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Another drug bites the dust: A review on the mechanisms of antimalarial drug resistance and current preventative intervention

Abstract

Malaria is a disease transmitted to humans through female mosquito bites. The disease is caused by a parasite that advances through a complex life cycle composed of unique stages depending on its changing host environments. Millions of people, primarily inhabitants of Africa and southeast Asia, succumb to the disease every year. In the past few decades, there has been a rise in resistance to current antimalarial therapies, sounding the alarm for intervention. Chloroquine, sulfadoxine-pyrimethamine, and artemisinin-based combination therapies are among the most potent pharmacological tools against the parasite, but mutations in *P. falciparum* have begun to disarm these drugs. This review summarizes mechanisms of action for various antimalarial drugs and carefully examines the genetic mutations in *Plasmodium falciparum* conferring drug resistance.

Introduction

Malaria has taken victims for thousands of years. The disease was likely reported as far back as 2700 BC in febrile individuals living in marshy areas (2). Later, ancient Romans noted this observation and coined the disease *mal'aria* meaning "bad air" (3). Modern understanding of malaria began in the 19th century when a French army doctor, Charles Louis Alphonse Laveran, analyzed the blood of an ill soldier and found crescent-shaped, transparent structures containing a small area of pigment (3). Future research would identify the pigment as hemozoin, a byproduct of hemoglobin digestion by the malaria-causing parasite. Laveran analyzed blood specimens in several malaria patients and ultimately identified the single-celled *Plasmodium* parasite that caused malaria (3).

Surgeon-Major Dr. Ronald Ross spent several years studying various mosquitoes, all of which were incapable of hosting malaria. It wasn't until his discovery on August 20th, 1897, when he examined an *Anopheles* mosquito that had fed on a patient infected with malaria, eventually giving rise to World Mosquito Day. He noticed a circular structure in its stomach wall. After dissection, it was confirmed that the circular body contained malarial pigment. Ross, confident that he had found malaria parasites growing inside of the mosquito, designed subsequent experiments using the parasite known to cause malaria in sparrows and crows, *Plasmodium relictum*. He found that the salivary glands

of mosquitoes that had fed on malaria-infected birds contained sporozoites, the infective form of the malaria-causing parasite. Ross was later awarded the Nobel Prize for his discovery of the mosquito stages of malaria (3).

It was known that plasmodium infected red blood cells (RBC), but little information was known about the breeding grounds for plasmodium in the human host. Researchers aimed to isolate specific organs or organ systems that provided such an environment. Studies conducted on rhesus monkeys by collaborators at the Ross Institute of the London School of Hygiene and Tropical Medicine found that the primate-specific malaria was localized to the liver. Interestingly, livers of human volunteers who were knowingly infected with *Plasmodium falciparum*, displayed similar parasite staging, thus the eventual discovery of parasite replication in human tissue (3).

The parasite responsible for malaria in humans can be of several species. The most prevalent species are *Plasmodium Ovale*, *Plasmodium Vivax*, and *Plasmodium Falciparum*. As *Plasmodium Falciparum* (*P. falciparum*) is the most common and severe, this review will primarily focus on drug resistance mechanisms in this species.

Malaria Parasite Life Cycle

Malaria is transmitted from human to human through a mosquito vector. The life cycle of the malaria parasite can be broken down into 3 stages; liver stage, blood stage, and mosquito stages. The liver and blood stage occur in the human host, and the mosquito stage involves maturation in the gut of the mosquito. An infected female mosquito takes a blood meal and simultaneously introduces sporozoites into the host bloodstream. The sporozoites travel through the blood and eventually invade liver hepatocytes where they differentiate into schizonts. Each schizont asexually replicates and after about 7-14 days, the schizont ruptures releasing thousands of merozoites back into the bloodstream, initiating the blood stage (5). Merozoites then hijack host erythrocytes where they progress from ring stage to trophozoite to schizont. Mature schizonts release new merozoites into the blood after the erythrocytes lyse. These new merozoites are able to infect new RBCs and continue the cycle. By signals not clearly identified, some ring stage parasites do not become new merozoites but rather they differentiate into gametocytes. Gametocytes only enter human blood circulation after they have matured. Immature gametocytes are sequestered in the bone marrow, where they develop for 6-8 days before release into the bloodstream (16). When subsequent mosquitos take a blood meal from an infected host, it is the mature gametocyte that gets taken up and undergoes sexual reproduction inside the mosquito. Further development occurs in the gut wall and the gametocyte eventually takes refuge in the salivary glands of the mosquito(5). Upon the next bite, sporozoites are again injected into a human and the cycle repeats. The identification of the malaria parasite life cycle

has given researchers specific areas to target when searching for a solution to stop malaria transmission.

