IN SILICO AND IN VITRO STUDIES OF ACETAZOLAMIDE AS A GAMETOCIDAL AGENT

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ABSTRACT
Malaria remains the leading cause of mortality around the world and is the most common disease in Africa and some countries in Asia with the highest number of indigenous cases with Plasmodium falciparum responsible for the majority of the disease burden. Plasmodium has a complex life cycle consisting of multiple stages in two hosts. During the erythrocytic life cycle, it follows one of two developmental fates: terminal sexual differentiation into gametocytes or asexual propagation. Transmission from an infected human host to a susceptible mosquito is done through highly specialized sexual stages gametocytes because they are the only form of malaria parasite that are transmissible to mosquito vector, hence the discovery of new antimalarials with transmission blocking activity remains a key issue in efforts to control malaria and eventually eradicate the disease. In this study the in silico and in vitro gametocidal properties of one of the Sulfonamide drugs Acetazolamide had been checked in order to find new antiplasmodial agent. In vitro cultivation of the RKL-9 (Chloroquine resistant) strain of P. falciparum was continued to develop sexual gametocytic early stages. The drug acetazolamide was screened for gametocidal assay in order to check its transmission blocking activity by targeting early sexual stages of the parasites. Three Plasmodium falciparum proteins Pfpg27, Pfs230 and PUF1 were selected for molecular docking with the acetazolamide as ligand. HEX 8.0.0 server was used as docking tool and the results obtained were analysed in Discovery studio visualizer. Our findings of in vitro studies revealed that the selected drug acetazolamide possess promising gametocidal activity with IC₅₀ = 4.812µg/ml. Hence the study can be used further for drug formulations as antimalarials targeting Gametocytic stages. Based on the in silico analysis the good binding scores -196.76, -173.87 and -193.29 (Kcal/mol) were obtained for all three selected Plasmodium falciparum proteins Pfpg27, Pfs230 and PUF1 with the ligand acetazolamide respectively. Thus the docking energy results of in silico studies also supported our in vitro results that the acetazolamide can be used as an antiplasmodial drug against the gametocytes. Further studies for the mechanism of action of the acetazolamide as a gametocidal agent can be done for preventing the transmission of malaria.

Keywords: Plasmodium falciparum, Gametocide, Acetazolamide, In-silic, In vitro.

INTRODUCTION
In the developing countries, Malaria is responsible for highest rate of morbidity and mortality. Despite several advances and progresses for the control of malaria, it is still an important global health problem. There is no significant fall in malaria burden globally from an estimated 239 cases in 2010 to 219 million new malaria cases in 2017 and thousands of death by malaria occurred globally[1]. It is surprising that the 80% of the burden is solely found in India and sub Saharan African countries as per WHO report 2018 [1].In a current scenario, malaria treatment is relied on very few drugs. Spread of resistance to first line antimalarial drugs including artemisinin based combination therapy calls for the search and novel drug development [2]. Efforts are required to focus on novel inhibitor compounds that can specifically target and block parasite growth and transmission of Plasmodium parasite. The merozoites leave the cycle of blood-stage asexual multiplication and differentiate into sexual erythrocytic stages forming male and female gamete precursors called gametocytes. Gametocytes are highly specialized form of the parasite responsible for its
transmission. The gametocytes are ingested when a female Anopheles mosquito bites an infected human and develop into mature cells called gametes and complete the sexual cycle in the mosquito that takes 10-18 days depending on species and temperature [3,4]. For attaining malaria elimination, preventing transmission is crucial and hence approaches targeting gametocytes are essential. Pfs230 is one of the major candidates and plays an important role in sexual-stage development of the parasite. The full length Pfs230 expressed in gametocyte (sexual-stage parasites in humans) is a 360-kDa protein. When a gametocyte is ingested by a mosquito, the parasite egresses from the erythrocyte and becomes a gamete. During this process, the first 442 amino acids of the Pfs230 molecule are cleaved and the remaining Pfs230 is exposed on the surface of gamete [5].

