HPD-INDUCED REVERSAL OF CHLOROQUINE RESISTANCE OF MALARIA

F. SOGANDARES-BERNAL**, J.L. MATTHEWS** & M.M. JUDY*

Hematoporphyrin Derivative (HPD) binding to P. berghei in the host and in vitro, and to P. vivax and P. falciparum in vitro, has been demonstrated by fluorescence and laser techniques. The binding of chloroquine to HPD has been demonstrated by fluorescence in P. berghei while in the host. Furthermore, resistance of P. berghei to chloroquine has been reversed by treatment with HPD followed by chloroquine.

Hematoporphyrin was first used in photodynamic studies by Hausmann (1908). He used hematoporphyrin hydrochloride as prepared earlier by Nencki & Zaleski (1900). Schwartz, Absolon & Vermund (1955) noted that the hematoporphyrin produced by the Nencki & Zaleski (1900) method was very crude and produced a more refined compound, hematoporphyrin derivative (HPD). They used an acetic acid-sulfuric acid mixture to acetylate hematoporphyrin they hydrolyzed this preparation under basic and near neutral conditions. Lipson, Baldes & Olsen (1961) prepared HPD, observed its fluorescence in tumors and, along with his colleagues (Lipon, Gray & Baldes, 1966), used this substance to treat tumors by photodynamic methods. By 1975's, Dougherty et al. reported eradication of mouse mammary tumors using HPD and red light (600-700nm). It was not until the early 1980's that the results of serious efforts concerned with the in vivo biological activities of the components of HPD appeared in the literature (Berenbaum, Bonnett & Scourides, 1982). Fig. 1 from Berenbaum, Bonnett & Scourides (1982) shows the structure and names of identified components of HPD prepared from hematoporphyrin dihydrochloride in DMSO/bicarbonate buffer.

Fig. 1: Structures and name of identified components of HPD (after Berenbaum, Bonnett & Scourides, 1982).

* Baylor Research Foundation (BRF), 3600 Gaston Avenue, Dallas, Texas 75246, USA.
** BRI and Department of Biology, Southern Methodist University, Dallas, Texas 75275, USA.

Centers for Infectious Disease and Laser Research, Baylor Research Foundation.
Fig. 2 shows Bonnett's et al. (1981) previous findings by HPLC analysis. By 1983, it is clear that at least three groups (Dougherty et al., 1983; Kessel & Chou, 1983; and Moan & Sommer, 1983) showed that in addition to the porphyrin monomers in HPD mentioned previously, some of the porphyrins are also present as dimers, oligomers, and aggregates under physiological conditions in vitro and in vivo. Berenbaum, Bonnett & Scourides (1982) had already postulated covalent bonding in dimer and oligomer formation. It has been stated by Moan & Sommer (1983) that components of HPD may be classified into three groups: 1) Monomers with a high fluorescence quantum yield, and sharp HPLC peaks, which are rapidly concentrated by cells in vitro; 2) Dimer and oligomer components with lower fluorescence quantum yield, and sharp HPLC peaks, which are more slowly (18h) incorporated into cells in vitro; and 3) Aggregated components of low fluorescence quantum yield with short retention times on a P-10 column and broad unresolved HPLC peaks, which when administered to patients are found concentrated in tumor cells in vivo.

Fig. 2: Preparative separation of components of HPD by HPLC (after Bonnett et al., 1981). The major components are identified by analytical HPLC comparisons and spectroscopic methods. Because of variations in $\lambda_{\text{max}}$ with structure, peak areas do not represent relative molar amounts of the various compounds.
It is not the purpose, nor the intent, of this presentation to review the extensive literature of hematoporphyrins, but the aforementioned serves to indicate to this audience that the HPD used in our experiments is a mixture of different compounds. In a manner similar to the study of HPD substances in cancer photodynamic therapy, it will be necessary to isolate the active components concerned with the results which we will demonstrate in the following.

