

Search for microbial metabolites targeting *Plasmodium falciparum* mitochondrial electron transport chain dehydrogenases as antimalarial lead compounds

Division of Infection Control Sciences · Department of Drug Discovery Sciences ·

Molecular Cellular Biology

Amila Pramisandi · DI17003

[Backgrounds]

Malaria remains one of the most life-threatening parasitic diseases worldwide. Recently, the World Health Organization reported 219 million cases and 435,000 deaths in malaria. The efficacy of all existing antimalarial drugs has been hampered by the rapid appearance of resistant parasite. Consequently, there has been a continuing and urgent need for the development of new and innovative antimalarial agents.

The mitochondrial electron transport chain (ETC) of *Plasmodium falciparum* plays a critical and essential role in the life cycle of the asexual blood stage parasite. Thus, it has been considered as potential drug target source. Mitochondrial ETC of *Plasmodium* contains five dehydrogenases which contribute to generating electrochemical gradient for ATP synthesis. *P. falciparum* L-malate:quinone oxidoreductase (*Pf*MQO) is a mitochondrial ETC dehydrogenase reported as a novel target of antimalarial drug. This enzyme involves in biological pathway of the parasite including the fumarate cycle, TCA cycle, and electron transport chain that are essential in parasite life cycle. MQO is absent in mammalian cells but it is conserved in several pathogens, therefore *Pf*MQO is considered to be an attractive drug target with novel mode of actions.

P. falciparum dihydroorotate dehydrogenase (*Pf*DHODH) is a mitochondrial ETC dehydrogenase, which is a rate-limiting step in pyrimidine *de novo* biosynthesis of the parasite. Since *Plasmodium* has no salvage pathway of pyrimidine, the protozoa completely depend on the pyrimidine *de novo* biosynthesis. Most organisms, including human and malarial parasites, possess a DHODH enzyme. Human DHODH (*Hs*DHODH) inhibitors are used as therapeutics for human autoimmune disease. Therefore, the inhibition specificity towards *Pf*DHODH is an essential aspect to guide the search for *Pf*DHODH inhibitors and possible development into antimalarial drug candidates.

Numerous *Pf*DHODH inhibitors with various chemical scaffolds have been identified by chemical library screening, structure-based virtual screening and chemical modifications of

existing inhibitors (triazolopyrimidine derivatives). However, only one *Pf*DHODH inhibitor, DSM265, is undergoing clinical trial of Phase IIa. Until now, no report has been published of work focusing on the search for *Pf*DHODH inhibitors derived from microorganisms.

Indonesia is a tropical country possessing variety of rainfall type, volcanic land, and transitional area between Asia and Australia. Those geographical conditions suggested wide diversity of Indonesian microbes. Since microbial metabolites have been known to show various biological properties and to have complex chemical diversity of the compounds and the novelty of structures, it is possible to search for new structures of *Pf*MQO and *Pf*DHODH inhibitors as antimalarial lead compounds.

[Method]

Enzymatic screenings of fungal and actinomycetes culture extracts and a natural product library were performed with *Pf*MQO and *Pf*DHODH. A dereplication method in *Pf*MQO inhibitors screening was developed to find chemically ubiquitous inhibitors. Utilizing the α -cyclodextrin (α -CD) treatment on the screening extracts, common free fatty acids (FFAs) that exhibited inhibitory activity against *Pf*MQO were eliminated. The candidate strains producing *Pf*MQO and *Pf*DHODH inhibitors were cultured in large-scale to isolate their active metabolites. The isolation of bioactive compounds targeting *Pf*MQO and *Pf*DHODH was carried out empowering a bioassay-guided fractionation by various column chromatography techniques. The characterization of the isolated compounds was performed using various spectroscopic analyses. Their biological properties against the *P. falciparum* 3D7 cells *in vitro* and their cytotoxicity against one human cell line, MRC-5, were also evaluated.

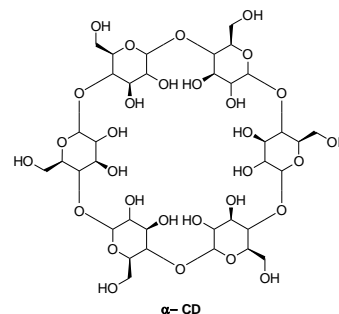
[Results]

