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Original Article

The Potential of β Carbolin Alkaloids to Hinder Growth and Reverse Chloroquine Resistance in *Plasmodium falciparum*

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Abstract

Background: Nowadays, scourge of malaria as a fatalistic disease has increased due to emergence of drug resistance and tolerance among different strains of *Plasmodium falciparum*. Emergence of chloroquine (CQ) resistance has worsened the calamity as CQ is still considered the most efficient, safe and cost effective drug among other antimalarials. This urged the scientists to search for other alternatives or sensitizers that may be able to augment CQ action and reverse its resistance.

Method: Three β-carbolin derivatives, namely, harmalin, harmol and harmalol were tested for their anti-plasmodial and CQ resistance reversal effects against *P. falciparum* 3D7 and K1. SYBRE Green-1 based drug sensitivity assay and isobologram analysis were used to screen the mentioned effects respectively.

Results: All of them showed moderate anti-plasmodium effect and harmalin was the most effective as compared to the others in reversing CQ resistance and tolerance.

Conclusion: The mentioned phytochemicals are not ideal to be used as conventional anti-malarials and only harmalin can be suggested to reverse CQ resistance in *P. falciparum* K1.

Introduction

β-carbolins are natural indol alkaloids with a common tricyclic pyrido-indol ring. They were isolated from a herbaceous perennial plant called *Peganum harmala* family (Zygophillacea)-(Syrian rue or harmal) (1).The plant had been used extensively in the traditional medicine of Middle east, India, China and Africa as emmenagogue, hallucinogen, snuff, abortifacient, antidepressant, anti-inflammatory, antimalarial, anti-leishmania and anti-microbial (1). Nowadays, many β-carbolins have been found to possess antibacterial, antiviral, antiparasitic and anticancer activities (1).

Chloroquine (CQ) is still the safest, cheapest and the most efficient among other antimalarials, (2) but unfortunately it started to lose its token as a potent drug due to emergence of chloroquine resistant and tolerant strains of *Plasmodium falciparum*. The former is associated with loss of drug response to higher doses while the latter is accompanied by higher incidence of the disease reoccurrence (3, 4). This issue has urged the scientist to search for other alternatives or agents that can chemosensitize CQ and reduce its resistance or tolerance (5).

In this communication, both the anti-plasmodium and CQ resistance reversal potencies of three different β-carbolins alkaloids; harmalin, harmol and harmalol were tested against both 3D7 (CQ sensitive) and K1 (CQ resistant) strains of *P. falciparum*.

Materials and Methods

Parasite culturing, maintenance and synchronization

Both CQ sensitive and resistant strains of *P. falciparum K1* and *3D7* were cultured in O+ red blood cells suspended in a Complete Malaria Culture Medium (cMCM) containing RPMI-1640, 25 mM HEPES (pH 7.4), 0.75 mM hypoxanthine, 0.5% albumax, 24 mM sodium bicarbonate, 11 mM glucose and 50 µg/L gentamicin. PH was maintained at 7.4 and the hematocrite level at 2%. The culture was incubated at 37 °C in a micro-aerophilic atmosphere containing 90% N_2, 5% CO_2 and 5% O_2. Furthermore, the medium was changed every 24 h and parasitemia was monitored using Giemsa stained thin blood smears (6, 7). Prior to drug screening, the parasites were synchronized using sorbitol synchronization technique described by (8).

Stock solution preparation

Stock solutions of 100 mM of chloroquine and each of the mentioned phytochemicals were prepared in PBS (pH 7.4) and a mixture of [methanol: Dimethylsulphoxide (DMSO) (1:1)] respectively.

