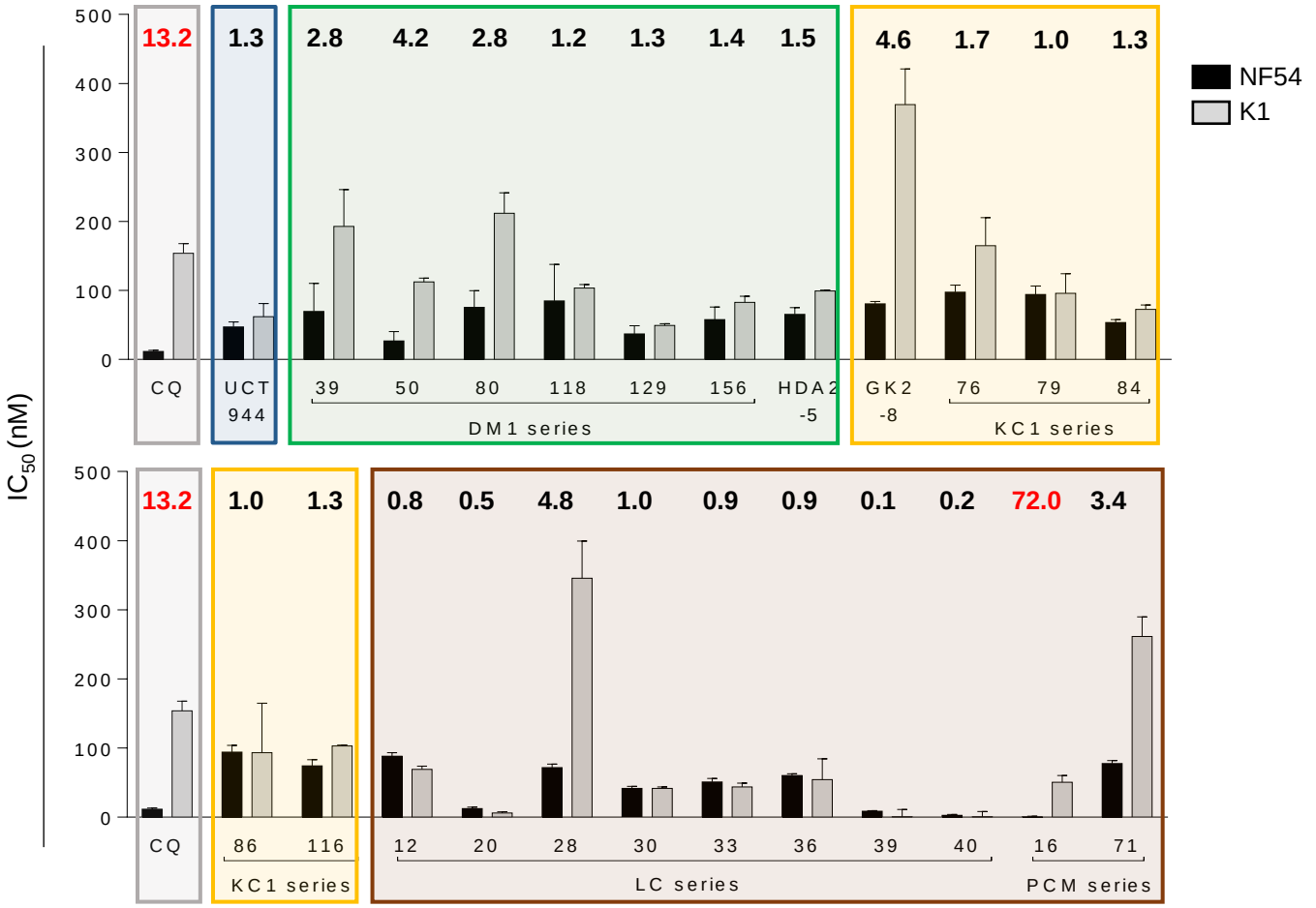


Figure 3.13. The efficacy of selected potent compounds across *P. falciparum* strains with different drug sensitivities.

The ability of potent novel compound to retain potency against *P. falciparum*, NF54 drug-sensitive (black bars) and K1 multidrug-resistant (grey bars) as indicated by resistance indices (RI). RI (bold numbers) is the IC_{50} fold change between strains (K1/NF54) with values above 10 in red text . Potent compounds are grouped in their classes: aminopyrazines (blue), astemizoles (green, imidazopyrazines (brown), pyrido(1,2-α)benzimidazoles (mustard) with RI of chloroquine (CQ) (grey) used as a control. Data are from three independent biological experiments of technical triplicates, n=3 ± SEM.

