

Iloneoside, an antimalarial pregnane glycoside isolated from *Gongronema latifolium* leaf, potentiates the activity of chloroquine against multidrug resistant *Plasmodium falciparum*

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ABSTRACT

The rapid spread of drug resistant malaria parasites has necessitated the search for novel antimalarials and chemosensitizers capable of reversing drug resistance in the parasites. A number of studies have revealed the resistance reversal activities of pregnane glycosides and the antimalarial activity of a pregnane glycoside obtained from *Gongronema* species. However, the pregnane (2) and pregnane glycosides (1, 3–4) isolated from *Gongronema latifolium* leaf have not been evaluated for these activities. This study was therefore carried out to evaluate the antiplasmodial and chloroquine resistance reversal activities of a pregnane and three pregnane glycosides isolated from *G. latifolium* leaf in vitro. The compounds were evaluated for their inhibitory activities against *P. falciparum* 3D7 (a chloroquine-sensitive strain) and *P. falciparum* W2 (a chloroquine-resistant clone) in vitro. The activities of chloroquine in separate combination with each of the compounds against *P. falciparum* W2 were also evaluated. Moreover, the interaction of the active compounds (1 and 4) with selected *P. falciparum* proteins (PfProteins) were evaluated *in silico*. The results revealed that only 1 and 4 were active against *P. falciparum* 3D7 and *P. falciparum* W2. Also, 2 and 3 did not exhibit chloroquine resistance reversal activity. Activity of chloroquine against *P. falciparum* W2 was potentiated by 1 by 3200% at concentrations higher than 0.625 µg/mL. Also, 1 and 4 demonstrated similar binding patterns and higher binding tendencies to the selected PfProteins compared to chloroquine. Thus, 1 (iloneoside) is an antimalarial pregnane glycoside which can potentiate the activity of chloroquine against multidrug resistant *P. falciparum*.

1. Introduction

Malaria, the most prevalent parasitic disease of humans is caused by *Plasmodium* species [1]. *P. falciparum* is the most virulent and it is responsible for almost all the morbidity and mortality recorded worldwide, with about 96% of the deaths attributed to malaria occurring in sub-Saharan Africa [2]. The spread of resistant strains of the parasite has rendered conventional antimalarial drugs ineffective [3]. Among such antimalarial drugs, chloroquine (CQ) has been the most used single drug for the treatment of malaria [4] and was popular because of its effectiveness and affordability until resistant strains started to emerge in the

1960 s [3]. It is relatively tolerated in the body and has its therapeutic effect at the dose of 10 mg/kg body weight but with a lower selectivity index [5]. Unfortunately, these desirable qualities of CQ have been seriously eroded by resistance on a global scale by malaria parasite [6]. While most strains of *P. vivax*, *P. malariae*, and *P. ovale* remain sensitive to CQ, more than 80% of field isolates of *P. falciparum* have been reported to be resistant to CQ [7]. Sadly, CQ-resistance is associated with cross resistance to other quinoline drugs, such as quinine and amodiaquine [8]. Moreover, there have been alarming reports of resistance to artemisinin by the parasite [9] and an increase in resistance of *P. falciparum* to artemisinin-based combination therapies (ACT) in

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Southeast Asia [10].

The resistance of the parasite to conventional drugs has necessitated the search for new antimalarial compounds with novel mechanisms of action. Various proteins have been identified as common to the different stages of the parasite and are now used as targets for drug discovery, examples of which are *P. falciparum* lactate dehydrogenase (*PfLDH*), enoyl-acyl-carrier-protein reductase (*PfECPR*), plasmepsin IX (*PfPMIX*) and phosphatidylinositol-4-kinase (*PfPI4k*) [11]. However, while the search for novel antimalarials is on-going, there is the need to also search for more agents that can reverse the resistance of the parasite to conventional antimalarials, especially the cheaper ones, such as CQ.

Some compounds have been reported to reverse antimalarial drug resistance. Fluoxetine has been reported to reverse mefloquine and CQ resistance while ketoconazole has also been reported to exhibit mefloquine resistance reversal activity in simian models [12,13]. Fluoxetine has been reported to exert this effect by inhibiting P-glycoprotein homolog 1 [13]. Some chlorpheniramine analogues have also been reported to reverse CQ resistance in *P. falciparum* Dd2 by inhibiting *PfCRT* [14]. Moreover, some pregnanes and their glycosides have been reported to exhibit multidrug resistance reversal activity in cancer caused by P-glycoprotein [14]; it has its counterpart (P-glycoprotein homolog 1) in malaria parasite which is also responsible for multidrug resistance in *P. falciparum*. Moreover, some pregnane glycosides of the aerial parts of *Carallumatu berculata* have been reported to exhibit antiplasmodial activity [15]. This suggests that some pregnane glycosides may possess the dual potential of killing the parasites directly and reversing the resistance of the parasite to other antimalarial drugs. A pregnane glycoside, gongroneside A, which was isolated from *Gongronema napalense* has been reported to be active against *P. falciparum* D6 and W2 [16], indicating that similar pregnane glycosides with antimalarial activity may be present in other species of the same genus.

Recently, a pregnane and three pregnane glycosides with antileukemic activities were isolated from *Gongronema latifolium* leaf, namely Iloneoside (1), marsectohexol (2), 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl]-17β-marsdenin (3) and 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-canaropyranosyl]-17β-marsdenin (4) [17]. However, these compounds have not been screened for their antiplasmodial and drug resistance reversal activities. This study was therefore carried out to evaluate the antiplasmodial and CQ resistance reversal activities of this pregnane and the three pregnane glycosides isolated from *Gongronema latifolium* leaf in vitro.

2. Methods

2.1. Chemicals

Chloroquine diphosphate, verapamil hydrochloride (Farmanguinhos, FIOCRUZ-RJ, Brazil), desipramine hydrochloride, trifluoperazine dihydrochloride, were purchased from Sigma Chemical Co., St. Louis, Mo, USA. Other reagents used were of analytical grade.

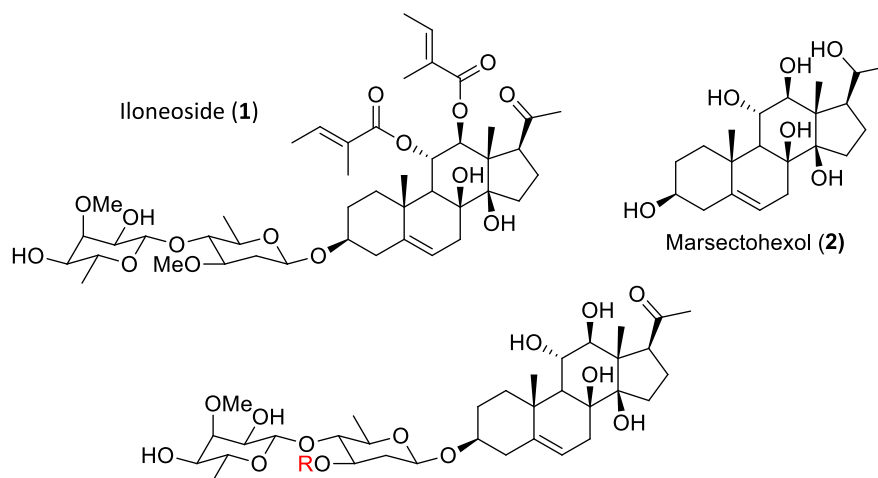
2.2. Phytochemicals

Iloneoside (1), marsectohexol (2), 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl]-17β-marsdenin (3) and 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-canaropyranosyl]-17β-marsdenin (4) were isolated from *Gongronema latifolium* leaf in our previous study using standard protocols [17]. The structures of 1–4 are presented in Figs. 1–3.

2.3. Evaluation of phytochemicals for antiplasmodial activity

The activities of 1–4 against *P. falciparum* 3D7 (chloroquine-sensitive strain) and *P. falciparum* W2 (chloroquine-resistant clone) were evaluated using the SYBR assay described by Smilkstein et al. [74], with minor modifications [18].

Briefly, the *P. falciparum* culture (maintained according to the method of Trager and Jensen (1976)) was synchronized using 5% sorbitol and 0.5% glucose to obtain the ring stages, which were immediately incubated with the test compounds (previously solubilized in 0.05% dimethyl sulfoxide, DMSO) at various concentrations [19]. The test compounds, in serial dilutions, were incubated with the parasite suspensions (0.5% parasitemia, 2% haematocrit) in 96-wells plates ('U' bottom). After 48 h at 37 °C, the culture supernatant was removed and replaced with 100 μL of lysis buffer solution [Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%; wt/vol), and Triton X-100 (0.08%; vol/vol)] followed by addition of 0.2 μL/mL SYBR Safe DNA gel stain (Invitrogen, Norcross, GA, USA). The plate content was transferred to a flat bottom plate and incubated in the dark for 30 min. Then, the plate was read in a fluorometer (Synergy H4 Hybrid Reader, Biotek) with excitation at 485 nm and emission at 535 nm. The fluorescence of uninfected erythrocytes was considered as background. Each test was performed in triplicate in at least two different experiments. The results were compared with those of control cultures in complete medium with



R = Me: 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl]-17β-marsdenin (3)
 R = H: 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-canaropyranosyl]-17β-marsdenin (4)

Fig. 1. Pregnane and pregnane glycosides isolated from *Gongronema latifolium* leaf.

no drugs. CQ was used in each experiment as an antiplasmodial standard.

The half-maximal drug inhibitory concentration (IC_{50}) was estimated by curve fitting charts using the Origin software (Origin Lab Corporation, USA) and compared to the parasite growth in the drug free medium.

2.4. Cytotoxicity tests

The cytotoxicity test was done using Buffalo Green monkey kidney cell line (BGM), and the test was performed as described by Aguiar et al. [20]. Cells were cultured in 75 cm² plates with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and gentamicin 40 mg/L, at 5% CO₂ atmosphere and 37 °C. The cells were trypsinized when the monolayer was confluent, washed with culture medium, distributed in a flat-bottomed 96-well plate (1×10^5 cells/mL) and incubated at 37 °C for 18 h to ensure cell adherence. The cells were properly diluted and incubated with 20 µL of the compounds at different concentrations (1–1000 µg/mL) for 24 h in a 5% CO₂ atmosphere at 37 °C. The neutral red assay as described by Borenfreund and Puerner [21] was used to evaluate cell viability by the accumulation of the dye in the viable cell lysosome. Neutral red solution (4 mg/mL, 200 µL), was added to the plates and incubated for 3 h. The supernatant was carefully removed, followed by the addition of 200 µL of 0.5% (v/v) formaldehyde and 1% CaCl₂ solution. After 5 min, the supernatant was removed, then 100 µL of an alcohol- acetic acid (50–1%) solution was added to extract the dye. The absorbance was read at 540 nm on an ELISA reader (Spectra Max340 PC384, Molecular Devices). Cell viability was expressed as the percentage of control absorbance obtained in untreated cells and the minimum lethal dose for 50% of the cells (MLD₅₀) was determined. Based on the values of cytotoxicity (MLD₅₀) and antimalarial activity (IC_{50}) the selectivity index (SI) of activity was calculated using the formula:

$$\text{Selectivity Index (SI)} = \frac{MLD_{50}}{IC_{50}}$$

2.5. Reversal of chloroquine resistance in *P. falciparum*

The method of Oduola et al., [77] was used to evaluate the CQ resistance reversal activities of the phytochemicals. Briefly, fixed sub-inhibitory concentrations of 2 and 3 (each at the concentrations of 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 µg/mL) and verapamil (0.0625, 0.125, 0.25, 0.5 and 1 µg/mL) were separately combined with CQ [at varying concentrations obtained by 2x serial dilution from the highest concentration (50 and 500 ng/mL for *P. falciparum* 3D7 and W2 strains respectively)] and evaluated for their activities against *P. falciparum* strain and clone in triplicates in each experiment. The antiplasmodial activity of CQ alone [using varying concentrations obtained by 2x serial dilution from the highest concentration (50 or 500 ng/mL)] was concurrently evaluated with those of other drug combinations.

The data were transformed and expressed as the response modification index (RMI) [22]. RMI was calculated as the ratio of IC_{50} of CQ in the presence of the phytochemical/verapamil to the IC_{50} of CQ alone. An RMI of 1.0 indicates no change in the IC_{50} of CQ upon addition of phytochemical/verapamil. An RMI of less than 1.0 represents chemosensitization (including possible synergy), while an RMI of more than 1.0 represents antagonism [23].