Malaria drug sensitivity assay

Malaria drug sensitivity assay was performed according to previous study (9). Aliquots of 50 µl of PRBCs suspension (synchronized at the ring stage at 2% parasitemia and 2% Hct) were uploaded to a 96 well flat bottomed microtiter plates; featured serial dilution of CQ or each phytochemical (1 nM to 1 µM) as well as drug, RBCs and PRBCs controls wells, were incubated for 48 h at 37 ºC with PRBCs (synchronized at the ring stage with 2% parasitemia and 2% Hct). Drug dilution was done so that DMSO concentration did not exceed its cytotoxic threshold against P cells and RBCs (<0.5%). Three plates were prepared for each drug and each dilution was done in triplicate. At the end of the incubation period, the plates were freeze-thawed and then 100 µL of SYBR green-I lysis buffer was added to each well. The mixture was incubated at room temperature for 1 h and finally the fluorescence was measured after 15 seconds of plate agitation (twice) in Victor Plate reader (Perkin Elmer, Salem, MA) at an excitation/emission wavelength of 485/535 nm. Each of the geometric mean of the first and second pass was used to exclude any measurement error (9).

Estimation of IC_{50} and IC_{90}

Both IC_{50} and IC_{90} for CQ against both *P. falciparum* K1 & 3D7 were determined using Microsoft excel 2007 software according to the recommended protocol of percentage of parasite inhibition versus log [drug concentration].

Drug combination assay and isobologram analysis

For drug combination assay, working solutions of CQ and each of the mentioned β-carbolin were prepared from their stocks at concentrations equivalent to 16 times of their IC_{50} such that their IC_{50s} fall in the fourth two-fold serial dilution. Then CQ solution was mixed with each test comp at 10:0, 7:3, 5:5, 3:7 and 0:10 (CQ/ test comp. in nM). Then the combinations were uploaded in triplicate and serially diluted for 8 times within a flat-bottomed 96 well plate. Control suspensions containing untreated RBCs and PRBCs were uploaded as well (10). After that, an equal volume of PRBCs suspension (synchronized at ring stage at 2% Hct and 2 % parasitemia) was added to the wells that contained (CQ/test comp.) Then the plates were incubated at the standard abovementioned conditions (section 2-1) for 48 h and treated in the same way as in the drug sensitivity assay to determine parasite growth and assess both IC_{50} and IC_{90} of each combination separately. For each combination ratio, both FIC_{50} and FIC_{90} (fractional inhibitory concentration) were calculated determining the ratio of IC_{50} or IC_{90} of each drug within the combination to those values when the drug was incubated alone. At the end, both IC_{50} and IC_{90} based isobologram curves were derived through plotting both FIC_{50} and FIC_{90} for each content of the combination (CQ and the test comp.) on Y and X axes respectively. The line that link the two FICs, is considered as the line of additivity so that if the plot falls on, the interaction is considered additive (Fig. 1).

If it falls above or below the line, then the interaction is considered either antagonism or synergy respectively. Some authors dictate that the interaction is considered as complete synergy, if the total FIC_{50} or FIC_{90} ($FIC_{50 \text{ or } 90}$ CQ+ $FIC_{50 \text{ or } 90}$ comp) is equal to or less than 0.5, antagonism if it is > 2, additive if it is 0.5-1 and indifferent if it is in the range of 1-2 (11).

Results

Drug sensitivity assay showed that *P. falciparum* 3D7 was quietly sensitive to CQ (IC_{50} = 22.3 ± 0.76 nM) while K1 was resistant (IC_{50}= 265 ± 3.3 μM**).**

Table 1: FIC50 & FIC90 based Isobologram analysis of harmalin, harmol and harmalol with chloroquine at different mixing proportions. Add., Indif., Syn. and Ant. mean additive, indifferent, synergistic and antagonistic actions respectively