3.6. Cytotoxicity of selected potent hit compound

The main criteria for antimalarial hits are an IC_{50}<1 µM and at least 10-fold selectivity for parasites over mammalian cells [122, 123]. The 23 potent compounds were screened against HepG2 hepatocellular cells to determine whether they met the selectivity criteria. All of the compounds were tested at 2 µM, screening the compounds at >20-fold higher than the antiplasmodial IC_{50}. Antimalarial drug CQ (2 µM) and EM (10 µM) were used as reference controls for cytotoxicity. Compounds that resulted in <50 % cytotoxicity met the selectivity criteria (Figure 3.14).

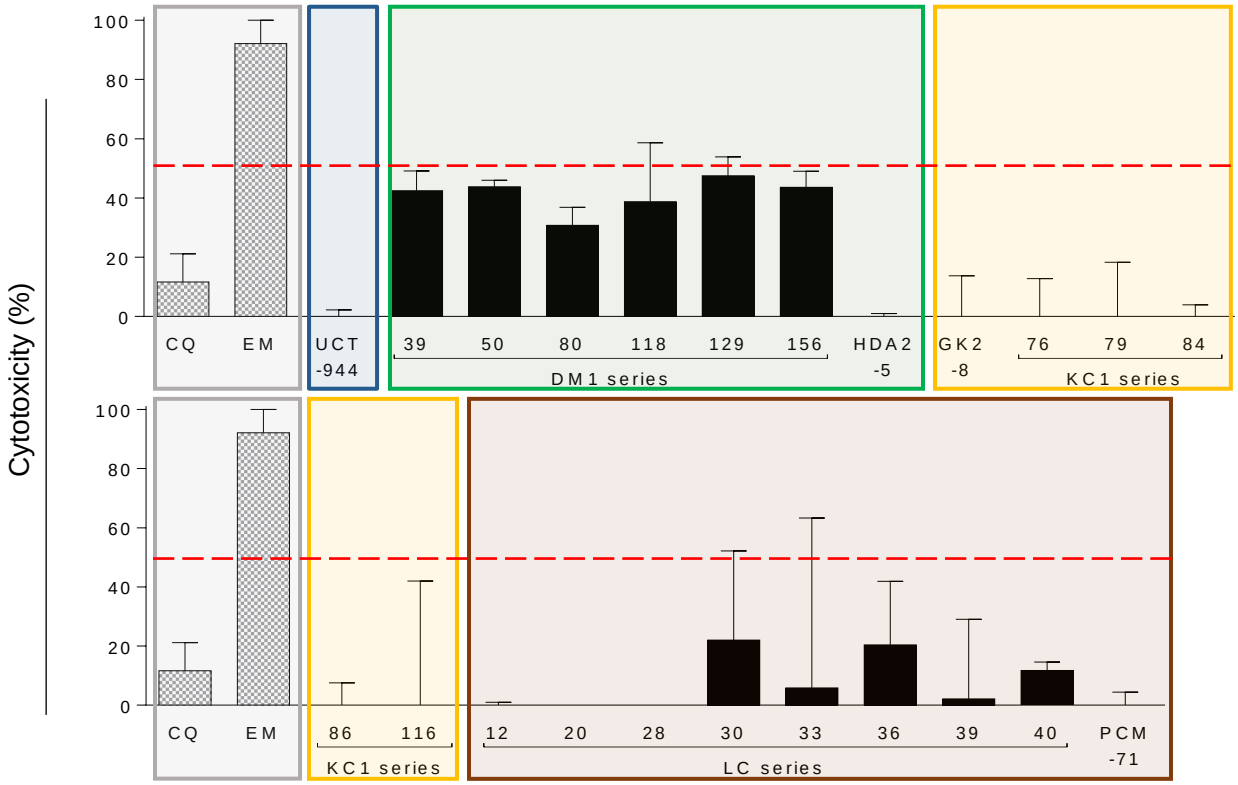


Figure 3.14. *In vitro* cytotoxicity and selectivity of potent compounds against hepatocellular carcinoma cells.

