Non lethal pathogenic diseases: changing the paradigm from a zero sum game to a symbiotic relationship, the case of malaria.

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Editor, Journal of Excipients and Food Chemicals

Received: May 1, 2013; *Accepted:* May 25, 2013

Review Paper

ABSTRACT

Non-lethal pathogens with a long human co-evolutionary history, may be reasonably expected to be more genetically diverse, and thus have a greater propensity to create resistant variants in response to vaccine deployment. Conversely, their human hosts would be expected to generate Darwinian balanced polymorphisms against parasite invasion that mitigate damage to both host and parasite alike. Food selection represents one of the most obvious routes for cultural adaptations to disease. In the case of malaria, so stark is the effect of food ingredients, that they can actually reverse malaria protective hemoglobin variant genotypes. In addition to the current paradigm of IC_{50} screening, which can produce parasiticidal drugs with the ability to save lives, yet with short clinical life spans, it is worthwhile to superimpose a knowledge of the Darwinian evolutionary trajectories of the host and the parasite on the drug development process to help select molecular targets and/or pathways that would be less amenable to the development of drug resistance. Altered drug screening procedures involving a multitude of molecules such as those typically found in extracts could prove worthy of this proposed 'evolutionary drug design' paradigm. Existing malaria eradication policies should be revisited and re-examined, with a view to working with, rather than against, natural selection.

KEY WORDS: Malaria, gametocytogenesis, vaccine, hemoglobin, Plasmodium, drug resistance, food ingredients, Darwinian selection

INTRODUCTION

A history of use of food ingredients in tropical and sub-tropical populations exposed to endemic malaria indicates a pattern that is consistent with suppression of only the asexual forms of the parasite without interference with gametocytogenesis, exposure of erythrocytes to increased oxidative burden, *in situ* generation of cytoadhesion suppressants (derived from the diet) and heme scavengers. This strategy, combined with (the mosquito) vector evolution non-intrusion, has allowed for (largely) asymptomatic co-existence of a majority of (post-childhood) individuals with the parasite for millennia. It is highly desirable that policies and medicines be respectively pursued and developed, which synchronize with, and take

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advantage of, Darwinian evolution. In this context, medicines that deliver cytoadhesion preventive, gametocytogenesis or red blood cell dehydration/oxidation stimulative molecules specifically to paracitized erythrocytes, in conjunction with policies that foster vector (mosquito) evolution capable of accepting a larger gametocyte/oocyte load, and parasite evolution without the presence of blood invasive forms, should be pursued.

Why resistance develops to drugs or vaccines thereby necessitating the search for the next 'wonder drug' in a vicious 'arms race' cycle

In cases where wild-type pathogens can transmit from vaccinated hosts, in the so called 'leaky vaccines', it is possible for increasingly virulent strains to emerge. Such virulent strains have rendered many veterinary vaccines ineffective (1). Serial passage experiments performed using Apical Membrane Antigen-1 (AMA-1), a component of several of the candidates for malaria vaccines in clinical trials, selected for more virulent strains in mice whose increased virulence was caused by changes in undefined pathogen loci, other than that at the *ama-1* locus (2). *Plasmodium falciparum* exhibits large genetic diversity as a result of a long evolutionary history of selective pressure by the human immune response system. Vaccine resistant malaria variants can therefore be expected to emerge with greater probability in response to surface antigen malaria vaccine deployment (3). The process of natural selection has already allowed *Plasmodium vivax* to create a variant which has the ability to invade Duffy negative red blood cells (4), a hitherto resistant genotype host population.