B-carbolin	Combined preparation CQ/ B-carbolin	CQ mean FIC_{50}	β-carbolin FIC_{50}	FIC interaction	Interaction type	CQ mean FIC_9	B carbolin FIC_{90}	FIC interaction	Interaction type
Harmalin	7:3	0.41	0.18	0.59	Syn	0.53	0.22	0.75	Add Add
	5:5	0.39	0.39	0.78	Add	0.50	0.48	0.98	Add
	3:7	0.44	1.02	1.45	Indif	0.56	1.23	1.79	Indif
Harmolol	7:3	0.89	0.40	1.29	indif	1.13	0.48	1.61	Indif
	5:5	0.87	0.92	1.79	Indif	1.10	1.09	2.18	Indif
	3:7	0.74	0.99	1.73	Indif	0.93	1.09	2.02	Indif
Harmal	7:3	0.73	0.30	1.03	Add	0.72	0.27	0.98	Add
	5:5	0.82	0.77	1.58	Indif	0.70	1.34	2.04	Indif
	3:7	0.62	1.41	2.03	Indif	0.63	1.34	1.97	Indif

Meanwhile, both of them were poorly sensitive to each of harmalin, harmalol and harmol (IC_{50}= 3.993 ±0.019, 17.632 ±0.094 and 14.47±0.075 µM, IC_{90}= 7.14± 0.03, 42.67±0.12 & 45.060 ± 0.09 µM respectively). Harmalin was seen to have the highest anti-plasmodium activity among the others. Results of isobologram analysis showed that only harmalin could have synergistically reduced CQ resistance when it was combined at 7:3 and produced an additive effect at 5:5 (CQ/harmalin). The other combinations did not show any interaction except for harmol which showed an additive interaction when it was combined at a ratio of 7:3 (CQ/harmol) (Fig. 1, Table 1).

The IC_{90} based isobologram shows that harmalin could have synergistically reduced CQ tolerance when it was combined with CQ at a ratio of 7:3 (CQ/harmalin) while harmol produced an additive effect at this ratio. Overall, according to the results of the two isobolograms, CQ/harmalin combination at a ratio of 7:3 represents the most optimum combination to reduce both resistance and tolerance in *P. falciparum K1* (Fig. 1, Table 1).