According to the findings of Sony Shrestha et al., 2016, during P. falciparum sexual development the Pf-family member Puf1 (denoted as PfPuf1 for the P. falciparum protein) plays a significant role. They conclude that PfPuf1 was expressed in all gametocyte stages and at higher levels in female gametocytes. PfPuf1 disruption did not interfere with the asexual erythrocytic cycle of the parasite but resulted in an approximately tenfold decrease of mature gametocytes. In the PfPuf1-disrupted lines, gametocytes appeared normal before stage III but subsequently exhibited a sharp decline in gametocytaemia. This was accompanied by a concomitant accumulation of dead and dying late-stage gametocytes, which retained normal gross morphology. In addition, significantly more female gametocytes were lost in the PfPuf1-disrupted lines during development, resulting in a reversed male-to-female sex ratio. These results indicate that PfPuf1 is important for the differentiation and maintenance of gametocytes, especially female gametocytes [6].

In the human malaria parasite P. falciparum, gametocyte maturation is a process remarkably longer than in other malaria species, accompanied by expression of 2–300 sexual stagespecific proteins. The work of Olivier et al., 2009 suggested that the Pfg27 involved in maintaining cell integrity in the uniquely long gametocyogenesis of P. falciparum [7]. The studies of Lobo et al., 1999 interprets that the parasites could have undergone sexual commitment and then failed to progress through the normal course of gametocyte maturation because of the lack of Pfg27 [8]. Further studies may elucidate the role of Pfg27 in the sexual development in P. falciparum, an apasrate that has a devastating impact on the health of more than 300 million people annually.

**ACETAZOLAMIDE**

The global emergence of drug-resistant malaria parasites necessitates identification and characterization of novel drug targets and their potential inhibitors. Primary Sulphonamide compounds are known to inhibit carbonic anhydrase (CA) enzyme activity in many organisms. Carbonic anhydrases (CAs), a group of ubiquitously expressed metalloenzymes, are involved in numerous physiological and pathological processes, including gluconeogenesis, lipogenesis, ureagenesis, tumorigenicity and the growth and virulence of various pathogens. In addition to the established role of CA inhibitors (CAIs) as diuretics and antiglaucoma drugs, it has recently emerged that CAIs could have potential as novel anti-obesity, anticancer and anti-infective drugs [9]. CA enzymes maintain an important physiological equilibrium: the hydration of carbon dioxide to bicarbonate anion and a proton: H₂O + CO₂ ⇌ HCO₃⁻ + H⁺ and are responsible for HCO₃⁻ and pH homeostasis, including within erythrocytes.

Malaria parasite CA inhibitors were first suggested as a potential new class of antimalarials in 1998 [10] and later the esterase activity of P. falciparum CA (PfCA) [11] shown to be inhibited by a series of Primary Sulphonamide compounds [12]. Further characterized PfCA from P. falciparum 3D7 identified that this protein as the first member of a new family of CAs (the η-CA family) [13]. Using purified recombinant PfCA expressed in E. coli, hydration of CO₂ was shown to be inhibited by clinically used PS compounds [14].

An in vitro study for checking the antimalarial activity of the selected drug acetazolamide had been conducted against the P. falciparum RKL-9 chloroquine resistant strain early gametocytic stages. For confirmation of the experimental results, the computational study was also performed using in silico molecular docking. In our study the in silico molecular docking analyses was also performed for the determination of binding affinity of the selected
drug acetazolamide with *P. falciparum* proteins Pf230, PUF1 and Pf27 associated with the maintenance and differentiation of the gametocytogenesis.

Materials and methods

**In vitro cultivation of RKL-9 chloroquine resistant strain**

*In-vitro* Cultivation of Malarial Parasites RKL-9 Chloroquine resistant strain was performed by the method of Trager and Jensen [15]. Complete parasite media (CPM) consisted of RPMI 1640 supplemented with HEPES, L-glutamine, NaHCO3, glucose (1g) and gentamycin. Conditions of incubation: 37°C, 92.5% nitrogen, 5.5% carbon dioxide, 2% oxygen. Parasites were cultured in O^−ve^ RBC’s and maintained in a CO2 incubator with daily media change until a parasitaemia of more than 5% ring stages were obtained.

