



Pyronaridine – the Current Antimalarial Standing Up to Parasite Resistance

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Pyronaridine, an aza-9-anilinoacridine schizonticide, was synthesized in China 54 years ago and since 2012 has been prescribed globally in combination with artesunate, marketed under the name Pyramax. Artesunate is an analog of artemisinin, a sesquiterpene lactone with an endoperoxide moiety, extracted from *Artemisia annua* L., and used as an herbal remedy in Chinese traditional medicine to treat jungle fever, also discovered in China at nearly the same time. Pyramax is one of the safest and most efficacious forms of artemisinin combination therapy (ACT) for treating uncomplicated *Plasmodium falciparum* malaria in adults and children. This narrative review explains the mechanism of action of pyronaridine, why it remains effective against *P. falciparum* even though the parasite has evolved resistance or tolerance to all other antimalarial drugs used in clinical practice, and suggests possible antiplasmodial deaza-pyronaridine (acridine) analogs that could be used should pyronaridine becomes ineffective.

Keywords: Antimalarial resistance, Artemisinin combination therapy, β -Hematin, Heme detoxification, Pynacrine, Pyronaridine

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Introduction

The World Health Organization (WHO) in 2023 estimated globally that there were 249 million cases of malaria compared to 233 million cases in 2019, with deaths occurring in 61% of children under 5 years of age.¹ Although there are a number of explanations for this rise in malaria morbidity and mortality, an unescapable fact is the development of parasite resistance, particularly by *Plasmodium falciparum*, to all antimalarials currently in clinical use, with the notable exception of pyronaridine.² This review discusses the structure and mode of action of pyronaridine, which may help explain the basis for the lack, to date, of parasite resistance to this drug, and also highlights the development of novel compounds based on the deaza-pyronaridine (acridine) scaffold, which could replace this antimalarial when inevitably it become ineffective.

In 1970, a group of medicinal chemists at the Institute of Chinese Parasitic Disease, Chinese Academy of Preventative Medicine, Shanghai synthesized a novel benzonaphthyridine, named pyronaridine, an aza-analog of the known antimalarial quinacrine (also known as mepacrine), which is a 9-aminoacridine, but containing a side chain modified from the antimalarial amodiaquine (Figure 1).³ In the following year, attention of the malaria drug community was focused on the discovery at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing of artemisinin (“qinghaosu”), the potent active sesquiterpene lactone containing a unique endoperoxide moiety responsible for its mode of action, which was isolated from *Artemisia annua* L. (sweet wormwood, “qinghao”), the extract of which had been advocated since the 1st century CE for treating jungle fever (malaria).⁴ Both pyronaridine and artemisinin have since been successfully used as monotherapies for malaria in China.

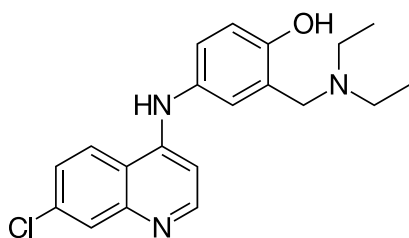
A single drug treatment for malaria is no longer advocated due (in general) to the rapid appearance of

resistance in the parasite, particularly *P. falciparum*, the most virulent of the 5 species of *Plasmodium* spp infecting humans, the other 4 being *P. malariae*, *P. ovale*, *P. vivax*, and *P. knowlesi* (a recently recognized zoonotic parasite originating from infected rhesus macaque).⁵ Since 2005 WHO has recommended all antimalarials used for treating *P. falciparum* infection to be in the form of a drug combination therapy.⁶ This usually consists of an artemisinin analog (a fast-acting drug targeting *Plasmodium* early ring stage, but with rapid blood clearance within a few hours) together with another drug with longer half-life (ie, with a more favorable pharmacokinetics profile). The dual drug administration is named artemisinin combination therapy (ACT).⁶ A pyronaridine-artesunate combination was approved on February 16, 2012, by the EU Committee for Medicinal Products for Human Use outside the EU and is marketed as Pyramax, a film-coated tablet formulation (180 mg of pyronaridine tetrphosphate and 60 mg of artesunate) for adults and children over 20 kg, and as a granule formulation (60 mg of pyronaridine tetrphosphate and 20 mg of artesunate) for children and infants between 5 and 20 kg, taken once-a-day for 3 consecutive days.^{7,8} Pyronaridine has a high blood-to-plasma distribution ratio due to its tendency to accumulate in blood cells (facilitating killing of intraerythrocytic parasites), a rapid oral absorption (a peak concentration within approximately 2 hours after oral administration), large volume of distribution and low total body clearance, resulting in a long terminal apparent half-life (13 - 15 days).^{7,8}