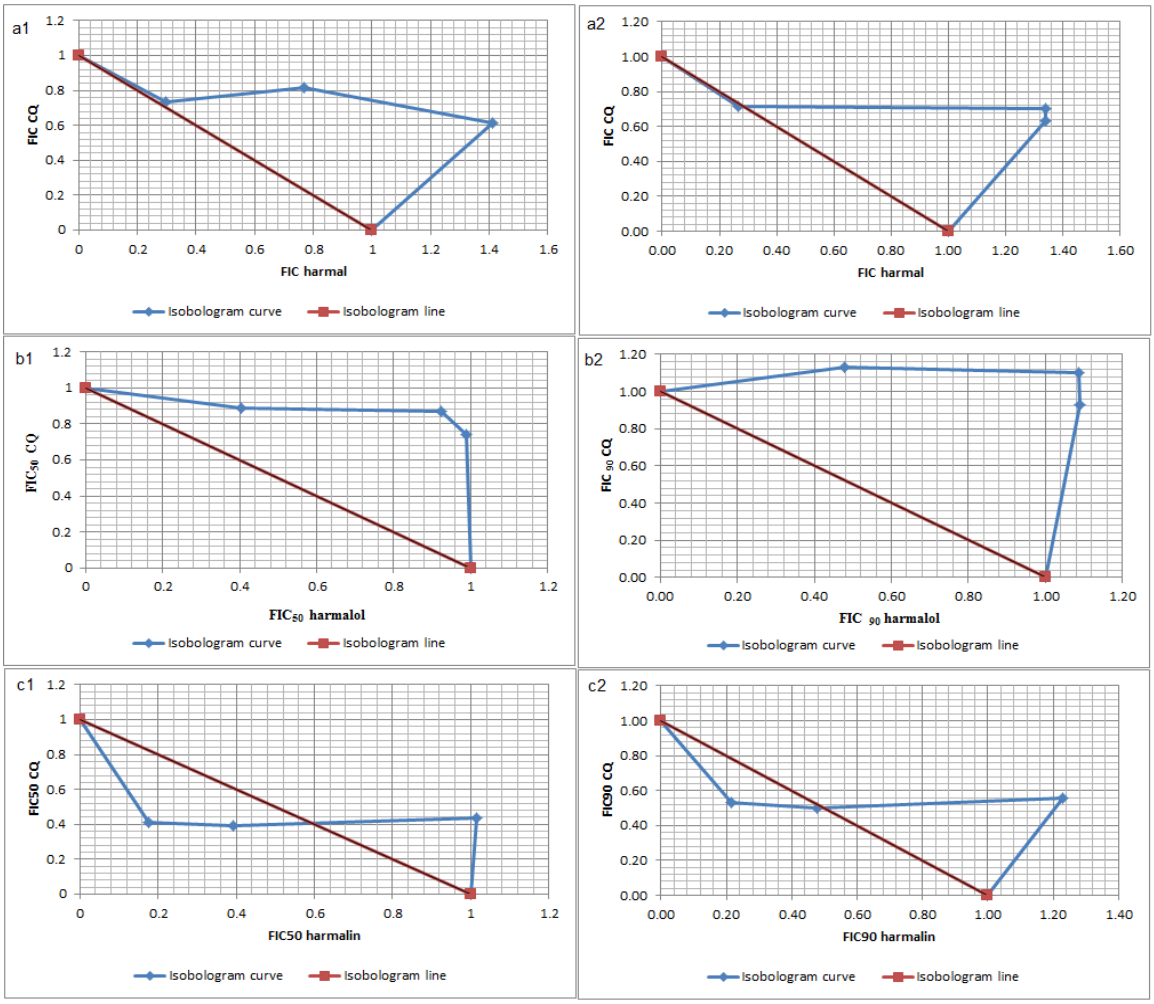

Fig. 1: Isobologram curves based showing the interaction of CQ with three chosen β-carbolins, namely; harmol, harmalol and harmalin. a, b and c represents both IC_{50} (a1, b1 &c1) and IC_{90} (a2, b2 & c2) based isobologram curves for the combinations of CQ with each of harmol, harmalol and harmalin respectively. The red line that connects the X and Y axes represents line of additivity. The interaction is considered as additive if the point fell on it, synergistic if it was below and antagonistic or indifferent if it was located above

Discussion

Medical importance of β- carbolin alkaloids has been studied extensively both In vitro and In vivo. They have been used profoundly in the folk Chinese, Arabic, Middle Eastern and African medicines to treat various ailments and infectious diseases including leishmaniasis and malaria (12).

In vitro effect of β- carbolins on human cancerous and non-cancerous cells had been studied previously. They were found to have a cytotoxic effect against cancer cell line as possessed genotoxic effect characterized by DNA intercalation, induction of chromosomal aberration, induction of DNA damage, formation of DNA adducts and inhibition of DNA excision repair (13, 14). Furthermore, they interfere with the intracellular enzymatic system as they were found to be inhibitors of topoisomerase enzyme (15), β-hydroxylase, monooxygenase, triosphosphate isomerase and cycline dependant kinase. Moreover, other studies had proven that they are effective inhibitors of heat shock protein system that protects protein scaffolds against any mis-folding. Their effect was more pronounced on cancer cells and they were reported to potentiate other cytotoxic drugs in cancer chemotherapy (16). It is noteworthy that different β- carbolin induce different extent of cytotoxic action on cancer cell lines (16).

In our study, we tried to apply the same model on plasmodium cells through combining the three mentioned β- carbolin derivatives with CQ and screening their anti-plasmodium effect and their potency to reverse CQ resistance in *P. falciparum* K1.