2.6. Evaluation of the activity of the combination of chloroquine with compounds 1 and 4 against *P. falciparum* W2

Fixed concentrations of 1 and 4 (each at the concentrations of 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 µg/mL) were separately combined with CQ [at varying concentrations obtained by 2x serial dilution from the highest concentration (500 ng/mL)] and evaluated for their

activities against *P. falciparum* W2 strains in triplicate in each experiment. The antiplasmodial activity of CQ alone [using varying concentrations obtained by 2x serial dilution from the highest concentration (500 ng/mL)] was concurrently evaluated with those of other drug combinations. The same protocol was adopted for the data transformation and RMI calculation as describe in Section 2.5.

2.7. In silico analysis

2.7.1. Sequence dynamics simulation

The FASTA format of the protein sequences for *Plasmodium falciparum* 3D7 plasmepsin IX (PfPMIX), phosphatidylinositol 4-kinase (PfPI4K), *Plasmodium falciparum* P-glycoprotein homologue (PfP-gh); (NCBI Reference Sequence: XP_001351656.1, XP_001348455.1 and CEO43617.1 respectively) on the National Center for Biotechnology Information (NCBI) nucleotide database were obtained. The simulation of the protein sequences was performed on the DynaMine server (<http://dynamine.ibsquare.be/submission/>) using default setting [24].

2.7.2. Homology modeling of *P. falciparum* (plasmepsin IX, P-glycoprotein homologue and phosphatidylinositol 4-kinase) and evaluation of model quality

The unavailability of the deposited 3D crystal structure of PfPMIX, PfPI4K and PfP-gh informed the reason for the homology modeling of the protein from its deposited protein sequence. The three models of PfPMIX, PfPI4K and PfP-gh were built using the SWISS-MODEL server (<https://swissmodel.expasy.org/>) [25]. The fully automated protein structure homology modeling server accessible via ExPASy web server, was used to develop a model [26]. The FASTA format of the protein sequence was fetched onto the SWISS-MODEL server. From the SWISS-MODEL template library (SMTL version 2020–07–15, PDB release 2020–07–10), a search for evolutionary related structures matching the target sequences as suitable templates was performed. A similarity search using the BLAST [27] and HHblits [28] databases simultaneously generated homologues of known PDB structures that were ranked based on QMEAN scoring function [26]. The top-ranked structures were selected and used as templates to build the PfPMIX, PfPI4K and PfP-gh models. The best model was refined by GalaxyRefine [29], a web-tool available on GalaxyWEB server (<http://galaxy.seoklab.org/>), and further refined by 3DRefine server (<http://sysbio.rnet.missouri.edu/3Drefine/>) [30]. The qualities of the refined models were then analyzed using ERRAT [31] ProSA [32] and by generating Ramachandran plots on PROCHECK [33].

2.7.3. Retrieval and preparation of protein structure for molecular docking

The three-dimensional (3D) structure of *P. falciparum* Lactate Dehydrogenase (PfLDH) (PDB ID: 1LDG) [34], *P. falciparum* enoyl-acyl-carrier-protein reductase (PfECPR) (PDB ID: 1NHW) [35] and *P. falciparum* Chloroquine Resistance Transporter (PfCRT) (PDB ID: 6UKJ) [36], were retrieved from the Protein Data Bank (<http://www.rcsb.org>). The crystal structures of the models and downloaded proteins were processed by removing existing ligands and water molecules while missing hydrogen atoms were added according to the amino acid protonation state at pH 7.0 utilizing Autodock Tools (Scripps Research Institute, La Jolla, CA). Thereafter, non-polar hydrogens were merged, while polar hydrogens were added to each protein. The process was repeated for each protein and subsequently saved into a dockable pdbqt format for molecular docking.

2.7.4. Ligand preparation

The Structure Data Format (SDF) structures of chloroquine was retrieved from the PubChem database and converted to PDB using Discovery Studio Visualizer version 16. The flexible structures of compounds 1–4 were drawn using Chemdraw Ultra 12.0 and were converted to mol2 chemical format using Open babel [37]. Using AutoDock Tools v1.5.6, polar hydrogen charges of the Gasteiger-types were assigned and nonpolar hydrogens were merged with the carbons and the internal

degrees of freedom and torsions were set to zero. The protein and ligand molecules were further converted to the dockable pdbqt format using Autodock tools.

2.7.5. Active site targeted molecular docking

An active site targeted docking of the test compounds and reference inhibitors against models and downloaded proteins was performed using AutoDock Vina [38] in PyRx (Python prescription) 0.8 [39]. For each of the docking steps, the ligands were imported via the OpenBabel [37], plug-in tool in PyRx 0.8. The Universal Force Field (UFF) was used as the energy minimization parameter and conjugate gradient descent as the optimization algorithm. The active regions of the 6 proteins as defined by the grid boxes that were used for docking studies are presented in Table 1. All the other parameters were kept as default. The molecular interactions were viewed with Discovery Studio Visualizer version 16.

3. Results

3.1. In vitro antiplasmodial studies and cytotoxicities

The antiplasmodial activities of **1** – **4** and verapamil are shown in Table 2. From the results, **1** exhibited the highest activity against *P. falciparum* 3D7 ($IC_{50} = 6.2 \mu\text{g/mL}$) and *P. falciparum* W2 ($IC_{50} = 2.1 \mu\text{g/mL}$), though lower than CQ. Also, **4** was active while **2** and **3** were not active against the two parasite strains. Verapamil was partially active against *P. falciparum* 3D7 and W2. The results revealed that **1** was the most cytotoxic effect out of the four compounds considered, followed by **2**. Only **1** and **4** had selectivity indices higher than 10 and were considered active. Moreover, **1** and **4** had lower resistance indices (RI) compared to chloroquine and verapamil, though the RI of **1** was higher than that of **4** (Table 2).

3.2. In vitro chemosensitizing activity of test compounds

The IC_{50} s of CQ at varying concentrations of verapamil, **2** and **3** against *P. falciparum* 3D7 (CQ-sensitive strain) and *P. falciparum* W2 (CQ-resistant clone) are shown in Tables 3 and 4 respectively. The RMI values for the combination of CQ with each of the three compounds evaluated against *P. falciparum* 3D7 were higher than 1, indicating antagonism (Table 3).

With respect to *P. falciparum* W2 clone, the potentiating activities of **2** (at higher concentrations above $1.25 \mu\text{g/mL}$) and **3** (at $20 \mu\text{g/mL}$) when separately combined with CQ were very low (with RMI being a little lower than 1) compared to that of verapamil (with RMI being very much lower than 1) (Table 4).

3.3. Activities of the combination of chloroquine with compounds **1** and **4** against *P. falciparum* W2

The results showed that the combination of **1** (at concentrations higher than $1.25 \mu\text{g/mL}$) and **4** (at $20 \mu\text{g/mL}$) separately with CQ

Table 1
Binding site coordinates of selected *Plasmodium falciparum* proteins.

	center_x	center_y	center_z	Size_x	Size_y	Size_z
Modeled PfPMIX	25.4	29.1	15.3	28.6	28.4	35.2
Modeled PfP4K	-14.8	356.9	80.0	25.0	29.9	19.8
PfECPR	11.8	100.2	27.4	27.0	27.7	27.4
PfLDH	28.8	24.4	36.0	27.2	22.2	25.1
PfCRT	146.19	170.34	141.85	23.46	15.76	20.80
Modeled PfP-gh	23.70	7.70	63.94	36.37	55.73	25.0

Plasmodium falciparum Chloroquine Resistance Transporter (PfCRT); *Plasmodium falciparum* P-glycoprotein homolog (PfP-gh); *Plasmodium falciparum* plasmepsin IX (PfPMIX); *Plasmodium falciparum* phosphatidylinositol 4-kinase (PfP4K); *Plasmodium falciparum* lactate dehydrogenase PfLDH and *Plasmodium falciparum* enoyl-acyl-carrier-protein reductase (PfECPR)

reduced the IC_{50} of CQ for *P. falciparum* W2 clone from 100.0 ng/mL to $< 3.13 \text{ ng/mL}$, with an RMI of < 0.03 , revealing a very significant synergy (Table 5).

3.4. In silico

3.4.1. Homology modeling and evaluation of model quality

A SWISS-MODEL template library (SMTL) search and sequence alignment identified a total of 50 templates for each model. This list was filtered down to five templates. Finally, plasmepsin X of *Plasmodium falciparum* 3D7 (PID: 6ORS), human phosphatidylinositol 4-kinase (PID: 4D0L) and *Mus musculus* P-glycoprotein (PID: 4Q9J), were selected as the best templates for PfPMIX (Leu 1080 – Leu 1542), PfP4K (Ile 229 – Lys 564) and PfP-gh (THR 1- MET 90), respectively. The selected templates for the three models had Global Model Quality Estimate (GMQE) scores of 0.628, 0.690 and 0.628, sequence coverage of 0.813, 0.556 and 0.31, sequence identities of 44.57%, 43.44% and 42.86%, and sequence similarities of 0.43, 0.421 and 0.43 for PfPMIX, PfP4K and PfP-gh models respectively [Figs. S2, S3 and S4 (supplementary data); Table 6]. The QMEAN Z-scores of the models generated were -3.03 , -2.04 and -4.22 respectively (Table 6).

Analysis of the refined models with PROCHECK and Protein Sequence Analysis tool (ProSA) revealed that the 3DRefined-models had higher improvement in quality and were, therefore, selected for subsequent steps. The Z-scores (the overall quality scores) of the models, in relation to the Z-scores of PDB experimentally determined native structures, obtained from ProSA-web analysis were -7.5 , -7.4 and -2.76 for PfPMIX, PfP4K and PfP-gh models respectively [Figs. S2, S3 and S4 (supplementary data); Table 6], thus falling within the range of those of the PDB native structures. In addition, the amino acid local energy profile plots showed that all the residues across the sequence had negative values [Figs. S2, S3 and S4 (supplementary data)], indicating favorable amino acid energy profiles, high accuracy and reliability of the models. From the Ramachandran phi/psi torsion angle plots, as generated from PROCHECK analysis, the residues in the favorable and disallowed regions indicated that the three models had good stereochemical quality and energetically favored geometry (Table 6). All the residues forming the binding sites studied in this work were in the favored regions of the Ramachandran plots. The overall quality factors (OQF) of 82.271%, 85.152% and 84.281% for PfPMIX, PfP4K and PfP-gh respectively obtained from ERRAT plot analysis of the models were above the generally accepted score of $> 50\%$. The structural alignment showed an RMSD of $< 3.0 \text{ \AA}$ (Fig. 2c; Table 6), indicating that the models had a very high structural similarity with the templates and thus were reliable and suitable for the study.

3.4.2. Binding affinities of compounds **1** and **4** to selected *Plasmodium falciparum* proteins

The binding affinities of the selected parasite proteins for CQ, **1** and **4** are shown in Table 7. From the results, *P. falciparum* proteins had higher binding affinities for **1** and **4** compared to chloroquine, except PfP4K which had lower binding affinity for **1** compared to chloroquine (Table 7).

3.4.3. Amino acid interactions of compounds **1** and **4** with selected *Plasmodium falciparum* proteins

The amino acid interactions of *Plasmodium falciparum* proteins with compounds **1** and **2** are represented in Table 7. The interaction of residues of the proteins with respective ligand groups were mainly through hydrogen bonds (below $3.40 \text{ \AA}$), hydrophobic interactions and few other bonds (Figs. 3–8 and Table 8).

A hydrogen bond was observed between TYR²³¹ of the modeled PfPMIX and the carbonyl of 11-O-tigloyl moiety of the 17 β -marsdeninaglycone of **1** (Fig. 3bi). The aglycone and the sugar moiety of **1** and **4** made several carbon-hydrogen bonds with TYR²²⁸, SER³⁸³, GLY²³⁰ and LEU³⁸⁵ of the modeled PfPMIX (Fig. 3bi & bii). A Pi-alkyl interaction was

Table 2Antiplasmodial activities and cytotoxicities of Compounds 1–4 isolated from *Gongronema latifolium* leaf.

Samples	Activity against <i>P. falciparum</i> (IC ₅₀ , µg/mL) ^a			Cytotoxicity against BGM kidney cells (MLD ₅₀ , µg/mL) ^b	Selectivity Index ^c	Active
	3D7 strain	W2 Clone	RI ^c			
1	6.2 ± 0.3	2.1 ± 0.9	0.34	35 ± 0	17	Yes
2	> 20	> 20	NA	132 ± 4	NA	No
3	> 20	> 20	NA	227 ± 12	NA	No
4	18.7 ± 1.5	8.0 ± 2.5	0.43	147 ± 28	18	Yes
Verapamil	20.5 ± 5.4	10.6 ± 0.1	0.52	238.9 ± 7.6	23	Yes
Chloroquine	0.024 ± 0.02	0.141 ± 0.009	5.88	51 ± 0	362	Yes

Values are Mean ± SD of two independent experiments, with each run in triplicates. aIC₅₀ = Concentration that inhibits 50% of parasite growth. lloleoside (1), marsectohexol (2), 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl]–17β-marsdenin (3) and 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-canaropyranosyl]–17β-marsdenin (4). bMLD₅₀ = Median lethal dose. BGM – Buffalo Green Monkey. *RI (resistance index) obtained from the ratio of the IC₅₀ of the selected parasite line to the IC₅₀ of the parent strain (Nzila and Mwai, [75]); **Selectivity Index obtained from ratio MLD₅₀/IC₅₀ (Bézivin et al., [76]), where SI ≤ 10 was indicative of toxicity; NA = Not Applicable.