Why over-reliance on IC_{50} values represents a non-sequitur to combat parasites in the long run

Whole plant extracts generally demonstrate lesser IC_{50} values than expected in terms of their 'active constituent' content. Such is the case with *Artemisia annua* extracts compared to the artemisinin molecule by itself. This phenomenon has been attributed to the fact that other ingredients in the extract(s) typically may activate/modulate mechanisms/pathways that increase the efficacy of the 'active constituent'. It has been suggested that some of these mechanisms/pathways involve monoamine oxidase, cytochrome P450 enzymes, antioxidant effect and multidrug resistance (5). Multiple mechanisms can therefore potentially change the inhibitory concentration requirements for active compounds. *In vitro* screening of single molecules for those that exhibit IC_{50} values below a specified threshold has consequently produced dismal results. To date, no new commercial malaria drug has emerged from *in vitro* screening of African plants.

African plants (whose extracts) that have been consumed by people that have been exposed to malarial parasites for several millennia seldom present with single molecules with 'acceptable' and commercially exploitable IC_{50}. This may be because direct parasiticidal effects may not be the goal of traditional African antimalarial therapy. As Maranz so eloquently puts it, it may very well be that, from the perspective of maintaining a sustainable, viable indigenous therapy over centuries, direct elimination of parasites is self-defeating (6).

Because parasiticidal activity is the only response measured by IC_{50}, the current paradigm of screening, by definition, selects single molecules that impose direct, life cycle invariant, selective pressure on parasites. It is not surprising therefore, that such a selection criterion leads to the 'discovery' of drugs with limited effective clinical life spans, before parasites become resistant *via* the bottleneck effect and subsequent selective sweep that may occur in as few as 80 vector generations (7). Resistance has emerged to Quinine and its derivatives, to sulfa drug combinations with pyrimethamine and to Artemisinin (8). In every known case of an effective anti-plasmodium drug entering widespread use, parasites have developed resistance (9).

The selective pressure exerted by *Plasmodium* has resulted in several host adaptations such as sickle cell disease, thalassemias and glucose-6-phosphate dehydrogenase (G6PD) deficiency. Interestingly, these evolutionary host adaptations do not seem to be designed to kill the invading organism, but rather to minimize the damage produced to the host during infection. For example, all of the hemoglobinopathies decrease the cytoadhesive capacity of parasitized erythrocytes. This is accomplished by displaying less and aberrantly presented PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein) on the erythrocyte surface (10, 11), which in turn is due to the interference with the ability of the *Plasmodium* to remodel erythrocyte actin (12). This mechanism mitigates the life-threatening aspects of infection that can result from the sequestration of such infected erythrocytes in the brain and other post capillary micro vessels (13). Contrary to suggestions in the literature (14), attenuated parasite infected RBC sequestration may not impose selection pressure on *Plasmodium* so long as gametocytogenesis is not threatened. It can be argued that in the case of homozygous carriers of mutated hemoglobin genes, this response to *Plasmodium* selective pressure represents a case of 'balanced polymorphism' (15) carried to extreme, such that the homozygotes' hematological disadvantage becomes considerably greater (instead of being balanced) than its resistance to malaria.

These protective hemoglobinopathies are also associated with increased transmission of the parasite from its human host to its mosquito vector (16, 17). From a natural selection point of view, if asexual parasite forms are preferentially targeted (by hemoglobin variants, for example), the fitness cost of gametocytes in terms of lost asexual reproduction is significantly reduced. The 'switch' from asexual to sexual transmission promoting forms would therefore be increased. As regards the cellular mechanism(s) of this switch, it has been shown that microRNAs' (in particular, miR-451) found in red blood cells carrying variant hemoglobin alleles translocate into *Plasmodium falciparum* and suppress cyclic-adenosine mono phosphate (cAMP)-dependent, Protein Kinase A-Regulatory domain (PKA-R) protein levels (18). The concomitant increase in PKA-C(catalytic domain) activity results in an induction of gametocytogenesis. Consistent with this mechanism, an upregulation of cyclic-guanosine mono phosphate (cGMP) has also been shown to stimulate gametocytogenesis (19).