Incomplete media of 90ml added to 10ml of O^−ve^ human plasma with CaCl2 treatment and stored it at 4°C. The parasite RKL-9 strain of *P. falciparum* was obtained from National Institute of Malaria Research (NIMR) Sector-8 Dawarka, New Delhi. The infected blood was centrifuged and supernatant was removed. The cells were washed and prepared 50% suspension with complete media. To the infected pellet added appropriate amount of uninfected cells to get an initial parasitaemia of 0.5-1%. Culture was kept in a CO2 incubator at 37°C. For the checking of parasitaemia the media was removed using sterile Pasteur pipette without disturbing the cells settled down. Mixed the cells without frothing and placed a small drop of blood on the slide and made a thin blood film (smear). Stained the slides in JSB-1 (16-18 sec), JSB-II (6-8 sec) stains and examined for parasitaemia and compared with initial Parasitaemia (100x Oil immersion lens). The growth was estimated by number of schizonts and rings in subsequent slides. When the parasitaemia obtained above 2-3% in the initial culture then proceeded for sub-culturing.

Determining the percentage of parasitized erythrocytes

The number of infected red blood cells in 1000 RBCs is converted to percentage. This method estimated the percentage of red blood cells infected with malarial parasites. The smear is scanned carefully, one ‘row’ at a time. The total number of red cells and the number of parasitized red cells tabulated separately. The 1000 red cells were counted and divided by the number of parasitized red cells by 10 to get the percentage [16].

Synchronization of culture

Synchronization is a process by which cells in a culture at different stages of the cell cycle are brought to the same phase. When cultures with 27%-60% of ring stage were synchronized using this procedure, 70-93% ring stages were obtained after 48hrs of culture and relative growth synchrony remained for at least two erythrocytic stages. The culture was synchronized using 5% aqueous solution of sorbitol. The degenerated stages were removed by centrifuging for 5 minutes at 1500 rpm. The supernatant was discarded and the pellet was washed thrice with incomplete media. The pellet was transferred into vials and complete media was added, mixed well and kept at 37°C in CO2 incubator [17].

Culturing of Gametocytes

In order to minimise parasite stress and spontaneous gametocyte production, asexual cultures were kept between 1 and 5% parasitaemia, at a maximum of 3% haematocrit, in a culture media volume of between 35- and 50-ml. Parasites were regularly sub-cultured at trophozoite or schizont stages to maintain synchronicity and to minimise gametocyte formation prior to stress induction. In addition, selection of ring stage parasites by incubation with 5% d-sorbitol (Sigma) in water was carried out to improve synchronicity. Medium was changed daily until the point of induction of gametocytogenesis. Cultures were monitored by microscopy using standard JSB I and JSB II blood smears. 2.5 ml culture was seeded with a 0.2% parasitaemia, at 6% haematocrit in a glass culture vials and added 100 µl of RBC’s on day 1. From day 4 onwards, media was replaced daily with 2.5ml of CPM to reduce the haematocrit to 3%. On day 9-11, 50Mm of N- acetyl glucosamine (NAG) was added to the cultures [18]. Early gametocytes obtained were isolated and used further for gametocidal assay.
Gametocidal Assay
The drug acetazolamide (Tablets I.P. 250 mg; Manufactured by Pfizer Limited) was dissolved in DMSO (1%) and added to triplicate wells of a 96 well plate. Early stage gametocytaemia of the parasite culture was adjusted to 5% by adding RBCs to the culture. 90µL of early stage purified gametocytes culture was pipetted into each treated well. The plates were put into an incubating chamber and gassed for 6 minutes. The incubating chamber was then placed in the incubator for 72 hours. Thin smears were prepared from each well and stained with JSB 1 and JSB II for 15 minutes. The slides were dried and observed under a 100X oil immersion objective lens to estimate gametocytocmia [19].