Table 3Activities of chloroquine in combination with Compounds 2 and 3 against *Plasmodium falciparum* CQ-sensitive 3D7 strain.

Drug Combination	Drug Concentration	IC ₅₀ (ng/mL) ^a (Mean ± SD)	RMI
CQ	50 ng/mL ^b	7.7 ± 0.3	
CQ + Sample 2	+ 20 µg/mL	8.1 ± 0.6	1.05
	+ 10 µg/mL	7.8 ± 1.1	1.01
	+ 5 µg/mL	8.1 ± 0.6	1.05
	+ 2.5 µg/mL	8.0 ± 0.6	1.04
	+ 1.25 µg/mL	10.4 ± 3.6	1.35
	+ 0.625 µg/mL	9.5 ± 2.1	1.23
CQ + Sample 3	+ 0.3125 µg/mL	8.6 ± 1.4	1.12
	+ 20 µg/mL	9.1 ± 0.3	1.18
	+ 10 µg/mL	8.5 ± 0.0	1.10
	+ 5 µg/mL	8.7 ± 0.1	1.13
	+ 2.5 µg/mL	8.5 ± 0.2	1.10
	+ 1.25 µg/mL	9.6 ± 0.8	1.25
CQ + Verapamil	+ 0.625 µg/mL	8.8 ± 0.0	1.14
	+ 0.3125 µg/mL	8.4 ± 0.1	1.09
	+ 1 µg/mL	8.4 ± 2.1	1.09
	+ 0.5 µg/mL	8.8 ± 2.1	1.14
	+ 0.25 µg/mL	8.6 ± 1.8	1.12
	+ 0.125 µg/mL	8.1 ± 0.2	1.05
	+ 0.0625 µg/mL	9.5 ± 1.0	1.23

^a Values are the means standard deviations for two independent experiments, each of which was performed in triplicate.

^b Chloroquine was tested in serial dilution (2x) from the highest concentration (50 ng/mL) in all combinations performed. 2 – marsectohexol; 3 – 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl]–17β-marsdenin. Response Modulation Index (RMI) – the ratio of IC₅₀ of CQ in the presence of the phytochemicals to the IC₅₀ of CQ alone. An RMI of less than 1.0 represents synergy, while an RMI of more than 1.0 represents antagonism.

also observed between the A ring of the aglycone of **1** and LYS³⁸¹ of the modeled *PfPMIX* (Fig. 3bi). The 2 hydroxyl groups on the allopyranosyl sugar of **4** formed hydrogen bonds with LEU³⁸⁵ and TYR²³¹, while the hydroxyl group of oleandropyranosyl formed 2 carbon-hydrogen bonds with SER³⁸³ and GLY²³⁰ (Fig. 3bii). The A and B rings of the aglycone of **4** formed Pi-alkyl interactions with LYS³⁸¹ and TRY²²⁸ (Fig. 3bii). The 4-hydroxyl group of the allopyranosyl sugar of **1** formed the only hydrogen bond with ASP²⁸⁰ of the modeled *PfPI4K* (Fig. 4bi). The methyl groups of the allopyranosyl and oleandropyranosyl sugar of **1** formed 2 alkyl interactions with CYS²⁸² of the modeled *PfPI4K* (Fig. 4bi). In the same vein the 18, 19-dimethyl groups of the 17β-marsdeninaglycone formed alkyl interactions with LYS²²⁹ and ILE²²⁷ of the modeled *PfPI4K* (Fig. 4bi). The oleandropyranosyl sugar of **1** formed Pi-Sigma bonds with TYR²⁷⁷ of the modeled *PfPI4K* (Fig. 4bi). The 19-methyl group of 17β-marsdenin of **4** formed an alkyl interaction with ILE²²⁷ of the modeled *PfPI4K* (Fig. 4bii). The methyl groups of the two sugars of **4** also formed two alkyl interactions with CYS²⁸² of the modeled *PfPI4K* (Fig. 4bii). The carbonyl group of the 12-tigloyl of **1**

Table 4Activities of combination of chloroquine with Compounds 2 and 3 against *Plasmodium falciparum* W2.

Drug Combination	Drug Concentration ^a	IC ₅₀ (ng/mL) ^a	RMI
CQ	500 ng/mL ^b	99.9 ± 0.2	
CQ + Comp 2	+ 20 µg/mL	87.0 ± 15.6	0.87
	+ 10 µg/mL	94.0 ± 21.2	0.94
	+ 5 µg/mL	95.5 ± 26.2	0.96
	+ 2.5 µg/mL	89.8 ± 21.6	0.90
	+ 1.25 µg/mL	102.0 ± 2.8	1.02
	+ 0.625 µg/mL	110.5 ± 2.1	1.11
CQ + Comp 3	+ 0.3125 µg/mL	114.5 ± 2.1	1.15
	+ 20 µg/mL	92.0 ± 11.3	0.92
	+ 10 µg/mL	108.0 ± 14.1	1.08
	+ 5 µg/mL	104.5 ± 6.4	1.05
	+ 2.5 µg/mL	118.5 ± 3.5	1.19
	+ 1.25 µg/mL	105.5 ± 2.1	1.06
CQ + Verapamil	+ 0.625 µg/mL	110.0 ± 8.5	1.10
	+ 0.3125 µg/mL	110.0 ± 2.8	1.10
	500 ng/mL ^b	204 ± 16	
	+ 1.0 µg/mL	≤ 7.8	< 0.04
	+ 0.5 µg/mL	≤ 7.8	< 0.04
	+ 0.25 µg/mL	17 ± 3	0.08
	+ 0.125 µg/mL	29 ± 14	0.14
	+ 0.0625 µg/mL	48 ± 19	0.23

^a Values are the means ± standard deviations for two independent experiments, each of which was performed in triplicate.

^b Chloroquine was tested in serial dilution (2x) from the highest concentration (500 ng/mL) in all combinations performed. RMI – the ratio of IC₅₀ of CQ in the presence of the phytochemicals to the IC₅₀ of CQ alone. 2 – marsectohexol; 3 – 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl]–17β-marsdenin. An RMI of less than 1.0 represents chemosensitization (including synergy), while an RMI of more than 1.0 represents antagonism.

interacted via a hydrogen bond with THR¹⁰¹ of *PfLDH* (Fig. 5bi). Several Pi-alkyl interactions were formed between Steroid ring A of **1** and PHE¹⁰⁰ and between β-D-oleandropyranosyl sugar of **1** and ILE⁵⁴ and ILE¹¹⁹ of *PfLDH*, while alkyl interactions were observed between the methyl groups on the allopyranosyl and 17β-marsdenin rings of **1** and LYS¹¹⁸ and MET³⁰ of *PfLDH* respectively (Fig. 5bi). The 4-hydroxyl group of the allopyranosyl of **4** interacted via strong hydrogen bonds with PRO²⁴⁶ and SER²⁴⁵ of *PfLDH* (Fig. 5bii). A carbon-hydrogen bond was formed between the (1→4) glycoside bond between the allopyranosyl and canaropyranosyl sugars of **4** and GLY²⁹ while the 3-O-methyl of the canaropyranosyl of **4** formed two hydrogen bonds with THR¹⁰¹ and GLY⁹⁹ of *PfLDH* (Fig. 5bii). Ring A of the steroid aglycone of **4** interacted via a Pi-alkyl bond with ILE⁵⁴ of *PfLDH*, while three alkyl interactions were formed between the methyl substituents of **4** and MET³⁰, PHE¹⁰⁰ and ILE¹¹⁹ of *PfLDH* (Fig. 5bii). The 14-hydroxyl group of the aglycone and the allopyranosyl sugar of **1** served as the hydrogen donors to LYS²⁸⁵ and ARG³¹⁸ of *PfECPR* respectively, while the carbonyl group of the 12-O-tigloyl on the aglycone, methyl and 3-O-methyl

Table 5

Activities of combination of compounds 1 and 4 with chloroquine against *Plasmodium falciparum* W2.

Drug Combination	Drug Concentration ^a	IC ₅₀ (ng/mL) ^b	RMI
CQ	500 ng/mL ^b	100.0 ± 4.2	
CQ + Comp 1	+ 20 µg/mL	< 3.13	< 0.03
	+ 10 µg/mL	< 3.13	< 0.03
	+ 5 µg/mL	< 3.13	< 0.03
	+ 2.5 µg/mL	< 3.13	< 0.03
	+ 1.25 µg/mL	9.0 ± 4.2	0.09
	+ 0.625 µg/mL	99.0 ± 5.7	0.99
CQ + Comp 4	+ 0.3125 µg/mL	104.0 ± 0.0	1.04
	+ 20 µg/mL	< 3.13	< 0.03
	+ 10 µg/mL	77 ± 11	0.67
	+ 5 µg/mL	103 ± 13	0.90
	+ 2.5 µg/mL	107 ± 26	1.21
	+ 1.25 µg/mL	116 ± 19	1.25
	+ 0.625 µg/mL	126 ± 24	1.39
	+ 0.3125 µg/mL	112 ± 24	1.25

^a Values are the means ± standard deviations for two independent experiments, each of which was performed in triplicate.

^b Chloroquine was tested in serial dilution (2x) from the highest concentration (500 ng/mL) in all combinations performed. 1 – Iloneoside; 4 - 3-O-[6-deoxy-3-O-methyl-β-D-allopyranosyl-(1→4)-β-D-canaropyranosyl]-17β-marsdenin. RMI – the ratio of IC₅₀ of CQ in the presence of the phytochemicals to the IC₅₀ of CQ alone. An RMI of less than 1.0 represents synergy, while an RMI of more than 1.0 represents antagonism.

Table 6

Quality check and validation scores for *Plasmodium falciparum* modeled proteins.

Parameters	PfPMIX	PfPI4K	PfP-gh
QMEAN Z-Score	-3.03	-2.04	-4.22
Global Model Quality Estimate (GMQE) score	0.628	0.690	0.16
Sequence coverage	0.813	0.556	0.31
Sequence identity (%)	44.57	43.44	42.86
Sequence similarity	0.43	0.421	0.43
Z-score	-7.54	-7.44	-2.76
Most favored regions (%)	87.8	95.9	91.7
Additional allowed regions (%)	8.2	3.7	8.3
Disallowed regions (%)	1.7	0.0	0.0
G-factor (%)	2.3	0.4	
Overall quality factor	82.271	85.152	84.281
RMSD	0.385	0.212	1.65

Plasmodium falciparum plasmepsin IX (PfPMIX); *Plasmodium falciparum* phosphatidylinositol 4-kinase (PfPI4K); *Plasmodium falciparum* P-glycoprotein homolog (PfP-gh); Protein Sequence Analysis (ProSA) Root Mean Square Deviation (RMSD) Protein Check (PROCHECK).

moiety of the allopyranosyl sugar of 1 formed carbon-hydrogen bonds with ALA³²⁰, ASN²¹⁸ and ALA²¹⁹ of PfECPR respectively (Figure 6bi). The A ring of the aglycone and the oleandropyranosyl ring of 1 formed Pi-alkyl interaction with ALA³¹⁹ of PfECPR (Figure 6bi). Alkyl interactions were formed by the 18, 19-dimethyl groups on the 17β-marsdenin, and methyl group of allopyranosyl of 1 with ALA²¹⁷, TYR²⁶⁷, and TRP¹³¹ of PfECPR respectively (Figure 6bi). The 21-carbonyl and 11-hydroxyl groups of the aglycone and 3-O-methyl group of allopyranosyl sugar 4 interacted via hydrogen bonds with LEU³¹⁵, SER³¹⁷ and GLY¹⁰⁶ respectively (Figure 6bii). Carbon-hydrogen bonds were formed between 11, 12-dihydroxyl groups of 17β-marsdenin of 4 with ALA³²⁰ and GLY¹⁰⁴ of PfECPR respectively (Figure 6bii). The 18, 19-dimethyl groups of the aglycone and 3-methyl group of canaropyranosyl sugar of 4 formed alkyl interactions with TYR²⁷⁷, ILE³²³, ALA²¹⁷, VAL¹⁴³ and TRP¹³¹ of PfECPR respectively (Figure 6bii). The A and B rings of 4 formed Pi-alkyl interactions with TYR¹¹¹ and LEU²⁶⁸ of PfECPR (Figure 6bii).