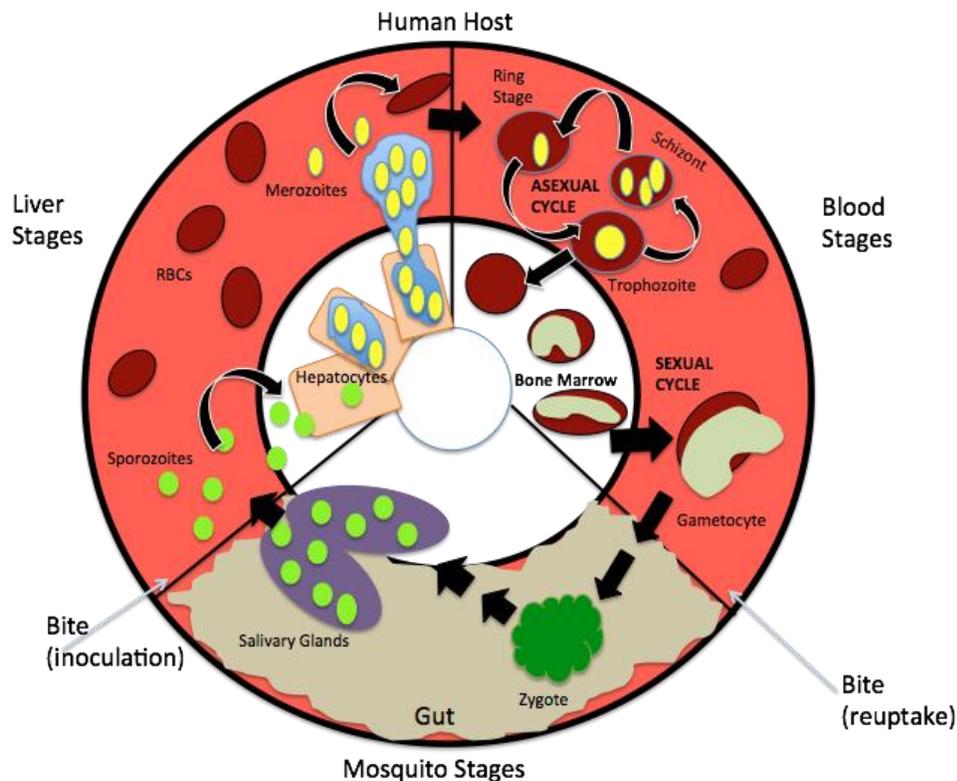


Figure 1. *P. falciparum* Life Cycle

The inoculation bite (left) is when the parasite is first introduced into the human bloodstream by the mosquito. From here, the parasite continues through the liver stage and then blood stage. Upon a second blood meal (right), a different mosquito bites the infected host and now harbors the parasite where it will develop inside the mosquito and infect another victim at its next blood meal.

Plasmodium Falciparum Mechanism of Host Cell Infiltration

Upon intraerythrocytic infection, *P. falciparum* must acquire nutrients to satisfy its cellular growth demands and cell survival. Initially, the parasite breaks down host RBCs and liberates hemoglobin (Hb). The hemoglobin is taken up into the parasite using its lysosome-like organelle, called the digestive vacuole. This digestive vacuole is distinctive because it does not contain the typical glycosidases or phosphatases seen in most digestive vacuoles, suggesting its sole function is hemoglobin degradation (9,17). Further degradation of the hemoglobin produces small peptides and eventual amino acids for use in parasite protein synthesis. However, hemoglobin is not an excellent source of amino acids, suggesting that the parasite digests hemoglobin for other benefits as well (15). This was demonstrated in a study where the parasite was placed

in culture surrounded by 20 essential amino acids and the parasite still degraded hemoglobin (17). During Hb digestion, irreversible, rapid oxidation of Fe²⁺ to Fe³⁺ forms a toxic byproduct, free ferriprotoporphyrin IX (FPIX) heme (5). FPIX heme, if left untouched, is injurious to the parasite cell membrane as malarial parasites lack the tools for processing FPIX via heme oxygenase pathway. To avert damage and promote stage growth, the parasite converts FPIX heme to nontoxic, crystalline hemozoin (Hz) (5). Hemozoin is the substance visible in cells, commonly known as malaria pigment.