It has been proposed that parasitized red blood cell specific, sickling mimetic drugs, be identified and/or synthesized (20). The efficiency of *Plasmodium* invasion has been shown to be inversely proportional to red blood cell dehydration regardless of whether such dehydration is experimentally induced or inherent in the particular RBC density distribution (21). It is not, however, apparent that promotion of sickling in parasitized erythrocytes would also enable the dismantling of PfEMP surface presentation or (the dismantling) of their increased cytoadhesive properties. It must first be determined whether infected erythrocytes containing non-mutated hemoglobin can be made to dehydrate (22), and further, whether such a reduction of intracellular erythrocyte volume, in itself, is sufficient to reduce and enhance, sequestration and spleenic degradation, respectively.

In this context, much can be learned from the pharmaceutical agents used to treat sickle cell disease (SCD). One of the mechanisms of the action of 5-Azacitidine and Hydroxyurea is by increasing fetal hemoglobin (23), as do the immunomodulatory agents, Lenalidomide and Pomalidomide. Fetal hemoglobin is immune to the 6Glu->Val or the 6Glu->Lys mutations in the β-chain because its composition does not comprise these chains. Instead it utilizes the γ-chains from the fetal gamma genes, HBG1 and HBG2. Interference with fetal hemoglobin silencing via inactivation of the BCL11A repressor in murine models has been shown to correct the hematologic and pathologic defects

associated with SCD through fetal hemoglobin induction (24).

Magnesium Pidolate acts via the inhibition of the ATPase dependent KCl cotransporter channel, while several imidazole antimycotics and the selective drug Senicapoc, cause blockage of the voltage gated Gardos channel. The favorable modulation of these channels leads to a decrease in cellular dehydration (25).

Certain peptide sequences of PfEMP1 have shown to induce strain transcending antibodies (26) and a recombinant sub-fragment of PfEMP1, which encompasses the CD36 binding domain, reverses the adhesion of PfEMP1 expressing RBCs to the vascular endothelium under physiological flow conditions (27). A 'small molecule' drug that binds with high affinity to a strain invariant (relatively) conserved domain(s) (28) of PfEMP1 conjugated with another drug that could induce dehydration of the target RBC, could potentially satisfy the 'live and let live' paradigm of the Plasmodium parasite without causing selective pressure.

How to live with a greater parasitic load and still be asymptomatic

Mutations in hemoglobin which have evolved against plasmodium infections primarily limit disease severity by preventing tissue damage without targeting the pathogen. Human sickle cell hemoglobin induces the expression of heme oxygenase-1 (HO-1), encoded by the gene *Hmox1*, in hematopoietic cells (29) via degradation of a cytoplasmic repressor of the transcription factor NF-E2-related factor-2 (Nrf2). This causes an increased nuclear translocation of Nrf2, which, in turn, binds to the stress-responsive elements in the *Hmox1* promoter to cause HO-1 expression to occur(30). HO-1 catabolizes free heme into biliverdin, iron and carbon monoxide (CO). CO inhibits the oxidation of cell free hemoglobin, consequently preventing heme release from cell free oxidized hemoglobin (31), thereby preventing the onset of severe malaria.

Paradoxically, *Hmox1* expression seems to be necessary for the proliferation of merozoites in the liver during the asymptomatic 'liver stage' of the disease because liver merozoite generation is significantly decreased when *Hmox1* is deleted by homologous recombination (32). These ambivalent effects of HO-1 on *Plasmodium* survival during various stages of the disease suggest that the parasite may have evolved in a manner that subverts the HO-1 system to promote both its own survival, as well as, that of the host, at least in those individuals that have been continuously exposed (HbS mutation in the heterozygous form). This evolutionary 'trade-off' is also reflected in the low levels of lethality (< 2%) due to *Plasmodium* infections (33), although the absolute numbers are unacceptably high. Interestingly, a greater genetic diversity of a non-lethal pathogen may serve as an indication not to design drugs and/or vaccines that exert selective pressure on it. This is because the greater the pathogen surface antigen genetic diversity, the greater is the probability of a long period of co-existence with the host. This may imply that the evolutionary trajectory of the pathogen is not lethal to the host.

