International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2016): 79.57 | Impact Factor (2015): 6.391

Resistance Mitigating Effect of *Artemisia Annua* Phytochemicals against *Plasmodium berghei* ANKA and *Plasmodium yoelii* in Swiss Albino Mice

Lucy N. Kangethe

Department of Biochemistry and Biotechnology, Technical University of Kenya P.O BOX 52428-00200 Nairobi, Kenya

Abstract: ***Malaria is a disease of global concern. Resistance of Plasmodium falciparum to drugs such as chloroquine and sulfadoxine-pyrimethamine is a major problem in malaria control. Artemisinin derivatives, particularly in combination with other drugs, are thus increasingly used to treat malaria, reducing the probability that parasites resistant to the components will emerge. Artemisinin resistance has recently been reported in the Thai-Cambodia border. The project was designed to demonstrate resistance-mitigating effects of phytochemical A. annuaof Artemisia annua relative to pure artemisinin against therodent malaria parasite Plasmodium bergheiAnkaand plasmodium yoelii. The in vivo experiments were done by inoculating the Swiss albino mice with the P. berghei ANKA parasite and P. yoelii and thereafter treated them with pure artemisinin and Artemisia annua phytochemicals. After 4 days parasitaemia was determined and the ED_{50} and the ED_{90} calculated and then the mice were passaged. The ED values got were utilized to determine the doses to be used for resistance development. The ED_{50}andED_{90} got for artemisinin with P. berghei ANKA was 1.43 and 7.18 mg/kg.day respectively while the ED_{50}andED_{90} got for the A. annua with P. berghei ANKA was 34.5 and 118 mg/kg.day respectively. The chloroquine resistant murine plasmodium (P.yoelii) values were as follows artemisinin ED_{50}andED_{90} got was 11.63 and 29.8 mg/kg.day respectively. The efficacy of dihydroartemisinin was also determined in order to compare with artemisinin and the ED_{50}andED_{90} got for DHA with P. berghei ANKA was 1.73 and 8.31 mg/ kg. day respectively. In order to determine resistance development the ED_{50} and ED_{90} were determined after every 10 cycles. This was compared with the values that had been obtained before exposure to the drug pressure. Relative index was calculated as final ED_{50} divided by the parental ED_{50}. The results indicated that there was incremental increase in the Relative index with increase in cycles in both P. yoelii and P. bergheianka. Resistance obtained in the two murine plasmodium parasites was found to be transient.***

Keywords: Malaria, Artemisinin, Artemisia annua, Plasmodium, Resistance development

1. Introduction

Resistance of *Plasmodium falciparum* to drugs such as chloroquine and sulfadoxinepyrimethamine is a major problem in malaria control. Artemisinin (ART) derivatives, particularly in combination with other drugs, are thus increasingly being used to treat malaria, reducing the probability that parasites resistant to the components will emerge. Although stable resistance to artemisinin has not been reported, its emergence would be disastrous because of the lack of alternative treatments. In 2001 WHO recommended use of artemisinin combined therapies as a measure against resistance development to artemisinin. Many countries have now introduced artemisinin (ART) derivatives as their first-line therapy, in combination with other drugs (such as mefloquine, amodiaquine, piperaquine, pyrimethamine/sulphadoxine or lumefantrine) (World Health Organization, 2006).

These artemisinin combination therapies (ACTs) present favourable pharmacokinetics and are thought to reduce the probability of mutations that underlie resistance and treatment failure emerging in parasite populations (White, 1999). Artemisinin has a short half-life but acts extremely quickly in reducing parasite densities and symptoms. The activation, mechanisms of action and targets of artemisinin derivatives have been vigorously investigated and debated (Olliaro *et al*, 2001; Meshnick, 2002; Krishna *et al*., 2006).

Artemisinin (is a compound from the plant *Artemisia annua*) and its derivatives contain a stable endoperoxide bridge, which, it is suggested, is cleaved by intraparasiticheme. The cleaved endoperoxide becomes a carbon- centered free radical which then functions as an alkylating agent, reacting with both heme and parasite proteins (Akompong et al 2000), Kamchonwongpaisan et al 1996, Ubalee et al 1999). A previous study with *P. falciparum* suggested that a sarcoplasmic and endoplasmic reticulum Ca2_ATPase (SERCA)-type protein encoded by a gene denoted*pfatp6* might be the major chemotherapeutic target of these drugs (Eckstein-ludwig et al 2003).

Traditionally prepared formulations of *Artemisia annua* (sweet annie, annual worm wood, or sweet worm wood) for malaria treatment has been utilized in China for over 2000years as a tea infusion with no reported resistance (Mueller 2000). *A. annua* has a very rich phytochemistry comprising several classes of compounds mainly monoterpenes, sesquiterpenes (including artemisinin) and flavonoids (Bhakuni *et al* 2002).