Search for microbial metabolites targeting *Pf*MQO

A high-throughput screening of *Pf*MQO inhibitors was performed on 4,400 microbial broth extracts derived from Indonesia and Japan. It obtained 51 (1.2%) of hits, which exhibited >50% inhibitory activity against *Pf*MQO using 2 μ l test sample per 200 μ l assay mixture and showed a dose-dependent manner. Three producing strains, Indonesian strain, *Trematosphaeria biappendiculata* BioMCC-f.I.1004, two Japanese actinomycetes strains, *Streptomyces* sp. K17-0004 and *Saccharomonospora* sp. KMA-0167, were cultured in large-scale and inhibitors against

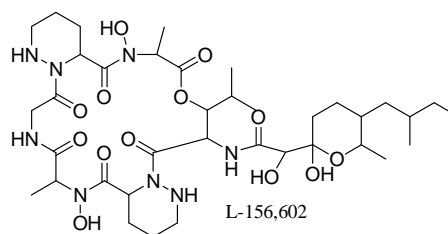
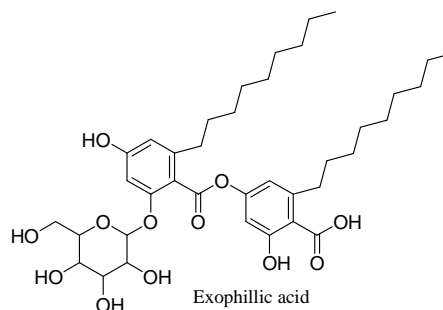
PfMQO were purified by liquid-liquid partition and several chromatographies. The *PfMQO* inhibitors obtained from all these three hits were linoleic acid and/or oleic acid.

Saturated and unsaturated FFAs have been found to show an IC_{50} value against *PfMQO* in micromolar order. Oleic acid and linoleic acid exhibited the most potent *PfMQO* inhibition activity with IC_{50} values of 3.8 and 3.9 μM , respectively. FFAs are general metabolites in microorganisms, therefore, a dereplication method to eliminate FFAs from the *PfMQO* inhibitors screening hits was developed by inclusion complex formation using α -CD. An optimum α -CD treatment was obtained as an equal volume addition of 15% α -CD aqueous solution to the methanol solution of microbial culture extracts. This α -CD treatment could eliminate the microbial culture extracts showing activities by FFAs, thus significantly reduce the number of hit rates from 5.2% to 0.2%.



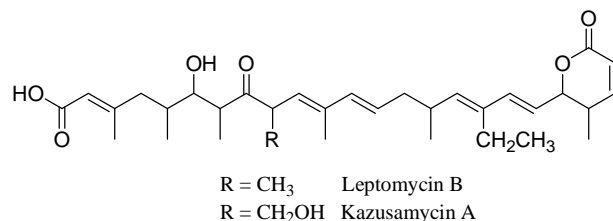
After the dereplication by α -CD, a producing strain of the hit sample, *Micromonospora* sp. KC16-65, was cultured. The culture extract was purified by liquid partition, silica gel chromatography, ODS chromatography, Sephadex[™] LH-20 gel filtration, and preparative TLC to afford four structurally related large molecular weight metabolites with MW >2000. All isolated metabolites exhibited an IC_{50} value of 0.65-0.75 $\mu g/ml$ against *PfMQO*. However, those chemical structures could not be elucidated due to loss of its productivity.

The screening of *PfMQO* inhibitors was also performed using 480 compounds of the Kitasato natural products library, and two inhibitors (0.4%), exophillic acid and L-156,602, showed dose-dependent manner with IC_{50} values of 1.1 and 5.5 μM , respectively. Though the L-156,602 producing strain, *Streptomyces* sp. AM-6112 was cultured in large-scale, it failed to produce the objective compound. The culture extract, which still



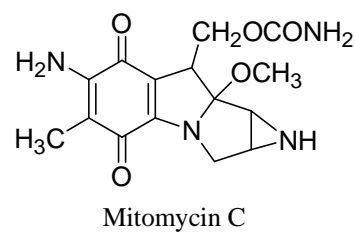
showed the *PfMQO* inhibition, was purified by ODS column chromatography, Sephadex[™] LH-20 gel filtration, and preparative TLC to give leptomycin B and kazusamycin A exhibiting inhibitory activity against *PfMQO* with IC_{50} values of 15 and 80 μM , respectively. They showed less potency of inhibitory activity against *PfMQO* than ferulenol, a plant-derived metabolite known as

the first *Pf*MQO inhibitor, with an IC₅₀ value of 0.06 μ M (Hartuti et al., 2018). Leptomycin B and kazusamycin A exhibited weak suppression against the *P. falciparum* cells growth.