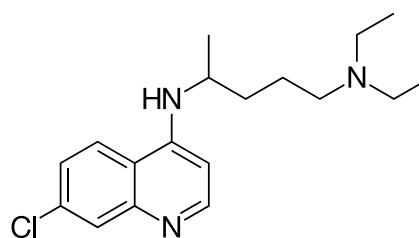
Status of Pyramax in Treating Uncomplicated *Plasmodium falciparum* Malaria

A recent meta-analysis of Pyramax in treating uncomplicated *P. falciparum* malaria, involving 10 randomized controlled trials, comprising 5711 participants (4465 from 13 sites in Africa and 1246 participants from

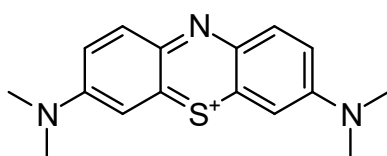
Figure 1. Antimalarial Analogs of Quinoline (Amodiaquine, Chloroquine) and Acridine (Methylene Blue, Pynacrine, Pyronaridine, Quinacrine)



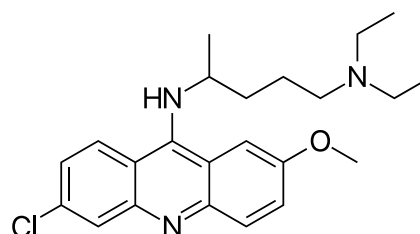
Amodiaquine



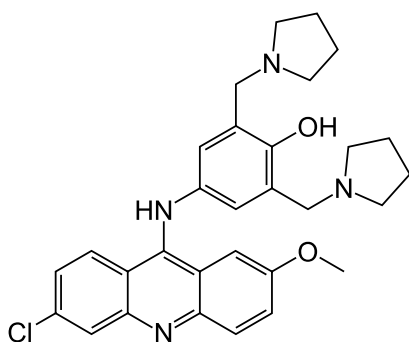
Chloroquine



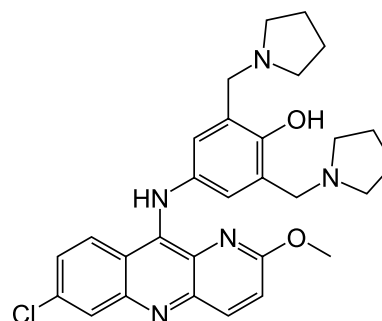
Methylene blue



Quinacrine



Pynacrine



Pyronaridine

5 sites in Asia, and including 541 children aged less than 5 years), concluded that pyronaridine-artesunate was efficacious against uncomplicated *P. falciparum* malaria, achieving a polymerase chain reaction (PCR)-adjusted treatment failure rate of less than 5% at days 28 and 42, and was at least as good as, or even superior than, other marketed ACTs.⁹ Pyronaridine-artesunate increased the risk of mild liver injury (moderate- to high-certainty evidence), but there was no evidence of any liver injury resulting from Pyramax treatment that was severe or

irreversible. Trials involving 904 children age 12 years and under showed that pyronaridine-artesunate treatment did not differ in terms of efficacy or safety from artemether-lumefantrine.^{9, 10}

Pyramax was also applicable for the treatment of blood-stage *P. vivax* malaria, but was unable to kill the latent liver hypnozoite stage.¹¹ A desired property of any antimalarial is that it should act at both asexual and sexual stages (ie, it should cure the patient and also prevent transmission). Although it was previously reported that

pyronaridine demonstrated *P. falciparum* gametocidal activity in vitro,¹² this effect was not observed with mature gametocytes in *P. falciparum*-infected patients.⁷ The in vitro experiments were conducted on young stage II and III gametocytes, which reside within the bone marrow and so may not be accessible to pyronaridine administered to the infected subjects.¹³

