Resisting resistance: is there a solution for malaria?

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Abstract

Introduction

Currently, widely used antimalarial drugs have a limited clinical lifespan due to parasite resistance development. With resistance continuously rising, antimalarial drug discovery requires strategies to decrease the time of delivering a new antimalarial drug while simultaneously increasing the drug's therapeutic lifespan.

Areas covered

Lessons learnt from various chemotherapeutic resistance studies in the fields of antibiotic and cancer research offer potentially useful strategies that can be applied to antimalarial drug discovery. In this review we discuss current strategies to circumvent resistance in malaria and alternatives that could be employed.

Expert opinion

We have been "beating back" the malaria parasite with novel drugs for the past 49 years but the constant rise in antimalarial drug resistance is forcing the drug discovery community to explore alternative strategies. Avant-garde anti-resistance strategies from alternative fields may assist our endeavors to manage, control and prevent antimalarial drug resistance to progress beyond beating the resistant parasite back, to stopping it dead in its tracks. Here we investigate the

development of strategies that are able to either overwhelm or outwit the parasite in its attempts to develop resistance.

Article Highlights box

- In most instances the malaria parasite develops drug resistance at a faster rate than a novel antimalarial drug can be developed.

- For antimalarial drug discovery to remain sustainable, the clinical lifespan of an antimalarial drug must as least exceed the time taken to develop the drug.

- Currently, antimalarial drug resistance is being controlled through the development of novel drugs, which are combined with an appropriate drug partner into a combination therapy.

- Polypharmacology (multitargeting) may be able to speed up the delivery of a novel antimalarial drug while simultaneously increasing the clinical lifespan of the drug.

- Unexplored targets such as the virulence potential, hijacked host factors and stress factors may deliver drugs that can effectively resist resistance.

- Other post-resistance strategies such as molecular decoys and chemogenomics may also prove valuable in curbing resistance.

1. Introduction

In 2016, we are contending with a parasite that risks the lives of 3.3 billion people in 97 countries; causing febrile malaria, particularly in young children, immuno-compromised patients or non-immune populations (e.g. travelers), pregnant women and the elderly [1]. With the announcement of the United Nations Millennium Development Goals in 2000 including: 1) combating HIV/AIDS (Human Immunodeficiency Virus), malaria and other diseases, and 2) prevent childhood mortality; efforts in the fight against malaria were renewed in a concerted manner. As malaria is still one of the three major causes of childhood mortality in Africa, the WHO's Global Malaria Action Plan (GMAP) [2] was adopted in 2007 by the global malaria community to galvanize coordinated efforts that aim not only at global malaria control but worldwide elimination and ultimately eradication of this disease. Malaria elimination will not be achieved by focusing solely on the treatment of the disease in humans (through current antimalarial chemotherapies) or on exclusion of the mosquito vector (through physical vector control mechanisms of *Anopheles*) but requires also blocking of transmission of the parasite between the human host and mosquito vector. The GMAP set forth specific goals including a 10-fold reduction in malaria incidences and associated deaths by 2030 and since its inception in 2007, has led to a dramatic (~50%) decrease in malaria incidence. The 2014 WHO report indicates 198 million cases of malaria resulting in 367 000–755 000 deaths annually [1]. Unfortunately, the heaviest burden on public health is felt in the economically constrained WHO African Region, where an estimated 90% of malaria deaths occur. However, outstanding success has been achieved in Africa with the prevalence of *P. falciparum* infections being halved and the incidence of clinical disease decreasing by 40% between 2000 and 2015 [3].

In totality, the successes of the past decade can be attributed to concerted and global efforts from multiple role players. Although alternative and innovative vector control strategies have been used for the past 10-15 years including insecticide impregnated bed nets for people at risk and indoor residual spraying to control vector populations [4], parasite control still remains largely dependent on chemical interference; both for prophylactic and therapeutic use. Vaccines aimed against the parasite have gained strengths with the vaccine RTS,S/AS01 providing partial but not long lasting protection in children [5] but is still not at a point where it will solely be able to control the parasite. It is exactly this dependence on antimalarials to control the parasite that highlights concerns for its sustainability, given the remarkable ability of the parasite to develop phenotypic and clinical resistance against all chemical entities used against it. History has clearly indicated that new antimalarials must be continually developed in the ensuing event of resistance development to the current antimalarial arsenal.

Several extensive reviews have been published in this regard [6-12]. This review therefore will not provide a comprehensive past history nor current status of antimalarial drug discovery, but will rather aim to introduce and interrogate potential strategies for resisting resistance already being implemented in other fields (e.g. antimicrobials and anticancer) as innovative and supplementary opportunities for antimalarial drug discovery.

2. Malaria parasites and the development of drug resistance

Plasmodium falciparum, the parasite that still causes ~90% of malaria cases in sub-Saharan Africa and results in the most deadly forms of the disease (its sister species infecting humans being *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) has evolved to relatively quickly, but highly efficiently, circumvent drug pressure through genetic adaptation to develop various

resistance mechanisms. To date, *P. falciparum* in particular has become resistant to all clinically used antimalarial therapeutics, including the most recently introduced artemisinins and its combinations (Figure 1)[10,13-15]. Five classes of chemicals have been used clinically for the treatment of malaria and include 1) the aminoquinolines (e.g. chloroquine, amodiaquine, piperaquine); 2) the aminoalcohols (mefloquine, halofantrine, lumefantrine); 3) antifolates (sulfadoxine, pyrimethamine, proguanil); 4) endoperoxides (e.g. artemisinin (ART) and derivatives) and 5) the hydroxynaphtoquinone, atovaquone [16].