The 1→4 glycosidic bond between the allopyranosyl and oleandropyranosyl sugar moiety of 1 formed a carbon-hydrogen bond with SER³⁸⁸, the first ring of the aglycone formed an alkyl interaction with ILE⁶⁶, the methyl end of 11 and 12-O-tigloyl substituents on the

aglycone of 1 formed alkyl and Pi-alkyl interactions with VAL⁷³, ILE⁶⁶, ALA³⁵⁵, ALA³⁵⁹, PHE³⁸², LEU⁶⁹, LEU³⁸¹ and LEU³⁸⁵ (Figure 7bi). The 6-deoxy-3-O-methyl-β-D-allopyranosyl sugar moiety of 4 formed 2 carbon-hydrogen bonds with SER³⁸⁸ and VAL³⁴⁸ of PfCRT while the first ring of the aglycone formed Pi-alkyl and alkyl interactions with TYR⁶² and ILE⁵⁵ of PfCRT (Figure 7bii). In the case of the modeled PfP-gh, the 3-O-methyl group of the allopyranosyl sugar moiety of 1 formed 2 hydrogen bonds with ASN²⁰ and SER¹⁸. The carbonyl oxygen of the 11-O-tigloyl substituent and the 14-hydroxyl group formed 2 hydrogen bonds with PHE¹⁰ while 8-hydroxyl group of the aglycone formed a hydrogen bond with LEU¹² of the modeled PfP-gh (Figure 8bi). The 3-O-methyl of oleandropyranosyl sugar of 4, the 11, 12-dihydroxyl groups of the aglycone formed 2 hydrogen bonds with SER⁴² while 14-hydroxy group and 21-carbonyl oxygen of the aglycone formed hydrogen bonds with ARG¹⁷ and ASN²⁰ of 4 (Figure 8bii).

4. Discussion

Some pregnane glycosides have been reported to exhibit antimalarial drug resistance reversal activities while some have also been reported to exert antimalarial activities [15,16]. In this study, the natural products exhibiting IC₅₀ values ≤ 10 µg/mL were considered active, those exhibiting IC₅₀ values ranging from 10 to ≤ 20 µg/mL were considered moderately active; natural products exhibiting IC₅₀ values > 20 µg/mL were considered inactive. From the results, 1 was active while 4 was moderately active against *P. falciparum* 3D7 while both compounds were active against *P. falciparum* W2 (with the most active, 1, being a hit compound against this clone having an IC₅₀ < 11 µM [40]).

However, 2 and 3 were not active against both parasite strains. Considering their structures, 1, 3 and 4 have the same pregnane glycoside skeleton (Fig. 1). Relative to 4, the presence of an additional tigloyl substituents at C-11 and C-12 of 1 may be responsible for its enhanced antiparasmodial activity. This corroborates the finding that replacing the hydroxyl group at C-11 and C-12 with some functional groups could enhance the activity of pregnane [41]. O-methylation of C-3 of the inner sugar (oleandrose) moiety of 1, compared to 4, may not contribute to its enhanced activity because the deletion of this methyl group in 4 made it active compared to 3 which possessed the same methyl group but was not active. However, the most active phytochemical (obtained from *G. latifolium* leaf) in this study, 1 (IC₅₀ = 2.48 µM), was not as active against *P. falciparum* W2 as grongoneside A (IC₅₀ = 1.39 µM) which was also isolated from a plant of the same genus, *G. napalense* [16]. This result confirms that plants of this genus are good sources of pregnane glycosides with antimalarial activities. The activities of 1 and 4 may still be enhanced with further structural modification, e.g. addition of more functional groups, in order to generate leads. For example, the presence of acetyl and perbenzoyl groups on the pregnane skeleton of pregnane glycosides obtained from *Caralluma berculata* has been reported to enhance the antiparasmodial activity of the phytochemicals [15].

The results revealed that iloneoside was more cytotoxic than other compounds. This result corroborates our earlier findings that iloneoside, a novel cytotoxic compound isolated from *Gongronema latifolium* leaf, was more cytotoxic than the four other compounds isolated from the leaf alongside with it [17]. The results revealed that only 1 and 4 had selectivity indices higher than 10, the benchmark for natural or synthetic products that can be used for antimalarial drug discovery. It, thus, suggests that the compounds did not just kill the parasite and host cells indiscriminately but were more specific for the parasite.

Activities of the combination of CQ separately with 2 and 3 against *P. falciparum* 3D7 were slightly lower than that of CQ alone, with RMI values of a little higher than 1, suggesting antagonism of the compounds at these concentrations. Activities of the combination of CQ separately with 2 (at higher concentrations) and 3 (at the highest concentration) against *P. falciparum* W2 clone were slightly higher than that of CQ alone, suggesting very low chemosensitization. Thus, compounds 2 and

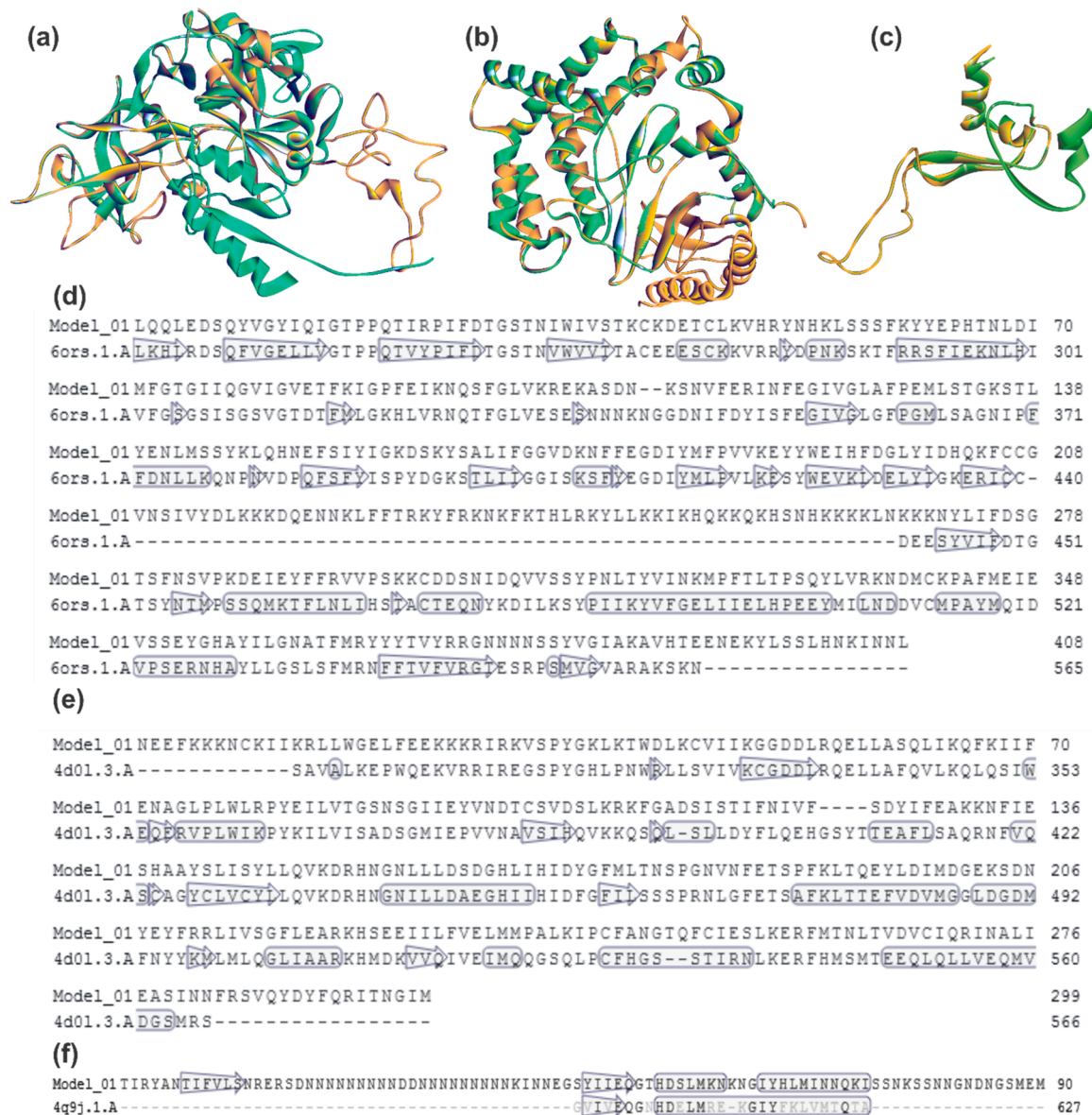


Fig. 2. Model-Template structural alignment of (a) *Plasmodium falciparum* Plasmepsin IX model (gold), *Plasmodium falciparum* Plasmepsin X: template (green); (b) *Plasmodium falciparum* Phosphatidylinositol 4-kinase model (green), human Phosphatidylinositol 4-kinase: template (gold); (c) *Plasmodium falciparum* P-glycoprotein homolog model (gold), *Mus musculus* P-glycoprotein homolog template (green); (d) Model-Template alignment of *Plasmodium falciparum* Plasmepsin IX; (e) Model-Template alignment of *Plasmodium falciparum* Phosphatidylinositol 4-kinase model; (f) Model-Template alignment of *Plasmodium falciparum* P-glycoprotein homolog.

Table 7

Binding energies of compounds 1 and 4 docked in the active sites of *Plasmodium falciparum* proteins.

Protein	Binding affinities (kcal/mol)		
	Comp 1	Comp 4	Chloroquine
PfPMIX	-7.1	-7.5	-5.5
PfP14K	-6.8	-6.9	-6.9
PfLDH	-7.4	-7.8	-6.0
PfECPR	-9.2	-10.4	-7.8
PfCRT	-7.3	-7.1	-7
PfP-gh	-6.7	-6.7	-4.9

Plasmodium falciparum plasmepsin IX (PfPMIX); *Plasmodium falciparum* phosphatidylinositol 4-kinase (PfP4K); *Plasmodium falciparum* lactate dehydrogenase PfLDH; *Plasmodium falciparum* enoyl-acyl-carrier-protein reductase (PfECPR); *Plasmodium falciparum* Chloroquine Resistance Transporter (PfCRT) and *Plasmodium falciparum* P-glycoprotein homolog (PfP-gh)

3 are not effective chemosensitizers; in fact they were antagonists at lower concentrations, with RMI values higher than 1.

Activities of the combination of CQ separately with 1 (IC₅₀ values of <3.13 µg/mL at higher doses) and 4 (IC₅₀ of <3.13 µg/mL at highest dose) against *P. falciparum* W2 was very high compared to that of CQ alone (IC₅₀ = 100 µg/mL), which is more than 3200% increase. The combination had an RMI of < 0.03, suggesting a very significant synergy, which potentiated the activity of CQ. The higher activity of 1 compared to that of 4 still reveals the importance of the substituents at C-11 and C-12 of 1 in potentiating the activity of CQ against *P. falciparum* W2 (a multidrug resistant clone, MDR). MDR is mediated by P-glycoprotein homolog 1 (P-gh) in malaria parasite. Chemosensitizers from different classes of natural and synthetic compounds have been reported to act synergistically to reverse CQ and multidrug resistance in *P. falciparum* [42]. Though there have been some reports on the activity of pregnane glycosides isolated from plants in reversing multidrug resistance in cancer cells [43] through inhibition of

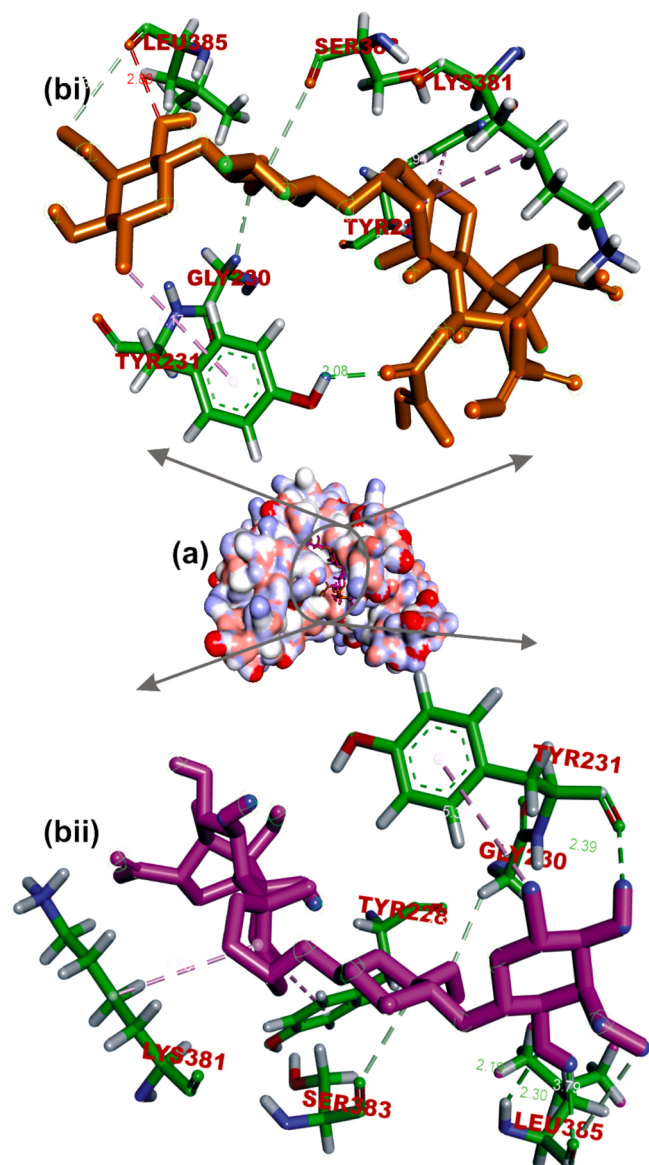


Fig. 3. Interaction of pregnanes with amino acids in the active site of modeled *Plasmodium falciparum* plasmepsin IX (PfPMIX) (a) surface representation (b) 3D interactive representation, ligands in stick representation are presented in different colors: (i) gold: 1 (ii) purple: 4. Types of interactions are represented by: Green-dotted line-hydrogen bonding; light purple-dotted line-hydrophobic interaction (pi-alkyl, alkyl and pi-stacking); purple-dotted line-pi-pi T-Shaped interaction; yellow-dotted line-pi-sulphur interaction and 3-letter abbreviation of amino acids are in red color.

