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

Lucy N. Kanguhe

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. annuao Artemisinin annua relative to pure artesinin against theroydon malaria parasite Plasmodium berghei/Ankaand plasmodium yoelli. The in vivo experiments were done by inoculating the Swiss albino mice with the P. berghei ANKA parasite and P. yoelli and thereafter treated them with pure artesinin and Artemisia annua phytochemicals. After 4 days parasitemia was determined and the ED50 and the ED90 calculated and then the mice were passaged. The ED values got were utilized to determine the doses to be used for resistance development. The ED50 and ED90 got for artesinin with P. berghei ANKA was 1.43 and 7.18 mg/kg/day respectively while the ED50 and ED90 got for the A. annua with P. berghei ANKA was 34.5 and 118 mg/kg/day respectively. The chloroquine resistant murine plasmodium (P.yoelli) values were as follows artesinin ED50 and ED90 got was 11.63 and 29.8 mg/kg/day respectively. The efficacy of dihydroartesinin was also determined in order to compare with artesinin and the ED50 and ED90 got for DHA with P. berghei ANKA was 1.73 and 8.31 mg/kg day respectively. In order to determine resistance development the ED50 and ED90 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 ED90 divided by the parental ED50. The results indicated that there was incremental increase in the Relative index with increase in cycles both in P. yoelli and P. berghei/anka. 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 sulfadoxine-pyrimethamine 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/sulfadoxine 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 intraparasitichemical. The cleaved endoperoxide becomes a carbon-centered free radical which then functions as an alkylating agent, reacting with both heme and parasite proteins (Akomo et al 2000). Ramchonwangpaisen et al 1996, Ubalie 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 2000 years as a tea infusion with no reported resistance (Mueller 2000). A. annua has a very rich phytochemistry comprising several classes of compounds mainly monoterpines, sesquiterpines (including artemisinin) and flavonoids (Bhakuni et al 2002).

While some of the genes involved in chloroquine and pyrimethaminesulfadoxine 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 pfmdrl and pfco, 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 pfatpase, ubiquininating 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 to pyrimethamine and *Plasmodium yoelii* (P. yoeliiyoelii (P. yoeliiyoelii 17x) obtained from the MR4 repository (MRA-865, MR4, ATCC Manassas, Virginia) were used.

Male, random-bred Swiss albino mice (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 10⁷ 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 ⁰C 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 were 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 (ED₅₀ and ED₉₀)

Fifty percent and 90% effective doses (ED₅₀ and ED₉₀) 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 artemisinin 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] X 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). ED₅₀ and ED₉₀ 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 10⁷ 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 to ED90, 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 2×ED₉₀. 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 ED₉₀ in the standard 4-DT which permits the calculation of an ‘index of resistance’, RSI₉₀ (defined as the ratio of the ED₉₀ of the resistant line to that of the sensitive, parent line).

The RSI₉₀ values were grouped into four categories, based on previous work by Melki and Richle (Melki and Richle, 1980): (1) RSI₉₀ = 1.0, sensitive, (2) RSI₉₀ = 1.01–10.0, slight resistance, (3) RSI₉₀ = 10.01–100.0, moderate resistance and (4) RSI₉₀ > 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 ED₉₀. 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. RSI₉₀ were computed as the ratio of the ED₉₀ 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>
<thead>
<tr>
<th>Table 1: ED$_{50}$ and RSI obtained with P. berghei Anka</th>
<th>Parasites exposed to artemisinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI at cycle 0</td>
<td>RSI at cycle 10</td>
</tr>
<tr>
<td>Art</td>
<td>1</td>
</tr>
<tr>
<td>Blend</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2: ED$_{50}$ and RSI obtained with P. berghei Anka exposed to A. annua</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI at cycle 0</td>
</tr>
<tr>
<td>Art</td>
</tr>
<tr>
<td>Blend</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3: ED$_{50}$ and RSI obtained with P. yoelii exposed to Artemisinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI at cycle 0</td>
</tr>
<tr>
<td>Art</td>
</tr>
<tr>
<td>Blend</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4: ED$_{50}$ and RSI obtained with P. yoelii exposed to A. annua</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSI at cycle 1</td>
</tr>
<tr>
<td>Art</td>
</tr>
<tr>
<td>Blend</td>
</tr>
</tbody>
</table>

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 Elfwal 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 (Elfwal 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


[13] Centre for Infectious Diseases, Royal Free and University College Medical School, London, UK