The most successful antimalarials used to date, as assessed by their clinically useful therapeutic lifespans before being retired due to the development of resistance, had their origins in medicinal plant extracts. For instance, chloroquine as synthetic derivative of the natural remedy quinine (from Chincona bark), showed resistance within 12 years (1945-1957) and lost most of its therapeutic efficacy after 32 years (by 1977), but in total had a useful lifespan of 50 years in specific parts of the world (Figure 1)[17]. Mefloquine resistance however, appeared within a few years after its introduction in the late 1970's. The discovery of artemisinin in 1972, originating from the long-used Chinese herb *Artemisia annua*, has led to ~40 years of clinical usefulness (1972 to ~2007) before the first signs of resistance emerged [18], with clinical resistance now present throughout Southeast Asia [19,20]. Both chloroquine and artemisinin target a broad range of essential biochemical functions within the parasite. By contrast, resistance against synthetic inhibitors aimed at single proteins like sulfadoxine and pyrimethamine (SP, antifolates) developed fairly quickly with the first resistant parasites against SP selected for within a year of its use (1967) and reduced their clinical usefulness to ~20 years (1967-1990's) [10,17]. Atovaquone similarly lost efficacy within ~6 years when used as single entity [21].
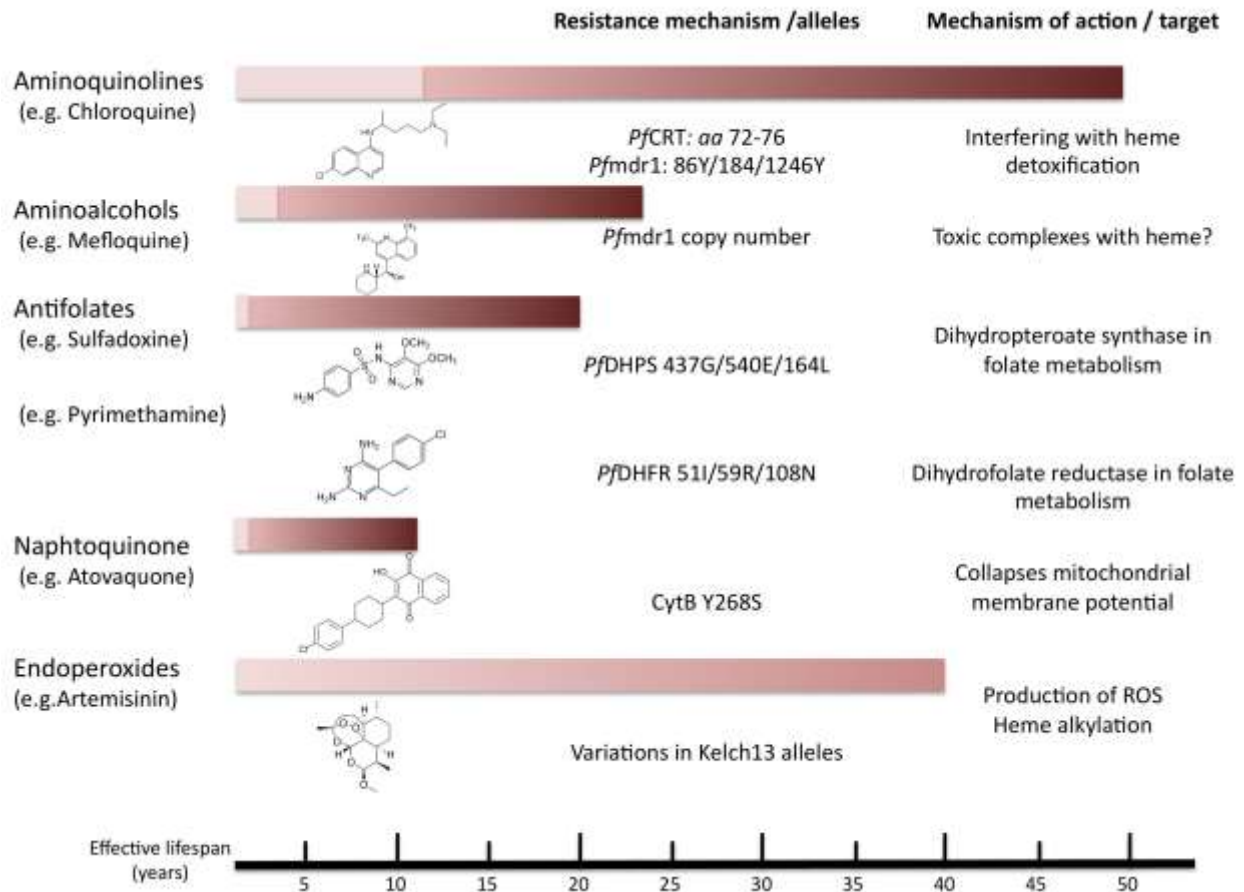
Figure 1. The effective clinical lifespans, resistance mechanisms and mechanisms of action of the main antimalarial drug classes. The five classes of antimalarial drugs that have been used for the clinical treatment of malaria include: the aminoquinolines, aminoalcohols, antifolates, endoperoxides and naphtoquinones. Chemosensitivity to the compounds are indicated by lighter shades and onset of resistance depicted by the change in color bar, with more intense colors correlating to increasing prevalence of resistance.

The development of resistance in malaria parasites has a clear genetic basis with the genome described as unusual, highly permissive and with great plasticity [15,22]. However, the precise mechanism(s) causing resistance development is not yet clearly defined. Initially it was hypothesized that a phenomenon referred to as ARMD (accelerated resistance to multiple drugs; the ability of a parasite strain to generate a resistant clone under drug pressure) was associated with resistance development as specific strains of *P. falciparum* have up to a 1000x higher

frequency to develop resistance to selected compounds [23]. The main contributing factor promoting the ARMD phenotype is reported to be the high mutation rate during parasite multiplication, associated with the low efficiency of the DNA repair mechanisms of specific parasite strains [23,24]. However, more recently, evidence has emerged that suggests that the core genomes of clones previously reported to have the ARMD phenotype (e.g. Dd2 strain), are indeed stable irrespective of drug pressure, suggesting that Dd2 clones did not acquire resistance through an intrinsically higher average mutation rate [26]. This is extended to some variable gene families (e.g. *var* genes) where recombination is implied as the major contibutor to genetic variation [27].