Isobologram technique was used to screen the effect of CQ combination with each compound. It had been used widely to deduce the type of interaction between two different compounds (10). Effect of each combination was tested on both IC_{50} and IC_{90} of the combined compounds. They are correlated with drug resistance and tolerance respectively. Both resistance and tolerance started to encounter different strains of *P. falciparum*. The former predestines using higher dose to eradicate the parasite whilst the latter requires protracting the treatment period (3, 4). Our results showed that *P. falciparum* was more sensitive to harmalin as compared to harmol and harmalol. Furthermore, harmalin was more effective in suppressing both CQ resistance and tolerance as seen in the results of the IC_{50} and IC_{90} based isobolograms. On the other hand, harmol had succeeded in ameliorating CQ tolerance without producing a pronounced effect on its resistance. Nevertheless, harmalol did not show any visible interaction when it was combined with CQ.

The anti-plasmodial effect of the mentioned β-carbolins and the ability of some of them to reverse CQ resistance in *P. falciparum K1* can be attributed to its re-known effects on cellular physiology. They had been found to arrest the cell cycle through DNA intercalation and DNA adducts formation, compromising DNA replication fidelity and DNA repair system and inhibition of DNA strands break (17-20). Furthermore, they were found to inhibit some vital enzymes involved in cellular replication, viz; topoisomerase (21), cycline dependant kinase (22). Not only is cellular replication a target for β-carbolins action, they were found to affect some other biological targets, such as; protein kinase C, sodium dependent uptake of the dibasic amino acids, oxygenase, epoxide hydrolase, hydroxylase, epoxide hydrolase and triosephosphate isomerase (23).

Effect of β-carbolins on these targets had been studied extensively on cancerous and non-cancerous mammal cells. It had not been studied on other undeveloped cells. Different derivatives have different intensity of action on each target and indeed, there is a variation in their effect on mammalian and protozoal cells. In spite of the similarity in their action, phytochemicals affect mammalian cells in a way different from their effect on protozoal cells due to the difference in the allosteric site

wherein the drug can combine. Further studies are recommended to find the interactive effect of the other carbolins and more investigations are required to determine the precise mechanism through which carbolins affect plasmodial targets.

CQ produces its action through prevention of hemozoin formation inside the plasmodial digestive vacuoles. Plasmodia digest hemoglobin inside the digestive vacuole to use its protein part as a source of amino acids and release heme as a toxic byproduct. Heme toxicity is prevented through its polymerization into an innocuous product called hemozoin. Heme capping by CQ prevents heme detoxification. To achieve this action, CQ needs to accumulate inside the digestive vacuole DV. Hindrance of CQ accumulation through inhibition of its influx or triggering its efflux is one of the mechanisms through which plasmodia exhibit CQ resistance. Agents that inhibit the transporter system that pumps CQ outside the vacuole or trigger CQ influx can be used to reverse CQ resistance (4).

A recent study has found that CQ can affect the DV membrane integrity at concentrations lower than what is required to hinder hemozoin formation. This promotes the DV membrane permeabilization and exodus of some of the DV hydrolytic enzymes especially those whose g.m.wt is low, viz; Cathepsin. Cathepsin exodus into the protoplasm induces the cascade of the programmed cell death process of apoptosis. The study denoted to the importance of apoptosis process in progression of CQ induced cell death (24).

The obtained synergism between CQ and harmalin is more likely to be attributed to its effect on this apoptotic pathway as the previous studies had pointed out to its powerful effect on arresting the cell cycle but no study had pointed to their effect on membranous drugs transporters. Anyway, further investigations are recommended to prove this notion and to justify the interactive effect of this β-carbolin with CQ.

Overall, Both CQ sensitive and resistant strains of *P. falciparum* 3D7 and K1 show weak to moderate response to harmalin, harmol and harmalol. Harmalin could have showed synergy through suppressing CQ resistance in K1, although the degree of suppression was not enough to reverse the resistance and render K1 to be CQ susceptible. On the other hand, both harmalin and harmol were effective in suppressing CQ tolerance.

Conclusion

In spite of their anti *Plasmodium* effect, β-carbolins are incapable of producing full eradication of malaria parasite. Nevertheless, some of them are effective in reducing CQ resistance and tolerance in the resistant strain of *P. falciparum*. Further studies are recommended to monitor their In vivo efficiency and their safety to be used for this purpose.

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