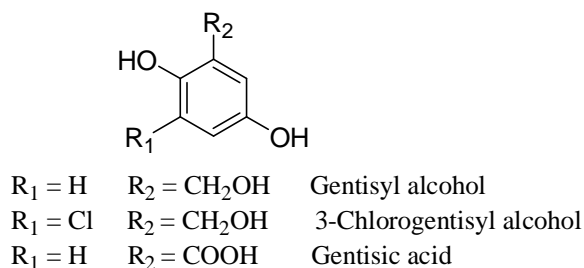
Search for microbial metabolites targeting *Pf*DHODH

The screening of *Pf*DHODH inhibitors was performed using 480 compounds of the Kitasato natural products library, and one inhibitor (0.2%), mitomycin C, showed an IC₅₀ value of 2.8 μ M against *Pf*DHODH. The IC₅₀ value was 46-fold smaller than that of the human ortholog, *Hs*DHODH. Mitomycins are known as DNA crosslinker agents and show potent cytotoxicity against the mammalian cells.



The screening of *Pf*DHODH inhibitors from 613 broth extracts of fungi and actinomycetes, which were isolated from Indonesia and Japan, was performed and two candidate broths (0.3%) were found. The hit extracts exhibited no inhibitory activity against the *Hs*DHODH and showed specific inhibition.

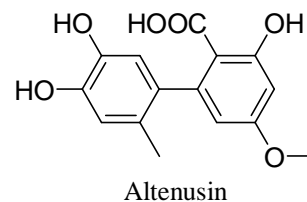
A Japanese soil fungal strain *Hypomyces pseudocorticiicola* FKI-9008 was cultured. A bioassay-guided purification was performed by silica gel column chromatography, Sephadex[™] LH-20 gel filtration, and ODS column chromatography to afford a *Pf*DHODH inhibitor,



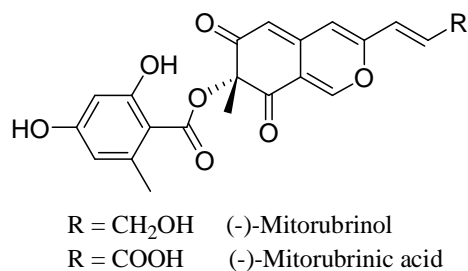
and it was identified as gentisyl alcohol by MS and NMR analyses. Gentisyl alcohol exhibited inhibitory activity against *Pf*DHODH and *Hs*DHODH with IC₅₀ values of 3.4 μ M and >4,500 μ M, respectively. A chlorinated derivative was also isolated and identified as 3-chlorogentisyl alcohol. However, 3-chlorogentisyl alcohol disturbed the *Pf*DHODH assay system prior to the enzymatic reaction, and the activity could not be measured in the assay system. A commercially available carboxylic derivative, gentisic acid, was also tested, and it showed an IC₅₀ value over 9,000 μ M against both *Pf*DHODH and *Hs*DHODH. Although gentisyl alcohol and 3-chlorogentisyl alcohol

showed suppression against *P. falciparum* 3D7 cells growth, both metabolites showed cytotoxicity against the human MRC-5 cells in much low concentration.

Subsequently, another soil fungal strain from Indonesia, *Talaromyces pinophilus* BioMCC-f.T.3979 was cultured. Isolation of active metabolites targeting *Pf*DHODH was performed by liquid partition, silica gel column chromatography, and SephadexTM LH-20 gel filtration repeatedly. Altenusin, with an IC₅₀



value of 5.9 μ M against *Pf*DHODH, was obtained as the most potent *Pf*DHODH inhibitor along with two azaphilone pigments identified as mitorubrinol and mitorubrinic acid with IC₅₀ values of 42 and 160 μ M, respectively. All isolated metabolites did not inhibit *Hs*DHODH and showed high specificity against *Pf*DHODH. Altenusin and mitorubrinol showed suppression against the *P. falciparum* 3D7 cells growth with IC₅₀ values of 5.4 and 18 μ M, respectively. However, they also showed cytotoxicity against MRC-5 cells.



This is the first study of *Pf*MQO and *Pf*DHODH inhibitors obtained from microbial metabolites. *Pf*DHODH inhibitors, gentisyl alcohol, altenusin, mitorubrinol, and mitorubrinic acid were found to be specific inhibitors for *Plasmodium* enzyme compared to the human ortholog. In addition, those isolated metabolites showed moderate suppression against the growth of *P. falciparum* 3D7 cells. Though they have cytotoxic effect against MRC-5 cells, it is possible to synthesize their derivatives with much potent enzyme inhibition, stronger efficacy *in vivo*, with minimal cytotoxic properties.

[References]

Hartuti et al., *Biochim. Biophys. Acta*, **1859**, 191-200 (2018).