P-glycoprotein [44], this is the first report on the potentiation of CQ activity against multidrug resistant *P. falciparum* by a pregnane glycoside at a very low concentration of 2.5 µg/mL. Lack of further increase in RMI with increasing concentration of 1 suggests a threshold effect.

Drug-resistant malaria parasites are believed to emerge through mutations in the active sites of drug targets [45] or from biochemical changes in the drug receptors [46]. The consolidation of *in silico* techniques with “wet-lab” experimentation in the field of drug discovery enables a deeper understanding of underlying molecular interactions integral in ligand–protein binding. The insight extracted from these molecular interactions may further supplement the interpretation of experimental results at an atomic-level [47]. In this study, six *P. falciparum* drug targets were further explored for interactions with 1 and 4 *in silico*.

Plasmepsin IX, is expressed at discrete stages in the parasite cycle.

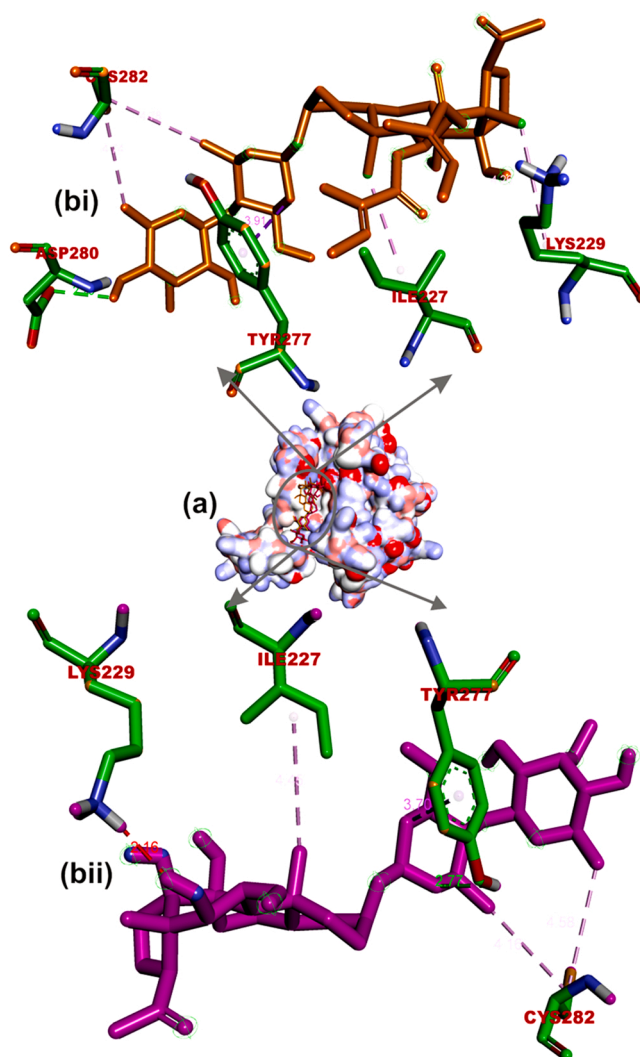


Fig. 4. Interaction of pregnanes with amino acids in the active site of modeled *Plasmodium falciparum* phosphatidyl inositol-4-kinase (PfPI4K): (a) surface representation (b) 3D interactive representation, ligands in stick representation are presented in different colors: (i) gold: 1 (ii) purple: 4. Types of interactions are represented by: Green-dotted line-hydrogen bonding; light purple-dotted line-hydrophobic interaction (pi-alkyl, alkyl and pi-stacking); purple-dotted line-pi-pi T-Shaped interaction; yellow-dotted line-pi-sulphur interaction and 3-letter abbreviation of amino acids are in red color.

These aspartic proteases have recently emerged as propitious antimalarial targets [48]. The lipid kinase, PfPI4K, has also been established as a promising drug target [49]. Inhibition of PfPI4K prevents membrane ingress that occurs during completion of the asexual erythrocytic stage of the plasmodial life cycle [50]. However, the crystal structure is not available till date, which has made *in-silico* drug design challenging; this informed the need of a model structure from the amino acid sequence of this target protein. Plasmepsin X of *Plasmodium falciparum* 3D7, human phosphatidyl inositol 4-kinase and *Mus musculus* P-glycoprotein were selected as the best templates for PfPMIX, PfPI4K and PfP-gly respectively. A template with sequence similarity of more than 0.3 with the sequence of interest is generally considered suitable and model obtained therewith is expected to be reliable and plausible [51]. In this study, sequence similarities of 0.43, 0.421 and 0.43 were obtained for PfPMIX, PfPI4K and PfP-gly models respectively, suggesting that they were suitable for the study. Validation of the predicted structures of the modeled PfPMIX, PfPI4K and PfP-gly proteins revealed a good model quality. The QMEAN score of −3.03, −2.04 and −4.22

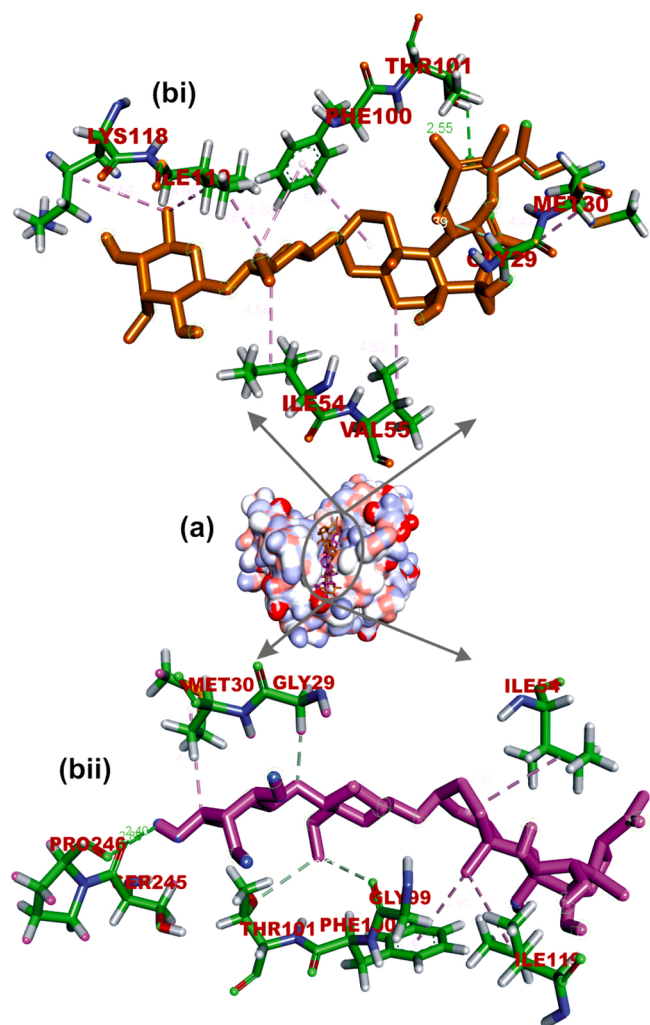


Fig. 5. Interaction of pregnanes with amino acids in the active site of *Plasmodium falciparum* lactate dehydrogenase (PfLDH): (a) surface representation (b) 3D interactive representation, ligands in stick representation are presented in different colors: (i) gold: 1 (ii) purple: 4. Types of interactions are represented by: Green-dotted line-hydrogen bonding; light purple-dotted line-hydrophobic interaction (pi-alkyl, alkyl and pi-stacking); purple-dotted line-pi-pi T-Shaped interaction; yellow-dotted line-pi-sulphur interaction and 3-letter abbreviation of amino acids are in red color.

for PfPMIX, PfPI4K and PfP-gh respectively indicated that the models had satisfactory agreement with experimental structures of similar size [52]. The ERRAT plot of non-bonded atomic interaction of protein structures indicated a higher OQF, implying higher model quality [56]. Most of the residues for the proteins were properly folded with very low error rate, hence, the high OQF. The conformation of the catalytic residues of the first two models (PfPMIX and PfPI4K) was in conformity with those obtained in previous studies [47,49,53]. Overall, the validation results demonstrated that the established model structures were reasonable structures that could be used for the docking studies. The modeled structures (PfPMIX, PfPI4K and PfP-gh) with three other *P. falciparum* proteins (PfLDH, PfECPR and PfCRT) were evaluated for their interactions with 1 and 4 using molecular docking techniques. The results of the docking studies for 1 and 4, which were docked in selected *P. falciparum* proteins, revealed that the six parasite proteins had higher binding affinities for 1 and 4 compared to chloroquine, though the latter was more active than them in in vitro studies. This is because these proteins have not been reported as the primary targets of chloroquine, which is mainly the inhibition of heme detoxification in the food vacuole of the parasites [54]. In the same vein, four of the parasite proteins had

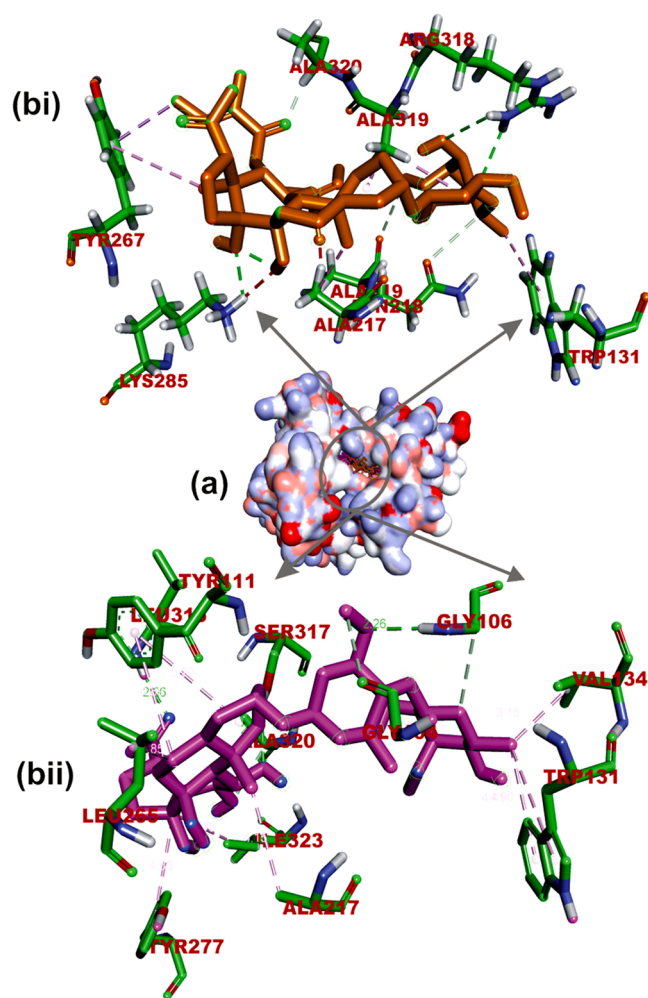


Fig. 6. Interaction of pregnanes with amino acids in the active site of *Plasmodium falciparum* enoyl-acyl carrier protein reductase (PfECPR): (a) surface representation (b) 3D interactive representation, ligands in stick representation are presented in different colors: (i) gold: 1 (ii) purple: 4. Types of interactions are represented by: Green-dotted line-hydrogen bonding; light purple-dotted line-hydrophobic interaction (pi-alkyl, alkyl and pi-stacking); purple-dotted line-pi-pi T-Shaped interaction; yellow-dotted line-pi-sulphur interaction and 3-letter abbreviation of amino acids are in red color.

higher binding affinities for 4 compared to 1, though the former was less active than 1 in in vitro studies. This suggests that the proteins may not be the primary target(s) of 1. The observed higher affinities for 4 may be as a result of 4 interacting with more residues at the active sites of the enzyme than 1 (Table 5). Results of the docking studies showed that 1 and 4 interacted with the catalytic aspartyl residues of PfPMIX in a binding mode similar to a known hydroxy-ethylamine derivative-inhibitor (49c) [55]. With respect to the complexes, PfPMIX-1 and PfPMIX-4 interactions were mainly via hydrogen bonding. Also, 1 and 4 fitted into the hydrophobic pockets S₁ and S'₁ just like the hydroxy-ethylamine derivative-inhibitor (49c) [55]. Interactions with the residues of this cavity are known to influence substrate specificity [55]. PfPMIX has been reported to be essential for invasion of merozoites [56]. It acts on the biogenesis of rhoptry secretory organelle of the merozoite. It has been reported to be involved in the processing of the rhoptry associated protein 1 (PfRAP1) and the apical sushi protein (PfASP) [56].