Being that the blood stage is the only stage in the parasite life cycle that causes disease, the proposed strategy will prevent or decrease the asexual reproductive cycle in the red blood cells without exerting significant selective pressure on either the gametocytes or the transmission rates of the parasite. Such an outcome can be achieved because stimulation of asexual growth has been shown to repress gametocytogenesis (34), hemoglobinopathies select for increased gametocyte differentiation and mathematical modeling indicates that higher gametocyte conversion rates result in lower peak asexual-form densities (35) as well as a counterintuitive decrease in absolute gametocyte load (36). Thus, the objectives of research may be to decrease the absolute levels of asexual parasites, increase the ratio of gametocytes to asexual parasites, to increase the rate of commitment to gametocytogenesis (but not necessarily to

transmission enhancement (30)), and to formulate and institute policies that do not actively seek to decrease the transmission rate of the parasite host.

In manuscripts titled, "Why so few transmission stages?" and "Why so few gametocytes", Taylor *et.al.* (37) and Talman *et.al.* (38) respectively argue that natural selection and/or selective pressure prefers variants which restrict gametocyte densities because a) of a host immune pressure through transmission blocking activity, and b) a greater number of gametocytes may inflict damage to their invertebrate (mosquito) host. Pharmacological intervention that causes increased gametocyte conversion rates is likely to manifest itself in concomitant evolution of the mosquito vector to accept a greater gametocyte (oocyte) load.

The transmission-drug resistance paradox

Pyrethoid insecticide treated mosquito nets (ITN) have been shown to reduce child mortality in Sub-Saharan Africa by 18% at negligible cost ($2.10 average annual cost) (39). The global malaria eradication program launched by the WHO in 1955 with the aim to eliminate malaria outside Sub-Saharan Africa was successful (at least in terms of transmission and vector reduction) because of dichlorodiphenyl trichloroethane (DDT) based indoor residual spraying (IRS). However, vectors can (and did) develop physiological and behavioral resistance to insecticides. Physiological resistance to pyrethoid insecticides has been seen (40). Behavioral resistance is exemplified by selective declines in the population of house-entering and indoor-feeding (endophagic) species relative to more outdoor-feeding (exophagic) species. For example, in the southwest Pacific, the percentage of early evening biting by *Anopheles farauti* prior to DDT-IRS implementation ranged from 11 to 40% with equal feeding indoors and outdoors. After DDT-IRS was implemented, that percentage increased to 70%, with the majority of biting occurring outdoors (41). Since the mosquito has a short generation time, genetic shifts in response to selective pressure induced by interventions may manifest rapidly.

Table 1 provides an (admittedly simplistic) scenario for estimating the spread of drug resistance as a function of transmission rate. Measurement of inbreeding coefficients for *P falciparum* in Tanzania and Papua New Guinea indicate that the transmission rate is inversely related to inbreeding (42, 43) although recent data appear to be inconsistent with this hypothesis and indicate a considerable amount of inbreeding (44) and/or relatedness (45) in high infectivity regions. The explanation provided for the inverse (transmission rate *versus* inbreeding) hypothesis is that individuals are less likely to concurrently carry genetically different infections acquired from different mosquitoes if there occur fewer infective mosquito bites per unit time. Therefore, there is a greater probability of mating between genetically related parasites. The caveats linked to this proposition (46), namely that resistance is multigenic and resistance genes are simultaneously rare and subject to low selection pressure, seem to be satisfied in the case of chloroquine, for which resistance did not appear until a decade after the drug became widely available in 1947. Coincidently, chloroquine resistance began to appear at about the same time as the WHO DDT-IRS malaria elimination program was launched in 1955. The question of whether resistance spread because of this policy specifically directed toward reducing transmission remains tantalizingly speculative.