The parasite develops *de novo* resistance (without the need for meiotic recombination of male and female forms of the parasite during mosquito transmission) when submitted to sub-lethal / sub-therapeutic concentrations of a drug either *in vitro* with enhanced evolution strategies [28], or following the natural acquisition of resistance in the parasite *in vivo* in animals or humans. This can take the form of either copy number variants (CNVs) or single nucleotide variants (SNVs) [29] and can occur directly in the drug target or may result in e.g. upregulation of transport mechanisms to export the drug and alleviate drug action. Several factors work in conjunction to influence the frequency by which resistance develops within parasite populations including parasite load and fitness cost to the parasites, patient immunity and drug pharmacokinetics (PK) / pharmacodynamics (PD) [15].

Malaria drug resistance mechanisms are additionally quite unique, as the parasite is capable of inducing resistance in the exact cellular target of the drug. This is in contrast to other diseases e.g. TB (*Mycobacterium tuberculosis* infections) and AIDS where the drug resistance phenotype is mostly induced due to enhanced and 'non-specific' efflux of drugs through

induction of multidrug resistance (MDR) transporters and is considered a serious problem in resistance development. Although the parasite uses MDR transporters as a resistance mechanism of certain classes of antimalarials, it is not necessarily the primary source of resistance development and therefore MDR only causes a problem in regional pockets [25]. This target-specific resistance mechanism could imply that resisting resistance to the parasite may need to be individualized for each drug class.

3. How is antimalarial resistance currently being managed?

Antimalarial drug resistance development is of major concern and is globally monitored with e.g. the World Wide Antimalarial Resistance Network [30], raising early alarms of resistance development / spread and informing the malaria community of potential efficacy loss of antimalarials. As such, it is imperative that we preserve and protect the lifespan of current clinically approved antimalarials or those within the clinical pipeline by sensible management strategies, correct deployment of drug interventions, continuous monitoring, preventing counterfeit drug exposure and involving all public health systems [10,21,31].

3.1 Continuous discovery of chemically and mechanistically novel antimalarial agents

The past decade has seen an unprecedented renewed focus on the discovery of new antimalarial entities through extraordinary collaboration between academia (parasitologists, medicinal chemists, pharmacologists, clinicians) and industrial / private partnerships (e.g. Medicines for Malaria Venture, MMV, [32]). Clearly defined target candidate profiles [6-11] streamlines the discovery process to identify new drugs able to cure infections with limited resistance development, increased compliance and short duration of clinical treatment, and *in*

lieu of malaria elimination also block malaria transmission. Additionally, we need new chemical entities to be used prophylactically (chemoprotection of vulnerable, non-immune populations) and prevent relapses of *P. vivax* or *P. ovale* infections. The antimalarial drug pipeline is now continuously populated with new chemotypes such as OZ439, ACT451840, MMV390048, DDD107498 etc. [10], which have entered pre-clinical or clinical investigations. The major requirements for any new chemical entity to be considered as a worthwhile antimalarial candidate whilst extending the effective therapeutic lifetime of the antimalarial and limiting the development of resistance include 1) the compound has to be chemically distinct, 2) the compound should target essential but novel biochemical entities / processes, 3) the drug target has to be known before clinical prioritization to decrease unnecessary investment in a number of chemical entities targeting the same drug target; if resistance develops it renders them all useless, and 4) unless single exposure radical cure can be claimed [6,7,33], all drugs should show an ability to be used in combination with other chemical entities [6]. Most importantly, for all new antimalarial entities, the risk of resistance development has to be assessed (Box 1) [34].

Box 1. Assessing the risk of resistance

Assessing the risk of resistance

Screening compounds for cross-resistance
1st screen: A standard multi-drug resistant *P. falciparum* strain (K1)
2nd screen: Multidrug-resistant *P. falciparum* panel (D6, HB3, 7G8, Dd2 V1/S, FCB and TM90C2B)
3rd screen: Field isolates of *P. falciparum* and *P. vivax*
Recommend discontinuation of compound if cross-resistance is present

Resistance selection *in vitro*
Minimal inoculum for resistance (MIR)
High risk : MIR of 10^5 parasites
Medium risk: MIR of 10^7 parasites
Low risk: MIR of 10^9 parasites

Resistance fitness cost (C-value: loss of fitness)
Elevated risk: $C \geq 0$
High risk: $C < 0$ (fitness advantage)

Determine gametocyte production
High risk: increased
Lower risk: decreased

Determine resistance mode-of-action
Analysis of genetic determinants to identify loci-specific marks associated with resistance phenotype

3.2 Expanding combination therapies

Combination therapies are well established for various disease states and infections including HIV, TB, cancer as well as malaria, with the potential advantage of combining different modes-of-action as well as delaying resistance development against either partner. Currently, the WHO recommends the use of antimalarials in fixed-dose regiments with partner drugs. Each of these partner drugs should still be effective in killing the parasite, with minimal signs of resistance. ACTs (<u>a</u>rtemisinin <u>c</u>ombination <u>t</u>herapies for the treatment of uncomplicated malaria) exploit the fast and potent action of the artemisinin component combined with a longer-lasting partner drug i.e. artemether-lumefantrine (Coartem®), artesunate-amodiaquine

(Coarsucam™), dihydroartemisinin-piperaquine (Eurartesim®) or pyronaridine-artesunate (Pyramax®) [35,36]. Several additional new ACT's are in Phase III clinical trials or being registered for market [10,37] but given the development of resistance against the artemisinin component, several other combinations are currently under investigation including non-artemisinin containing formulations, such as trimethoprim-sulfamethoxazole, fosmidomycin-piperaquine and new leads like OZ439-piperaquine [37].

The antibacterial field has relied on combination therapy to curb resistance development, particularly in TB [38], and includes combinations of more than 2 partner drugs. This, in theory, would result in targeting different activities in the organism, thereby more effectively curbing the development of resistance against any of the partner components. However, therapeutic action and dosing becomes increasingly complex and requires in-depth understanding of drug-drug interactions and how this influences PK / PD of each component in combination to enable rapid and extended decrease in parasite load. SP was used in the early 1980s in combination with mefloquine, but efficacy of this triple combination could never be clearly shown as the parasite populations in which it was used already indicated a level of SP resistance [37].

In context of malaria elimination, one scenario that may be envisaged is that antimalarial therapeutics (already a combination of two partner drugs) would need to be combined with a third drug with transmission blocking capacity but with a completely different pharmacologic profile. It remains to be seen to what extent the boundaries of drug combinations will be able to be pushed whilst conforming to malaria target candidate profiles.