Cytotoxicity caused by 48 h exposure to 2 µM of the novel potent compounds from the aminopyrazines (blue), astemizoles (green, imidazopyrazines (brown), pyrido(1,2-α)benzimidazoles (mustard) classes. Emetine (EM) used as a reference control for cytotoxicity and chloroquine (CQ) used as a control for good selectivity. A maximum cytotoxicity of 50 % (red dashed line) was used as the cut-off for acceptable cytotoxicity. Data are from three biological experiments performed in technical duplicates ± SEM.

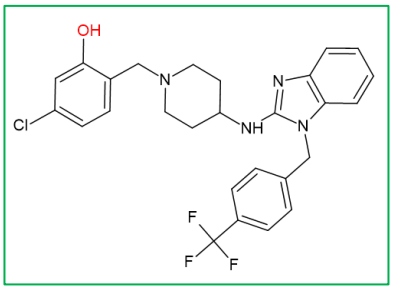
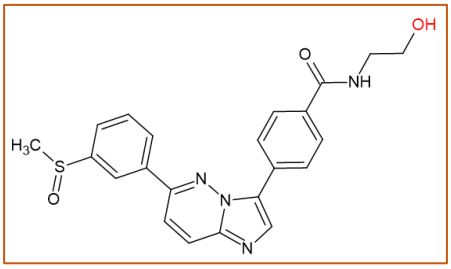
The PB class showed the lowest cytotoxicity (<20 %) at 2 µM for all of the compounds. The majority of the ASTs were more cytotoxic (30-45 % cytotoxicity) than the IP and PBs. Nonetheless, all of the compounds showed <50 % cytotoxicity, indicating that the potent compounds were at least 20-fold selective towards the *P. falciparum* parasite over mammalian cells. Since all of the compounds met the criteria for acceptable cytotoxicity, no cytotoxicity dose-response was investigated.

Selected hits were then profiled for their possible attachment to the NP backbone. Since an esterification process was used to chemically conjugate the compound to the NP backbone by way of an acid-labile linker, the novel compound needed to have an alcohol (OH) group. From the 23 potent hits only 2 compounds, LC12 (AST class) and HDA2-5 (IP class), possessed and alcohol group in their side chains. LC12 and HDA2-5 were further profiled *in silico*.

3.7. *In silico* predictions of selected compounds

Since neither LC12 nor HDA2-5 showed cytotoxicity *in vitro,* the logS (aqueous solubility a pH 7.4), logP (hydrophilicity at pH 7.4) and MW was determined *in silico*. The compounds had to meet the following criteria in order to potentially result in good absorption and bioavailability: MW<500 Da, logP<5 and logS>-5.8. For consistency, three different programmes, Osiris Datawarrior v 5.0.0, Mol Soft v 1.5.1 and ALOGPS v 2.1 were used to determine the parameters of LC12 and HDA2-5 using SMILES provided by UCT (Table 3.2).

Table 3.2. *In silico* predictions of potent compounds with hydroxyl groups.
The Molecular weight (MW), aqueous solubility (logS, pH 7.4 at 25 °C) and hydrophilicity (logP, pH 7.4 at 25 °C) of novel compounds with hydroxyl groups, LC12 and HDA2-5, were determined using Osiris Datawarrior v 5.0.0, Mol Soft v 1.5.1 and ALOGPS v 2.1.

Compound	HDA2-5			LC-12		
Structure						
MW	514.98			420.49		
Programme	Datawarrior	Mol Soft	ALOGPS	Datawarrior	Mol Soft	ALOGPS
logS[a]	-5.34	-5.87	-5.30	-4.38	-2.50	-3.71
logP	5.79	6.16	5.23	1.49	1.72	1.60

[a] logS is calculated as the logarithm of solubility measured in mol/L (M).

The *in silico* values for LC12 revealed an acceptable MW and a moderate solubility. Furthermore, a high hydrophilicity (logP<5) was predicted, even meeting the requirements for oral and intestinal absorption (logP between 1.38 and 1.8). On the contrary, HDA2-5 predictions were that of low solubility and low hydrophilicity across all three platforms. Furthermore, the MW was above the 500 Da Lipinski limit. Between LC12 and HDA2-5, the latter was the least soluble and did not meet MW and hydrophilicity criteria. For this reason, HDA2-5 would benefit the most from the NP system.

