Why are Artemisia infusions prophylactic? The herbicidal hypothesis.

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Σωκράτης

Our association IFBV-BELHERB has received numerous anecdotic reports on the prophylactic effects of Artemisia plants. This effect has been documented in scientific papers. Patrick Ogwang from Uganda (Ogwang PE, et al. Trop J Pharm Res. 2012;11:445–53) showed that an infusion of *Artemisia annua* consumed once weekly reduced the risk of *Plasmodium falciparum* episodes due to a yet unidentified constituent. All this is an important lead as classical antimalarial drugs like quinine (D.Shanks, Am Soc Trop Med Hyg, 2016, May), chloroquine (T Sahu et al., Frontiers in Microbiology, 2015, 1, 283), artemisinin are not prophylactic. They only act on the erythrocytic stage but have no impact on the liver stage invasion. They are just designed to kill the parasites in the erythrocytes, but they leave a bloody battlefield and a depressed immune system. Some even enhance the gametocytogenesis.

Many medicinal plants used against malaria in endemic areas are aimed to treat the acute symptoms of the disease such as fevers and their action is limited to these symptoms. In some endemic areas of the Brazilian Amazon region one medicinal plant seems to be an exception: *Ampelozyziphus amazonicus*, localla named “Indian beer” used to prevent the disease when taken daily as a cold suspension of powdered dried roots (VF Andrade-Neto et al., Int J Parasitology, 2008, 38, 1505-11). In infections induced by sporozoites, chickens treated with extracts of this plant were partially protected against *Plasmodium gallinaceum*. Some animals did not become infected, whereas others had a delayed prepatent period, lower parasitemia and a reduction in mortality. A delay in the time until parasites establish blood stage infection is not a minor effect. It reduces the risk of severe and cerebral malaria.

In a previous document published on www.malariaworld.org “Pentacyclic triterpenes in antimalarial plants, a new paradigm” we alerted to the important role these acids play in malaria control. And indeed *Ampelozyziphus amazonicus* is very rich in pentacyclic triterpenes, mainly betulinic acid (D do Carmo et al., Pharmacognosy Magazine, 2015, 11, 244-250). Betulinic acid, maslinic acid, oleanolic acid, ursolic acid and others seem thus to be responsible for the prophylactic activity of these plants. But that does not explain why.

Another good example for the antimalarial and prophylactic effect is *Phyllantus amarus*. This plant is well known for these properties in Ghana (R Appiah-Opong et al., Ghana Medical Journal, 2011, 45, 143-146), in Burkina Faso (M Traore et al., Phytother Res. 2008. 22, 550-1, in Nigeria, RDCongo (L Tona aet al., J Ethnopharmacol. 2004, 93, 27-32), but also in China, India, Brazil. The aqueous extract shows suppressive and curative properties similar to standard antimalarials like chloroquine, or artesunate, but it also shows prophylactic properties by delaying the onset of infection (T Ajala et al., Asian Pacific J Trop Med 2011). The plant is very rich in pentacyclic triterpenes.

DO PENTACYCLIC TRITERPENES ACT AS HERBICIDES AGAINST THE PLASMODIUM APICOPLAST

The apicoplast is a plastid organelle, homologous to chloroplasts of plants or algae, that is found in apicomplexan parasites like Plasmodium or Toxoplasma. In hindsight it seems incredible that such an organelle with a nonmammalian metabolism could so long have concealed its identity in parasites that have received as much scientific

attention as Plasmodium (RF Waller et al., Curr Issues Mol Biol 7, 2005, 57-80). It is now recognized that this large group of parasites had a photosynthetic ancestry and were converted into parasitism early in the evalution of animals. Apicoplast function is necessary for both intraerythrocytic and intrahepatic development. Recently it was found that the apicoplast is also present in the gametocytogenesis, in the sexual stage of *Plasmodium falciparum*. But only the female macrogametes have an apicoplast, the male microgametes don't (N Okamoto et al., Eukaryot Cell 8(1) 2009, 128-132).

Attempts have been made to find antimalarials which selectively attack the apicoplast. Several antibiotics are known to interfer with this organelle. They do not kill parasites in the first generation, but the progeny of drug-treated parasites suffer a delayed death, probably because they lose their apicoplast and the second generation of merozoites is unable to penetrate erythrocytes. Antibiotic treatment specifically inhibits the biogenesis and inheritance of the apicoplast in *Plasmodium falciparum* liverstage, resulting in continuous liver stage maturation but subsequent failure to establish blood-stage infection. This process of maturation of numerous merozoites in the liver induces potent immune protection for subsequent infections. This prophylactic protection obtained by several antibiotics was demonstrated to be exceptionally robust (J Friesen et al., www.ScienceTranslationalMedicine.org, 2010, 2).

Many herbicides also have an activity against the malaria protozoan (S O Duke, Weed Science, 58, 2010, 334-339). Herbicides interfer with plant cells by interrupting mitosis and the formation of multinucleated cells. A good example is the action of herbicides from the nitroanilin family (trifluralin, oryzalin). They have a strong action on *Plasmodium falciparum*. The herbicide amiprophosmethyl also has antimalarial activity and a significant action on schizogony, i.e. the formation of multinucleated parasites. A more surprising example is the effect of the well known glyphosate on several apicomplexa like *Plasmodium falciparum, Toxoplasma gondii* (F Roberts et al., *Nature* **393**, 801-805, 1998).