3.3 Chemosensitizers

Chemosensitization has been proposed in drug resistant cancer lines as a means to enable cells to respond to drug treatment [39] and has been investigated for reversal of chloroquine resistance in malaria parasites, with especially the calcium channel blocker verapamil in the presence of chloroquine resulting in sensitizing previously resistant strains of the parasite [40]. More recently, dual-acting sensitizers [41,42] also enables the efficacy of chloroquine in 'resistant' lines [43]. However, beyond these interesting examples, the concept of chemosensitization has not met with the expectations of delivery of antimalarials fully able to overcome resistance.

4. Resisting resistance: new innovations.

Innovative strategies are being developed to resist resistance in the extensively studied fields of antibiotic and anticancer chemotherapeutic and here we assess their applicability to antimalarial drug discovery. Although bacteria and parasites are vastly different organisms, with many differences in their acquisition of resistance, there are several characteristics that share similarity e.g. between malaria, TB and HIV [25], and parasites and cancer cells [44,45]. These shared characteristics may serve as a viaduct offering unconventional but perhaps valid strategies for the field of antimalarial research in a 'piggyback' approach.

Several approaches have been suggested from the antibacterial and anticancer fields to resist resistance including using polypharmacology (multitargeting and combination therapies) as well as new innovations relying on either targeting unexplored alternatives or responding to the exact mechanisms causing resistance (Figure 2).
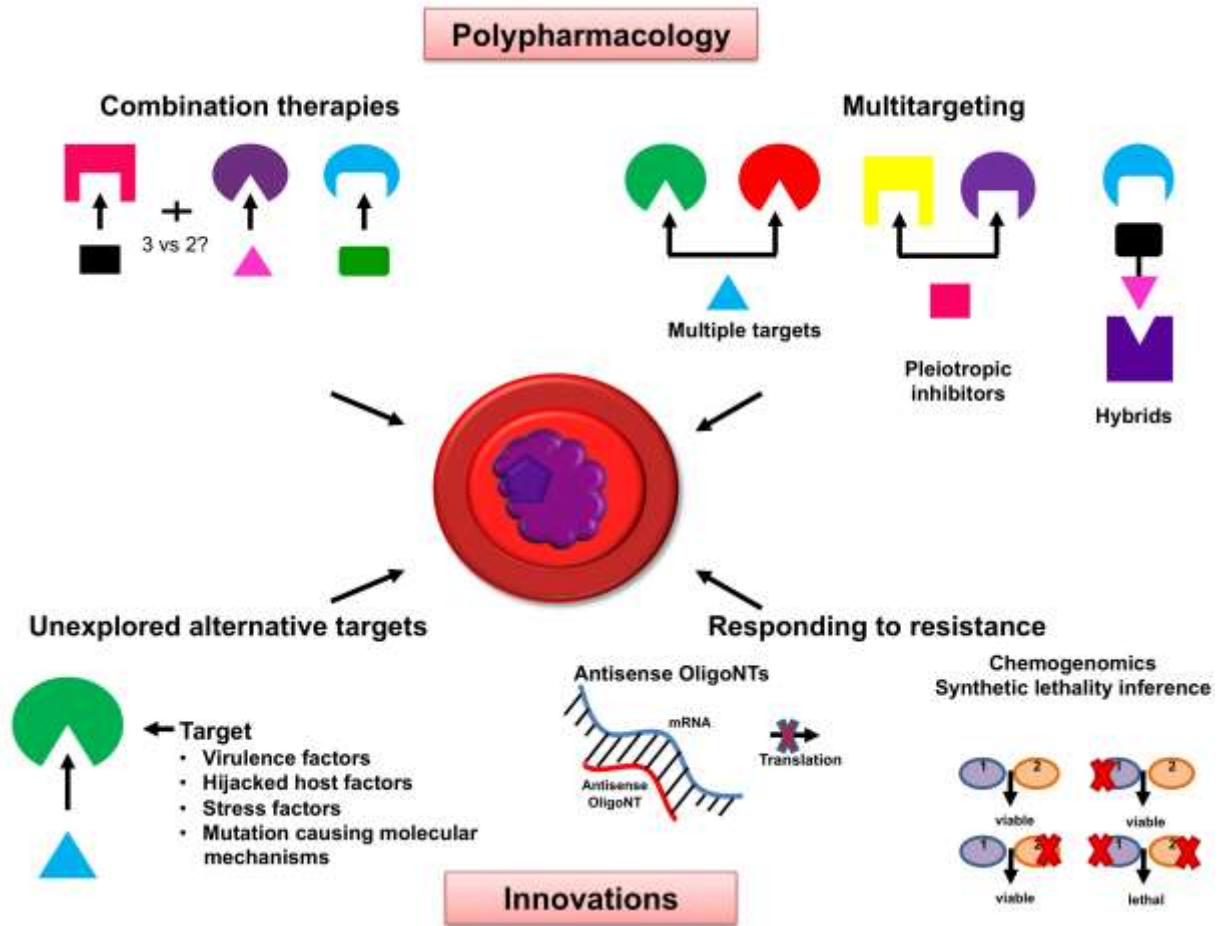
Figure 2. Resisting resistance strategies highlighted from the fields of antimicrobial and anticancer research.
Several alternative anti-resistant strategies have been recommended such as 1) expanding current antimalarial combination therapies from 2 drug partners to 3 drug partners; 2) utilizing polypharmacology to target multiple targets with a single inhibitor; these inhibitors may target multiple related targets or exhibit pleiotropic activity; 3) inhibiting unexplored alternative targets that decrease selective pressure by targeting non-essential factors involved in virulence potential, non-essential hijacked host factors and stress responses; 4) employing strategies that involve scaffolds (antisense oligonucleotides) that can be readily altered to keep up with the high plasticity of the *Plasmodium* genome; or utilizing synthetic lethality inference as a means to selectively target drug-resistant malaria strains.