Given that pyronaridine does not kill *Plasmodium* liver stages, it is unlikely to be recommended as a malaria prophylaxis, but it could be a suitable candidate to enhance efficacy of current malaria vaccines. In 2021, WHO recommended use of the pre-erythrocytic malaria vaccine RTS,S/AS01 (Mosquirix) for the prevention of *P. falciparum* malaria in children 5 to 17 months of age living in regions with moderate-high malaria transmission.¹⁴ The RTS,S vaccine consists of a portion of the *P. falciparum* circumsporozoite protein (CSP) gene, which includes part of the central repeat domain and the C-terminal flanking region fused to a hepatitis B virus surface gene to generate a virus-like particle, and is delivered together with an AS01 adjuvant. However, the vaccine efficacy against clinical malaria decreased from 74% at initiation of vaccination to 28% after 12 months. More recently (2023), WHO has also recommended R21/Matrix-M™ vaccine, which is similar to RTS,S but lacks the hepatitis B virus surface gene, therefore providing a higher density of CSP epitopes on the particle surface, which produces higher levels of anti-CSP antibodies, and is formulated with Matrix-M™ adjuvant.¹⁵ In children 5 to 36 months of age, R21/Matrix-M™ vaccine had an efficacy of 75% at the primary vaccination, which waned only to 68% after 1 year. Interestingly, it was recently reported that a combination of the RTS,S/AS01 vaccine with malaria chemotherapy enhanced the vaccine efficacy.¹⁶ As *falciparum* parasites have the ability to rapidly develop resistance to (almost all) antimalarials used, pyronaridine would be the drug of choice should this strategy become widely adopted.

Pyronaridine Mode of Action

Pyronaridine mode of action (MOA) is not obvious from a cursory inspection of its structure (Figure 1). Morphological examination using electron microscopy of pyronaridine-treated *P. falciparum* parasites shows a marked enlargement of the food vacuole and the formation of intravacuolar vesicles and membranous whorls suggesting the site of pyronaridine MOA lies at the membrane or within the digestive vacuole.¹⁷ Reexamining pyronaridine structure indicates the presence of the chloroquine signature 7-Cl-4-NH-quinoline scaffold, which is also present in the 7-Cl-4-NH-quinoline congeners amodiaquine and quinacrine (Figure 1). Hence, using standard in vitro assays of chloroquine MOA it was possible to demonstrate that pyronaridine mirrored all the chloroquine properties tested, namely, inhibition of β -hematin formation (at a concentration equal to that of chloroquine), production of a hematin complex with a drug:hematin stoichiometry of 1:2, enhancement of hematin-induced red blood cell lysis (but at 1/100 of the chloroquine concentration), and inhibition of glutathione-dependent degradation of hematin.¹⁸ Of note, to date, there is no direct evidence that pyronaridine MOA in cellulo is located within the parasite digestive vacuole. In a recent review, Bailly¹⁹ noted that as pyronaridine was able to intercalate DNA, it could also act as a topoisomerase II (topo II) inhibitor. Using a KCl-SDS precipitation assay to detect the presence of protein-DNA complexes within parasite cells, an indicator of topo II inactivation, Auparakkitanon et al²⁰ reported that pyronaridine did not generate such protein-DNA complexes compared to the well-known topo II inhibitor amsacrine, an antitumor drug.²¹

The main source of amino acids required by malaria parasite metabolic processes comes from the digestion of host hemoglobin imported into the parasite digestive vacuole.²² This process results in the release into the parasite vacuole compartment of large quantities of



toxic Fe[II] heme (that is rapidly oxidized to Fe[III] hematin), which the parasite detoxifies by forming hemozoin, an inert crystalline material composed of hematin dimers (and other minor components).²³ There has been much debate whether chloroquine MOA was by forming a soluble chloroquine-hematin complex that was unable to form hemozoin or that the complex binds to the hemozoin crystal itself thereby inhibiting its further growth.²⁴ Using time-resolved in situ microscopic surface observation of β -hematin crystal, a synthetic form of hemozoin, Olafson et al²⁵ showed that chloroquine- and pyronaridine-hematin complexes bind to the growing faces of the β -hematin crystal, competitively inhibiting attachment of hematin dimers and hence crystal growth. Given the differences in the structure between chloroquine and pyronaridine, the chloroquine-hematin complex was shown preferentially to bind β -hematin crystal “axial” $\{011/0\bar{1}\bar{1}\}$ growing faces, thereby inhibiting further extension and producing short and wide crystals. On the other hand, pyronaridine-hematin complex binds to both “upper/lower” $\{100/\bar{1}00\}$ and “side” $\{010/0\bar{1}0\}$ growing β -hematin crystal faces generating crystals tapering at both axial termini.²⁵ It is worth noting that the formation of drug-hematin complexes does not necessarily predict their MOAs in the heme detoxification process. For example, amodiaquine forms a hematin complex that does not strongly interact with the β -hematin crystal surface yet exhibits suppression of crystal growth without any morphology aberrations, which is brought about by decreasing the pool of hematin dimers required for crystal proliferation.²⁵