PART III: Evaluation of the tNP with novel compound as therapeutic agent

3.8. Conjugation of HDA2-5 to tNP decreases the antiplasmodial activity

HDA2-5 was conjugated to the NP backbone with targeting ligand, GSRSKGT, to make tNP-HDA2-5. The antiplasmodial activity of tNP-HDA2-5 was then tested to determine whether an improvement would occur. The IC_{50} of the tNP-HDA2-5 was adjusted to represent only the amount of the compound in the tNP (Figure 3.15).

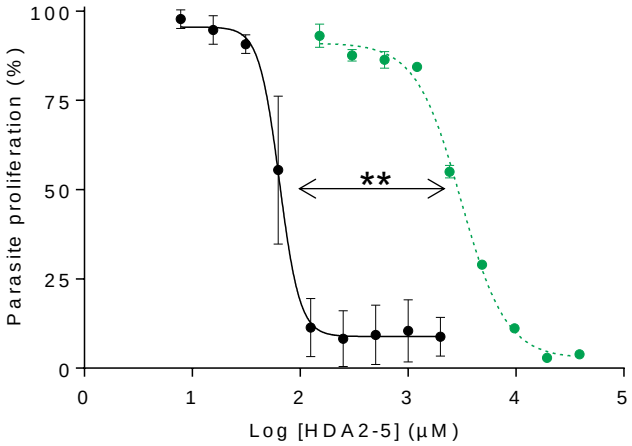


Figure 3.15. The effect of targeted nanoparticle (tNP) on the antiplasmodial activity of novel compound HDA2-5.
The IC_{50} shift for HDA2-5 (●) and tNP-HDA2-5 (■) as determined against asexual intraerythrocytic *P. falciparum* parasites. Data are from technical triplicates for three independent biological replicates, n=3 ± SEM. Where not visible, error bars fall within the symbols. Significant differences were determined using the paired Student's *t*-test (*$P < 0.01$).

Similar to the trend observed for the interrogative antimalarial drugs HCQ and AL, tNP-HDA2-5 lowered activity of HDA2-5 by a significant ($P<0.01$, n=3) 46.7-fold, decreasing this to moderate activity (IC_{50} between 1 µM and 5 µM) (Figure 3.15).

The next step was to determine whether HDA2-5 possessed any haemolytic activity and whether haemolysis would be decreased by conjugation to the targeted NP backbone (tNP-HDA2-5).

3.9. Nanoparticles decrease haemolysis of novel compound HDA2-5

To ascertain whether tNP-HDA2-5 would decrease haemolysis of HDA2-5, the haemolytic activity was determined at 5 µM and 10 µM of HDA2-5 proportion (Figure 3.16).

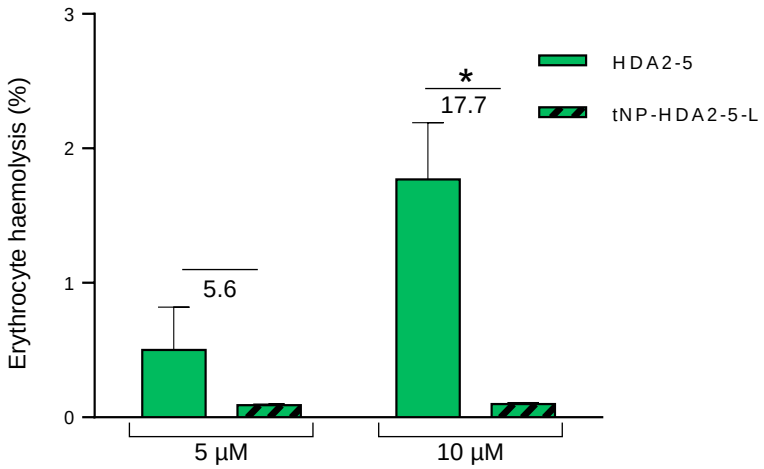


Figure 3.16. Effect of targeted nanoparticle (tNP) on the haemolysis of novel compound HDA2-5. Inhibitory effect at 5 µM and 10 µM of HDA2-5 and tNP-HDA2-5 on erythrocytes determined using the haemolysis assay. The tNP-HDA2-5 was represented by the proportion of novel compound HDA2-5 in the tNP. Significant differences were determined using the paired Student's *t*-test (**P*<0.05) where n=3, ± SEM.