The hypothesis has been set forth by others that the 4-aminoquinoline, chloroquine, binds to *Plasmodium berghei* ferriprotoporphyrin IX and interferes with the ionic gradient of malaria-infected red blood cells, resulting in lysis of the host cell (Chou & Fitch, 1980, 1981a, b; Chou, 1980; Chevi & Fitch, 1980; Fitch & Chevi, 1981). Jeannipatkul & Panijpan (1980) have confirmed the binding of chloroquine to ferriprotoporphyrin IX and Sirawaraporn, Panijpan & Yuthavong (1982) have demonstrated chloroquine-binding to the parasite membranes. Yuthavong (1980) has also indicated that most of the binding to chloroquine is in the parasite and not in the host cell. Chevi & Fitch (1980) have suggested that ferriprotoporphyrin IX-chloroquine compounds delay the transformation of ferriprotoporphyrin IX into malarial haeme pigments. It is difficult at this time to provide generalizations for the mode of action of chloroquine which would apply to all species of mammalian malaria. On the one hand, certain strains of chloroquine
resistant malaria such as \textit{P. berghei} do not accumulate haeme pigments, leading Fitch and his co-workers (supra cit) to assume that the chloroquine resistance may be due to the absence (or perhaps reduction) of the chloroquine binder ferritrotoporphyrin IX. On the other hand, there are highly chloroquine resistant strains of \textit{P. falciparum} which digest host cell haemoglobin and incorporate it as haeme pigment. These different features suggest, perhaps, either different reasons for chloroquine resistance by malaria, or point to the distinct possibility that we do not know enough of the different haeme substances and their transitional forms in the living parasite.

The availability of Photofrin I\textsuperscript{®} (Hematoporphyrin Derivative) provided by Photofrin, Inc., a subsidiary of Johnson and Johnson Co., has led us to examine the possibility that any one of these compounds might be incorporated by the malarial parasite. Preliminary experiments demonstrating the incorporation of HPD by the parasites \textit{Plasmodium berghei in vitro and in vivo}, and \textit{P. vivax} and \textit{P. falciparum in vitro} are reported. Reported are also preliminary studies showing that HPD-Chloroquine is effective in the reduction of parasitemia in mice infected with chloroquine resistant \textit{P. berghei}.

MATERIALS AND METHODS

Source of HPD — HPD in concentrations of 5 mg/ml was obtained from Photofrin Inc. The compound listed as Photofrin I\textsuperscript{®}, a proprietary one, was at the time under Stage 3 U.S. FDA license for experimental use in cancer therapy of human beings.

Source of Mouse Malaria — \textit{P. berghei} NYU strain was originally obtained from Dr. R.O. McAlister, formerly of the Department of Biology, Southern Methodist University (SMU). This strain had been consistently maintained at SMU in ICR/Timco mice for a series of 575 subpassages prior to our using the parasites. The infections (3x10\textsuperscript{6} parasites) are currently passed i.p. by syringe every seven days into each ICR/Timco mice weighing approximately 30 g. The mice are fed Rodent Blox (Wayne Petfood Division, Continental Grain Co., Chicago) and watered \textit{ad libitum}. \textit{P. berghei} red blood cells from the mice were collected in sterile heparinized tuberculin syringes by heart puncture of previously CO\textsubscript{2} anesthetized mice, then introduced into sterile tubes and covered with screw caps.

Source of Human Malaria — \textit{Plasmodium vivax} and \textit{P. falciparum} were collected by heparinized Vacutainer\textsuperscript{®} from the cubital veins of infected human beings in Amazonas Territory of Venezuela, in or near the vicinity (within 60 km) of Puerto Ayacucho which borders the Orinoco River. Infected patients were identified by personnel of the Venezuela Ministry of Health (VMH) who immediately radioed the presence of a patient, prior to treatment, and we were driven to the site where a blood sample was collected prior to treatment. In other instances, we visited villages in the company of personnel from the Malaria Division and conducted our own surveys. Infected blood was transported to Puerto Ayacucho where it was processed for study.

Demonstration of Binding of HPD to Malaria: Exposure of Malaria to HPD — \textit{In vitro} exposure of red blood cells containing the malarial parasites was done simply by introducing HPD in various dilutions (sterile 0.85% NaCl diluent) directly into sterile capped tubes containing aliquots of heparin-treated blood from the infected patients or from infected ICR/Timco mice.

\textit{In vivo} exposure of the malaria parasite were conducted in mice only and consisted of i.p. exposure of infected (experimental) and uninfected (control) mice to doses of 12.5 mg/kg of HPD at different time intervals post infection. A second control group of mice consisted of those not administered the drug. Blood for experimental and control groups was either collected from the tip of the tail or by heart puncture using a 25 ga needle mounted on heparinized tuberculin syringes.