The MOA of artemisinin and its analogs (collectively termed ART) also lies within the parasite food vacuole where they interfere with the heme detoxification process. ART are pro-drugs, requiring activation through cleavage of the endoperoxide bond via a Fe[II]-mediated Fenton reaction. ART are susceptible to reaction with Fe[II] heme in vitro and in cellulo (where almost all

Fe[II] hemes are located within the parasite food vacuole), a process that first involves the generation of an O-centered (O[•]) ART radical followed by rapid rearrangement into a C-centered (C[•]) form that alkylates Fe[III] hematin.²⁶ Time-resolved in situ atomic force microscopy revealed that the hematin-ART adducts produce large amounts of nucleation of nonextendable nanocrystals, which are incorporated into the larger growing crystals, thereby preventing further generation of β -hematin crystals.²⁷ Thus, some 40 years after their advent and separate applications to treat malaria in China, the 2 drugs were united in a combined therapy (Pyramax) to combat this disease outside the country. Interestingly, they have similar MOAs (ie, act in the parasite food vacuole to derail heme detoxification), which is a vital and unique process present (to the best of the author’s knowledge) only in malaria parasites.

As there was no previous report from China of malaria parasite resistance to artemisinin, although used as a monotherapy (7-day drug regimen), thus it came as a shock to the malaria control community that *P. falciparum* in the Greater Mekong Subregion had evolved tolerance (also variously termed resistance, partial resistance, and reduced sensitivity) to ART, manifested in a patient by a longer parasite clearance time on the first day of a 3-day ACT regimen.²⁸ Of note, resistance to ACT requires ART tolerance accompanied by co-resistance of the partner drug. The confusion regarding the nomenclature of ART “tolerance/partial resistance/reduced sensitivity” stems from the observation that the affected *P. falciparum* parasites did not show the standard 10 folds or higher EC₅₀ (concentration to inhibit in vitro growth by 50%) values but displayed a dormancy (or delay) in ring-stage development, from which these early-stage parasites recover after a few hours and resume normal, but accelerated development to attain the 48-hour intraerythrocytic growth cycle (at least under standard culture conditions).²⁹



In vivo, resumption of normal development appears to be a stochastic process, the mechanism of which remains unknown.³⁰

As regards pyronaridine, there has been, to date, no report of overt resistance by *P. falciparum* worldwide. One of the first clinical trials of pyronaridine outside China was conducted in 1996 in Thailand, which showed that pyronaridine alone at a total dose of 1800 mg prescribed over 5 days was well-tolerated in 101 patients suffering from acute uncomplicated malaria and had activity against multidrug-resistant falciparum malaria. The patients were admitted to a university hospital in a nonendemic region of the country (Bangkok) for 28 days to avoid the possibility of reinfection.³¹ However, the authors did not recommend use of the drug alone in Thailand due to the high (12%) recrudescence rate. Nevertheless, there has been no increase in EC_{50} and EC_{95} values in recrudescing parasites.³¹ A more recent study of 118 parasite isolates collected from endemic areas along the Thai-Cambodian and the Thai-Myanmar border regions from 1989 to 2014 found no significant changes to EC_{50} values from a previous study of isolates collected from eastern and northern Thailand 30 years prior.^{32, 33} However, of 296 isolates collected between April 2008 and August 2012 from 21 malaria-endemic countries in Africa, 16 isolates (5%) showed ex vivo reduced susceptibility to pyronaridine, which was associated with a K76T mutation in the *pfert* gene (discussed below) ($P = .004$). However, the odds ratio for reduced susceptibility to pyronaridine associated with the K76T mutation was 4.47 (95% confidence interval [CI], 1.39 - 18.84) compared to that for chloroquine of 96.4 (95% CI, 41.8 - 244.8) for the same set of parasite samples.³⁴