The haemolysis caused by HDA2-5 was less than 2 % at 5 µM and 10 µM. Although already low, tNP-HDA2-5 further decreased the haemolysis caused by HDA2-5 to a negligible proportion. This was highlighted at 10 µM where the decrease in haemolysis was significant (*P*<0.05, n=3, paired Student's *t*-test) with a 17.7-fold difference between HDA2-5 and tNP-HDa2-5.

3.10. Targeted nanoparticle tNP-HDA2-5 is biocompatible to HepG2 cells

The cytotoxicity on HepG2 cells was also determined for HDA2-5 and tNP-HDA2-5 at 10 µM. There was no detectable cytotoxicity at 10 µM for either HDA2-5. Instead, the cytotoxicity of tNP-HDA2-5 was determined at >10X the antiplasmodial IC_{50} for tNP-HDA2-5 to determine biocompatibility (Table 3.3).

Table 3.3. *In vitro* cytotoxicity of targeted nanoparticle with novel compound HDA2-5 as therapeutic agent (tNP-HDA2-5) on HepG2 mammalian cells.
Inhibitory effect at 40 µM of tNP-HDA2-5 on HepG2 cells determined using the LDH release assay. Significant differences were determined using the paired Student's *t*-test (**P*<0.05) where data were obtained for n=3, ± SEM.

	IC_{50} (µM)[a]	[HDA2-5][b]	Cytotoxicity (%)[c]	CC_{50} (µM)	SI[e]
tNP-HDA2-5	3.00 ± 0.09	40 µM	4.45 ± 1.23	>40[d]	>13.7*

[a] Antiplasmodial IC_{50} of tNP-HDA2-5.
[b] Concentration of HDA2-5 in tNP-HDA2-5 used to treat HepG2 cells.
[c] Cytotoxicity observed at 40 µM treatment.
[d] Cytotoxic half-maximal concentration (CC_{50}) represent as greater than concentration used. A 50 % cytotoxicity was not reached at selected concentration.
[e] SI is the selectivity index calculated as CC_{50}/IC_{50} whereby an SI>10 are significant (paired Student's *t*-test, **P*<0.05).

As expected, tNP-HDA2-5 was more than 10-fold selective for *P. falciparum* parasite than HepG2 cells *in vitro*. This meets the criteria as set by the MMV [123]. The results confirm that tNP-HDA2-5 was biocompatible to mammalian cell types and would not result in overt cytotoxicity.

Chapter 4: Discussion

Treating the symptom-causing asexual intraerythrocytic stages of *P. falciparum* parasites remains a vital fight against mortality rates. However, many antimalarial drugs and drug combinations present side effects during treatment [94] due to various reasons including low aqueous solubility, insufficient metabolic or physical stability and dose-related toxicity. As a result, patients become non-compliant which may in-turn result in the development and spread of resistant strains. The interest of drug delivery systems such as NPs is a growing field in malaria research, which can be used to avoid various drug issues. However, this is the first study that aims to simultaneously exploit two aspects in malaria infections: (1) site-specific drug release in the specialised FV of *P. falciparum* parasites by way of an acid-labile linker, and (2) enhance affinity to *P. falciparum*-infected erythrocytes through the targeting ligand.

The structure of the NPs presented here consisted of a hydrophilic PVP and hydrophobic PVL backbone, both of which are non-toxic [97, 109, 110]. The data confidently indicates biocompatibility and safety of the NP backbone, particularly to HepG2 mammalian hepatocytes and non-infected erythrocytes. This was a good finding, indicating that even when the NP backbone is left behind after drugs are released, it would be well tolerated until clearance from host circulation.

The drugs were conjugated to the NP backbone through an acid-labile linker in an effort to ensure that the NPs and tNPs would remain intact in any host environment with a physiological pH, including plasma and mammalian cells [99]. Under an acidic environment, such as the FV [130] of *Plasmodium* parasites, the acid-labile linker was to degrade, releasing the drug in a specific compartment. Figure 4.1 illustrates drug release studies conducted prior to this project, whereby NPs were exposed to a physiological pH 7.4 and pH 5.0, mimicking the acidic environment of the FV. According to the results from figure 4.1, after 72 h (1.5 IDC life cycle duration) most of the AL (75-80 %) would be released in the FV whereas only 15-20 % could be potentially released elsewhere, showing minimal signs of premature and non-target release.