Several herbicides including those of the cyclohexanedione family act by perturbing the apicoplast fatty acid biosynthesis (C Goodman et al., Int J Parasitol. 2014, 44, 285-289). This synthesis is dispensable in blood stages of human and rodent malaria, but vital for for the liver stage.

Artemisia annua has strong allelopathic properties as was documented by Mediplant in Switzerland. In other words the plant becomes invasive and inhibits the growth of other plants or cash crop on fields where Artemisia has been planted for the extraction of artemisinin. A recent paper from Iran (MH Bijeh Keshhavarzi et al, J Biol & Envir Sci. Nov 2014) describes the allelopathic effects of *Artemisia annua* on lettuce *Lactuca sativa.* The aqueous extract on an outside plot significantly reduced germination percentage and rate, fresh and dry weight. Another paper (Seyed Mohsen et al., Annals of Biological Research, 2011, 2-6, 687-69) describes the allelopathic effect of *Artemisia annua* aqueous extracts on vegetables and plants like *Portulaca olearcea* (pursley), *Chenopodium album* (goose-foot), *Avena ludoviciana* (oat), *Plantago ovata* (plantain). For the latter the effects are noticeable on germination percentage, germination rate, plumule length, radicle length, wet weight, dry weight. A Chinese paper (Shen He et al., Ying Yong Sheng Tai Xue Bao. 2005 Apr;16(4):740-3) had previously studied the allelopathy of different plants. *Artemisia annua* affected the seedling height and fresh weight of radish, cucumber, wheat and maize around 50%.

But the allelopathy of Artemisia remains a controversial issue. Although in vitro trials on seed germination of various plants show an impact of artemisinin and flavonoids, this remains far from field effects noticed and may only offer a partial explanation, A

rtemisinin and flavonoids are hardly soluble in water and rapidly degraded in the soil.

A recent paper offers an explanation which is very attractive proposing the role of pentacyclic triterpenoids in the allelopathic effects of *Alstonia scholaris*. (Wang CM[1], J Chem Ecol. 2014 Jan;40(1):90-8). *Alstonia scholaris* is a tropical evergreen tree native to South and Southeast Asia. Alstonia forests frequently lack understory species. However, potential mechanisms, particularly the allelochemicals involved remain unclear. They identified allelochemicals of *A. scholaris*, and clarified the role of allelopathic substances from A. scholaris in interactions with neighboring plants and showed that the allelochemicals from leaves, litter, and soil from *A. scholaris* were identified as pentacyclic triterpenoids, including betulinic acid, oleanolic acid, and ursolic acid. In the field, ursolic acid accumulated abundantly in the soil in *A. scholaris* forests, and suppressed weed growth during summer and winter. *A. scholaris* pentacyclic triterpenoids influence the growth of neighboring weeds by inhibiting seed germination, radicle growth, and functioning of the photosystem.

These molecules are stable in powder form and in water for months (P Puttarak et al., Natural Product Sciences, 2016, 22, 1-20)

If the pentacyclic triterpenes have an allelopathic, herbicidal effect, this offers a completely new hypothesis and opportunity for destroying the malaria parasite in all its forms, from hepatocytes to gametocytes.

THE SKIN AS BARRIER

As the primary interface between the body and the outside environment, the skin protects the host against invading organisms.

It has always been assumed that sporozoites rapidly exit the injection site and enter the blood circulation. But it was demonstrated by PCR that the majority of the infective sporozoites remain in the skin for hours (L M Yamauchi et al., Cellular Microbiology, 2007, 9, 1215-22). The same authors had shown in preliminary studies no decrease in the sporozoite loads in the skin up to 3h. These findings imply that there is ample time for host and parasite to interact at the inoculation site and that tailored treatments of the skin might inhibit the survival of the sporozoites before they enter the bloodstream. At 37°C the time required for sporozoite penetration in hepatoma cells is 3 hours (VF Andrade-Neto op.cit.). Malaria-specific $CD8^+$ T cells are primed in the skin draining lymphs (S Chakravarty et al., Nat Med 2007, 13, 1035-1041). A significant reduction in anti-sporozoite $CD8^+$T cell response was observed in animals that had their draining lymph nodes removed prior to sporozoite infection. Pentacyclic triterpenes could play a role. Topical application of ursolic and maslinic acid for example significantly reduces epidermal inflammation, NF-κB, Cox-2 and skin tumor proliferation (Jiyoon Cho et al., Oncotarget, 6-36, 39292-305).

The high concentration of pentacyclic triterpenes in the skin of fruits or barks of trees evidently has the function to repel parasites and molds and to prevent their entry into the fruit or plant. Betulinic acid acts as an antifeedant (SG Jagadesh et al., J Agric Food Chem. 1998, 46, 2297-99). It affects the growth of larvae and pupae (V Lingampally et al., Asian J Plant Sc and Res. 2012, 2, 198-206).

