INHIBITION OF A SPECIFIC ANTIMALARIAL MOLECULAR TARGET AND CORRELATION WITH THE ACTIVITY AGAINST *P. falciparum* AS SELECTION CRITERIA OF POTENTIAL ANTIMALARIALS FROM NATURAL SOURCES

Katalina Muñoz [*], Jelver Sierra[1], Geyson Fernandez [1], Fernando Alzate [1], Gabriel J. Arango [1], César Segura [2], Karent E. Bravo [1].

[1]Grupo de Investigación en Sustancias Bioactivas –GISB- Corporación Académica para el Estudio y Control de Patologías Tropicales CAPET. Facultad de Química Farmacéutica. [2]Grupo de Malaria. Universidad de Antioquia.

Summary

Malaria is the world's most important parasitic infection, ranking among the major health and developmental challenges for the poor countries of the world (1). It is a disease caused by parasites of the genus *Plasmodium* and it is the responsible of almost 2.7 millions of deaths each year. One of the different strategies in order to find new treatment alternatives is using the pharmacological knowledge available about mechanism of action of antimalarials available, as well as potential targets to be attacked in the discovery of new molecular entities from our biodiversity. In this work two species of Colombian flora were studied, *Monnina angustata* (Polygalaceae) and *Symbolanthus pterocalyx* (Gentianaceae), these species have restricted growing in Colombia and its biogeography limits. A bioguided study was developed according to which the chemical fractionation was carried out looking for the ability for inhibit β-hematin formation, synthetic substance identical to hemozoin which is formed inside the food parasitic vacuole as heme detoxification strategy. The inhibition of the β-hematin formation triggers oxidative stress process mediated by monomeric and dymerics forms of ferriprotoporphirin IX that results in highly toxic effects for parasite. This model was used in order to determine the inhibition percentage and that IC_{50} of extracts and fractions compared against chloroquine. Finally, the activity of the fractions which were inhibitor of β-hematin formation was correlated with other model that allow make a radioisotope determination of cellular viability using FCB-1 strain of *P. falciparum* cultured in presence of radiolabeled hypoxanthine. It was obtained an important correlation between the activity against the molecular target and on the parasite (r_p=0.7186, valor P*).

Key words: hematin, antimalarial, natural products, *Monnina angustata* and *Symbolanthus pterocalyx*

Methods

Identification of plants and extract preparation Both plants *Monnina angustata* and *Symbolanthus pterocalyx* were collected and plenty identified by a biologist expert in plant taxonomy. The samples are stored in the University of Antioquia Herbarium (Herbario Universidad de Antioquia) HUA imens are Part of plants (2-3 Kg) selected for biological study were dried, ground and submitted to a maceration process with Hexane for 48 h at 25 °C. The Extract obtained was evaporated under vacuum.

Inhibition of β-Hematin formation assay

This assay is performed according to Baelmans et al. method, lightly modified. In a series of Eppendorff tubes are mixed 100μL of haemin (Sigma) solution 6.5 mM previously dissolved in 0.2 N NaOH, 50μL of glacial acetic acid, 50μL of H_2O and 200 μl buffer sodium acetate 3 M, everything is incubated at 60°C for 60 minutes in water bath in order to allow the formation of β-hematin. Then the tubes are centrifuged at 12000 RPM for 15 minutes and the supernatant is discarded. The pellet obtained must be washed twice with DMSO and discarded. The final pellet is dissolved in 600 μL of NaOH 0.1 N, an aliquot is diluted in a final volume of 3.000 μL NaOH 0.1 N and monitored by its absorbance at 360nm by an spectrophotometer (Spectronic Genesys 2). In the case of testing samples the amount of water is replaced by the sample dissolved in DMSO. The results are expressed as percentage of inbhibition of β-hematin formation following the next equation:

$$\%\,Inhibition = 100\,x\left[1-\left(\frac{Ab_{\,sample}}{Ab_{\,control}}\right)\right]$$

Where Ab_{sample} is the absorbance of the sample which is testing and $Ab_{control}$ is the absorbance of the control. The inhibitory activity is expressed as IC50 and is calculated by

GraphPad Prism demo version 4.00 for Windows (GraphPad software, Inc, San Diego CA 2003).

