Prevention of Sporogony of *Plasmodium vivax* in *Anopheles albimanus* by Steroids of *Solanum nudum* Dunal (Solanaceae)

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The sporontocidal activity of three steroids (SN-1, SN-2 and SN-4) from *Solanum nudum* Dunal (Solanaceae) was determined against naturally circulating isolates of *Plasmodium vivax* in *Anopheles albimanus*. Laboratory-reared *Anopheles albimanus* mosquitoes were infected with *P. vivax* from gametocytogenic blood of volunteers resident in Buenaventura, Valle del Cauca (Colombian Pacific Coast) by using an artificial membrane feeder. Prior to mosquito feeding, gametocytogenic blood was centrifuged, plasma was separated, packed blood red cells were washed with RPMI 1640 and then resuspended in non-immune AB serum, then the steroids were added at different doses. On day 7 after infection, the presence and number of oocysts in mosquitoes was determined. The steroid SN-2 reduced the infection of mosquitoes by 90% and the mean number of oocysts by 60%. These data confirmed that the experimental steroid is capable of interrupting the sporogonic development of *P. vivax* in *Anopheles albimanus*. This experimental steroid has potential for transmission blocking in vivax malaria. Copyright © 2006 John Wiley & Sons, Ltd.

*Keywords: P. vivax; An. albimanus; sporogony; transmission blocking; S. nudum; steroids.*

INTRODUCTION

Sporogony in *Plasmodium* takes place in a mosquito vector and results in the production of sporozoites that migrate to salivary glands and are transmitted to humans by the bite of an infected mosquito. Disruption of *Plasmodium* sporogony has been considered as an alternative to control the malaria problem since it can be applied to block the transmission of the parasite (Butcher, 1997; Coleman et al., 1994; Coleman et al., 2001; Ponsa et al., 2003; Teklehaimanot et al., 1985). Drugs with effects on the sexual stages (gametocytoidal activity) or on the sporogony (sporontocidal activity) have transmission blocking activity. There are few drugs with transmission blocking activity, the most representative are primaquine, tafenoquine and some artemisinine derivatives which can potentially interrupt malaria transmission (Butcher, 1997; Coleman et al., 2001; Ponsa et al., 2003; Price et al., 1996).

The increase of *in vivo* treatment failure of chloroquine and sulfadoxine/pyrimethamine in *P. falciparum* malaria has been described in many studies (Blair et al., 2001; 2002; 2003; Castillo et al., 2002; Kazadi et al., 2003; Osorio et al., 1999; Roche et al., 2003). Asia and America have *P. vivax* as the most prevalent specie (Mendis et al., 2001). In Colombia, *P. vivax* causes more than 60% of the malaria cases (Carmona-Fonseca, 2003). On the other hand, vivax malaria which is usually considered as a benign infection, has now reported complications (Beg et al., 2002; Carlini et al., 1999; Echeverri et al., 2003; Gonzalez et al., 2000; Pukrittayakamee et al., 1998; Valecha et al., 1999).

Due to increasing parasite drug resistance, intensive efforts are being made to discover new and effective antimalarial alternatives. Traditionally, plants have been a source of antimalarials. The people of Tumaco (Nariño), on the Colombian Pacific Coast, commonly use the plant *Solanum nudum* Dunal (Solanaceae) to treat fevers. Extracts of this plant have shown *in vitro* antimalarial activity against asexual blood forms of *P. falciparum* (Cardona, 1997; Saez et al., 1998). After characterization of the secondary metabolites, five steroids from extracts of the leaves and stems of the plant were reported, named SN-1, SN-2, SN-3, SN-4 and SN-5, an isomer of SN-4 (Fig. 1) (Saez et al., 1998). These compounds showed a significant growth inhibition ranging from 12% to 71% of asexual blood forms *P. falciparum*, strain FCB-2 (Pabon et al., 2002). While *in vitro* mutagenesis (Pabon et al., 2003) and *in vivo* clastogenic studies (Alvarez et al., 2004) of some *S. nudum* derivatives, were negative.

This study evaluated the ability of SN-1, SN-2 and SN-4 to block the sporogony of *P. vivax* in *An. albimanus*. The criteria used to assess the sporontocidal activity of the steroids included the percentage of mosquitoes with oocysts and the mean number of oocysts per infected mosquito.

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PREVENTION OF SPOROGONY OF P. VIVAX BY S. NUDUM

Figure 1. Molecular structure of Solanum nudum steroids. * SN-5 is a SN-4 isomer.