4.1 Polypharmacology: a numbers game

Polypharmacological strategies include the use of single chemical entities that either target related activities or completely unrelated targets or could rely on combinations of chemical entities in hybrid molecules affecting a variety of biological mechanisms [46]. Additionally, drug

repurposing, or the repositioning of a drug for a different application for which it was originally designed for, provides a quicker and less expensive option of source material with obvious polypharmacological action [47]. Polypharmacology is proposed to speed up the process of delivering candidates into clinical practice but additionally also prolongs the development of resistance (Box 2). Additionally, multitarget drug overcomes drug-drug interaction issues, simplifies treatment regimens and compliance and enables PK / PD predictions.

Box 2. The opportunities and challenges of polypharmacology [112-114].

Polypharmacology: 'yae or nay'

Opportunities

- Treat complex multigenic diseases through multitargeting
- Decrease probability of resistance development
- Less pre-clinical factors to consider than two single targeting drugs in combination therapies
- Easier manufacture and formulation of single active pharmaceutical ingredient (multitarget drugs)

Challenges

- Off-target effects
- Partial understanding of pathways/ mechanisms at a molecular level
- Optimizing potency at multiple target sites simultaneously- may only be able to achieve low potency at targets
- Identifying multitarget inhibitors

As mentioned above, drug combinations are already the mainstay for antimalarial therapeutics. However, polypharmacology could allow for the inhibition of multiple targets

within a single life stage of the parasite or have the advantage of targeting multiple life cycle stages as well. In this manner, resistance development could be reduced by intensifying the number of target inhibitions, thereby increasing the difficulty to develop full resistance without lethally disrupting vital parasitic functions.

4.1.1 Inhibiting multiple related targets

Large protein families sharing similar mechanistic biochemistry have been proposed to be good targets for polypharmacological drugs. For example, protein kinases represent promising drug targets for a variety of diseases [48-51] with several clinically used drugs for human diseases [52]. Imatinib, as example of a multikinase inhibitor, [53] has revolutionized treatment of chronic myelogenous leukemia due to its low toxicity, high level of activity, continuing durability and multitargeting ability of Abl tyrosine kinases [54]. Protein kinases are essential to malaria parasite growth, maturation and differentiation [55] and inhibitors of single kinases (e.g. PI4K[56]) is leading the antimalarial discovery profile, but if viewed from a polypharmacological perspective, could hold a lot more promise in resisting resistance as well [55].

Beyond the kinases as multitarget example, proteases, ribosomal proteins, transporters / channels, structural proteins (e.g. tubulins) and protein families involved in epigenetic mechanisms have been identified as having multitarget potential [57]. Members of these protein families are currently considered as recently validated or revived antimalarial targets within the MMV pipeline including falcipain-cystein proteases 2-3 and aspartic protease plasmepsins I, II, IV [10].

4.1.2 Compounds with pleiotropic actions

Multitarget TB drugs e.g. SQ109 have been shown to have the ability to target multiple biochemical activities including the MmpL3 transporter and enzymes involved in menaquinone biosynthesis and electron transport and thereby potently kill the bacillus. Additionally, this pleiotropic drug had very low rates of spontaneous drug resistance development, making it an ideal tool for resisting resistance [58].

Polyamines have also been described as a class of pleiotropic bioactive molecules due to their essential nature in well-regulated cell growth / development in most organisms by targeting a variety of cellular effector sites through their highly specific and spatially oriented cationic nature [59,60]. The polyamine scaffold has been described as a universal template / pharmaceutical skeleton key for pleiotropic drugs [61], with numerous studies validating polyamine-based agents [62] as selective antiproliferative [63], antiparasitic agents [64], antiprion chemotherapeutics [65] and neuro-protectants [66]. Given the complex nature of a multifactorial disease, an effective multitarget polyamine analogue is designed by inserting appropriate pharmacophores on the nitrogen atoms or on the linker connecting these atoms in the polyamine scaffold [61]. Polyamines and their analogues have been shown to be readily taken up by malaria infected erythrocytes [67] and analogues with (bis)urea and (bis)thiourea substituents are potently and selectively active on the parasite (IC_{50} = 26 nM; selectivity indexes >7000-fold) [68]. Pluriplarmacology is further evident with the (bis)urea polyamine chemotype targeting parasite asexual proliferation through multiple mechanisms, and (bis)thiourea analogues uniquely blocking transmissible sexual forms of the malaria parasite [69]. Importantly, when asexual parasites are exposed to this pleiotropic scaffold, no recrudescence or viable resistant

mutants were generated (unpublished results), suggesting that these promising multitarget inhibitors may serve as "resistance-refractory" antimalarial candidates.

4.1.3 Creating multitarget scaffolds: hybrids

An alternative to using pre-existing multitarget scaffolds is the rational design of multitarget hybrid drugs, defined in this context as the covalent association of independently active drugs that result in enhanced activities. Hybrids have been used in malaria to directly target the parasite's resistance mechanisms and of particular interest has been the hybridization of quinolines [70,71] with artemisinin (and derivatives), synthetic peroxides and novel inhibitory motifs (e.g. chalcones, β-lactams, HDAC inhibitors etc.), all of which results in activity against chloroquine-resistant and -sensitive *P. falciparum* strains. Furthermore, hybrids composed of a chloroquine-like moiety and a resistance reversal-like moiety have shown to be orally active with good *in vitro* and *in vivo* antimalarial activity [72].

One striking example is of quinine dimers that resulted in not only enhanced activity of the drugs but additionally cleverly also resulted in the inhibition of the parasite's resistance mechanism to this class of compounds [73]. These hybrids were not transported from the digestive vacuole and thereby have dual activities ensuring killing of the parasite.

4.2 Unexplored alternative targets

Resistance in its simplest terms is the opposition offered by one force to another, implying that to remove resistance, the primary force that causes that resistance needs to be targeted. From an antimalarial perspective, the primary force acting on the parasite is the inhibition of essential pathogenic targets/processes but this relies on mechanisms of DNA modifications. If the inhibition of essential targets is inextricably linked to resistance

development, then perhaps we need to rethink our targeting strategy to curb resistance. The concept of circumventing the parasites' radar by inhibiting non-essential targets / processes or essential processes that mediate resistance development as a means to debilitate the parasite could therefore provide alternative strategies.