Fluorescence — Blood samples of control and experimental groups were incubated in the dark for varied time periods at room temperature. Blood cells were gathered by sterile Pasteur pipette and a drop of blood placed on 25 mm x 75 mm microscope slides and thin smears prepared in the conventional manner. The slides were air-dried and immediately placed into black plastic light-tight slide boxes until examined by fluorescence microscopy at most within a few hours of preparation, except for those brought to the U.S. for further and more detailed examination.

Fluorescence was demonstrated in a darkened room in the field by darkfield transmitted fluorescence with the aid of a Leitz Labolux microscope fitted with an HBO 200 burner and 400 nM excitation filter and 570 nM absorbance filter. Smears prepared in the U.S. were examined
with the same Labolux microscope or by reflected fluorescence using Zeiss Photomicroscope, Leitz Ortholux, and Olympus Vannox-T microscopes equipped with the equivalent burner and filters indicated above (except in the case of the Zeiss photomicroscope in which a 460nM long-wave pass filter was used). In any case, low fluorescence (Cargille Type A, no 23 e 1.5180) immersion oil (Cargille Laboratories, Cedar Grove, N.J.) was applied directly to the blood film in the traditional manner and the preparations were examined at magnifications of 1000X to 1500X.

Laser Activation — HPD is a photopactive compound and will release singlet oxygen when excited by appropriate wavelength radiation (Weisshaut, Gomer & Dougherty, 1976). A helium-neon laser (632.8nM) with power of 7.0 milliwatts was fitted with a lens constructed from a solid glass rod so that the beam could be focused along the entire length of a microhematocrit tube which was then rotated in the same plane as the beam. Preparations of sterile 0.85% NaCl-washed red blood cells from patients infected with P. vivax (estimated 5% parasitemia of RBCs) were drawn into standard unheparinized microhematocrit tubes, then irradiated with the laser for different time intervals. After irradiation of controls (uninfected RBCs treated with HPD) and experimental samples, the samples were centrifuged and the microhematocrit values observed, as well as the color of the supernatant solution. Blood from infected (estimated 40% parasitemia of RBCs) and uninfected mice previously treated with HPD by different regimens was collected, as described above, and irradiated as described for washed human RBCs. It was assumed that the microhematocrit values would be lowered in those samples in which the RBC membranes were altered due to damage by singlet oxygen. Furthermore, the presence of hemoglobin freed from the RBC could be easily detected visually in the supernatant solution. As a tool for assessment of hematocrit change, a microhematocrit index was developed:

\[
\text{Microhematocrit Index} = \frac{H(O) - H(R)}{H(O)} = \frac{\text{Hematocrit Before Irradiation} - \text{Hematocrit After Irradiation}}{\text{Hematocrit Before Irradiation}}
\]

Development of Chloroquine-Resistant Mouse Malaria — Mice dying of P. berghei infection were administered different dosages of Aralen (chloroquine hydrochloride) in physiological saline. Malaria was isolated from certain survivors and subpassaged to uninfected ICR/Timco mice. This procedure was continued for several months, each time increasing the number of chloroquine exposures of the infected mice until the malaria could tolerate at least three daily i.p. injections of 0.4mg/kg each without affecting the lethal outcome of the infection. Malaria obtained as described above was designated chloroquine-resistant P. berghei.

HPD-Chloroquine Binding by Fluorimetry and In Vivo — Fluorimetry — Varied quantities of HPD were reacted with varied amounts of chloroquine in quartz cuvettes. The quenching of HPD fluorescence (405nM excitation, 615nM emission) was observed as the concentration of chloroquine was increased relative to HPD. In Vivo — Mice infected for 10 days were administered HPD doses 12.5mg/kg 4hrs. prior to the administration of chloroquine (0.4mg/kg body wt.). A tail blood sample was smeared on slides prior to and after the administration of chloroquine to determine if the same HPD-chloroquine fluorescence quenching effect observed in vitro could be duplicated in vivo.

Exposure of Chloroquine-Resistant P. berghei to HPD With and Without Chloroquine — Mice ICR/Timco (30g) were administered a dose of approximately 3x10⁸ parasites i.p. Several groups were run with different drug regimens, but the groups reported here for demonstration purposes were purposes were composed of five mice each. These groups are special in that the inoculum used to infect all mice was derived from a single infected mouse.

