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Glabridin induces oxidative stress mediated apoptosis like cell death of malaria parasite *Plasmodium falciparum*

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ABSTRACT

Plants are known as the source of novel agents for developing new antimalarial drugs. Glabridin is a polyphenolic flavonoid, a main constituent in the roots of *Glycyrrhiza glabra* possesses various biological activities. However, its anti-plasmodial activity is unexplored. In the present work, it is for the first time demonstrated that glabridin inhibits *Plasmodium falciparum* growth in vitro with an IC_{50} 23.9 ± 0.43 μM. Glabridin showed poor cytotoxicity in vitro with an IC_{50} 246.6 ± 0.88 μM against *Vero* cell line and good selectivity index (9.6). In erythrocytic cycle, trophozoite stage was found to be most sensitive to glabridin. In silico study showed that glabridin inhibits *Pf* LDH enzyme activity by acting on NADH binding site. Glabridin induced oxidative stress by the generation of reactive oxygen and nitrogen species. Glabridin could induce apoptosis in parasite as evidenced by the depolarization of mitochondrial membrane potential ($\Delta\psi_m$), activation of caspase like proteases and DNA fragmentation. These results indicate that glabridin exhibits antiplasmodial activity and is suitable for developing antimalarial agent from a cheap and sustainable source.

1. Introduction

Malaria kills about a million people each year, including young children under the age of five and pregnant women [1]. Day to day increasing resistance in the malaria parasite *Plasmodium falciparum* against artemisinin based drugs is challenging to malaria control programs [2] and demands a non-stop attempt to develop new antimalarial leads. Plant based drugs or natural products and their derivatives represent about half of all drugs in clinical use today [3]. The discovery of novel antiplasmodial lead molecules of pharmaceutical interest from natural products is increasing after the successfulness of quinine and Artemisinin.

Glabridin [(*R*)-4-(3,4-dihydro-8,8-dimethyl)-2*H*,8*H*-benzo[1,2-*b*:3,4-*b*]dipyran-3yl)-1,3-benzenediol] is a polyphenolic flavonoid, the main ingredient of the root extract of *Glycyrrhiza glabra* (Licorice) plant. Licorice is a well-known medicinal herb used in Ayurveda as a medicine and its extracts are also used as flavoring and sweetening agents in various food products [4]. The licorice root possesses several bioactive components, including glycyrrhizin, glycyrrhetinic acid, glabridin and isoliguiritigenin etc. [5]. Glabridin is a principal constituent with multiple biological activities, such as anti-proliferative, antimicrobial, antiviral, antifungal, antihepatotoxic, anti-oxidant or radical scavenging activities, anti-inflammatory, estrogen-like activity, anti-ulcer, as well as neuroprotective and antiosteoporosis activities etc. [5–9]. Glabridin is reported as a novel anticancer agent inhibiting migration, invasion, angiogenesis and the Rho signaling pathway [10].

Oxidative stress is the result of imbalances in cellular redox regulation and the inability of the antioxidant defense system to regulate stress causing agents like reactive oxygen species (ROS) or other free radicals [11]. ROS and reactive nitrogen species (RNS) at the physiological level regulate intracellular signaling and act as redox messenger, while at high levels cause oxidative burst that leads to the disruption of cellular homeostasis in various cells. The accumulation of ROS induces oxidative damage of membrane lipids, nucleic acid and proteins that ultimately leads to cell death [12]. Induction of oxidative stress is a major molecular mechanism of various drugs like chloroquine, quinine, mefloquine, primaquine, artemisinin [13] and ciprofloxacin [14] etc. Previous studies showed that several molecules induce oxidative stress leading to the induction of programmed cell death (PCD) e.g. arsenic trioxide [15], 2-methoxyestradial [16], Monensin [17], Elesclomol [18], and Bilirubin [19]. Many studies reported the occurrence of programmed cell death in unicellular parasites including *P. falciparum* [19–22]. A putative metacaspase gene (PfMCA1) has also