*In silico* studiesHex Software
In the present study, Hex (version 8.0.0) software was used as a docking tool. Protein-ligand docking and Protein-Protein docking is a very useful approach to find the binding interaction of target protein with single or multiple ligands. Based on the literature review for our *insilico* studies we have selected Acetazolamide as ligand and Pfg27, PfPuf1 and Pfs230 *Plasmodium falciparum* gametocytic proteins as our target proteins.

Uniprot
The uniprot knowledge base is a large resource of protein sequence associated detailed annotation. The database contains over 60 million sequences, of which over half a million sequences have been curated by experts who critically review experimental and predicted data for each protein. The remainder are automatically annotated based on rule systems that rely on the expert curated knowledge. UniProt is a long-standing collection of databases that enable scientists to navigate the vast amount of sequence and functional information available for proteins.

Swiss model
Homology modelling of all three selected *P. falciparum* proteins (Pfg27, PfPuf1 and Pfs230) was done using SWISS MODEL online software. SWISS MODEL is a database of 3D protein structure models generated by the SWISS–MODEL homology modelling pipeline. The aim of the SWISS MODEL Repository is to provide access to an up-to-date collection of annotated 3D protein models generated by automates homology modelling for all sequences in SwissProt and for relevant model organisms. Regular updates ensure that target coverage is complete, that models are built using the most recent sequence and template structure databases and that improves in the underlying modelling pipelines are fully utilized [20].

Discovery Studio Visualizer
The ligand proteins interactions after docking were visualized under the BIOVIA Discovery Studio Visualizer.

PubChem
Acetazolamide was selected as ligands for this study and was obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in 3D structure data format (SDF) [21].

Results and Discussion

*In vitro* cultivation of *Plasmodium falciparum* RKL-9 Asexual Stages
*In vitro* cultivation of malarial parasites was performed according to Trager and Jensen (1976) standardized method with some modifications. The parasites (*P. falciparum*) were obtained from National Institute of Malaria Research (NIMR) Sector-8 Dwarka, New Delhi. For the present study we had used chloroquine resistant strain RKL-9. After synchronization with 0.5% sorbitol culture was continued to grow for next 48 hrs to develop 5% ring stages in 10% haematocrit. Slides having thin smears were stained in JSB-1 (16-18 sec), JSB-II (6-8 sec) and examined for parasitaemia and compared with initial parasitaemia (100x Oil immersion lens). The growth was estimated by number of schizonts and rings in subsequent slides.
In vitro cultivation of P. falciparum Gametocytic Stages (RKL-9)

Synchronized culture containing 5% ring stage parasitaemia was continued for gametocytes cultivation following standardized method of Fivelman [15].

Gametocytes play an important role in transmission of malaria because they are responsible for the continuation of cycle. Gametocidal assay was performed for selected drug acetazolamide showed good gametocidal activity. Early stages gametocytes were treated with acetazolamide for gametocidal assay by the standardized method for 72hrs described by Amoah [19]. The control gametocytes taken were 30. After treatment, as concentration of the drug increases the numbers of gametocytes seen decreased. The IC50 value for acetazolamide obtained was 4.812µg/mL calculated using HN-Nonlin software.

In silico Results

Homology modelling of all three selected P. falciparum proteins (Pfg27, PfPuf1 and Pfs230) was done using SWISS MODEL online software. After homology modelling the structures of the proteins were downloaded in pdb format for further docking as shown in Figure-3. The validation of proteins structure was done through Ramachandran Plot analysis. The ligand Acetazolamide structure in sdf format was downloaded from Pubchem as shown in Figure-4.
In the present study, Hex (version 8.0.0) software was used as a docking tool.