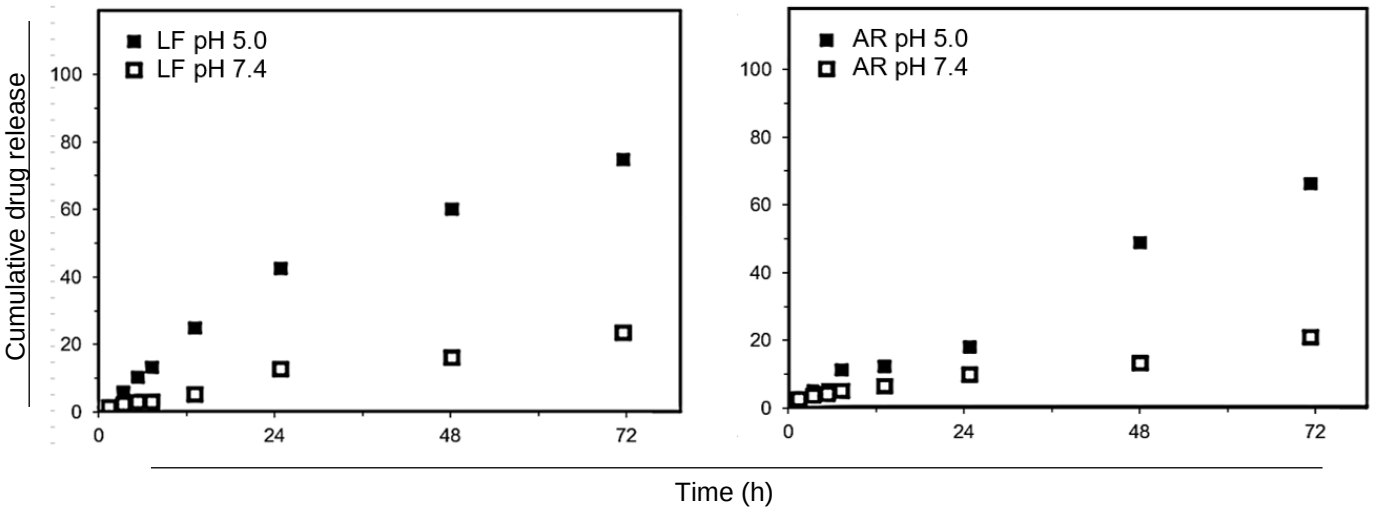


Figure 4.1. Release of drugs from the NP backbone under acidic and physiological pH.
Cumulative drug release of (A) LF and (B) AR from NPs at acidic pH 5 (black scatter) mimicking the environment of the FV and physiological pH 7.4 (white scatter). Graphs were obtained from Dr Lisa Fortuin, SU.

The first measure that successful drug release had occurred was the fact that antiplasmodial activity was observed for the NPs and tNPs whereas there was no activity for the NP backbone. This could be an indication that the NPs and tNPs did indeed reach infected erythrocytes and, subsequently, the FV of *P. falciparum* parasites. However, the tNPs significantly decreased antiplasmodial activity, contrary to what was hypothesised. The significant loss in antiplasmodial activity may be explained by the drug release rate (Figure 4.1) and the nature of the FV.

A complete FV is only observed in the mid trophozoite stages (only after ~28 hpi). Prior to this, only small vesicles are observed starting in the late ring stages [52]. During the early schizont stages, the FV begins to shrink and feeding slows down. By the late schizont stages the FV is completely separated from daughter merozoites [48]. Should release be possible in the small vesicles then a maximum of 50 % drug release is possible as there is a 26-32 hr window. However, this is unlikely. With the assumption that a complete FV is required for release then drugs can only be released during the mid-trophozoite stages and before the late schizont stages, where the FV is most active. This leaves a 10-14 hr window within which drug release needs to happen. Only 20-30 % of the drug would be released in the FV within this time-frame (Figure 4.1). Regardless of which time-frame is most accurate, the tNP antiplasmodial IC_{50} presented here was calculated on the assumption that 100 % drug release would occur. The determination of an accurate IC_{50} is, therefore, complicated by the fact that it is not possible to determine how much of the drug is actually released in the FV *in vitro* and the best model is that of the *in vitro* drug release experiment

at pH 5. Even with this “loss” in activity the NPs and tNPs were moderately active (IC_{50} between 1 µM and 5 µM). Therefore, the NP does not necessarily decrease antiplasmodial activity but rather that a slow-release mechanism is used. In a positive light, this means that when erythrocytes rupture during merozoite release, the released tNPs that still have drugs can enter newly infected erythrocytes killing the parasite.