Chloroquine Use in the Treatment of Malaria and Related Resistance Mechanism

Initial malaria treatment began with the use of chloroquine in 1946, after widespread clinical trials found no substantial toxic side effects. Its relatively low production and distribution costs combined with high clinical efficacy made it one of the best weapons against malaria (6). Since its inception, widespread use of chloroquine applied a forceful selection pressure to *P. falciparum* and the parasite was forced to adapt. About 13 years later, in 1959, the first reported cases of chloroquine resistance emerged from South America and again from southeast Asia one year later (6,1) From these foci, the chloroquine-resistant parasite rapidly spread to other continents in all directions. Its arrival in Africa is of special concern because this region faces financial and clinical compliance blocks that leave chloroquine as the only affordable and practical pharmacological treatment method accessible for infected individuals.

Antimalarial activity of chloroquine is made difficult by the changes in parasite life cycle, specifically when looking at intraerythrocytic vs exoerythrocytic stages. Each stage exists in a unique chemical environment and is surrounded by specialized enzymes and degradation processes. Chloroquine (CQ), a weak base, acts on the parasite in the intraerythrocytic stage. Once administered, CQ freely diffuses across the membrane of the parasite digestive vacuole. Here, the chloroquine binds up the free ferriprotoporphyrin IX (FPIX) heme, preventing its conversion to Hz (5). Additionally, the acidic vacuolar pH of about 5- 5.4 protonates CQ, preventing diffusion out of the vacuole (9, 17). The chloroquine-FPIX heme complex accumulates within the vacuole and eventually poisons the parasite with its own toxic metabolic byproducts.

P. falciparum chloroquine-resistance transporter (PfCRT) and PfMDR as a co-modulator

Unfortunately, resistance to CQ emerged not long after its widespread use. Analysis of antimalarial-resistant parasites showed decreased concentrations of chloroquine in the digestive vacuole. Several allelic studies and genetic crosses identified an association with polymorphisms in the *pfCRT* gene with lower levels of chloroquine accumulation in the vacuole. The *pfCRT* gene codes for a 10-transmembrane helices PfCRT protein consisting of 424 amino acids that inserts in the membrane of the digestive vacuole and

assists in transport across the membrane. It is known that the PfCRT variants contain anywhere from 4-10 non-synonymous mutations, making this protein extremely complex (11, 13). Although many polymorphisms have been identified, replacement of K76 by 76T seems to be one mutation that is routinely present in chloroquine-resistant parasites (1). It is suggested that the loss of the positive lysine residue accounts for the acquisition of the ability of mutant PfCRT to export protonated chloroquine from the vacuole. Junge et al. conducted a proteoliposome study in which it was discovered that CQ-resistant *P. falciparum* showed higher CQ export activity, but CQ-sensitive accumulated higher concentrations of CQ. This concluded that CQ-resistant variants bypass toxic effects of CQ by increased transport activity out of the digestive vacuole (11). Subsequent research demonstrated that although chloroquine transport was lost when removing K76T mutation, wild type pfCRT introduced to K76T mutation was not enough to permit chloroquine export from the cell (9). Other factors such as dosing, pre-existing immunity, and whether or not the infection was caused by chloroquine-tolerant or chloroquine-resistant parasites also play a role in treatment success. A study found that individuals without the Thr76 mutations were highly likely to find success in chloroquine treatment, while individuals with the mutation indicated a 33% treatment failure rate (7). Current literature identifies mutant PfCRT as a marker for chloroquine treatment failure, but recognizes there must be additional players modulating resistance.

The *P. falciparum* multidrug resistance gene (pfmdr) has been heavily studied as a co-modulator for chloroquine-resistance. This gene codes for a p-glycoprotein homolog that is a transmembrane protein in mammals, which is also involved in membrane transport. Studies suggest that the N86Y mutation encourages chloroquine-resistance but several others such as Y184F, S1034C, and D1246Y also exist and influence derivatives similar to CQ (1). Increases in gene copy numbers are associated with decreased clinical efficacy after treatment with other antimalarial drugs as well. Mutations in the pfmdr gene have also been associated with cancer resistant drugs in mammals (1).