Parasite Culture

P. falciparum strain FcB1/Colombia, were maintained in continuous culture on human erythrocytes in RPMI medium containing 7% (v/v) heat-inactivated human serum under an atmosphere of 3% CO_2, 6% O_2, 91% N_2, at 37°C, as described by Trager and Jensen (2).

Anti-*Plasmodium* activity

Susceptibility assays were performed using a modification of the semi automated microdilution technique of Desjardins *et al* (3) Stock solutions of test compounds were prepared in DMSO. Those solutions were serially diluted twofold with 100 µl culture medium in 96-well plates. Asynchronous parasite cultures (100 µl, 1 % parasitemia and 1 % final hematocrite) were added to each well and incubated for 24 hours at 37°C prior to the addition of 0.5 µCi of [^{3}H] hypoxanthine per well. After a further incubation of 24 hour, plates were frozen and thawed. Cell lysates were then collected onto glass-filter papers and counted in a liquid scintillation spectrometer. The growth inhibition for each sample concentration was determined by comparison of the radioactivity incorporated in the treated culture with that in the control culture (having the same final % of DMSO) maintained on the same plate. The concentration causing 50% growth inhibition (IC_{50}) and 90% growth (IC_{90}) were obtained from the drug concentration-response curve and the results were expressed as the means ± the standard deviations determined from several independent experiments. The DMSO concentration never exceeded 0.1% (v/v) and did not inhibit the parasite growth.

Mammalian cell culture and harvesting

Human cell line U937 was cultured under standard conditions in RPMI 1640, supplemented with 10% fetal bovine serum (FBS). Cells were subcultured each two days, prior to each experiment, cultures were collected and at least 2 x10^6 cells were seeded into 75 cm^2 flasks and cultured for 24 h.

Cytotoxicity assay

The MTT assay is based on the protocol described for the first time by Mosmann (4) and optimized for the cell line used in the experiments. Cells undergoing exponential growth were harvested, and seeded in 96-well microplates at a density of 1 x 10^4 cells per well, thereafter cells were added with the test fractions prepared in fresh medium and incubated for 48 h, when treatment were replaced with fresh medium containing MTT 0.83 mg/ml and incubated at 37°C for 4 h. The formazan was dissolved by adding DMSO. Optical densities were read at 550 nm with background subtraction at 630 nm in a Power Wave X microplate reader. The ‘‘control’’ was the absorbance of cells in medium only, and assigned 0 % in plots of toxicity.

MTT assays were performed in triplicated wells at least three times each. Results of IC50 were interpolated of a toxicity regression curve and are presented as mean of triplicates and standard deviation.

Results and Discussion

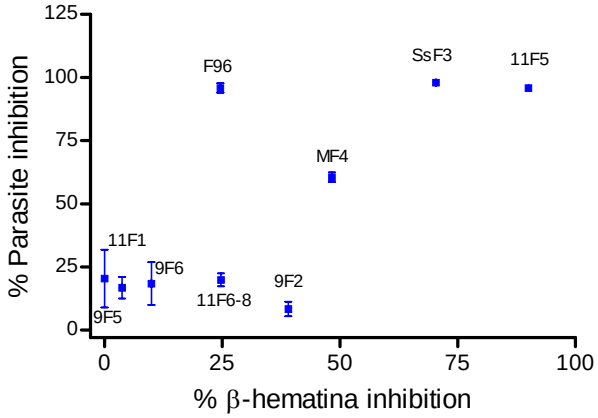
Understanding the mechanism of action of known drugs is an important priority (5). Complex formation between chloroquine/ferriprotoporphyrin IX (Fe(III)PPIX) and artemisinins/(Fe(III)PPIX) have been widely studied and characterized by using

different strategies like U.V spectrophotometer, ESI/MS and NMR (6-9) , those interactions appears to involve inhibition of the incorporation of Fe(III)PPIX into hemozoin, the detoxification process used by malaria parasite in order to avoid the damage caused by heme in its monomeric and dymeric form.