Table 1 Drug resistance in malaria

Transmission rate	Inbreeding (selfing)	Inbreeding coefficient	Spread of Chloroquine resistance
-	+	+	+
+	-	-	-

The effects of food ingredients: millennia of *in vivo* testing

Geographic malaria 'belts' include Mesoamerica and Amazonia, Sub-Saharan Africa, the Indian subcontinent and South East Asia. The pulp and seeds of *Theobroma Cacao*, Malvaceae have been used as constituents of drinks, beverages and tobacco dating back to 1100 BC. Chronic consumption of cocoa beverages has been shown to correlate with a significant decrease in plasma levels of soluble vascular cell adhesion molecule-1 (ICAM-1)(47). ICAM-1 expressing endothelial cells are the primary binding sites for parasite infected erythrocytes in cerebral malaria (48). Increases in epicatechin, a flavonoid found abundantly in *Cacao* beans have been shown to correlate with an increase in plasma nitric oxide (49), whose beneficial effect in cerebral malaria has been shown to associate with improved brain microvascular hemodynamics (50). Epicatechin is also rich in the nuts obtained from the Kola tree (51), native to the rainforests of Africa with a recorded use dating back centuries.

Tuberous rhizomes of perennial herbs belonging to the family Zingiberaceae contain curcuminoids, a class of compounds loosely classified as natural phenolic substances. These plants are distributed throughout the tropical regions of Asia, Africa and the Americas and are utilized in many culinary preparations. Turmeric is used as a spice in curries in India, fingerroot and galangal in China and Southeast Asia, ginger and cardamom are universal spices ubiquitous throughout south and South East Asia. The sesquiterpene, zerumbone from ginger, the flavonone, Pinocembrin from fingerroot and curcumin from turmeric are heme oxygenase-1 inducers(52-54). Interestingly, curcumin induces apoptosis in human A549 lung adenocarcinoma cells via disorganization of the architecture of actin microfilaments and a decrease in the levels of F-actin (55). Although human RBCs are not subject to apoptosis, targeted delivery of curcuminoids to parasitized RBCs may attenuate PfEMP1 surface display via this mechanism.

The oldest specimens of peanuts, found in Peru, have been dated to the 5th millennium BC. Pre-Columbian Mesoamerican cultures depict the peanut frequently in their art. They are usually eaten raw or roasted and/or made into sauces, peanut butter or peanut flour/powder. The Cashew tree is native to Northeastern Brazil, the name is derived from the indigenous Tupi name, acaju. The Tupi language itself dates back to the 5th millennium BC. The mongongo nut has been consumed by the San Bushmen of the Kalahari Desert for over 7000 years. The nuts of trees indigenous to South America and Africa contain significant amounts of the amino acid, arginine. Both nitric oxide, and arginine (the substrate for nitric oxide synthase) are low in malaria while the level of plasma arginase is elevated. Nitric oxide reduces endothelial cytoadherence of infected erythrocytes by down regulating basal ICAM-1 expression (56). Inhaled nitric oxide, significantly improved survival in a murine cerebral malaria model, compared with artesunate treatment alone (57). There is significant crosstalk between nitric oxide and carbon monoxide, NO donors upregulate HO-1gene expression in a variety of tissues (58). The significance of the selection pressure imposed by arginine on *P falciparum* gametocytes (59) may have been attenuated by the absence of historical vector control/elimination efforts.

Selection of foods represents one of the easiest mechanisms for cultural adaptation to disease (60). It would require astute observation to infer that a dietary component (fava beans) that exacerbated the symptoms of a disease (G6PD deficiency), which, in turn, rendered individuals resistant to malaria, could itself have a malaria resistive effect (61) when ingested by G6PD competent genotypes. In fact, so significant is this effect that a greater consumption of

organic cyanogen-rich foods, such as cassava and fava beans by populations experiencing holoendemic malaria regionally selects out hemoglobin S (sickling) gene frequencies (62) and provides significantly greater protection to populations with heterologous hemoglobin gene variants (63). This is the basis for the suggestion of using thiocyanates as anti-sickling agents (64) because they eliminate the Darwinian evolutionary need for erythrocytes to sickle in response to *Plasmodium* invasion. Fava bean is a species of bean native to North Africa, Southwest and South Asia. It is believed to have become incorporated in the eastern Mediterranean diet ~ 6000 BC. Cassava was first domesticated along the southern border of the Amazon basin (65) ~ 8000 BC.