Thus, 1 and 4 have the potential of inhibiting merozoite invasion into erythrocytes, though this is subject to in vitro and in vivo studies. In the same vein, 1 and 4 were docked into the ATP binding pocket of PfPI4K. Also, 1 and 4 interacted with the residues of the hydrophobic region (composed of residues like ILE) and the hinge region (composed mainly

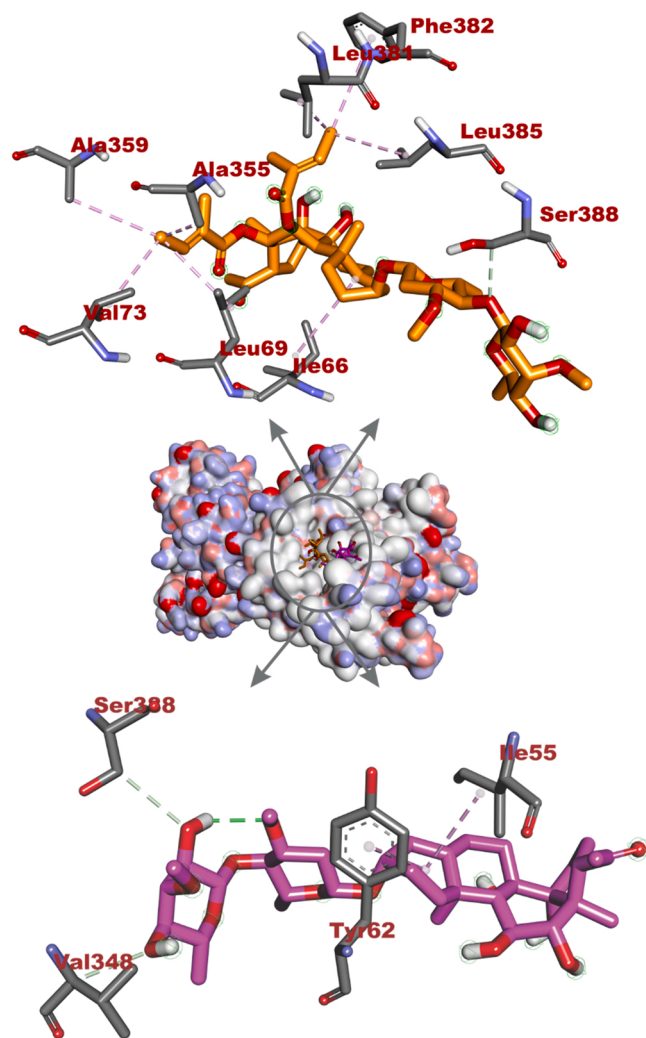


Fig. 7. Interaction of pregnanes with amino acids in the active site of *P. falciparum* Chloroquine Resistance Transporter (PfCRT): (a) surface representation (b) 3D interactive representation, ligands in stick representation are presented in different colors: (i) gold: 1 (ii) purple: 4 Types of interactions are represented by: Green-dotted line-hydrogen bonding; light purple-dotted line-hydrophobic interaction (pi-alkyl, alkyl and pi-stacking); purple-dotted line-pi-pi T-Shaped interaction; yellow-dotted line-pi-sulphur interaction and 3-letter abbreviation of amino acids are in red color.

of charged residues like HIS, LYS and ASP). The residues located in the hinge region interacted via hydrogen bonds with 1 and 4. This binding mode is similar to that of the highly potent selective inhibitor of PfPI4K, namely KAI407 [57]. Interaction with the ATP binding site, just like the imidazopyrazines, a class of compounds that target the PfPI4K, is known to inhibit the intracellular development of multiple *Plasmodium* species at each stage of infection in the vertebrate host [58,59] and PfPI4K has been reported to possess the potential of being an effective target for the eradication of malaria globally [57]. Due to the fact that PfPI4K had higher affinity for 1 and 4, the potentials of these compounds (and their structural modifications) in inhibiting this enzyme in vitro and in vivo can still be explored, with the aim of getting novel drugs that can immensely contribute to the global eradication of malaria.

Lactate dehydrogenase from *P. falciparum* (PflDH) has been considered as a potential molecular drug target [60], because of its critical role in production of reducing equivalent, which culminates in the production of ATP. Chloroquine interacted specifically with PflDH in the NADH binding pocket, occupying a position similar to that of the adenyl ring of the coenzyme, hence, acting as a competitive inhibitor for

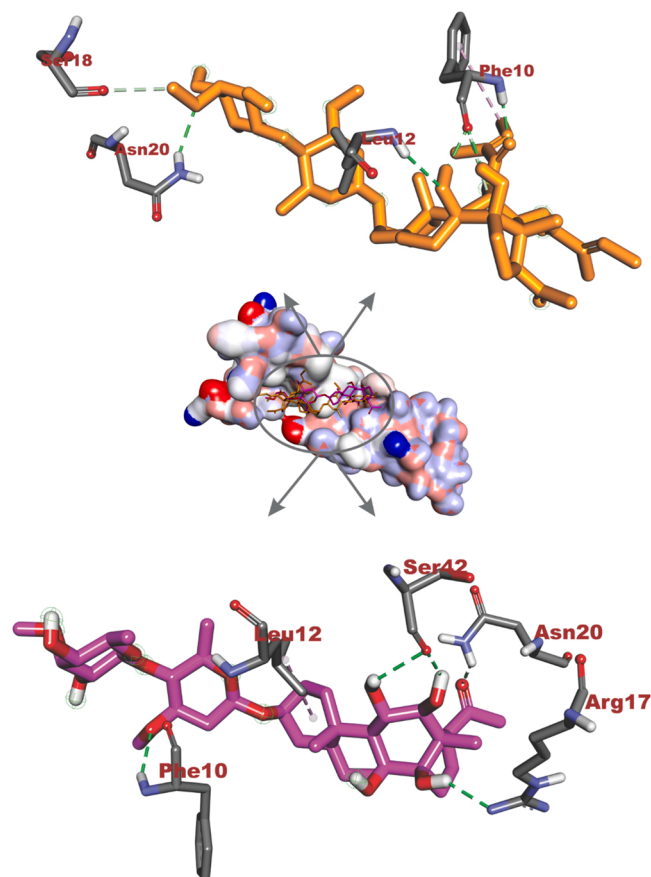


Fig. 8. Interaction of pregnanes with amino acids of modeled *P. falciparum* P-glycoprotein homolog (PfP-gH): (a) surface representation (b) 3D interactive representation, ligands in stick representation are presented in different colors: (i) gold: 1 (ii) purple: 4 Types of interactions are represented by: Green-dotted line-hydrogen bonding; light purple-dotted line-hydrophobic interaction (pi-alkyl, alkyl and pi-stacking); purple-dotted line-pi-pi T-Shaped interaction; yellow-dotted line-pi-sulphur interaction and 3-letter abbreviation of amino acids are in red color.

this critical glycolytic enzyme [61,62]. Also, 1 and 4 were docked into the substrate specificity loop (92–112) of LDH structure, which is extended by five more residues in PflDH at residue 108 (108 A–E). In a comparative binding mode analysis of 1, 4 and CQ (native co-crystallized ligand), it was observed that 1 and 4 were positioned in a lipophilic pocket formed by the hydrophobic residues ILE⁵⁴, ILE⁹⁸, ILE¹²³, PHE⁵², PHE¹⁰⁰ and TYR⁸⁵. Coincidentally, this form of association is considered competitive with NADH binding; a feature reported for gossypol and 8-deoxy hemigossypic acid derivatives [63,64]. *Plasmodium* species relies on glycolysis for its supply of energy because its cytochromes are not functionally well organized to ensure oxidative phosphorylation [65]. Since host erythrocytes lack mitochondria, *Plasmodium* species depends on its lactate dehydrogenase (LDH) to regenerate NAD⁺ which it needs to keep glycolysis on-going [66]. Therefore, inhibition of PflDH by 1 and 4 may result in limited or complete stoppage of ATP production and therefore the death of the parasite [63]. PfECPR, a critical enzyme in type II fatty acid biosynthesis, is a promising target for drug discovery against hepatocyte-stage *P. falciparum* [67]. PfECPR reduces the trans-2-enoyl bond of enoyl-acyl carrier protein (enoyl-ACP) substrate to saturated acyl-ACPs and it plays a vital role in completing the successive rounds of fatty acid elongation process. In a similar binding mode as triclosan (the co-crystallized ligand), 1 and 4 were docked into the fatty acyl binding pocket. The binding mode for triclosan is very similar to that of nicotinamide ring of NAD⁺, showing the same stacking interaction and comparable hydrogen-bonding pattern [68]. VAL²²², MET²⁸¹,

Table 8

Interacting amino acid residues of selected *Plasmodium falciparum* proteins with 1 and 4 isolated from *Gongronema latifolium* leaf.

Compounds	Pf PROTEIN	Residues involved in hydrogen bonding (bond distance, Å)	Residues involved in hydrophobic interactions
Comp. 1	PfPMIX	LEU ³⁸⁵ (2.19, 2.29) TYR ²³¹ (2.08) SER ³⁸³ (3.25, 3.73) GLY ²³⁰ (2.66) TYR ²²⁸ (2.73)	LYS ³⁸¹
Comp. 4		LEU ³⁸⁵ (2.19, 2.29) TYR ²³¹ (2.39) SER ³⁸³ (3.29) GLY ²³⁰ (2.54)	
Comp. 1	PfPI4K	ASP ²⁸⁰ (2.75)	LYS ²²⁹ CYS ²⁸² ILE ²²⁷ TYR ²⁷⁷
Comp. 4		PRO ²⁶⁴ (3.41) ARG ²⁶¹ (2.67) LYS ²⁴⁸ (2.25, 3.4) TRP ²⁵⁹ (2.12)	ARG ²⁶¹ LYS ²⁴⁸ TRP ²⁵⁹
Comp. 1	PfLDH	THR ¹⁰¹ (2.57)	MET ³⁰ ILE ⁵⁴ VAL ⁵⁵ PHE ¹⁰⁰ LYS ¹¹⁸ PHE ¹⁰⁰
Comp. 4		PRO ²⁴⁶ (2.80) SER ²⁴⁵ (2.39) GLY ²⁹ (2.36) THR ¹⁰¹ (3.57) GLY ⁹⁹ (3.66)	MET ³⁰ PHE ¹⁰⁰ ILE ¹¹⁹ ILE ⁵⁴
Comp. 1	PfECPR	ARG ³¹⁸ (2.54, 3.03) LYS ²⁸⁵ (2.17) ALA ²¹⁹ (2.36) ASN ²¹⁸ (3.46) ALA ³²⁰ (1.97)	TYR ²⁶⁷ ALA ³¹⁹ TRP ¹³¹
Comp. 4		LEU ³¹⁵ (2.54, 3.03) SER ³¹⁷ (2.17) GLY ¹⁰⁶ (2.36) GLY ¹⁰⁴ (3.46) ALA ³²⁰ (1.97)	TYR ²⁷⁷ ILE ³²³ LEU ²⁶⁵ ALA ²¹⁷ TYR ¹¹¹ VAL ¹³⁴ TRP ¹³¹
Comp. 1	PfCRT		VAL ⁷³ ILE ⁶⁶ ALA ³⁵⁵ ALA ³⁵⁹ PHE ³⁸² LEU ⁶⁹ LEU ³⁸¹ LEU ³⁸⁵ SER ³⁸⁸ TYR ⁶² ILE ⁵⁵ SER ³⁸⁸ VAL ³⁴⁸
Comp. 4			
Comp. 1	PfP-gH	PHE ¹⁰ (2.08) LEU ¹² (2.90) ASN ²⁰ (2.43) SER ¹⁸ (3.63)	
Comp. 4		PHE ¹⁰ (2.77) ASN ²⁰ (2.27) SER ⁴² (2.88) ARG ¹⁷ (2.61)	LEU ¹²

Plasmodium falciparum plasmepsin IX (PfPMI); *Plasmodium falciparum* phosphatidylinositol 4-kinase (PfP4K); *Plasmodium falciparum* lactate dehydrogenase (PfLDH) and *Plasmodium falciparum* enoyl-acyl-carrier-protein reductase (PfECPR); *Plasmodium falciparum* Chloroquine Resistance Transporter (PfCRT) and *Plasmodium falciparum* P-glycoprotein homolog (PfP-gH)

residues 218–219, the peptide backbone residues 217–231 and the side chain amino acids ASN²¹⁸, VAL²²², TYR²⁷⁷ and MET²⁸¹ have been reported to be significantly involved in the strong binding of both NAD⁺ and triclosan [35]. Also, 1 and 4 interacted with LYS²⁸⁵, TYR²⁷⁷, and TYR²⁶⁷ residues in a similar manner as celastrol, an inhibitor of PfECPR [67]. These suggest that 1 and 4 may inhibit fatty acid synthesis in the parasite.