P. falciparum isolates with chloroquine resistance were first described by Krogstad et al³⁵ to be due to enhanced efflux and not reduced uptake of chloroquine, resulting in a lower level of the drug in the food vacuole of resistant parasites. The transporter responsible for

chloroquine export is a food vacuole mutant membrane transporter, named *P. falciparum* chloroquine resistance transporter (*PfCRT*), the normal function of which is to transfer short peptides from the food vacuole to the parasite cytosol for further enzymatic degradation into free amino acids.³⁶ Interestingly, the only homolog of *PfCRT* is a plant transporter, CRT-like transporter (CLT), located in the plastid membrane for the export of glutathione (a tripeptide) from plastid to the cytosol.³⁷ *PfCRT* needs to acquire a set of nonsynonymous single nucleotide variants to become a chloroquine transporter. A ubiquitous amino acid substitution in the chloroquine-resistant alleles, regardless of origin, is the K76T mutation accompanied by additional region-specific multiple mutations. For example, the Southeast Asian *P. falciparum* Dd2 CRT isoform harbors 8 mutations compared to the chloroquine-sensitive strain.³⁶ It is expected that it will take some period of time before the *pfert* gene acquires the requisite set of mutations to achieve a comparable ability to ferry pyronaridine out of the food vacuole.

Using a mouse malaria model where stable pyronaridine-resistant *P. berghei* ANKA lines were generated by in vivo drug pressure, no point mutation was discovered in the *pbmdr1* gene, which codes for multidrug resistance transporter 1 (MDR-1), but the expression level of the gene increases concomitantly with rise in resistance, suggesting that elevated transcription levels may be linked to pyronaridine resistance in this murine parasite model.³⁸ MDR-1 is located in the parasite food vacuole membrane and imports various antimalarials, such as amodiaquine, chloroquine, mefloquine (a quinoline analog), and piperazine (a bis-chloroquine derivative) into the vacuole, and resistance to these drugs have been linked (in part) to point mutations of the *pfmdr1* gene.³⁶ However, it is counter-intuitive to directly link elevated expression of *pbmdr1* gene with pyronaridine resistance, as this transporter, if responsible for the transport of the drug



into the food vacuole, should cause an increase in pyronaridine sensitivity. Thus, either *PbMDR-1* is a pyronaridine transporter and the abovementioned resistance phenomenon is due to an accompanying epistasis with another locus, or that *PbMDR-1* is not related to pyronaridine import into the food vacuole and a different explanation must be sought. Nonetheless, to extend the useful lifespan of Pyramax, it would be perspicacious not to introduce this ACT into regions where parasite resistance to quinoline antimalarials currently exists or did in the past.

Pyronaridine in Triple Artemisinin Combination Therapy

Given the emergence of ART tolerance/partial resistance in *P. falciparum* and the spread of its signature genetic markers across Asia and even into some parts of eastern sub-Saharan Africa, suggestions have been raised to include another drug to existing and/or future ACTs, termed triple (T)ACT, to prolong the useful lifespan of the ACTs.³⁹ The rationale of having two partner drugs to ART in TACT is that if the parasite develops resistance to one partner drug, the other should still be efficacious, because it is presumed extremely unlikely that a parasite would evolve resistance to both partner drugs over a similar period, particularly if the 2 drugs are chosen to have different MOAs. The main problem in finding 2 antimalarials for inclusion in a TACT is the avoidance of any undesirable side effects (eg, antagonism between the 2 partner drugs or epistatic effects that may reduce TACT potency).⁴⁰ Given the recalcitrance of pyronaridine to parasite resistance, an obvious TACT to develop is by including another antimalarial to Pyramax. However, cross-resistance of pyronaridine to amodiaquine was reported in *P. falciparum* isolates (n = 161) from Senegal, Africa. However among *P. falciparum* isolates (n = 183) from Cameroon, Africa, were observed a synergy

between pyronaridine and primaquine, additive effects with 4-aminoquinolines (chloroquine and monodesethylamodiaquine, the active metabolite of amodiaquine) and weak antagonism with antifolates (pyrimethamine and cycloguanil), halofantrine (a phenanthrene analog) or mefloquine (a quinoline analog).^{41, 42} Using a multidrug-resistant *P. falciparum* K1 line (from Thailand), a weak antagonism was observed between pyronaridine and concanamycin A, a potent antiplasmodial macrolide ($EC_{50} = 0.2$ nM) that inhibits a V-type H^+ -ATPase located in the parasite digestive vacuole membrane.⁴³ Tu (discoverer of artemisinin) and her colleagues have advocated the continued usage of ACTs, but with vigilant monitoring for early appearance of treatment failure and/or of drug resistance genetic marker(s) (if known).⁴⁴