HEPATOCYTES

Unlike many other microbial organisms that utilize the phagocytic properties of their host for invasion, sporozoites actively invade hepatocytes. They even pass through several hepatocytes prior to the final hepatocyte in which they develop. The reason f or this process is not well understood; it is likely that the sporozoites choose the b

est environment for their differentiation into merozoites. Sporozoites have specialized secreting organelles in the apical region which play a central role in host cell invasion. The migration through several hepatocytes increases sporozoite competency for the formation of these apical organelles.

To survive and develop in the parasitophorous vacuole inside the hepatocyte the parasite has developed several strategies including depletion of CD-8 lymphocytes and suppression of NF-κB to prevent cell death. The circumsporozoite protein (CSP) plays a key role in this inhibition of NF-κB.

Memory $CD8^+$T cell populations residing in the liver provide the first line of defence against naïve infections, but more even in subsequent infections and are the key players of the recall response. Any immune strengthening approach, including vaccines, requires the generation of a robust, stable memory population. In particular, $CD4^+$cell help was shown to be required for $CD8^+$memory cell responses against malaria (She-Wak Tse et al., Mem Inst Oswaldo Cruz 2011, 106, 172-178). $CD4^+$helper T cells are critical orchestrators of immune responses. $CD4^+$T cells decrease the threshold required for protective immunity and this immunity may last for months (NW Schmidt et al., PNAS, 2008, 105, 14017-22).

It was shown that oleanolic acid upregulates the $CD4^+$and $CD8^+$populations (J Wang et al., International Immunopharmacology 2012, 14-4). It was also found that betulinic acid increased the total number of thimocytes, splenocytes, lymphocytes. It is a potential biological response modifier and may strengthen the immune response of its host (Y Jine Polish J Veterinary Sc. 2012, 15, 305-13). This is in line with our findings in Katanga where we confirmed that administration of capsules containing Artemisia leaf powder raised the $CD4^+$ (Constant Kansongo Tchandema, personal communication). This strengthening of the immune system by Artemisia plants may also be related to the pentacyclic triterpenes they contain.

Delayed apoptosis of infected hepacytes is another strategy of defence of the sporozoites. Blocking apoptosis allows the parasite to complete its full cycle and the merozoite burden is strongly enhanced. It fully exploits host cell resources and ultimately produces tens of thousands of merozoites which are released from the hepatocyte. Sensitizing the infected hepatocyte to apoptosis may substantially reduce parasite burden. Recent evidence suggests that Plasmodium infected hepatocytes are similar to cancer cells. The authors demonstrate that the anticancer drug obatoclax reduced the number of infected hepatocytes by > 70%, but had limited effect on uninfected cells (A Kaushansky et al., Cell Death and Disease, 4. E762). Many pentacyclic triterpenes, like betulinic acid, are used in cancer treatment. It seems worthwile to study their effect in malaria infection.

Several antibiotics also have an effect during the hepatocytic stage. Others, including tetracyclines and clindamycin used for the treatment of malaria, have little action on the pre-erythrocytic stages.

An extensive study has been made on the antibiotic thiostrepton and the effects of this drug on life-cycle stages of the malaria parasite *in vivo*. Preincubation of mature infective sporozoites with thiostrepton has no observable effect on their infectivity. Sporozoite infection both by mosquito bite and sporozoite injection was prevented by pretreatment of mice with thiostrepton. Thiostrepton eliminates infection with erythrocytic forms of *Plasmodium berghei* in mice. (M Sullivan et al., Mol Biochem Parasit 2000 109, 17-23).

After its release from the hepatocyte the merozoites penetrate the erythrocytes very rapidly. Parasite entry into erythrocytes is a complex, dynamic process. The invading merozoite orients its apical end toward the junction of invasion. Invagination of the

erythrocyte bilayer then results in engulfment of the parasite. Merozoites without their apicoplast are unable to penetrate red blood cells.

GAMETOCYTES

A study of the Walter Reed Army Institute already in 1992 found that the herbicide Trifluralin showed strong anti-malarial effects not only on *Plasmodium falciparum* in cultures, but also transmission blocking by inhibiting gametocyte maturation and viability (J Nath et al., 19th Army Science Conference, 1994).

Thiostrepton (op.cit) treatment of infected mice reduces transmission of parasites by more than ten-fold, indicating that the plastid has a role in sexual development of the parasite. These results also indicate that the plastid function is accessible to drug action *in vivo* and important to the development of both sexual and asexual forms of the parasite.

Supply of the isoprenoid building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) is the essential metabolic function of the apicoplast for isoprenoid biosynthesis, particularly during gametocytogenesis. When IPP supplementation was removed early in gametocytogenesis, developmental defects were observed, supporting the essential role of isoprenoids for normal gametocytogenesis. Furthermore, mosquitoes infected with gametocytes lacking the apicoplast developed fewer and smaller oocysts that failed to produce sporozoites. This finding further supports the essential role of the apicoplast in establishing a successful infection in the mosquito vector (J Wiley et al., Eukaryotic Cell 2015, 14, 128-133)

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