Abbreviations: ROS, Reactive oxygen species; RNS, Reactive nitrogen species; NO, Nitric oxide; pLDH, Parasite lactate dehydrogenase; *Pf* LDH, *Plasmodium falciparum* lactate dehydrogenase; $\Delta\psi_m$, Mitochondrial membrane potential; NBT, Nitroblue tetrazolium; PES, Phenazine ethosulphate; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; APAD, 3-Acetyl pyridine adenine dinucleotide; SNP, Sodium-nitroprusside; FBS, Fetal bovine serum; CM-H_2DCFDA, Chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate; DCF, Dichlorofluorescein; CCCP, Carbonyl cyanide 3-chlorophenylhydrazone; DEVD, L-aspartyl-Lglutamyl-L-valyl-L-aspartic acid amide.

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been identified with an important role in programmed cell death [23,24]. The well known drugs chloroquine and etoposide induced DNA fragmentation in a drug sensitive strain of *P. falciparum* [25].

In this study, we for the first time report the antiplasmodial activity of glabridin and its possible mechanism of action. We found that glabridin inhibits *Pf* LDH activity and shows parasitostatic effect on parasite blood stages. We showed experimental evidences suggesting that glabridin induces oxidative stress in parasite that lead to depolarization of $\Delta\psi_m$, activation of caspase like proteases, DNA fragmentation and causes apoptosis like parasite death.

2. Material and methods

2.1. Reagents

Glabridin (Cat. No-G 9548), Hypoxanthine, Triton X-100, L-lactic acid, 3-acetyl pyridine adenine dinucleotide, Nitroblue tetrazolium, Phenazine ethosulphate, Methylthiazolyldiphenyl-tetrazolium bromide, antibiotic–antimycotic solution (100×), Phosphate Buffered Saline, D-sorbitol, DMSO, Sodium-nitroprusside, Chloroquine diphosphate, Artemisinin, and Doxorubicin hydrochloride, were purchased form Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium, Albumax II, Fetal bovine serum and Fungizone were purchased from Gibco BRL (Grand Island, NY, USA). Griess reagent kit, (G-7921) Chloromethyl 2′, 7′-dichlorodihydrofluorescein diacetate, and Enzchek Caspase-3 assay kit (Cat. No-E13184) were purchased from Molecular probe/Invitrogen (Carlsbad, CA, USA). MitoScreen Flow Cytometry kit (Cat. No-551302) and APO-BrdU apoptosis detection kit (Cat. No-556405) were obtained from BD Biosciences (Franklin Lakes, New Jersey, USA).

2.2. P. falciparum culture

The chloroquine sensitive *P. falciparum* strain NF-54 was cultivated in human O^+ red blood cells using RPMI-1640 medium supplemented with 25 mM HEPES, 0.2% $NaHCO_3$, 370 μM hypoxanthine, 40 μg/mL gentamycin, 0.25 μg/mL Fungizone, and 0.5% Albumax II at 37 °C using the method described previously [26]. Culture was maintained in a standard gas mixture consisting of 5% CO_2. Culture medium was changed after every 24 h and routinely monitored through Geimsa staining of thin blood smears. The culture was synchronized by 5% D-sorbitol treatment to obtain ring-stage parasites [27].

2.3. Antiplasmodial activity

Glabridin was dissolved in DMSO and further diluted with culture medium to achieve the required concentrations (final concentration of DMSO <1%). Parasite growth was determined spectrophotometrically in control and drug-treated cultures using a parasite lactate dehydrogenase assay (pLDH) as described previously with minor modifications [28]. Briefly, a synchronous ring stage culture with 1.5% parasitemia and 2% hematocrit was incubated in flat bottom 96-well tissue culture plate with different concentrations (100 to 1 μM) of glabridin at 37 °C for 72 h. Artemisinin and Chloroquine diphosphate salt was used as positive control. After incubation, plates were subjected to three 20 minute freeze–thaw cycles to release cell content. Parasite culture was carefully mixed and aliquots of 20 μL were taken and added to another flat bottom 96-well plate containing 100 μL of Malstat reagent (0.125% Triton X-100, 130 mM L-lactic acid, 30 mM Tris buffer and 0.62 μM APAD) and 25 μL of NBT–PES (1.9 μM NBT and 0.24 μM PES) solution per well. The plate was incubated in dark