Using the model of β-hematin inhibition as a screening bioguided of potential antimalarials from natural products, results a very interesting option in order to make correlations with the activity against the parasite. This model has shown to be a good tool when the species were selected and the different fractions that were tested. Once we analyzed the activity between both models it is possible to notice a correlation statistically significant (r_p=0,7186, value P*) (Graphic 1). However the use of this model should not be understood as a direct measure of an antiplasmodial activity (Table 1), in fact it can be observed the case of the sample F96 where no correlation is observed between β-hematin inhibition 24,625% ± 0,079 and antiplasmodial activity but a high antiplasmodial activity 95,846% ± 1,873, it can be due to a different mechanism of action in any case related with inhibition detoxification. The IC_{50} for Choroquine in β-hematin inhibition model correspond to 1,837 mg/mL ± 0,006761 (data not shown). The citotoxicity over human cell line U937 was determined just for 11F5, 9F96, MF4 and was no possible to determine for SsF3 because of solubility problems (Table 2), it is important to notice that antiplasmodial value of IC_{50} in all cases is lower than the citotoxicity one, this parameter to have into a count for selectivity.

Table 1. Data of percentage of inhibition of β-hematin formation and *P. falciparum* inhibition growing

Fraction	% inhibition β-hematin (2,5 mg/mL)	%Inhibition parasite (50 µg/mL)
	Mean	Mean
11F1	3,729 ± 0,034	16,816 ± 4,251
11F5	90,028 ± 0,037	95,808 ± 1,050
11F6-8	24,719 ± 0,069	19,873 ± 2,533
9F2	39,017 ± 0,007	8,324 ± 2,878
9F5	0	20,452 ± 11,414
9F6	9,937 ± 0,115	18,416 ± 8,478
9F96	24,625 ± 0,079	95,846 ± 1,873
SsF3	70,403 ± 0,055	97,989 ± 0,823
MF4	48,277 ± 0,059	60,519 ± 1,927



Graphic 1. Pearson Correlation test between inhibition of β-hematin formation and antiplasmodial activity

Table 2. Antiplasmodial activityIC_{50} (μg/mL) and Citotoxicity U937 IC_{50} (μg/mL)

Fraction	Antiplasmodial activityIC_{50} (μg/mL)	Citotoxicity U937 IC_{50} (μg/mL)
11F5	27,55±1,58	83,87 ± 12,55
9F96	36,23 ± 13,52	212,23±0,33
SsF3	11,97± 4,14	ND
MF4	37,17± 8,49	58,59 ± 6,96

Reference

1. Sachs J, Malaney P: The economic and social burden of malaria. Nature 2002;415(6872):680-685.
2. Trager W, Jensen JB: Human malaria parasites in continuous culture. Science 1976;37:673-675.
3. Desjardins RE, Canfield CJ, Haynes JD, Chulay JD: Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother 1969;16(6):710-718.
4. Mosmann T: Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods 1983;65(1-2):55-63.
5. Olliaro P, Wirth D: New targets for antimalarial drug discovery. Journal of Pharmacy and Pharmacology 1997;49(SUPPL. 2):29-33.
6. Egan TJ: Interactions of quinoline antimalarials with hematin in solution. Journal of Inorganic Biochemistry 2006;100(5-6):916-926.

7. Messori L, Piccioli F, Eitler B, Bergonzi MC, Bilia AR, Vincieri FF: Spectrophotometric and ESI-MS/HPLC studies reveal a common mechanism for the reaction of various artemisinin analogues with hemin. Bioorganic & Medicinal Chemistry Letters 2003;13(22):4055-4057.
8. Robert A, oit-Vical F, Meunier B: The key role of heme to trigger the antimalarial activity of trioxanes. Coordination Chemistry Reviews 2005;249(17-18):1927-1936.
9. Robert A, Coppel Y, Meunier B: NMR characterization of covalent adducts obtained by alkylation of heme with the antimalarial drug artemisinin. Inorganica Chimica Acta 2002;339:488-496.