4.2.1 Targeting 'virulence potential'

The inhibition of virulence factors to resist antimicrobial drug resistance attempts to disarm the pathogen rather than halting pathogen growth, which could serve to decrease the selection pressure for the development of drug resistance [74]. *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) is a critical multigene family virulence factor expressed on the surface of infected erythrocytes [75], enabling cytoadherence of infected erythrocytes and causing severe disease. *Pf*EMP1 as virulence target could serve as a starting point to screen for inhibitors that are able to target epigenetic enzymes, such as *Pf*SETvs (variant-silencing SET), which regulates the expression of *Pf*EMP1, thereby debilitating its strategies to evade the host's immune system [76]. Alternatively, nanomimics that present specific host cell receptors on their surface bind to egressed daughter merozoites, preventing their ability to invade new erythrocytes and making them completely accessible to the immune system. This strategy might offer alternative treatment options for severe malaria or a new way to modulate the immune response [77]. Moreover, this strategy makes it nearly impossible for the parasite to evade these "pseudo" receptors on the nanomimic without compromising its regular route of entry into the erythrocyte.

4.2.2 Targeting non-essential, hijacked host factors

Intracellular pathogens exploit host factors to survive in hostile environments and one creative way to sidestep resistance is to avoid targeting the pathogens altogether and rather target the non-essential host factors that have been hijacked by the pathogen to elicit a therapeutic

response [78]. Host proteins are usually well conserved compared to the diverse targets produced by the genetic variability of many pathogens, making targeting easier. Furthermore, the evolution required by the parasite to re-direct its entire infection / virulence strategy to compensate for an absent host factor is exceedingly greater than adapting a parasite drug target [78] or induction of MDR transporter within itself. This strategy has successfully been used in treating several bacterial and viral pathogens [78]. Malaria parasites hijack human host proteins to boost their antioxidant defense repertoire [79], or allow erythrocyte invasion through human erythrocyte receptors [80]. Several human erythrocyte receptors have been associated with parasite erythrocyte invasion including sialic acid, complement receptor 1, and basigin. Basigin has demonstrated to be an essential human erythrocyte receptor required for parasite invasion [81,82].. A recombinant chimeric antibody (Ab-1) against basigin, which inhibited the *Pf*RH5-basigin, parasite-host interaction was shown to successfully block erythrocyte invasion by all parasite strains tested. Notably, Ab-1 rapidly cleared an established *P. falciparum* erythrocyte infection with no overt toxicity in an *in vivo* murine model. Collectively, the authors demonstrated that antibodies or other therapeutics targeting the host factor basigin may be a successful treatment for patients infected with multidrug-resistant *P. falciparum* [80].

4.2.3 Targeting stress responses

Several cancer studies established resistance-promoting adaptive responses through activation of pro-survival mechanisms to escape drug pressure [83]. In addition, studies investigating the mechanisms of resistance to the antibiotic, vancomycin, the first-line treatment against drug-resistant *Staphylococcus aureus*, revealed that susceptible *S. aureus* exposed to vancomycin respond by activating expression of genes involved in cell wall stress responses. Interestingly, vancomycin-resistant *S. aureus* have the same stress response genes activated, even

in the absence of vancomycin exposure. These findings suggest that this mechanism of resistance to vancomycin involves the permanent activation of a stress response in resistant bacteria [84]. A recent study revealed that malaria parasites resistant to artemisinin exhibit an enhanced cell stress response with lower levels of ubiquitinated proteins and delayed onset of cell death compared to artemisinin susceptible parasites, suggesting the involvement of a proteasome-engaging cell stress response [85]. Clinically used proteasome inhibitors strongly synergize artemisinin activity against both sensitive and resistant parasites. Continual activation of such stress responses in wild-type parasites due to drug exposure could ensure a permanent feature, even in resistant mutants. The stress response may serve as a pro-survival mechanism that 'buys time' for the sensitive parasite to develop resistance and the pro-survival mechanism may be maintained for its beneficial protective effect. Furthermore, dormancy or stress-induced quiescence is a key characteristic of bacterial persistence against a range of antimicrobials [86]. Indeed, in malaria parasites, long-term escalating artemisinin exposure extends the range of parasites able to enter quiescence and tolerate artemisinin toxicity. This new pluriresistance phenotype is highly reminiscent of multidrug tolerance of persister bacteria. Therefore, an additional avenue to increase the clinical lifespan of resistant drugs and also prevent drug resistance of wild-type malaria parasites would be to add drugs that eliminate pro-survival pathways, i.e. compensatory stress responses/ stressed induced quiescence thereby speeding up the "death event" and decreasing the available time for resistance development.

4.2.4 Targeting the molecular mechanisms causing resistant mutants

Malaria parasites are able to induce genetic level mutations, either in the form of CNVs or SNVs during the asexual replication cycle, leading to the selection of mutant forms of the parasite able to survive drug pressure and leading to resistance phenotypes. The molecular

mechanisms of mutant induction use a number of activities in concert in a canonical process to enable double stranded DNA breaks and repair. Although by no means fully understood, at least a few orthologs of the main proteins controlling homologous recombination (HR) and end-joining have been predicted [87] and these could serve as potential novel drug targets that would have the added advantage of disabling the parasite to form resistant mutants due to drug pressure. This concept has been exploited in the cancer field, with drugs targeting HR proteins (e.g. BRCA, RAD51 and poly(ADP)ribose polymerase 1) effective as mono- or combination therapies in drug-resistant cancer phenotypes [88].

5. Responding to resistance

Innovative strategies are being proposed to counter resistance by potentially using the 'therapeutical' itself as a tool that can be readily altered to keep up with the rapid pace of resistance evolution. Furthermore, new 'drugs' can be designed to inhibit targets that are linked to the drug resistance mutation thereby creating a lethal phenotype in potentially any drug-resistant strain.