RESULTS

Demonstration of HPD Binding by Fluorescence — Fig. 4 shows a malarial organism, P. berghei, from an animal treated with HPD, i.p. 4hrs. after exposure. Fig. 5 shows the same organism viewed (in Fig. 4) with the aid of the fluorescence microscope (400nM excitation, 570nM absorbance). Fig. 6 shows P. berghei from an infected animal not receiving HPD. Fig. 7 shows the same organism (in Fig. 6) viewed with the aid of the reflected fluorescence microscope (400nM excitation, 570nM absorbance). Note that there is no parasite autofluorescence. Problems with the microscope camera system in Venezuela precluded our obtaining adequate photographic evidence of in vitro binding of HPD to P. vivax and P. falciparum, but visual observations were as for P. berghei.
Table I and II shows the results of laser activation coupled with \( \bar{x} \) packed cell volume % (i.e., microhematocrit) as a means of analyzing in vitro binding of HPD to malarial organisms in man and the \( \bar{x} \) packed cell volume % index in the mouse. Table III shows binding in vivo (i.e., in the infected host) in the mouse demonstrated by laser activation coupled with \( \bar{x} \) packed cell volume % index.
REVERSAL OF CHLOROQUINE RESISTANCE BY HPD

TABLE I

<table>
<thead>
<tr>
<th>Group (No. of individual samples)</th>
<th>Condition of host</th>
<th>Irradiation Dose (J/cm²)</th>
<th>( \bar{x} ) Packed Cell V %</th>
<th>Hemolysis (% of Samples)</th>
<th>Approx. % RBC's infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (7)</td>
<td>uninfected</td>
<td>0</td>
<td>44</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 - 16</td>
<td>45 S.D. ± 1.27</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>2 (7)</td>
<td>infected</td>
<td>0</td>
<td>35.17 S.D. ± 1.6</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.2 - 16.7</td>
<td>33.0 S.D. ± 1.0</td>
<td>100</td>
<td>5</td>
</tr>
</tbody>
</table>

TABLE II

<table>
<thead>
<tr>
<th>Group No. **</th>
<th>Condition of RBCs*</th>
<th>Irradiation Dose (J/cm²)</th>
<th>Change in Hematocrit (H(0) - H(R))/H(0)</th>
<th>Approx. % RBCs infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T</td>
<td>0</td>
<td>0.39 ± 0.07</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.2</td>
<td>0.44 ± 0.07</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>TW</td>
<td>0</td>
<td>0.17 ± 0.07</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>0.55 ± 0.07</td>
<td>40</td>
</tr>
</tbody>
</table>

* Treated with HPD (T); Treated with HPD and washed and resuspended to original volume in 0.85% NaCl (TW); **Group n = 10 @

TABLE III

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Irradiation Dose (J/cm²)</th>
<th>HPD Administration Days Pi/hrs pre-irradiation</th>
<th>Change in Hematocrit (H(0) - H(R))/H(0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>13 Da infection no HPD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>0.08 ± 0.07*</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td></td>
<td>0.04 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td></td>
<td>-0.04 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>0.08 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>13,16 Da/3 hrs.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td></td>
<td>0.39 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td></td>
<td>0.27 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>0.43 ± 0.07</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>13,16 Da/1 hr.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>0.26 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td></td>
<td>0.30 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td></td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td></td>
<td>0.30 ± 0.07</td>
</tr>
</tbody>
</table>

* S.D.

Fig. 8 shows the results of the percentage of RBCs infected in chloroquine-resistant mouse malaria as compared with the RBCs of animals untreated/treated with chloroquine alone, and HPD and chloroquine in different time regimens. Fig. 9 demonstrates mouse malaria which has bound HPD in vivo, while in the host RBCs. Fig. 10 shows the quenching of fluorescence
observed following the administration of chloroquine to the same previously HPD-treated infected mouse. The results demonstrated above substantiate the fluorometric findings *in vitro* where it was found that addition of chloroquine of up to $5 \times 10^{-5}$ M concentration to $5 \times 10^{-3}$ g/lit HPD in 0.07M phosphate buffer resulted in quenching to 35% of its initial value.

Fig. 8: Percentage of mouse erythrocytes infected in *P. berghei* Group 1 represents no HPD, Group 2 chloroquine only, Group 3 HPD only, Group 4 HPD and chloroquine da 3 of malaria, Group 5 HPD and chloroquine da 5 of malaria and Group 6 HPD and chloroquine da 7 of malaria.