MATERIALS AND METHODS

Collection of plants. Authorization for collection of S. nudum Dunal (Solanaceae) was obtained from the Program International Tumaco/Colombia located in Cali (Valle-Colombia). With the help of local healers and natives, stems and leaves of S. nudum, common name ‘Zapata’, were collected on the creeks of Chagüí river of Tumaco (Nariño-Colombia) (78°30′ log. W; 1°43′15″ lat. N. 15 m altitude). S. nudum is a 2 m high shrub, with white flowers, yellow stems and green fruits which grows as a weed. Specimens were prepared and deposited at the Herbarium of the Universidad de Antioquia under voucher number 554 file 61181 of the Colombian flora catalogue. The identity of the species was carried out at the Herbarium of Universidad Nacional and Universidad de Nariño, and further confirmation was made at the New York Herbarium.

Compound preparation. Isolation and purification of the steroids from the plant has been described previously (Cardona, 1997; Pabon et al., 2002; Saez et al., 1998). Ground air-dried aerial parts were extracted by percolation with petroleum ether, followed by CH₂Cl₂ and finally with AcOEt. The obtained extract was passed through a silica chromatography column with an elution system of CH₂Cl₂/EtOAc. The structural verification of the steroids was confirmed by proton nuclear magnetic resonance spectroscopy. Since the steroids are insoluble in water (at non-toxic concentrations), polyvinyl pyrrolidone 10000 mw (PVP-10) was used. Stock solutions were diluted in distilled water and stored at 4 °C protected from light until use.

Mosquitoes and parasites. Anopheles albimanus (Buenaventura strain) was laboratory reared at the Instituto de Inmunología del Valle (Santiago de Cali, Colombia), under 80% relative humidity and at 28 °C. Females 3–4 days old and unfed with blood were used in the assays. Parasites were obtained from patients infected with P. vivax, with gametocytæmias of 0.1%–0.6%. Patients were informed of the study objectives and venous blood samples were obtained in a sterile tube containing EDTA and were kept at 37 °C until processing.

Assays. For blood samples, the plasma was removed and the packed red cells were washed twice with RPMI 1640. 150 μL of packed red cells was resuspended into non-immune human AB serum and steroids (at different doses) to a final volume of 300 μL. The control consisted of washed red cells and non-immune human AB serum. Assays consisted of four batches of 100 mosquitoes each: one for control (untreated) and the others for 50, 100 or 200 μg/mL dose of experimental steroids. PVP-10 was evaluated at 200, 400 and 800 μg/mL doses. Each preparation was transferred to an artificial membrane feeder, which was maintained at 37 °C by a circulating water bath, and was offered to a batch of mosquitoes for 30 min. The fed mosquitoes were kept for 7 days and a cotton pad moistened with 10% glucose solution was placed on top of the cages daily (Hurtado et al., 1997). The midguts were dissected 7 days after the infective blood meal and oocysts were detected microscopically after staining with 30% Mercurochrome. Experimental and control batches up to 60 midguts were dissected and the number of oocysts per midgut was determined.

Statistical analysis. Steroids and PVP-10 were assayed in duplicate and the results represent the mean of two independent assays. For steroids and controls, the mean number of oocysts and the percentage of infected mosquito midguts were calculated. Statistical differences in the mean number of oocysts were analysed by the Kruskal-Wallis test for independent variables, and
RESULTS

Eight assays were conducted; two for each compound (PVP-10, SN-1, SN-2 and SN-4), and all were successful, as determined by oocyst formation in mosquitoes of the control batch. The percentage of infected mosquitoes in the control batches ranged between 22% and 48% and the mean number of oocysts ranged between 2.1 and 9.1 (Table 1).

A statistically significant association between the dose and percentage of infected mosquitoes was observed for SN-1 and SN-2 ($p < 0.001$), whereas for SN-4 no association was found ($p = 0.109$) (Table 1, Part A). At the highest dose (200 $\mu$g/mL) of SN-1 or SN-2, only 1% of the mosquitoes were infected, while in the control batches the percentages of infected mosquitoes ranged between 28% and 39%, respectively (Table 1, Part A).

The mean number of oocysts in mosquitoes treated with either SN-1 or SN-4 was not significantly different from that of the control batch (Table 1, Part B). However, a significantly lower number of oocysts was observed in infected mosquitoes treated with SN-2 (mean of three doses: 1.3; $n = 13$) when compared with infected mosquitoes of the control batch (mean: 3.1; $n = 44$) ($p = 0.018$) (Table 1, Part B).