5.1 Antisense oligonucleotides (ASO)

The antibacterial field further highlights the use of antisense oligonucleotides as a potential means to develop a line of highly adaptable antibiotics [38]. Antisense oligonucleotide (ASO, ~20 bp single-stranded cDNA) binds to their target mRNA, to specifically inhibit gene expression and decreased levels of the target protein [89]. Antisense antibiotics are amenable to target drug resistance-associated mutations in the target nucleic acid sequence. By utilizing next generation sequencing technology, any mutation caused at DNA or RNA level in response to ASO exposure could easily be determined within hours resulting in adapting the ASO to the new

nucleic acid sequence of the resistant target [38] and thus ensuring a quick delivery of new drugs to fight resistant pathogens. At present, two antisense drug have been approved by the U.S. Food and Drug Administration; namely, Formivirsen (Vitravene) as a treatment for cytomegalovirus (CMV) retinitis [90] and Mipomersen (Kynamro) for homozygous familial hypercholesterolemia [91]. The genome of the malaria parasite is ~80% AT bp [92] and is substantially different from the human genome; which may provide opportunities to use sequence specific inhibitors to target the parasite's genome [93]. ASOs have been used in the malaria parasite for various applications [94,95] but with some delivery issues associated with uptake of charged ASOs into the malaria parasite [93] that could possibly be overcome with the use of peptide nucleic acids (PNA), as a potentially more metabolically stable and neutral alternative [96].

5.2 Nanotechnology and molecular decoys

Nano-drug delivery has been described as a solution to diseases where drug uptake is problematic and entails inclusion of drugs inside biocompatible nano-vehicles. Beyond enhancing drug delivery, nano-technology is now also being explored as a mechanism by which drugs could be masked to escape resistance development [97]. Co-delivery nano-systems aim to target multidrug-resistant cancers through inhibition of drug resistance transporters, which enhance chemotherapeutic efficacies. These nano-systems have been described as 'molecular decoys' and have been used to deliver combination therapies, siRNAs and antisense therapies and can also be designed to target specific cellular localizations. Molecular decoys have further been proposed as a solution to overcoming resistance and in the antimicrobial field for instance, co-use of fragments of the antibiotic results in the 'decoy' being exported by the resistance pumps, giving the actual antibiotic time for action [98]. This type of strategy may be interesting in the event of drugs being effluxed away from their site of action via the action of resistance-

mediating transporters, which can include *Pf*CRT or *Pf*MDR1 but would not necessarily have an impact on resistance mechanisms based on mutations in the cellular target.

5.3 Synthetic lethality inference

Synthetic lethality refers to a gene pair in which the mutation of either component is not lethal; however, if both the genes in the pair are mutated it results in death or a substantial fitness cost for the organism, these two genes are denoted as synthetic lethal (SL)[99]. The inhibition of SL proteins has been successfully applied in the field of cancer therapeutics [100,101], and has been investigated as a means to discover new antibiotic combination partners. SL combines the advantages of multitargeting with the inability of an organism to overcome pressure on essential biochemical activities. In *Plasmodium*, synthetic lethality inference has been suggested as a new therapy to treat drug-resistant malaria [102]. If one gene of a *Plasmodium* SL gene pair has a mutation that causes antimalarial drug resistance, a drug that targets the other gene of the SL pair would create a lethal phenotype and could be used as a successful treatment for drug-resistant strains of malaria. Prospective *Plasmodium* SL gene pair candidates were identified through yeast–human–*Plasmodium* ortholog filtering, antimalarial drug resistance mutations screening and first neighbors (their SL gene partner) inferred from yeast SL genes to identify pertinent antimalarial drug targets [102]. Identifying inhibitors against these drug targets may prove to be an alternative approach to selectively target drug-resistant malaria and allows for the potential identification and targeting of the inferred SL partners of antimalarial drug resistance genes acquired due to the selective pressure of any new antimalarial drug.

5.4 Exploiting evolutionary fitness constraints

Resistance development has an associated fitness cost as escape pathways become limiting and these few remaining survival mechanisms could be identified as new druggable processes. For instance, mutations in HIV-1 reverse transcriptase confers virus resistance to nucleoside reverse transcriptase inhibitors; however, these resistant mutants are now additionally hypersensitive to other HIV therapeutics due to an inability to incorporate natural nucleotide substrates [103]. Therefore, compounds that selectively target resistant phenotypes, combined with compounds that target sensitive phenotypes should result in targeting the essential metabolic pathway as well as resistance-associated escape pathways. This strategy has recently been introduced to the field of antimalarial discovery where such combinations were shown to efficiently kill parasites in the short-term with the added advantage to assist in shaping evolution away from drug resistance development [104].

6. Defining a target and chemical space relevant to resisting / responding to resistance.

Association of druggable protein targets through genomic signature analyses to their chemical partners in systems wide chemogenomics strategies is useful in drug discovery endeavors. The association of a drug response phenotype to large functional genomics datasets (transcriptomes, proteomes) identifies genes / proteins involved in chemosensitivity or drug resistance. The latter has been employed in understanding resistance mechanisms in cancer and identifying novel drug-target combinations with the goal of overcoming resistance [105,106].

Chemogenomic target prediction of ~20,000 antimalarial hits, identified in three independent phenotypic screening campaigns using orthologous genomic relationships and

chemically compatible target combinations, led to the identification of a priority set of 64 antimalarials that target 39 high-ranking proteins; 85% of this target space falls within multitarget profiles [57]. These compounds could serve as a multitarget pool displaying polypharmacology and is worth investigation for their potential as resistance-resistant antimalarial candidates. Integrated approaches linking polypharmacology to proteochemometric profiling also promise to identify target and potency indicators, and could be extended to resistance predictors [107].

Chemogenomic profiling of drugs across a collection of resistant mutants can assist in classifying promising compounds with unknown mechanisms of action and indicate resistance development. This has recently been applied to connect drug mechanism of action of the artemisinin family to gene functions and metabolic pathways [108].

The TDR Targets database [109] specifically functions to link functional genomics datasets to query strings and has been used to identify priority drug-target combinations for *P. falciparum* parasites [110,111]. If this could be extended and associated to resistance resisting profiles, it could serve as a primary filter for druggable targets in malaria parasites.