While some of the genes involved in chloroquine and pyrime thaminesulfadoxine resistance are known (Djimde *et al* 2001, Hayton et al 2004), those determining the responses to artemisinin are yet to be identified. For instance, two genes, originally proposed to modulate sensitivity to chloroquine in *P. falciparum*, have also been investigated in the context of artemisinin resistance. These are *pfmdr1* and *pfcrt*, encoding membrane transporter proteins, which are localized in the membrane of the parasite's food vacuole (Cowman 1991, Fidock 2000). Other genes that have been suggested to be associated with artemisinin resistance in *P. falciparum*include *pf*atpase, ubiquinating gene, and K13 propeller gene (Quattara *et al* 2015).

2. Materials and Methodology

2.1. Parasites, hosts and test compounds

To select artemisinin and *Artemisia annua* resistance, two strains of Plasmodium, a strain of *P. berghei* ANKA, resistant topyrimethamine and *Plasmodium yoeliiyoelii* (*P. yoeliiyoelii* 17x) obtained from the MR4 repository (MRA-865, MR4, ATCC_ Manassas, Virginia) were used.

Male, random-bred Swiss albinomice (20 ± 2 g), were obtained from KEMRI which were maintained in the animal house. They were each infected intra-peritoneally with donor blood containing approximately 2 X 10^7 parasite red blood cells (PRBC) in 0.2 ml inoculum. Infection was assessed by microscopic estimation of the proportion of infected erythrocytes in Giemsa-stained thin smears made from tail-vein blood. The animals were housed in experimental room in a standard Macrolon type II cages clearly labeled with experimental details at 22 ^{0}C and 60–70% relative humidity and fed on commercial rodent feed and water ad libitum.

Chloroquine which was used as a control drug was purchased from Sigma Chemical Co. (Poole, UK), while Artemisinin was obtained from Sigma Chemical Co. On the day of administration, the drugs was freshly prepared by dissolving in DMSO and then in water. *Artemisia annua* was obtained from Tanzania.

2.2. Determination of 50 % and 90 % effective-dose level (ED_{50} and ED_{90})

Fifty percent and 90% effective doses (ED_{50} and ED_{90})were measured in a quantitative standard method '4-daytest' (4-DT), in which the parasites were exposed to four, daily, drugdoses (Peters, 1975).The drug pressure tests were carried out by treating once with the already determined doses which were measured using the '1-day test' (1-DT), in which the parasites are exposed to a single drug dose (Vennerstrom *et al*.2004).All the experiments from the 1st passage of the *A. annua* and the artemisin in pressure were carried out at the Kenya Medical Research Institute (KEMRI), Nairobi, Kenya, using the 4-DT. Drugs were administered by oral route on day 1, (24 h post-infection) in the 1-DT or starting on the day 0, (4 h post-infection) and continuing for a total of four daily doses, days 0–3 (24, 48 and 72 h post-infection) in the 4-DT. Parasite count was estimated by microscopic examination of Giemsa-stained thin smears prepared from tail snips on day 3, 72 h post-infection in the 1-DT or on day 4, 96 h post-infection in the 4-DT. Percentage chemo suppression of each dose was then calculated as (A _ B)/A] _ 100], where A is the mean parasitaemia in the negative control group and B is the parasitaemia in the test group (Tona *et al.,* 2001). ED_{50} and ED_{90}were estimated using a linear regression line.

2.3. Procedures for exerting drug-selection pressure and assessing the level of resistance

After inoculation (2 x 10^7 parasitized red blood cells contained in 0.2 ml inoculums) in 5 mice, on day zero (D0), mice were then orally treated once with the drug at concentration equivalent toED90, 72 h post-infection (D3). Thereafter, parasitaemia was monitored until it reached 2–5%, when a mouse was selected for donation of PRBC to the next naive group of five mice.

During the first 4 passages of the drug pressure, after parasite inoculation (D0), mice (a group of 5) were treated once with the drug at concentration equivalent to 2xED_{90}. The first treatment was carried out 72 h post-infection (D3). Drugs were administered orally with the use of a cannula. After treatment, parasitaemia was monitored until it reached2% when a mouse was selected for donation of PRBC to the next naive group of five mice.

The level of resistance was evaluated at different intervals by measurement of ED_{50}in the standard 4-DT which permits the calculation of an 'index of resistance', RSI_{50} (defined as the ratio of the ED_{50} of the resistant line to that of the sensitive, parent line).