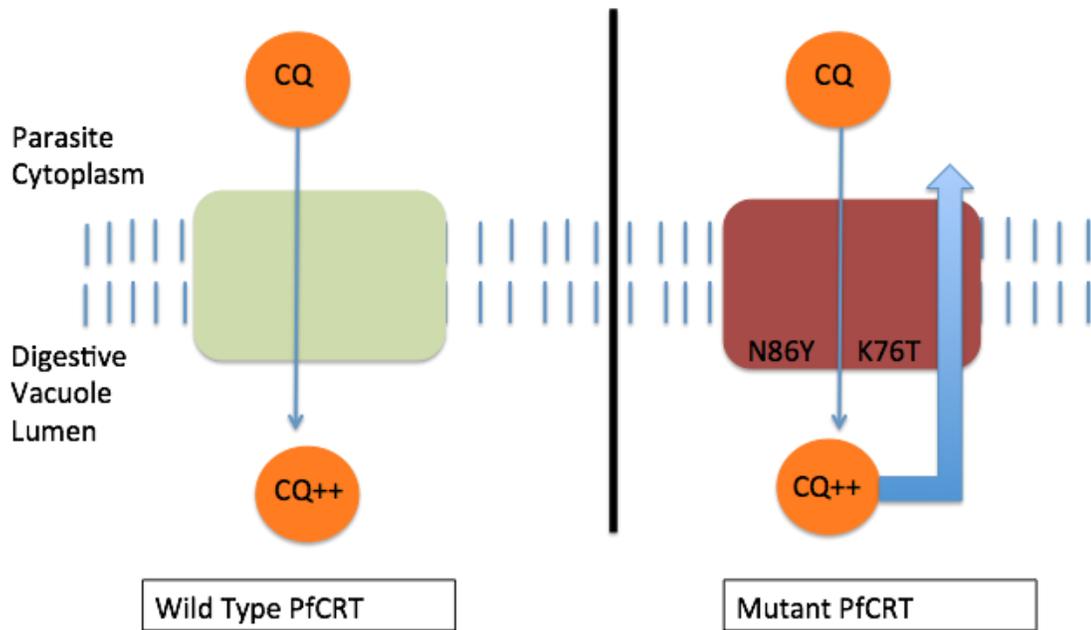


Figure 2. Digestive Vacuole Membrane

(Left) PfCRT transmembrane protein embedded in the digestive vacuole membrane of *P. falciparum* facilitating entry of chloroquine (CQ) into the vacuole. (Right) Mutated PfCRT with mutated *pfmdr* gene promoting efflux of protonated chloroquine from the vacuole.

P. falciparum dihydrofolate reductase-thymidylate synthase (PfDHFR-tr) and P. falciparum dihydropteroate synthetase (PfDHPS) as Second Line Defense

Resistance to CQ treatment soon emerged and the second line of defense was developed: a coformulation therapy, sulfadoxine-pyrimethamine (SP). The two drugs act synergistically on the folate synthesis pathway in the parasite (1). An important gene, *pfdhps*, codes for the PfDHPS enzyme that catalyzes the parasitic reaction synthesizing dihydrofolate, a precursor required to make pyrimidines (11). Pyrimidines are a class of bases used to synthesize RNA and DNA. PfDHPS has 5 known mutations (S436A/F, A437G, L540E, A581G, and A613T/S) and inhibits the action of the sulfa drugs, rendering sulfa drugs useless in the SP coformulation therapy (11). Sulfa drugs normally inhibit folate synthesis.

A co-modulating gene in SP resistance is *pfdhfr-ts*, which codes for the PfDHFR enzyme. The two primary responsibilities of this enzyme are to reduce dihydrofolate into tetrahydrofolate as well as synthesize dTMP via thymidylate synthase. (11) Eventually, these enzymes also assist in pyrimidine production in the parasite. The pyrimethamine component of SP is an antifolate that inhibits dihydrofolate reductase, therefore,

preventing pyrimidine synthesis in the parasite. Several point mutations in PfDHFR at N511, C59N, and I164L confer pyrimethamine resistance by reducing the binding affinity of the enzyme for its substrate (7, 11). Combinations of these mutations result in varying levels of resistance.