Of the six proteins, only two (PfCRT and PfP-gH) are well characterized and established proteins involved in parasite resistance to chloroquine, though some ATPases and helicases, such as PfMLH (MutL homolog) and PfUvrD involved in mismatch repair pathway, have been implicated in the development of resistance by the parasites to antimalarials [69]. PfCRT is a major determinant of drug resistance in malaria parasites, especially to CQ and piperazine (PPQ), resulting from distinct sets of point mutations [36]. PfP-gH is the product of the PfMDR1 (multidrug resistance 1) gene and is similar to the mammalian cancer drug resistance associated P-glycoprotein (P-gp) [70,71]. PfP-gH has been studied as a marker of resistance to multiple antimalarials, including chloroquine and its analogues [72]. These two proteins were studied for drug-resistance inhibitory activities of the pregnane compounds while the other four were studied for other parasite enzyme inhibitory activities.

1 and 4 interacted more strongly than chloroquine with active site residues of 7G8 isoform of PfCRT bearing the mutated THR76 [36]. The pregnanes interacted with some of the residues in the ligand binding cavity of the transporter, with the most prominent being the interaction with SER³⁸⁸ [36]. Recently a computational study revealed a lower binding affinity of CQ to the mutated PfCRT at Lys76Thr [73]. Due to the fact that 1 and 4 did not bind to THR76, the higher affinity of PfCRT for them compared to chloroquine is not mediated by their direct interaction with the mutated amino acid. PfP-gH had higher binding affinity for 1 and 4 than chloroquine; they may therefore serve as inhibitors or blockers of this protein, thereby reversing chloroquine resistance and enhancing the effectiveness of chloroquine in killing the parasite, as was observed with P-gH inhibitors/blockers which were reported to be effective in reversing multidrug resistance in *P. falciparum* [13]. The potentiation of the activity of chloroquine against *P. falciparum* W2 by 1 and 4 may be as a result of their interaction with PfP-gH more than PfCRT because they had similar binding energies for PfCRT compared to chloroquine while they had lower binding energies for PfP-gH compared to chloroquine.

5. Conclusion

The results of this study have shown that iloneoside (1), a ditigloylated pregnane glycoside, possesses antiparasmodial activity and potentiated the activity of CQ against a resistant strain of *P. falciparum*. The *in silico* study showed that 1 was firmly docked into the catalytic site and interacted with the catalytic residues of the selected Pfproteins in the same binding mode as the reference inhibitors. Thus, iloneoside, a pregnane glycoside obtained from *Gongronema latifolium* leaf, is a hit antimalarial compound, which can potentiate the activity of CQ against multidrug resistant strains of *P. falciparum*.

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CRediT authorship contribution statement

J.O. Adebayo was involved in the Conceptualization; I.P. Ceravolo, G.A. Gyebi and J.P. Penna-Coutinho were involved in the investigation; J.O. Adebayo, I.P. Ceravolo, G.A. Gyebi and J.P. Penna-Coutinho were involved in formal analysis; J.O. Adebayo and A.U. Krettli were involved in the Supervision; J.O. Adebayo, G.A. Gyebi, E.O. Olorundare, A.S. Babatunde and M. Koketsu were involved in initial isolation of compounds. J.O. Adebayo, A.U. Krettli, I.P. Ceravolo, G.A. Gyebi and J.P. Penna-Coutinho were involved in the preparation of manuscript.

Conflicts of interest/Competing interests

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molbiopara.2022.111474.

References

- [1] Y. Nishimoto, N. Arisue, S. Kawai, A.A. Escalante, T. Horii, K. Tanabe, T. Hashimoto, Evolution and phylogeny of the heterogeneous cytosolic SSU rRNA genes in the genus *Plasmodium*, *Mol. Phylogenetics Evol.* 47 (2008) 45–53.
- [2] WHO, World Malaria Report 2021, World Health Organization, Geneva, 2021. (<https://www.who.int/publications/i/item/9789240040496>).
- [3] M. Schlitzer, Antimalarial drugs—what is in use and what is in the pipeline, *Arch. der Pharm.: Int. J. Pharm. Med. Chem.* 341 (3) (2008) 149–163.
- [4] G. Edwards, Antimalarial chemotherapy: Mechanisms of action, resistance and new directions in drug discovery, *Br. J. Clin. Pharmacol.* 52 (4) (2001) 464.