The group of mosquitoes treated with PVP showed significant differences in the percentage of infected mosquitoes ($p < 0.001$) but not in the mean number of oocysts ($p = 0.299$) (Table 1). The percentage of infected mosquitoes observed in the batches under treatment (experimental batches) ranged between 14% and 68% and the mean number of oocysts ranged between 5.97% and 11.64%. In the control batch, 48% of mosquitoes were infected and the mean number of oocysts was 9.11 (Table 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Infected mosquitoes with oocysts/total number of mosquitoes dissected (%)</th>
<th>$\chi^2$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 50 100 200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN-1</td>
<td>45/120 (38) 25/87 (29) 4/126 (3) 1/121 (1)</td>
<td>85.6</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>SN-2</td>
<td>44/114 (39) 3/117 (3) 9/109 (8) 1/120 (1)</td>
<td>99.3</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>SN-4</td>
<td>24/109 (22) 13/126 (10) 21/119 (18) 19/110 (17)</td>
<td>6.04</td>
<td>0.109</td>
</tr>
<tr>
<td>PVP-10*</td>
<td>74/154 (48) 104/152 (68) 11/48 (23) 31/222 (14)</td>
<td>125.1</td>
<td>$&lt; 0.001$</td>
</tr>
</tbody>
</table>

* Polyvinyl pyrrolidone 10000 MW. Doses 200, 400 and 800 $\mu$g/mL.

Table 1. Effect of S. nudum steroids on P. vivax in An. albimanus

Part A. Percentage of infected mosquitoes with P. vivax oocysts according to treatment

Table 1. Mean number of P. vivax oocysts in infected mosquitoes according to treatment

Part B. Mean number of P. vivax oocysts in infected mosquitoes according to treatment

DISCUSSION

Drugs with direct action on the sexual stages of Plasmodium (gametocytocidal) or on stages in the vector cycle (sporontocidal) are considered useful in the reduction or blocking of malaria transmission and are thus alternatives to control malaria (Butcher, 1997; Coleman et al., 1994; Coleman et al., 2001; Ponsa et al., 2003; Teklehaimanot et al., 1985).

In evaluation of the transmission blocking activity, criteria such as the percentage of mosquitoes with oocysts, oocysts diameter, percentage of mosquitoes with sporozoites and the number of sporozoites are usually used, but to date the importance of such criteria is unclear and should be discussed. The transmission blocking activity of three S. nudum steroids was evaluated using as criteria the percentage of mosquitoes with oocysts and the mean number of oocysts per infected mosquito. The steroids SN-1 and SN-2 showed a significant reduction of the percentage of mosquitoes with oocysts but only SN-2 also showed a significant reduction of the mean number of oocysts (Table 1) reducing by 90% the percentage of mosquitoes with oocysts and the mean number of oocysts by 60%.

When compound PVP-10, used to solubilize the steroids, was evaluated alone, a reduction of the percentage of mosquitoes with oocysts was seen at doses of 400 and 800 $\mu$g/mL. This situation is difficult in the analysis of the steroids activity, however, it is clear that the mixture SN-2 + PVP-10 showed greater inhibition of the percentage of mosquitoes with oocysts than the PVP-10 alone and this mixture also affected the number of oocysts, suggesting that SN-2 has transmission blocking activity. Further studies are needed to determine the influence of PVP-10 on the percentage of infected mosquitoes and how it modulates the steroids activity.

Although the steroid SN-1 reduced the percentage of infected mosquitoes, no difference was observed in the mean number of oocysts when compared with controls. This suggests that SN-1, as well SN-4, have no

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apparent effect on blocking malaria transmission. This is common to most of the antimalarials available at present, only primaquine, tafenoquine and some artemisinine derivatives have been shown to block transmission (Butcher, 1997; Coleman et al., 2001; Ponsa et al., 2003; Price et al., 1996).

These experiments did not discriminate among the different forms of the parasite (gametocyte, gamete, zygote or ookinete). However, taking into account that gametocytes are the first stage to enter in direct contact with the compound it is proposed that the effect observed may be gametocytocidal. A similar observation was also made by other authors with different compounds (Coleman et al., 2001).

Few methods have been developed for evaluating the ability of compounds to block transmission (Coleman et al., 2001; Teklehaimanot et al., 1995) and the majority of them have been done in murine Plasmodium or P. falciparum (Butcher, 1997). In this work, the method was applied to P. vivax in An. albimanus, a species of Plasmodium responsible for most cases of malaria in Asia and America (Carmona-Fonseca, 2003; Mendis et al., 2001). Coleman et al. (2001) determined the efficacy of seven compounds against P. vivax in An. dirus, but due to the lack of a continuous culture of P. vivax, they had to expose volunteers direct to biting mosquitoes (Coleman et al., 2001; Ponsa et al., 2003). Our method avoids direct human exposure to mosquitoes and allows for direct evaluation of activity of different compounds on clinical isolates of P. vivax. This protocol allows for direct evaluation of the activity of steroids on clinical isolates as does the Coleman et al. (2001) method.

The steroids isolated from S. nudum displayed an antiplasmodial effect against blood stages of P. falciparum (Pabon et al., 2002). The results suggest that steroids of S. nudum have potential as transmission blocking agents against P. vivax.

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