Figure 3: 3D Structures of *P. falciparum* homology models

Figure 4: 3D Structures of Acetazolamide (CID_1986)

Molecular Docking studies
Molecular docking technique was employed to dock the selected Acetazolamide against three *P. falciparum* proteins (Pfg27, PfPuf1 and Pfs230) using Hex (version 8.0.0) to locate the interaction between the ligand and the target proteins. Using different *in-silico* tools like Hex (version 8.0.0), Discovery Studio Visualizer and Marvin View, the results obtained revealed a beneficial specific binding interaction between the target gametoocyte stage specific proteins and selected ligand acetazolamide which may be able to block the differentiation of the sexual stages to mature gametocyte stages hence blocking transmission by targeting these specific proteins. Docking score was calculated for all three proteins (Pfg27, PfPuf1 and Pfs230).

Molecular Docking analysis

(A) **Pfg27 and Acetazolamide:** The ligand showed binding affinity representing conventional Hydrogen Bond interactions and the responsible amino acid residues for protein-ligand interactions were Asp 123 and Trp 127. Docking score obtained for the Pfg27 and Acetazolamide was -196.76 (Kcal/mol).

Figure 5: Binding poses of Acetazolamide with target protein Pfg27
(B) **Pfs230 and Acetazolamide:** The ligand showed various binding affinity. The amino acid Lys 1132, Glu 1135 and Ile 1170 representing conventional Hydrogen Bond interactions. The amino acid Glu 1134 representing Pi-Alkyl and Attractive charge interaction. The amino acid Asp1169 for Carbon hydrogen bond interaction. Docking score obtained for the Pfs230 with Acetazolamide was -173.87 (Kcal/mol).

![Figure 6: Binding poses of Acetazolamide with target protein Pfs230](image)

(C) **PfPuf1 and Acetazolamide:** The ligand showed various binding affinity. The amino acid Glu 1031 representing conventional Hydrogen Bond interactions. The amino acid Lys 1032 representing Pi-Alkyl interaction. The amino acid Glu 995, Lys 998, Glu 999, Ile 1002, Arg 1003, Gly 1029, Asn 1030 and Val 1033 for Carbon hydrogen bond interaction. Docking score obtained for the Pfs230 with Acetazolamide was -193.29 (Kcal/mol).
According to Krungkrai [12] the infected red cells contained carbonic anhydrase ~2 times higher than those of normal red cells. The parasite enzyme activity was sensitive to well-known sulfonamide-based inhibitors of both bacterial and mammalian enzymes, sulfanilamide and acetazolamide. Human red cells infected with the parasite have more enzymatic activity than do uninfected red cells. The activity increases with the parasite maturation from ring to trophozoite and to schizont developmental stages in human red cells. These activities increase parallel with other metabolic activities as the parasite develops and the biomass increases. The product of malarial enzymatic catalysis, H2CO3, will serve as a precursor of the pyrimidine biosynthetic pathway operating in the parasite. The specific enzyme inhibitors, acetazolamide and sulphanilamide, show antimalarial activities by interfering with both intracellular development and the invasion process against the in vitro growth of P.falciparum in a stage-dependent manner[12]. Our in silico studies also promises good binding score of our selected ligand acetazolamide with the Pfg27, Pfs230 and PUF1 Plasmodium falciparum proteins.

CONCLUSION
The burden imposed by malaria protozoan parasites to human populations worldwide is due to the severity of the disease, particularly when caused by the species Plasmodium falciparum, and to the efficient transmission of the parasite between humans and the Anopheles mosquito vectors. Gametocidal assay was performed to check the efficacy of the drug acetazolamide on the early gametocytic stages of P. falciparum. After performing the assay results obtained were analysed and using HN-NonLin software IC50 value was calculated. The IC50 value obtained for acetazolamide was 4.812μg/ml. The results of in silico studies also supported our in vitro results that the acetazolamide can be used as an antiplasmodial drug targeting Gametocytic stages. Further studies for the mechanism of action of the acetazolamide as a gametocidal agent can be done for preventing the transmission of malaria.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest.

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