The RSI_{50}values were grouped into four categories, based on previous work by Melki and Richle (Merkli and Richle, 1980): (1) RSI_{50} = 1.0, sensitive, (2) RSI_{50} = 1.01–10.0, slight resistance, (3) RSI_{50} = 10.01–100.0, moderate resistance and (4) RSI_{50}> 100.0, and high resistance.

2.4. Stability study

The stability of artemisinin and the *A. annua* resistant line was evaluated by measuring drug responses after making 10 drug free passages followed by measurement of ED_{50};. Stable resistance was defined as the maintenance of the resistance phenotype when drug-selection pressure was removed for at least 10 passages inmice (Gervais *et al.,* 1999).

2.5. Resistance studies

The activity of artemisinin and the *A. annua* against both drug sensitive and resistant lines (after 10 drug free passages) was assessed in the 4-DT. RSI_{50} were computed as the ratio of the ED_{50} of the resistant line to that of the sensitive, parent line. Resistance was classified into three categories as previously described.

2.7. Ethical considerations

The study was conducted in accordance with KEMRI guidelines on animal care and use. Additionally; the study followed the internationally accepted principles for laboratory animal use and care, as found in WHO guidelines). Specifically 21 gauge needles were used in the animal experiments. Mice that died during the experiment as well as those that were sacrificed by exposure to chloroform fumes were at the end of the experiment were put in plastic bags and incinerated. Permission to carry out the study was granted by KEMRI'S Scientific Steering Committee and the Ethical Review Committee. (Study SSC No. 1340/08).

3. Results

The following results were obtained with *Plasmodium berghei* Anka and *Plasmodium yoelii.*

The Relative Sensitivity Index for *Plasmodium berghei* Anka exposed to Artemisinin increased 14.34 fold after 10 cycles and 24.9 fold after 20 cycles, when the same parasite was tested with the *A.annua* the RSI after 10 and 20 cycles was 2.0 and 3.08 respectively (Table 1 and 2). When *P.berghei* was exposed to the A. annua and tested with Artemisinin the increase after 10 and 20 cycles was only 2.39 and 5.9 respectively. The *P.berghei* that was exposed to *A. annua* and tested with *A. annua* had a very slight increase after 10 and 20 cycles of 1.65 and 1.9 respectively. This indicates that the *A. annua* has resistance mitigating effect.

Table 1: $ED_{50}$and RSI obtained with *P. berghei* Ankaparasites exposed to artemisinin

	RSI at cycle 0	RSI at cycle 10	RS1 at cycle 20	RSI at cycle 10rev	RSI at cycle 0 rev
Art	1	14.34	24.9	12.39	3.49
Blend	1	2.07	3.08	2.29	1.42

Table 2: $ED_{50}$and RSI obtained with *P.berghei*Anka exposed to *A. annua*

	RSI at cycle 0	RSI at cycle 10	RSI at cycle 20	RS1 at cycle 10 rev	RSI at cycle 0rev
Art	1	2.39	5.9	10.69	0.60
Blend	1	1.65	1.9	1.07	0.89

Table 3: $ED_{50}$and RSI obtained with *P.yoelii* exposed to Artemisinin

	RSI at cycle 0	RSI at cycle 10	RSI at cycle 20	RS1 at cycle 10 rev	RSI at cycle 0rev
Art	1	1.41	2.06	1.255	0.65
Blend	1	1.1	1.45	1.256	0.59

Table 4: $ED_{50}$and RSI obtained with *P.yoelii* exposed to *A. annua*

	RSI at cycle 1	RSI at cycle 10	RSI at cycle 20	RS1 at cycle 10 rev	RSI at cycle 0 rev
Art	1	1.24	0.77	0.73	0.63
Blend	1	0.94	0.56	0.51	0.61

Interesting again is the fact the *P.yoelii* had a lower RSI in comparison with the *P. berghei*. RSI for *P. yoelii* parasites exposed to Artemisinin was 1.41 and 2.06 after 10 and 20 cycles respectively. After removal of drug pressure resistance reduced as indicated by the reversal RSI values in Table 3 and Table 4. This indicated that the resistance was only transient and not stable.

4. Discussion

Our study shows Artemisinin resistance in *P. berghei* ANKA can be selected before 20 continuous cycles of drug pressure. To select for resistance the 2% relapse technique (2% RT) in which a single and high drug dose is administered at the time of each passage) has been successful in the laboratory (Peters and Robinson, 1999). When drug pressure was applied RSI went up indicating resistance build up which was highest in parasites exposed to artemisinin. It was also observed that the parasites exposed to the *A. annua* had the lowest RSI increment.

5. Conclusion and Recommendation

Findings showed that the *A. annua* mitigated against resistance development and this correlates with the findings of Elfawal where the whole plant overcomes resistance to artemisinin and also demonstrated that the whole plant treatment was more resilient to resistance than the pure artemisinin (Elfawal *et al* 2015).