Fig. 9: Fluorescence of *P. berghei* following i.p. administration of HPD to mouse host. Unstained preparation. 400nM excitation, 460nM longwave length pass filter. Fig. 10: Quenching of fluorescence observed after administration of chloroquine following HPD exposure of an infected mouse.
DISCUSSION

The preliminary experiments reports herein show that HPD component(s) bind to P. berghei, in vitro and in vivo, and in vitro to P. vivax and P. falciparum. The specific component(s) of HPD responsible for the binding has (have) not yet been identified, but is suspected to be one of the monomers due to its rapid uptake by the malaria cells. This suspicion is born out of Moan & Sommer's (1983) observation that monomers of HPD have high fluorescence quantum yield, sharp HPLC peaks, and are rapidly concentrated by cells in vitro. We noted both high fluorescence quantum yield and rapid uptake by malarial cells in vitro.

Results of microhematocrit studies in the mouse (Tables II and III) show a reduction in the hematocrit index levels when HPD is presumably bound to the parasites and irradiated with a red laser-light. This may reflect the destruction of infected RBCs when activated by laser light with subsequent singlet oxygen release and destruction of the infected RBC membranes. The mice in question had 40% parasitemia levels. It may be argued that the decrease in the hematocrit indices after irradiation demonstrates binding of HPD to the malaria parasite. Such differences are not as clear with the human malaria (Table I) since the parasitemia level was estimated to be about 5%. This low number of infected cells, as compared with the mouse model is within the error to be expected by the microhematocrit method used. Hemolysis was, however, noted in all of the HPD treated samples which were irradiated, suggesting that if the parasitemia levels would have been higher, microhematocrit differences between controls and experiments would have been more obvious.

Chloroquine has been demonstrated to bind chemically to HPD component(s) by fluorimetry and NMR techniques. Moreau et al. (1985) has measured shifts in NMR spectra of chloroquine upon the addition of monomeric porphyrin in HPD. The model has been interpreted as the chloroquine binding to the porphyrin in such a way that the ring of each species are stacked face to face. Decreased HPD fluorescence upon binding with chloroquine would be consistent with the Moreau et al. (1985) model. Quenching of HPD fluorescence demonstrated by fluorometric techniques is in agreement with findings in vivo where HPD taken up by P. berghei residing in circulating red blood cells can be quenched by the administration of chloroquine.

Utilizing the mouse model, the parasitemia by P. berghei selected to resist a minimum of three daily doses of 0.4mg/kg of chloroquine demonstrates a drastic reduction when treated with HPD prior to the administration of chloroquine.

Further work is being conducted in our laboratories dealing with the specific component(s) of HPD responsible for binding to the malarial organisms and to chloroquine.

ACKNOWLEDGEMENTS

A study of this type has numerous colleague-contributors. We remain forever indebted to Ciudadano Dr. Jose L. Gonzalez Herrera, at the time Minister of Health and Social Assistance, Venezuela, for his friendship, hospitality, and personal aid in the field.

Other Venezuelan colleagues who aided us in Puerto Ayacucho include: Ciudadanos Drs. Luis Rojas P., Medico Director Sub-Regional T.F.A.; Armando Gomez, Medico Adjunto Administrativo; Juana Pimentel, Medico Rural Isla de Raton; Messrs. Otilio Luna, Enfermero Salud Publica; Alfredo Lefebre, Inspector Sanitario; Miguel Conde, Inspector Sanitario; Evaristo Dopa, Auxiliar de Enfermeria Isla de Raton; Felix Galletti, Motorista Isla de Raton, Ciudadano Dr. Rolando Sifontes F., former Director, and Ciudadana Edelmira Mora, Technician, The Venezuelan Malaria Division in Maracay worked in the field with us and provided us highly valued assistance, coordination, and advice. Ciudadanos and Professors Maria C. de Blanco, former Director of the Instituto Nacional de Higiene and Arnoldo Gabaldon, former Director of the Venezuelan Malaria Division, remain valued friends and advisors in Venezuela.

Studies in the United States were aided by Ms. Joan Roberts, Supervisor of Animal Facilities at Southern Methodist University. Messrs. Richard Thompson and K.R. Weishaupt, and Dr. T.J. Dougherty helped us in numerous ways.

The financial aid of Photofrin, Inc. is hereby acknowledged.

REFERENCES