The fact that food ingredients can reverse malaria protective hemoglobin variant genotypes is a powerful reason to explore parasitized red blood cells targeting specific molecules derived from such diets. Targeted drug delivery of synthetic vectors or antibodies to (largely) invariant regions of PfEMP1 surface antigens carrying this food ingredient derived payload will result in even more specific therapies without causing selective pressure on the parasite. As examples, curcumin, miR-451, thiocyanates, RBC dehydrating agents (KCl cotransporter or voltage gated Gardos channel *openers*) and xanthurenic acid (66, 67) could be delivered to parasitized RBCs' *via* attachment to PfEMP1 antibodies.

CONCLUSION

The elation of having conceived a new idea can be tempered by finding out that the idea is not really 'new', just not explicitly so stated. Such is the case with the proposition presented in this paper, i.e., to consider a malaria 'control' strategy. The 'control' does not imply a WHO 'containment' moniker (68) as expounded in the global malaria control strategy in Amsterdam in 1992, rather, the opposite. It denotes a) the invention of medicines that selectively kill the asexual forms of the malaria parasite, b) compounds that facilitate the switch to sexual parasites i.e., gametocytogenesis and c) non-interference with vector which includes the gradual withdrawal and eventual reversal of ITN and spraying policies worldwide. The published literature includes references; albeit very few, to attenuating the symptoms of malaria caused by asexual parasite load by promoting their switch to sexual transmissive forms. Cautionary notes are interspersed in vaccine reviews that point out that any vaccines against malaria are bound to be 'leaky'. The necessity of targeting vector behavior, in addition to monolithic elimination strategies such as ITN and spraying, have been discussed. However, in none of these manuscripts is the idea further developed and stated explicitly, namely that selective pressure is better directed against only the asexual form of *Plasmodium*. Malaria may be best treated as a manageable disease associated with asymptomatic (made so by drugs that selectively target the asexual stages of the parasite without interfering with transmission) *in vivo* sexual parasite load.

The design/development of medicines against parasitic diseases has historically not taken into account the evolutionary trajectories of the parasite and the vector(s)/host(s). Bringing knowledge gleaned from a natural Darwinian selection to bear on this problem may help select a particular policy and/or molecular target/pathway that would be less amenable to the development of drug resistance, thereby prolonging the clinical use time of the drug, maximization of investor return and amelioration of disease. For example, in the case of malaria, molecules that can kill and spare, asexual and sexual forms respectively, or those that can increase gametocytogenesis at the expense of asexual merozoite load, or those that can otherwise exert selective pressure on *Plasmodium* to evolve without the virulent 'blood stage' may be expected to possess longer clinical use times. The paradigm of 'evolutionary drug design' must also include the

vector, being integral to the completion of the parasite life cycle. Policies that are directed toward mosquito eradication or containment can be modulated such that this invertebrate vector may evolve to accept a greater gametocyte load and oocysts. As detestable and counterintuitive as this (non-intervention to prevent mosquito bites) sounds, it would be much better in the long run to live with an 'innocuous' blood sucking vector that did not cause disease, than to live with a virulent drug resistant one that did.

Natural selection may have selected for hemoglobin variants that attenuate malarial 'blood stage' symptoms so long as transmission is not impeded. We can invent and formulate, medicines and policies respectively, that will reduce (and preferably eliminate) mortality while allowing for asymptomatic levels of blood parasites, and decrease the selective pressures on both; the sexual forms of malarial parasites and the vector.

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