We know that a large number of people are using *Artemisia annua* in various forms for treatment of many ailments. We also know that *Artemisia annua* has the potential to help solve some of the serious health issues in the world but scientific data about individual phytochemicals in the plant and their combinations is also missing. There is need to study phytochemicals in *A. annua* and also to determine individual constituents activity with Plasmodium.

References

[1] Ouattara A, Kone A, Adams M, Fofana B, Maiga AW, Hampton S, Coulibaly D, Thera MA, Diallo N,
[2] Dara A, Sagara I, Gil JP, Bjorkman A, Takala-Harrison S, Doumbo OK, Plowe CV, Djimde AA. (2015). Polymorphisms in the K13-propeller gene in artemisinin-susceptible *Plasmodium falciparum* parasites from Bougoula-Hameau and Bandiagara, Mali. *American Journal of Tropical Medicine and Hygiene.* 92(6):1202-6.
[3] Mostafa A. Elfawal, Melissa J. Towler, Nicholas G. Reich, Pamela J. Weathers and Stephen M. Rich(2015). Dried whole-plant *Artemisia annua* slows evolution of malaria drug resistance and overcomes resistance to artemisinin PNAS, 112 (3) 821-826
[4] Peters W., Robinson B. L. The chemotherapy of rodent malaria. LVI. Studies on the development of resistance to natural and synthetic endoperoxides. Annals of Tropical Medicine and Parasitology. 1999;93:325–329.
[5] Mueller M. S, Karhagomba I. B, Hirt H. M, and Wemakor E. (2000). The potential of Artemisia annua as a locally produced remedy for malaria in the tropics :agricultural, chemical and clinical aspects. *Journal of Ethnopharmacology* 73 487-493.
[6] Vennerstrom JL, Arbe-Barnes S, Brun R, Charman SA, Chiu FC, Chollet J, Dong Y, Dorn A, Hunziker D, Matile H, McIntosh K, Padmanilayam M, Santo Tomas J, Scheurer C, Scorneaux B, Tang Y, Urwyler H, Wittlin S, Charman WN. (2004)Identification of an antimalarial synthetic trioxolane drug development candidate**.**
[7] Gervais, G. W., Trujillo, K., Robinson, B. L., Peters, W., and Serrano, A. E. (1999). *Plasmodium berghei:*Identification of an*mdr*-like gene associated with drug resistance. *Experimental Parasitology***91,** 86–92
[8] Merkli, B., Richle, R. W., 1980. Studies on the resistance to single and combined antimalarials in the Plasmodium berghei mouse model. ActaTropica 37, 228–231.
[9] Tona, L., Mesia, K., Ngimbi, N. P., Chrimwami, B., Okond'ahoka, Cimanga, K., deBruyne, T., Apers, S., Hermans, N., Totte, J., Pieters, L., Vlietinck, A. J., 2001. *In-vivo* antimalarial activity of *Cassia*

occidentalis, *Morinda morindoides*and *Phyllanthusniruri*. Annals of Tropical Medicine and Parasitology 95, 47–57.
[10] Ouattara A, Barry AE, Dutta S, Remarque EJ, Beeson JG, Plowe CV. 2015). Vaccine. 2015 22;33(52):7506-12. Designing malaria vaccines to circumvent antigen variability.
[11] World Health Organization, 2006). http://www. who. int/malaria/publications/atoz/meeting_briefing19april.pdf
[12] Controlling malaria: challenges and solutions PieroOlliaro, Walter R. J. Taylor and Jean Rigal UNDP/World Bank/World Health Organization Special Program for Research & Training in Tropical Diseases (TDR), Geneva, Switzerland
[13] Centre for Infectious Diseases, Royal Free and University College Medical School, London, UK
[14] Controlling malaria: challenges and solutions PieroOlliaro, Walter R. J. Taylor and Jean Rigal
[15] OlliaroP, Walter R. J, Taylor 1, Riga J. (2001). Controlling malaria: Challenges and solutions
[16] Trop Med Int Health. 2001 Nov;6(11):922-7.
[17] MeshnickS. R, (2002). Artemisinin: mechanisms of action, resistance and toxicity. International Journal of Parasitology. 4;32(13):1655-60.
[18] Krishna S, Woodrow C. J., Staines H. M., Haynes R. K., PuijalonO. M-(2006). Re-evaluation of how artemisinins work in light of emerging evidence of in vitro resistance. Trends in Molecular Medicine 12, 5: 200-205
[19] White, N. J., van Vugt, M., Ezzet, F., 1999. Clinical pharmacokinetic and pharmacodynamics and pharmacodynamics of artemether-lumefantrine. Clinical pharmacokinetic 37, 105–125.