Abstract of Dissertation submitted by Endah Dwi Hartuti

Title: Identification of 3,4-Dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine Derivatives as Novel Selective Inhibitors of *Plasmodium falciparum* Dihydroorotate Dehydrogenase

Japanese title: 熱帯熱マラリア原虫ジヒドロオロト酸脱水素酵素の新規選択的阻害剤の同定

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Introduction:

Significant morbidity and mortality caused by protozoan parasitic infection pose serious threats to global health. Among parasitic diseases, malaria is the most devastating, with approximately 409,000 deaths reported during 2019. Great effort has been spent to control malaria; however, the emergence of parasites resistant to practically all antimalarial drugs hampers the control and elimination of malaria. The majority of malaria cases reported are caused by *Plasmodium falciparum*. This parasite has a complex life cycle involving intermediary (human) and definitive (mosquitoes) hosts. The pyrimidine *de novo* biosynthesis pathway is an attractive antimalarial drug target. Humans can acquire pyrimidines through both *de novo* and salvage pathways, while the genes necessary for the salvage pathway are not present in *P. falciparum*. Thus, *P. falciparum* is entirely dependent on the *de novo* pathway for the supply of cellular pyrimidine. In addition, the pyrimidine *de novo* pathway is connected to the ETC at the level of ubiquinone through a reaction catalyzed by dihydroorotate (DHO) dehydrogenase (DHODH), the fourth and rate-limiting step of this pathway. DSM265 is potent PfDHODH inhibitors and showed promising results in a phase 2a study by showing rapid parasite clearance after single-dose treatment, however it is no longer included in the Medicine for Malaria Venture (MMV) portfolio, possibly due to side effects. Thus, the identification of PfDHODH inhibitors with different chemical structures and better safety profiles than DSM265 is needed.

Materials and Methods:

The screening of 40,400 compounds was conducted in 384-well plates by adapting the end-point assay reported before. First, 1 µL of 200 µM compound was transferred into 384-well plates using a Benchtop Multi-Pipetter EDR-384SR. The same volume of dimethyl sulfoxide (DMSO) was added to columns 1 and 2 as negative controls (0% inhibition), while

18% (w/v) sodium dodecyl sulfate (SDS) was added to columns 23 and 24 as positive controls (100% inhibition). Next, 38 µL of assay mix (100 m M HEPES-NaOH pH 7.5, 5% (v/v) glycerol, 150 mM NaCl, 0.05% (v/v) Triton X-100, 15 µM decylubiquinone (dUQ), 120 µM 2,6-dichlorophenolindophenol (DCIP), and 20 nM PfDHODH were dispensed into all wells and mixed at 600 rpm for 1 min by a MixMate®® (Eppendorf). The reaction was started by the addition of 5 µL of 1.8 mM L-DHO as the substrate and mixed as described above for 20 sec. The absorbance at 600 nm was recorded using a SpectraMax®® Paradigm®® Multi-Mode Microplate Reader before (t0) and after 20 min (t20) incubation at room temperature. The readings at t0 were subtracted from t20, and PfDHODH inhibition was calculated as the inhibition relative to the negative and positive controls in a single-point assay. Hits were defined as compounds inhibiting more than 50% of PfDHODH activity at 4.5 µM. The IC50 values of the hit compounds were determined using the same assay system containing serial dilutions of each compound in triplicates (22.7, 6.8, 2.27, 0.68, 0.227, 0.068, 0.023, 0.007, 0.002, 0.0007 µM) using GraphPad Prism 8.0 software.

Results:

His6-SUMO-tagged PfDHODH was successfully expressed and purified from the *Escherichia coli* membrane. After digestion by SUMO protease, we purified the tag-free enzyme with a specific activity of 22.3 µmol/min/mg (K_{cat} = 17.5 s−1), which was used for screening 40,400 compounds from the Kyoto University chemical library. The quality of our screening was evaluated by calculating the following parameters: Z'-factor (0.88 ± 0.09), signal window (SW, 49.2 ± 20.3), signal-to-noise ratio (S/N = 85.0 ± 26.0), signal-to-background (S/B = 115.3 ± 28.6), and negative coefficient of variation (CV = 2.38 ± 1.85%), all of which were excellent. After the initial screening at 4.5 µM, we identified 43 compounds that meet the hit criteria (>50% inhibition), for a hit rate of 0.11%. According to the library policy, the chemical structures are disclosed only for the hits. After analysis of their chemical structures, we classified the hits as derivatives of 3,4-dihydro-2*H*,6*H*-pyrimido[1,2-*c*][1,3]benzothiazin-6-imine (DPBI) or 3,4-dihydro-2H-benzo[4,5]isothiazolo[2,3-a]pyrimidine (DBIP).

Discussion:

Kyoto University chemical library is open-access and composed of many original compounds and diverse scaffolds. Our efforts led to the identification of a new class of PfDHODH inhibitors that share DPBI and DBIP moieties. Those derivatives have previously been reported to inhibit the proliferation of human immunodeficiency virus (HIV), hepatitis C virus, and herpes simplex virus. The 43 PfDHODH inhibitors identified in this study showed no inhibition against the human enzyme or mammalian mitochondrial complexes I–III and II–III, except for 2, which weakly inhibited complex I–III activity. This result suggests that those compounds have little or no effect on the ETC of host mitochondria. The majority of those inhibitors were active against *P. falciparum* 3D7 and displayed low toxicity to mammalian normal and cancer cell lines.

In this study, we have identified PfDHODH inhibitors with new chemical scaffolds that inhibited the growth of the *P. falciparum* 3D7 strain. Further activity profiling, such as activity against multi-drug resistant strains (Dd2 or K strains), liver-stage parasites, as well as in vivo models (*P. berghei*) will be required for the future development of the compounds described in this study.

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