7. Conclusion

Unprecedented efforts have been made and outstanding successes have been achieved in alleviating the malaria burden world wide. It has been estimated that in the period 2000-2015, malaria control interventions have averted ~663 million clinical cases of malaria. Of the control measures implemented, insecticide-treated nets, were by far the largest contributor to reduce clinical cases by 68%. It is estimated that current chemotherapeutics (ACTs) accounts for 22% of

clinical cases averted [3]. *P. falciparum* has extraordinary genomic plasticity and its adaptive

nature is the reason why there is only a single vaccine with moderate efficiency and parasite

resistance to every antimalarial drug in existence. Lessons learnt from past antimalarial

resistance development (Box 3), have demonstrated the importance of combination therapies

Box 3. Lessons learnt from past resistance development [31].

Lessons learned from past resistance development

- Drug misuse, wrong formulations and counterfeit drugs all contribute to sub-therapeutic dosing and selects for resistance development through genetic mutation of parasite populations
- The parasite rapidly becomes resistant to all drugs used as single entities targeting single biological activities
- Drugs with multiple targets / pleiotropic actions have the longest lifespan of clinical efficacy and these have mostly been modified from natural compounds
- Specific parasite populations seem to be genetically more plastid than others and can be defined as 'hotspots' for resistance development
- The parasite's 'permissive genome' means that resistance might be lost in time in a population where drugs were removed; only in isolated and highly controlled cases could this mean re-use of old drugs
- Early detection and monitoring of spread of resistant parasites is imperative to identify wide-spread treatment failure, necessitating removal of the drug
- Active responses by the complete malaria community to the first indicators of antimalarial failure might ensure prolonged use of that entity

since the parasite rapidly becomes resistant to all drugs used as single entities targeting single

biological activities. However, drugs with pleiotropic action / multiple targets have had the

longest clinical lifespan and are usually modified from natural compounds found in plant matter

or tree bark. Alternative approaches from the fields of cancer and antibiotic research can be

divided into strategies that either aim to resist resistance or those that respond to the presence of

resistance. Strategies that resist resistance currently include polypharmacology (the multitargeting of either related targets or pleiotropic actions) and inhibition of unexplored alternative targets (virulence potential, hijacked host factors, stress responses and the molecular mechanisms that cause resistant mutants). Potential resistant-responsive strategies include antisense oligonucleotides (amendable to the resistance-associated mutation), molecular decoys (exported by resistance pumps, allowing the therapeutic time for action), synthetic lethality inference (targeting the synthetic lethal gene partner of the resistance-associated gene) and exploiting the evolutionary fitness constraints of the parasite (drug combinations that targeting both essential metabolic and escape pathways).

8. Expert Opinion

Drug resistance may arguably constitute the greatest challenge facing malaria control. As such, the challenge for antimalarial drug discovery is to find ways to increase and protect the current and future value of chemotherapeutics to combat malaria. Several factors may contribute to the current levels of ineffectiveness of chemotherapeutics in malaria control such as access to the drugs, patient compliance and / or drug resistance. Inevitably, the misuse of drugs, wrong formulations and counterfeit drugs all lead to subtherapeutic dosing, which readily selects for resistance conferring mutations in parasite populations. The active responses by the entire malaria community to the first indicators of antimalarial failure could safeguard prolonged use of that entity. An ideally effective resistance-proof antimalarial drug would remove the circulating parasite reservoirs in humans and so end transmission of the parasite leaving only the dormant hypnozoite forms to eradicate.

With resistance rising against the current first-line treatment for malaria it becomes imperative to find more effective ways of prolonging the limited clinical lifespans of antimalarials and the discovery of new entities that may have a "built-in" anti-resistance capacity. In brief: with regards to resistance, antimalarial drug discovery needs to continue to broaden its horizons. We have been "beating back" the resisting malaria parasite with novel drugs (recently in combination therapies) for the past 49 years. Even though we have successfully decreased malaria prevalence and related mortality, there has been a rise in antimalarial drug resistance. We should not equate the lowered prevalence and mortality rates of malaria with lower severity; a less prevalent disease generally receives less attention and perhaps reduced funding. If we do not continue to move with concerted momentum malaria may progress from a less prevailing disease to an untreatable one. As a community we need to consider avant-garde anti-resistance strategies from alternative fields to assist our endeavors to move beyond beating the resistant parasite back, to stopping it dead in its tracks. Examples from numerous chemotherapeutic resistance studies in the fields of antibiotic and anticancer research may be able to offer alternative strategies applicable to antimalarial drug discovery. From the strategies discussed in this review (Section 4: Resisting resistance), we foresee a great deal of promise for compounds that are able to either overwhelm the parasite through inhibiting multiple targets (polypharmacology) within a single / multiple life stage(s) or entities that can outwit the parasite by circumventing its radar targeting alternative parasite processes or hijacked host factors. The processes of either overwhelming or outwitting the parasite should in theory serve to prolong these therapies by making it exceedingly difficult for the parasite to evolve compensatory mechanisms to survive these types of inhibition. Although these strategies have met with success

in the antimicrobial and anticancer field their true potential in *Plasmodium* has not yet fully been investigated.

From a short-term perspective, in addition to the current drug derivatization approach, we may need to consider strategies that respond to resistance development by rapidly altering drugs to accommodate the newly developed resistant drug targets, ensuring a constant supply of drugs to treat drug-resistant malaria. Molecular decoys may provide an innovative way to protect vulnerable drugs and modulate the host's immune response to clear parasites; whereas, synthetic lethality inference and exploiting evolutionary fitness may present a highly effective way to clear drug-resistant malaria.

If the current antimalarial drug discovery endeavors also embrace avant-garde anti-resistance strategies we are likely to enter a new period where the treatment of malaria may be revolutionized through a collection of innovative therapies. Their success in turn depends on an in-depth understanding of parasite biology to unravel the complexity of the modes of action of drugs, the biological response of the parasite and its evasion strategies. Fortuitously, evolving post-genomic technologies such as chemical genomics, genome editing, chemical and systems biologies combined with single cell approaches, are starting to provide insights into the *Plasmodium's* plasticity, MOAs of drugs, their targets, affected pathways and resistance mechanisms and to reveal novel targets. Effective strategies to counter or resist resistance may enable us to stem the tidal wave of drug resistance development giving us the necessary breathing-space to keep up with the latter and to lessen the malaria burden.

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