- [5] W.R.J. Taylor, N.J. White, Antimalarial drug toxicity, *Drug Saf.* 27 (1) (2004) 25–61.
- [6] P.J. Guerin, P. Olliaro, F. Nosten, P. Druilhe, R. Laxminarayan, F. Binka, W. L. Kilama, N. Ford, N.J. White, Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development, *Lancet Infect. Dis.* 2 (9) (2002) 564–573.
- [7] H. Ginsburg, Should chloroquine be laid to rest? *Acta Trop.* 96 (1) (2005) 16–23.
- [8] H. Tinto, C. Rwagacondo, C. Karema, D. Mupfasoni, W. Vandoren, E. Rusanganwa, A. Erhart, C. Van Overmeir, E. Van Marck, U. D'Alessandro, In-vitro susceptibility of *Plasmodium falciparum* to monodesethylamodiaquine, dihydroartemisinin and quinine in an area of high chloroquine resistance in Rwanda, *Trans. R. Soc. Trop. Med. Hyg.* 100 (6) (2006) 509–514.
- [9] A.M. Dondorp, R.M. Fairhurst, L. Slutsker, J.R. MacArthur, J.G.B. MD, P.J. Guerin, T.E. Wellems, P. Ringwald, R.D. Newman, C.V. Plowe, The threat of artemisinin-resistant malaria, *N. Engl. J. Med.* 365 (12) (2011) 1073–1075.
- [10] F. Arley, B. Witkowski, C. Amaratunga, J. Beghain, A.-C. Langlois, N. Khim, S. Kim, V. Duru, C. Bouchier, L. Ma, A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria, *Nature* 505 (7481) (2014) 50–55.
- [11] S. Kumar, T. Bhardwaj, D. Prasad, R.K. Singh, Drug targets for resistant malaria: historic to future perspectives, *Biomed. Pharmacother.* 104 (2018) 8–27.
- [12] C.-P. Wu, D.A. van Schalkwyk, D. Taylor, P.J. Smith, K. Chibale, Reversal of chloroquine resistance in *Plasmodium falciparum* by 9H-xanthene derivatives, *Int. J. Antimicrob. Agents* 26 (2) (2005) 170–175.
- [13] M.F.M. Khairul, T.H. Min, J.H. Low, C.H.C. Nasriyyah, A.N. A shikin, M. N. Norazmi, M. Ravichandran, S.S. Raju, Fluoxetine potentiates chloroquine and mefloquine effect on multidrug-resistant *Plasmodium falciparum* in vitro, *Jpn. J. Infect. Dis.* 59 (5) (2006) 329.
- [14] K.J. Deane, R.L. Summers, A.M. Lehane, R.E. Martin, R.A. Barrow, Chlorpheniramine analogues reverse chloroquine resistance in *Plasmodium falciparum* by inhibiting PfCRT, *ACS Med. Chem. Lett.* 5 (5) (2014) 576–581.
- [15] E. Abdel-Sattar, F.M. Harraz, S.M.A. Al-Ansari, S. El-Mekkawy, C. Ichino, H. Kiyohara, A. Ishiyama, K. Otoguro, S. Omura, H. Yamada, Acylated pregnane glycosides from *Caralluma tuberculata* and their antiparasitic activity, *Phytochemistry* 69 (11) (2008) 2180–2186.
- [16] A. Libman, H. Zhang, C. Ma, B. Southavong, K. Sydara, S. Bouamanivong, G.T. Tan, H.H. Fong, D.D. Soejarto, A first new antimalarial pregnane glycoside from *Gongronema napaleense*, *Asian J. Tradit. Med.* 3 (6) (2008) 203.
- [17] G.A. Gyebi, J.O. Adebayo, O.E. Olorundare, A. Pardede, M. Ninomiya, A.O. Saheed, A.S. Babatunde, M. Koketsu, Ilongeone: a cytotoxic ditigloylated pregnane glycoside from the leaves of *Gongronema latifolium* Benth, *Nat. Prod. Res.* 32 (24) (2018) 2882–2886.
- [18] E. Siqueira, I. Ceravolo, M. Kohlhoff, A. Krettl, C. Zani, Synthesis and antiplasmodial activity of 2-methyl-3-carboxyl-naphtho [2, 3-B] furan quinone derivatives, *J. Med. Chem. Drug Des.* 1 (2) (2018).
- [19] C. Lambros, J.P. Vanderberg, Synchronization of *Plasmodium falciparum* erythrocytic stages in culture, *J. Parasitol.* (1979) 418–420.
- [20] A.C.C. Aguiar, R.D.M. Santos, F.J.B. Figueiredo, W.A. Cortopassi, A.S. Pimentel, T.C. C. França, M.R. Meneghetti, A.U. Krettl, Antimalarial activity and mechanisms of action of two novel 4-aminoquinolines against chloroquine-resistant parasites, *PLoS One* 7 (5) (2012), e37259.
- [21] E. Borenfreund, J.A. Puerner, A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90), *J. Tissue Cult. Methods* 9 (1) (1985) 7–9.
- [22] D.E. Kyle, A.M. Oduola, S.K. Martin, W.K. Milhous, *Plasmodium falciparum*: modulation by calcium antagonists of resistance to chloroquine, desethylchloroquine, quinine, and quinidine in vitro, *Trans. R. Soc. Trop. Med. Hyg.* 84 (4) (1990) 474–478.
- [23] A. Oduola, A. Sowunmi, W. Milhous, T. Brewer, D. Kyle, L. Gerena, R. Rossan, L. Salako, B. Schuster, In vitro and in vivo reversal of chloroquine resistance in *Plasmodium falciparum* with promethazine, *Am. J. Trop. Med. Hyg.* 58 (5) (1998) 625–629.
- [24] E. Cilia, R. Pancsa, P. Tompa, T. Lenaerts, W.F. Vranken, The DynaMine webserver: predicting protein dynamics from sequence, *Nucleic Acids Res.* 42 (W1) (2014) W264–W270.
- [25] M. Biasini, S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T. G. Cassarino, M. Bertoni, L. Bordoli, SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information, *Nucleic Acids Res.* 42 (W1) (2014) W252–W258.
- [26] A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello, R. Gumienny, F. T. Heer, T.A.P. de Beer, C. Rempfer, L. Bordoli, SWISS-MODEL: homology modelling of protein structures and complexes, *Nucleic Acids Res.* 46 (W1) (2018) W296–W303.
- [27] C. Camacho, G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, T. L. Madden, BLAST+: architecture and applications, *BMC Bioinforma.* 10 (1) (2009) 1–9.
- [28] M. Remmert, A. Biegert, A. Hauser, J. Söding, HHblits: lightning-fast iterative protein sequence searching by HMM-HMM alignment, *Nat. Methods* 9 (2) (2012) 173–175.
- [29] L. Heo, H. Park, C. Seok, GalaxyRefine: protein structure refinement driven by side-chain repacking, *Nucleic Acids Res.* 41 (W1) (2013) W384–W388.
- [30] D. Bhattacharya, J. Nowotny, R. Cao, J. Cheng, 3Drefine: an interactive web server for efficient protein structure refinement, *Nucleic Acids Res.* 44 (W1) (2016) W406–W409.
- [31] C. Colovos, T.O. Yeates, Verification of protein structures: patterns of nonbonded atomic interactions, *Protein Sci.* 2 (9) (1993) 1511–1519.
- [32] M. Wiederstein, M.J. Sippl, ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins, *Nucleic Acids Res.* 35 (suppl_2) (2007) W407–W410.
- [33] R. Laskowski, M. MacArthur, D. Moss, J. Thornton, SFCHECK: a unified set of procedures for evaluating the quality of macromolecular structure-factor data and their agreement with the atomic model, *J. Appl. Crystallogr* 26 (1993) 283–291.
- [34] C.R. Dunn, M.J. Banfield, J.J. Barker, C.W. Higham, K.M. Moreton, D. Turgut-Balik, R.L. Brady, J.J. Holbrook, The structure of lactate dehydrogenase from *Plasmodium falciparum* reveals a new target for anti-malarial design, *Nat. Struct. Biol.* 3 (1996) 912–915.
- [35] R. Perozzo, M. Kuo, J.T. Valiyaveetil, R. Bittman, W.R. Jacobs Jr., D.A. Fidock, J. C. Sacchettini, Structural elucidation of the specificity of the antibacterial agent triclosan for malarial enoyl acyl carrier protein reductase, *J. Biol. Chem.* 277 (15) (2002) 13106–13114.
- [36] J. Kim, Y.Z. Tan, K.J. Wicht, S.K. Erramilli, S.K. Dhingra, J. Okombo, J. Vendome, L.M. Hagenah, S.I. Giacometti, A.L. Warren, Structure and drug resistance of the *Plasmodium falciparum* transporter PfCRT, *Nature* 576 (7786) (2019) 315–320.
- [37] N.M. O'Boyle, M. Banck, C.A. James, C. Morley, T. Vandermeersch, G. R. Hutchison, Open Babel: An open chemical toolbox, *J. Cheminform.* 3 (2011) 33.
- [38] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading, *J. Comput. Chem.* 31 (2) (2010) 455–461.
- [39] S. Dallakyan, A.J. Olson, Small-molecule library screening by docking with PyRx, *Chemical biology*, Springer (2015) 243–250.
- [40] J. Bero, M. Fréderich, J. Quetin-Leclercq, Antimalarial compounds isolated from plants used in traditional medicine, *J. Pharm. Pharmacol.* 61 (11) (2009) 1401–1433.
- [41] G. Cioffi, R. Sanogo, A. Vassallo, F. Dal Piaz, G. Autore, S. Marzocco, N. De, Tommasi, Pregnane Glycosides from *Leptadenia p yrotechnica*, *J. Nat. Prod.* 69 (4) (2006) 625–635.
- [42] D.A. van Schalkwyk, J.C. Walden, P.J. Smith, Reversal of chloroquine resistance in *Plasmodium falciparum* using combinations of chemosensitizers, *Antimicrob. Agents Chemother.* 45 (11) (2001) 3171–3174.
- [43] B.Y. Hwang, S.E. Kim, Y.H. Kim, Y.S. Hong, J.S. Ro, K.S. Lee, J.J. Lee, Pregnane glycoside multidrug-resistance modulators from *Cynanchum wilfordii*, *J. Nat. Prod.* 62 (4) (1999) 640–643.
- [44] S.Y. Chung, M.K. Sung, N.H. Kim, J.O. Jang, E.J. Go, H.J. Lee, Inhibition of P-glycoprotein by natural products in human breast cancer cells, *Arch. Pharm. Res.* 28 (7) (2005) 823–828.
- [45] J.L. Venerstrom, E.O. Nuzum, R.E. Miller, A. Dorn, L. Gerena, P.A. Dande, W. Y. Ellis, R.G. Ridley, W.K. Milhous, 8-Aminoquinolines active against blood stage *Plasmodium falciparum* in vitro inhibit hematin polymerization, *Antimicrob. Agents Chemother.* 43 (3) (1999) 598–602.
- [46] M. Foley, L. Tilley, Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents, *Pharmacol. Ther.* 79 (1) (1998) 55–87.
- [47] G. Munsamy, M.E. Soliman, Unveiling a new era in malaria therapeutics: a tailored molecular approach towards the design of plasmepsin IX inhibitors, *Protein J.* 38 (6) (2019) 616–627.
- [48] A.S. Nasamu, S. Glushakova, I. Russo, B. Vaupel, A. Oksman, A.S. Kim, D. H. Fremont, N. Tolia, J.R. Beck, M.J. Meyers, Plasmepsins IX and X are essential and druggable mediators of malaria parasite egress and invasion, *Science* 358 (6362) (2017) 518–522.
- [49] S. Fienberg, C.J. Eyermann, L.B. Arendse, G.S. Basarab, J.A. McPhail, J.E. Burke, K. Chibale, Structural Basis for Inhibitor Potency and Selectivity of *Plasmodium falciparum* Phosphatidylinositol 4-Kinase Inhibitors, *ACS Infect. Dis.* 6 (11) (2020) 3048–3063.
- [50] J.E. Burke, A.J. Inglis, O. Perisic, G.R. Masson, S.H. McLaughlin, F. Rutaganira, K. M. Shokat, R.L. Williams, Structures of PI4KIIIβ complexes show simultaneous recruitment of Rab11 and its effectors, *Science* 344 (6187) (2014) 1035–1038.
- [51] Z. Xiang, Advances in homology protein structure modeling, *Curr. Protein Pept. Sci.* 7 (3) (2006) 217–227.
- [52] P. Benkert, M. Biasini, T. Schwede, Toward the estimation of the absolute quality of individual protein structure models, *Bioinformatics* 27 (3) (2011) 343–350.
- [53] D.N. Nair, V. Singh, D. Angira, V. Thiruvengadam, Structural investigation and in-silico characterization of Plasmepsins from *Plasmodium falciparum*, *Front. Hum. Neurosci.* 10 (2016) 460.
- [54] A.V. Pandey, H. Bisht, V.K. Babbarwal, J. Srivastava, K.C. Pandey, V.S. Chauhan, Mechanism of malarial haem detoxification inhibition by chloroquine, *Biochem. J.* 355 (2) (2001) 333–338.
- [55] B. Mukherjee, F. Tessaro, J. Vahokoski, I. Kursula, J.B. Marq, L. Scapozza, D. Soldati-Favre, Modeling and resistant alleles explain the selectivity of antimalarial compound 49c towards apicomplexan aspartyl proteases, *EMBO J.* 37 (7) (2018), e98047.
- [56] P. Pino, R. Caldelari, B. Mukherjee, J. Vahokoski, N. Klages, B. Maco, C.R. Collins, M.J. Blackman, I. Kursula, V. Heussler, A multistage antimalarial targets the plasmepsins IX and X essential for invasion and egress, *Science* 358 (6362) (2017) 522–528.
- [57] C.W. McNamara, M.C. Lee, C.S. Lim, S.H. Lim, J. Roland, A. Nagle, O. Simon, B. K. Yeung, A.K. Chatterjee, S.L. McCormack, Targeting *Plasmodium* PI (4) K to eliminate malaria, *Nature* 504 (7479) (2013) 248–253.
- [58] A.-M. Zeeman, S.M. Van Amsterdam, C.W. McNamara, A. Voorberg, E.J. Van Der Wel, A. Klooster, E.J. Van Den Berg, D.M. Remarque, G.-J. Plouffe, A. Van Gemert, Luty, KAI407, a potent non-8-aminoquinoline compound that kills *Plasmodium cynomolgi* early dormant liver stage parasites in vitro, *Antimicrob. Agents Chemother.* 58 (3) (2014) 1586–1595.

- [59] B. Zou, A. Nagle, A.K. Chatterjee, S.Y. Leong, L.J. Tan, W.L.S. Sim, P. Mishra, P. Guntapalli, D.C. Tully, S.B. Lakshminarayana, Lead optimization of imidazopyrazines: a new class of antimalarial with activity on *Plasmodium* liver stages, *ACS Med. Chem. Lett.* 5 (8) (2014) 947–950.
- [60] T.J. Egan, K.K. Ncokazi, Quinoline antimalarials decrease the rate of β -hematin formation, *J. Inorg. Biochem.* 99 (7) (2005) 1532–1539.
- [61] K.K. Ncokazi, T.J. Egan, A colorimetric high-throughput β -hematin inhibition screening assay for use in the search for antimalarial compounds, *Anal. Biochem.* 338 (2) (2005) 306–319.
- [62] D.S. Wishart, C. Knox, A.C. Guo, D. Cheng, S. Shrivastava, D. Tzur, B. Gautam, M. Hassanali, DrugBank: a knowledgebase for drugs, drug actions and drug targets, *Nucleic Acids Res.* 36 (suppl_1) (2008) D901–D906.
- [63] R. Connors, F. Schambach, J. Read, A. Cameron, R.B. Sessions, L. Vivas, A. Easton, S.L. Croft, R.L. Brady, Mapping the binding site for gossypol-like inhibitors of *Plasmodium falciparum* lactate dehydrogenase, *Mol. Biochem. Parasitol.* 142 (2) (2005) 137–148.
- [64] D.L. Vander Jagt, R. Royer, Gossypol prototype of inhibitors targeted to dinucleotide folds, *Curr. Med. Chem.* 7 (4) (2000) 479–498.
- [65] T. Planche, A. Dzeing, E. Ngou-Milama, M. Kombila, P. Stacpoole, Metabolic complications of severe malaria, *Malaria: Drugs, Disease and Post-genomic, Biology* (2005) 105–136.
- [66] F. Mi-Ichi, S. Takeo, E. Takashima, T. Kobayashi, H.-S. Kim, Y. Wataya, A. Matsuda, M. Torii, T. Tsuboi, K. Kita, Unique properties of respiratory chain in *Plasmodium falciparum* mitochondria, *Trop. Dis.* (2003) 117–133.
- [67] L. Tallorin, J.D. Durrant, Q.G. Nguyen, J.A. McCammon, M.D. Burkart, Celastrol inhibits *Plasmodium falciparum* enoyl-acyl carrier protein reductase, *Bioorg. Med. Chem.* 22 (21) (2014) 6053–6061.
- [68] R. Perozzo, M. Kuo, J.T. Valiyaveetil, R. Bittman, W.R. Jacobs, D.A. Fidock, J. C. Sacchettini, Structural Elucidation of the Specificity of the Antibacterial Agent Triclosan for Malarial Enoyl Acyl Carrier Protein Reductase*, *J. Biol. Chem.* 277 (15) (2002) 13106–13114.
- [69] M. Ahmad, R. Tuteja, Emerging importance of mismatch repair components including UvrD helicase and their cross-talk with the development of drug resistance in malaria parasite, *Mutat. Res. /Fundam. Mol. Mech. Mutagen.* 770 (2014) 54–60.
- [70] C.M. Wilson, A.E. Serrano, A. Wasley, M.P. Bogenschutz, A.H. Shankar, D.F. Wirth, Amplification of a gene related to mammalian *mdr* genes in drug-resistant *Plasmodium falciparum*, *Science* 244 (4909) (1989) 1184–1186.
- [71] A. Cowman, The P-glycoprotein homologues of *Plasmodium falciparum*: Are they involved in chloroquine resistance? *Parasitol. Today* 7 (4) (1991) 70–76.
- [72] J.P. Gil, S. Krishna, *pfm-dr1* (*Plasmodium falciparum* multidrug drug resistance gene 1): a pivotal factor in malaria resistance to artemisinin combination therapies, *Expert Rev. Anti-Infect. Ther.* 15 (6) (2017) 527–543.
- [73] C. Patel, D. Roy, A computational study of molecular mechanism of chloroquine resistance by chloroquine resistance transporter protein of *Plasmodium falciparum* via molecular modeling and molecular simulations, *Physchem* 1 (3) (2021) 232–242.
- [74] M. Smilkstein, N. Sriwilaijaroen, J.X. Kelly, P. Wilairat, M. Riscoe, Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening, *Antimicrob Agents chemother* 48 (5) (2004) 1803–1806.
- [75] A. Nzila, L. Mwai, In vitro selection of *Plasmodium falciparum* drug-resistant parasite lines, *J. Antimicrob Chemother* 65 (3) (2010) 390–398.
- [76] C. Bézivin, S. Tomasi, F. Lohézic-Le Dévéhat, J. Boustie, Cytotoxic activity of some lichen extracts on murine and human cancer cell lines, *Phytomed* 10 (6–7) (2003) 499–503.
- [77] A.M. Oduola, A. Sowumi, W.K. Milhous, T.G. Brewer, D.E. Kyle, L. Gerena, R. N. Rossan, L.A. Salako, B.G. Schuster, In vitro and in vivo reversal of chloroquine resistance in *Plasmodium falciparum* with promethazine. *The Am J Trop. Med. Hyg* 58 (5) (1998) 625–629.