Artemisinin Combined Therapy (ACT)

The rapid spread in SP-resistant *P. falciparum* necessitated the development and implementation of yet another weapon in the fight against malaria. As resistance to CQ monotherapy and SP emerged, artemisinin-based combination therapies (ACTs) have become the gold standard treatment for malaria. Artemisinin is derived from the dried leaves of the sweet woodworm herb. The active ingredient, qinghaosu, was isolated in the 1970s and has been an effective agent against many organisms in the genus *Plasmodium* (1, 12). This combination therapy consists of 1 rapid-acting parent drug paired with 1 longer-acting partner drug of a different class. Parent drugs tend to be derivatives of artemisinin, including dihydroartemisinin, artesunate and artemether, while partner drugs are often derivatives of several drugs. Lumefantrine, mefloquine, and amodiaquine are among the most common partner drugs used in ACTs (1, 5). The parent drug acts on the parasite in the gametocyte stage to reduce parasitemia quickly within the first 1-4 days. Activation of the slower acting partner drug follows and is responsible for clearing the remaining parasites (5, 11). It is also recognized that more data is needed to determine plasma conversion rates of each drug in the combination therapies (13).

Artemisinins act on *P. falciparum* in a variety of ways. One of the most notable is by inhibiting the phosphatidylinositol-3-kinase (PfPI3K), which is an enzyme that assists in endocytosis and transportation of host hemoglobin within the cytoplasm of the parasitic organism (14). PfPI3K inhibition results in loss of hemoglobin transport to the parasitic digestive vacuole for degradation, therefore, preventing free amino acid accumulation for further parasitic growth.

Emerging Resistance to ACTs

The first region of the world to receive ACTs, southeast Asia, was one that experienced low levels of malaria transmission and exposure to new infection after completion of therapy was unlikely. Infections in these areas were very susceptible to ACTs and often cleared completely (1). Contrastingly, ACTs administered in high transmission areas, including sub-Saharan Africa, saw a dramatic decrease in efficacy. These results can be attributed to the fact that people in this region are often exposed to new infections during the window of time when the amount of partner drug in the body is decreasing (1, 13). It is in this critical time period where ACT resistance has begun to emerge. A 2006 study carried out in Africa indicated that exposure to new infections during this time

period resulted in selection of parasites that were resistant to the long-acting partner drug as there was selection pressure for remaining parasites able to evade the partner drug (1). Administration of sub therapeutic drug levels reports inaccurate drug efficacy and allows resistance to occur at a rapid rate.

Artemisinin resistance can be classified as a delayed clearance phenotype (DCP), meaning the time taken for the elimination of malaria parasites is prolonged. Studies have identified that the resistance mechanisms developed in the parasite are specific to the ring stage of the malaria parasite life cycle (13). Artemisinin is activated by the binding of FPIX heme, a byproduct of Hb metabolism to its endoperoxide moiety. As low levels of FPIX heme exist in the environment prior to hemoglobin digestion, artemisinin resistance is incurred early in parasitic development, in the ring stage (13, 15). As metabolism continues, the levels of FPIX heme increases and ultimately activates artemisinin for therapeutic use.

Currently, the exact mechanism of DCP is unknown, but a genetic marker in *P. falciparum* for artemisinin resistance has been identified; a mutation with C580Y in PfKelch13 (1, 5, 13). PfKelch13 is a protein adaptor to E3 ubiquitin ligase. Kelch family proteins have been found in other species and have been associated with organization and protein interaction. Additional studies must be completed to fully understand kelch13 functions (11). Artemisinin-resistant strains of *P. falciparum* show the C580Y mutation paired with elevated levels of phosphatidylinositol-3 kinase (PfPI3K). This enzyme produces phosphatidylinositol-3-phosphate (PI3P), which is a lipid involved in cellular exocytosis. Upon polyubiquitination of PfPI3K and subsequent binding to PfKelch13 protein, proteolysis of PfPI3K would normally occur. However, with a PfKelch13 mutation, polyubiquitination of PfPI3K and binding to PfKelch13 occurred less frequently leading to an increase in kinase levels and PI3P (14). Additional studies showed that in an absence of PfKelch13 mutations, elevated PI3P also induced artemisinin resistance but was responsive to regulation by PfKelch13. As a result, the lipid PI3P product is thought to be the key factor of artemisinin resistance, while PfPI3K must be a target enzyme for malaria treatments going forward.