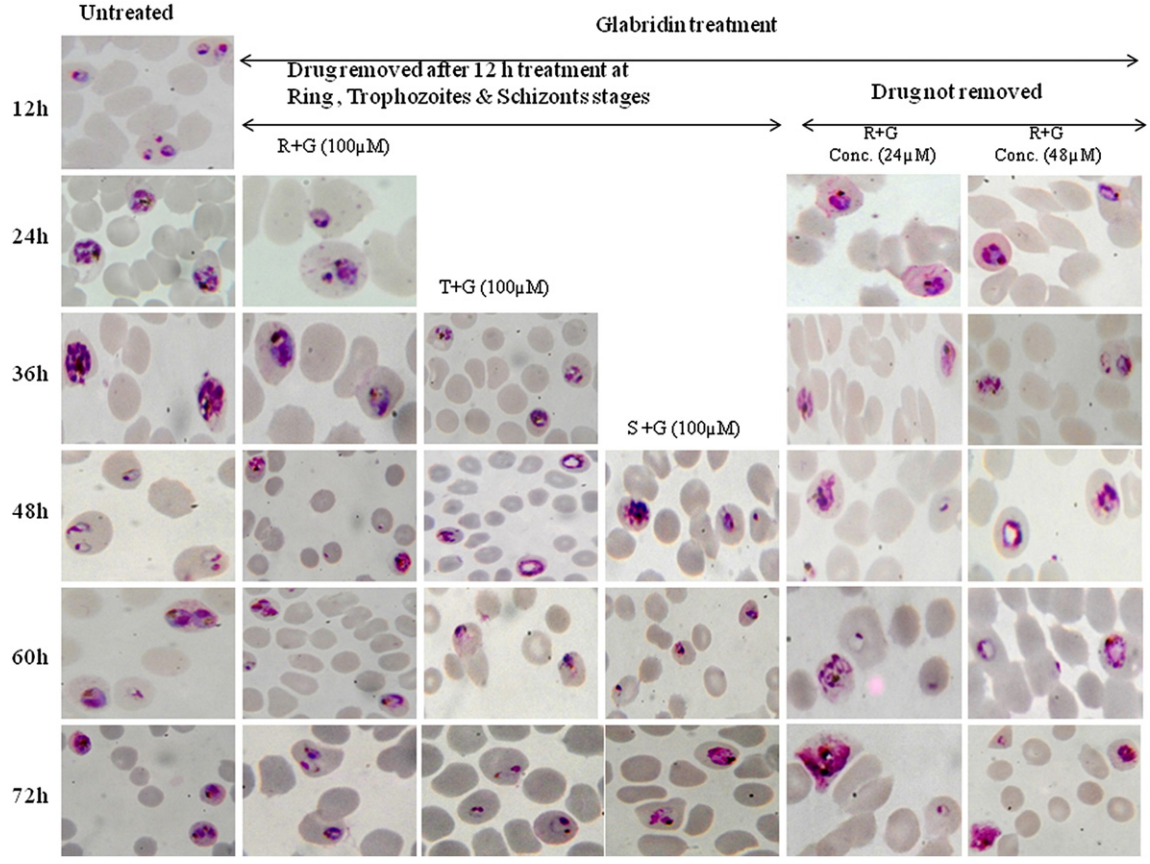

Fig. 1. Microscopic observation after glabridin treatment at different blood stages of *P. falciparum*. Ring, trophozoite and schizont stage cultures at 5% parasitemia were treated with 100 μM glabridin and removed after 12 h corresponding to each stage. For continuous treatment the ring stage synchronized culture was treated at 24 μM and 48 μM concentrations of glabridin. Morphological changes were observed after 12 h interval up to 72 h. R + G = Ring stag treated with glabridin; T + G = Trophozoite stage treated with glabridin; S + G = Schizont stage treated with glabridin.