Novel Drug Development Based on Deaza-Pyronaridine (Acridine) Scaffold

Should the inevitable evolution of malaria parasite resistance to pyronaridine occur, an acridine analog of pyronaridine, named pynacrine (Figure 1), has been synthesized, which is as potent as pyronaridine in inhibiting *P. falciparum* growth in culture as well as having all other in vitro properties of hematin targeting, but a 50-fold less effectiveness in inhibiting β -hematin formation, suggesting that pynacrine has other off-target(s) effects.⁴⁵ Previously, a series of 9-aniline analogs of amsacrine was shown to inhibit *P. falciparum* growth in culture by targeting hematin in vitro (using the same set of criteria described above) and interestingly, also acting as parasite DNA topo II inhibitor in vitro and in situ.⁴⁶

However, the crisis brought on by malaria parasites' rapid evolution of resistance (or partial resistance) to all other clinical antimalarials in current application, has hastened efforts to revitalize former drugs, such as quinacrine and methylene blue, a thia-analog of acridine (Figure 1). Quinacrine, the first synthetic



antimalarial drug with potent blood schizonticidal activity, was used extensively during the Second World War by allied forces in North Africa and the Far East, but it has serious side effects, thereby discouraging further development of antimalarials with an acridine scaffold, and quinacrine was rapidly replaced when synthetic chloroquine became available.⁴⁷ However, promising derivatives of quinacrine have recently been synthesized, with compounds having 9-NH linked to aromatic cores via urea linker, to quinolizidinylalkyl moieties or to furyl and alkyl groups showing low $\mu\text{M EC}_{50}$ values.⁴⁸

Methylene blue was the first synthetic antimalarial to be used when Ehrlich and Guttman, in 1891, administered the compound to treat 2 malaria patients in a Berlin hospital, and since has been prescribed for malaria treatment globally until the early 20th century, when it was replaced by newer synthetic antimalarials.⁴⁹ In a systematic review of 1504 malaria patients (2/3 children) in Africa, the American continent, Asia, and Europe, Lu et al⁵⁰ reported that methylene blue was consistently highly effective in all endemic areas and demonstrated a strong effect on *P. falciparum* gametocyte reduction. In glucose-6-phosphate dehydrogenase (G6PD)-deficient African patients, methylene blue caused a slight, but not clinically significant drop in hemoglobin level. Oral administration resulted in a maximal plasma concentration within 2 hours, with a plasma half-life of around 20 hours. Thus, methylene blue could be included in TACT to provide gametocidal activity. A recent study using dynamic molecular modeling showed methylene blue to be a strong competitive inhibitor of *P. falciparum* glutathione reductase, which catalyzes the reduction of this compound to produce parasitotoxic reactive oxygen species, mainly H_2O_2 .⁵¹ In addition, methylene blue has high efficacy against the *P. vivax* asexual stages and inhibits transformation of vivax zygotes into ookinetes.⁵²

Other approaches, such as developing acridine-based compounds with dual MOAs against *Plasmodium* either by the same molecule (eg, interference of heme detoxification in the food vacuole as well as being a DNA topo II inhibitor in organelles with DNA replication capability as exemplified by the 3,6-diamino-9-anilinoacridines),⁴⁶ or as hybrid molecules (eg, acridine-quinoline combinations),⁵³ should assist in guiding the future development of acridine-based molecules for the treatment of malaria.

Conclusions

It would be naïve to expect the current lack of malaria parasite resistance to pyronaridine will persist until malaria is eliminated. This review has described recent efforts to develop modifications to quinacrine as possible replacements for pyronaridine, including the work on pynacrine, its acridine analog. The importance of substituents with OCH_3 at position 2 and Cl at position 6 of the acridine scaffold for efficacious antimalarial activity has become clear. Another alternative antimalarial suggested is methylene blue, a thia-acridine that also possesses gametocidal activity. Future developments of 2- OCH_3 -6-Cl thia-acridines and other forms of aza-acridines should not be neglected, otherwise the lessons learned from pyronaridine would have gone to waste.

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Conflict of Interest

The author declares no conflict of interest.



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