This is important in the identification of artemisinin-resistant strains as well as for tracking the spread of the mutant parasite. ACTs continue to remain the top choice for treatment of malaria, however, one of its greatest strengths may lead to its demise. The common component in all combinations of ACTs is artemisinin, so the spread of artemisinin-resistance throughout the parasite life cycle stages threatens worldwide treatment success.

Current Preventative Intervention

In addition to researching mechanisms of drug resistance after parasitic infection, scientists have also begun to delve into the area of prevention in the form of vaccines. Although scientists have elucidated several targeted mechanisms of drug resistance, there are still many gaps in our armament against malaria. GlaxoSmithKline (GSK) has manufactured the only known first-generation vaccine for malaria, which has recently finished phase III clinical trial, the largest known malaria vaccine clinical trial in Africa. This vaccine, RTS,S/AS01, is intended to provide partial protection rather than complete protection against malaria in children 6 weeks of age and older. In other words, vaccinated children may still contract malaria if transmission is high enough.

Final results of Phase 3 showed that the vaccine prevented 4 in 10 cases of malaria and 3 in 10 cases of severe malaria in a four-year time frame among the children who received 4 doses of the vaccine. This shows that the vaccine impacts incidence rather than the overall number of people developing the disease. Long-term efficacy of the vaccine declined over time and more studies are needed to determine steps and dosing to improve this efficacy. The positive results of Phase 3 prompted the WHO to endorse recommendations to implement the malaria vaccine implementation programme (MVIP), involving 3 sub-Saharan African countries. In 2019, vaccinations began in Malawi, Ghana, and Kenya and Phase 4 studies will be conducted in these pilot areas for effectiveness and potential side effects. The program is expected to last through 2023 and will be a major determinant in the WHO's decision of whether or not to employ the vaccine on a more wide-spread scale.

The basis of the immunological mechanisms of the vaccine is summarized here. The stage of *P. falciparum* inoculated into the human bloodstream is the sporozoite. A protein called circumsporozoite protein (CS) covers the sporozoite surface and modulates entry into hepatocytes. The protein consists of central NANP repeats and flanking regions of non-repeats. The vaccine includes RTS,S which is a chimeric protein composed of CS NANP repeats fused to the hepatitis B virus surface antigen and AS01 adjuvant composed primarily of lipids that enhance immune response. Administration of the vaccine increases serum levels of anti-NANP IgG antibodies and ultimately reduces the rate of hepatocyte invasion and development of new blood stage infections. (18,19)

Additional questions have been raised regarding the role of cell-mediated immune (CMI) response. The available data suggests that protection is primarily due to antibodies, but there is some evidence to support contribution from CD8+ T cells in killing intracellular hepatocyte infection (18). Any modifications to the existing vaccine should aim, at a minimum, to match the potency of the IgG response over the CMI response.

Conclusion

The world as malaria knows it has drastically changed over the past several thousands of years as scientists have developed a wider and stronger array of defenses. Researchers have elucidated malarial parasite life cycles and mechanisms of attack and paved the way for use of targeted drug therapies. Despite these efforts, the *P. falciparum* parasite continues to display its strength and resistance to pharmacological intervention. The major mutations in the *P. falciparum* genome conferring antimalarial resistance such as PfCRT, PfDHPS, and PfKelch13 have raised the stakes once again. Researchers retaliate with the release of new combination therapies and identification of molecular markers of drug resistance. The molecular markers are helpful in locating emergence from various foci and can then be monitored to ensure administration of appropriate treatments for a given region of the world.

Concern regarding drug resistance remains the location to which the resistance reaches. Emergence of chloroquine and ACT-resistant strains pose exceptional threat to inhabitants of malaria endemic areas in Africa. These regions lack resources for and accessibility to newly developed drugs for malaria treatment. Antimalarial drug resistance remains one of the greatest challenges in the prevention and control of malaria. Although studies of parasitic and drug mechanisms have significantly propelled our current knowledge in targeted treatment methods, additional focus on vector control and vaccine development is essential in eradicating malaria. Improper control of vectors and inadequate vaccination could lead to neutralization of progress achieved thus far in the fight against malaria.

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