Drugs that do not reach their intended target efficiently, require higher doses for a therapeutic effect to occur [88]. It was hypothesised that tNPs would enhance delivery to infected erythrocytes, exploiting both active and passive methods (NPP). Active NP delivery systems rely on receptors present on the surface of diseased cells. In the same way, the addition of the targeting ligand (GSRSKGT) was intended to enhance delivery of NPs (tNPs) to *P. falciparum*-infected erythrocytes. This mechanism would in-turn improve the antiplasmodial activity of the NPs as non-specific tissue distribution would be limited. The data suggest that the targeting ligand did not enhance delivery to infected erythrocytes as proposed, since the antiplasmodial activity of the tNPs did not fare better than that observed for the NPs. One explanation for the significant decrease in antiplasmodial activity due to the tNPs as compared to therapeutic agents is the possibility that most of the tNPs did not make it into the FV sufficiently. However, a more plausible explanation is that the tNPs relied heavily on passive delivery such an NPP, since the targeting ligand was not sufficient. If so, then most of the tNPs would be taken up from 22 hpi since the NPP form as parasites mature to the trophozoite and schizont stages. This further substantiates that less than 50 % of the drug would be released in the short time-frame.

Since it is likely that only passive delivery occurred through NPP, then some of the tNPs would have made it into non-infected erythrocytes and HepG2 cells, much like the NPs. Despite this non-specific tissue targeting, the NPs and tNPs significantly decreased haemolytic toxicity against non-infected erythrocytes and cytotoxicity on HepG2 hepatocellular mammalian cells with no significant difference observed between the tNPs and NPs. This observance may be due to the acid-labile linker preventing non-specific release of drugs since the FV (pH 5) was the intended release site. The lowered *in vitro* toxicity of the drugs is a positive point for treatment for two reasons: Firstly, the toxicity of the drugs would not affect patients since the NP backbone masks these properties and any unused NPs would be well-tolerated until completely excreted. Secondly, even if tNPs were to enter other cell types (at a much lower concentration than infected erythrocytes), the drug would not be released from the NP backbone. Therefore, cell types that

are not the intended target would not be negatively affected with regards to toxicity. This could possibly translate to decreased side-effects and patient non-compliance.

Combination therapy is used to overcome the rapid emergence of resistance since the individual partners are potent and have different MoA i.e. ACTs [40]. However, each individual drug in a combination treatment can decrease bioavailability due to one of various shortcomings. AL is combination is an example of this: AR has a short-half life and LF is lipophilic and requires an increased lipid diet [87]. Furthermore, compounds may also target other cell types in the human host. The NPs alleviated solubility issues by masking the drug from the external environment. Furthermore, circulation time was enhanced through the NP backbone alleviating half-life issues, and the acid-labile linker ensuring that alternate cell types would not be affected. Overall, tNPs may result in improved oral bioavailability and high drug doses would not be required. This will further increase the chances of patient compliance and consequently decrease the chances of resistance forming. Most drugs get cleared rapidly from circulation or degraded, with some drugs having a plasma half-life of a few hours. Polymeric conjugates, such as the one described here, are not cleared so rapidly [131, 132] and can, therefore, clear residual parasites with no need for additional dosages while also improving patient tolerability. This means that a potent drug with discovered issues can be incorporated into the NP without having to go through the process of modification. Furthermore, tNPs could also be used in combination with a lower non-toxic dose of antimalarial drugs or combinations.

The AL combination has been reported to show gametocytocidal activity [58], therefore, NPs can make their way into infected erythrocytes and release drugs in the FV of sexually committed asexual intraerythrocytic parasites before sexual differentiation occurs. However, the window may be small as the NPs would have to enter the FV before differentiation begins. Furthermore, NPs have a long plasma stability rate as a result of the components of the NP backbone [131, 132], and one dose can be taken in at high drug concentrations to be released when parasites are present. This may also be beneficial for *P. vivax* infections where dormant hypnozoites can become problematic at a later time (TCP-3 activity). The advantage of the ability of the NPs to avoid clearance is short-term chemoprotection (TPP2) where the activated NPs can be administered before malaria season to people in appropriate locations or travellers who plan to visit malaria endemic areas.

The slow release of the therapeutic agent is due to the acid-labile linker, therefore, a linker that releases the drug at a faster rate may be explored. Burst drug release, whereby a large drug concentration is released suddenly and simultaneously is usually avoided for nanocarriers as it could be harmful to the patient due to possible sudden exposure [133]. Since NPs can only release drugs in the active FV (acidic pH 5) of *P. falciparum* parasites, a sudden burst release would only be detrimental for the parasites and consequently *Plasmodium*-infected erythrocytes. Therefore, harm to other healthy tissue such as non-infected erythrocytes would not be a major concern. If successful, an acid-labile linker that degrades more rapidly would make it possible for encapsulated drugs to be incorporated along with conjugated drugs while retaining sufficient release within the limited time-frame of the FV. This would be beneficial for combination therapy coupling compounds which have different MoA, such as novel compound HDA2-5 (inhibition of *B*-hematin formation) [134] and a novel kinase inhibitor compound such as those presented here.

To further substantiate the findings of the targeting ligand, localisation studies for the tNPs and NPs need done. High performance liquid chromatography or mass spectrometry can both be utilised to this end as attempts at fluorescence microscopy were unsuccessful. These methods may be able to prove that the tNPs are taken up into infected erythrocytes further determining whether the tNPs likely use an active mechanism or rely solely on passive targeting. Other future endeavours regarding the tNPs include *in vivo* pharmacodynamics evaluations in mice as well as incorporation of other targeting ligands that not only attract tNPs to *P. falciparum*-infected erythrocytes but also towards the FV once inside the erythrocytes.

In conclusion, a comparative evaluation of novel NPs revealed several central features that could be beneficial to malaria treatment with some modifications. Firstly, the data suggests that tNPs are stable under physiological pH and only release drugs under acidic pH pressure. Secondly, the data also suggests that the tNPs decrease the toxicity of drugs to cells that are not the intended target. Furthermore, that the tNPs retain some antimalarial activity although significantly less than the drug. Unfortunately, the data also suggests that the selected targeting ligand did not improve targeting to erythrocytes and hence antiplasmodial activity, contrary to what was hypothesised.

Chapter 5: Conclusion

Despite the successful rolling out of chemotherapeutics, the existence of resistant strains to all antimalarial classes poses a threat to this success. In response to this, rigorous drug discovery initiatives have resulted in new compounds, with novel MoA making it into clinical trials. However, many approved drugs come with underlying pharmacological issues that decrease treatment potential [85, 86]. Thus, there is a need to explore other methods of drug delivery such as NPS. NPs of various formations has been studied in malaria but those that utilise the physiology of the *P. falciparum* specialised FV against itself is a novel concept. In summary, a new NP system that utilises environmental response to release drugs in the FV only, has been described. Since the NPs have a peptidic targeting ligand (tNPs) and an acid-labile linker, it was hypothesised that the novel tNPs would improve antiplasmodial activity and toxicity of antimalarial drugs.

We sought to determine whether tNPs would improve antiplasmodial activity and toxicity of antimalarial drugs and be useful to improve performance of antimalarial candidates. The NP backbone was safe for mammalian and *P. falciparum* cell lines, revealing safety to non-target host cells. The data also revealed that the NPs and tNPs significantly decreased the toxicity of known antimalarial drugs, AL and HCQ, showing potential to decrease side effects. Furthermore, the tNPs and NPs improved the toxicity of the novel compound selected from whole cell phenotype screening, revealing that novel compounds with issues can be used without derivatisation. Although our findings showed that the novel acid-labile tNPs improved toxicity and solubility, the antiplasmodial activity was decreased. Therefore, the hypothesis is not supported in its entirety.

Low aqueous solubility of the drugs was masked by the amphiphilic NP backbone and the acid-labile linker ensured target-specific drug release [135]. Therefore, target-specific, environmental responsive amphiphilic NP conjugated drug delivery systems, such as the one described here, may be an essential alternative to costly and time-consuming modifications of novel drugs with various pharmacokinetic shortfalls. Furthermore, NPs with novel drugs may also play a future role in malaria treatment that ensures patient compliance. A single dose alternative that does not require a specialised diet may be beneficial for the impoverished malaria endemic regions. Therefore, the NP conjugations described in this project provide an attractive starting point for novel and clinically approved antimalarial drugs.

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