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NOTE

Antimalarial tropones and their *Plasmodium falciparum* glyoxalase I (*pf*GLOI) inhibitory activity

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During our screening program to discover new antimalarial compounds, we isolated several tropone-related compounds from the culture broth of *Penicillium* sp. FKI-4410.[1] Among them, puberulic acid showed potent antimalarial activity against *Plasmodium falciparum* K1 strain, with an IC_{50} of 0.051 μM. It has also been reported that some tropone derivatives show yeast glyoxalase I (*y*GLOI) inhibitory activity[2–5] and Barnard *et al.*[3] reported that several tropone-related compounds with *y*GLOI inhibitory activity showed *in vitro* antimalarial activity.

GLOI (EC 4.4.1.5) is an isomerase that catalyzes the formation of *S*-D-lactoylglutathione from the hemimercaptal adducts that form spontaneously between methylglyoxal (MG) and reduced glutathione (GSH) to detoxify MG in a glycolytic system. *P. falciparum* mainly depends on glycolysis for energy production, involving monomeric *P. falciparum* glyoxalase I (*pf*GLOI) containing two zinc ions, in contrast to dimeric human GLOI. Therefore, it is thought to be a target enzyme for development of a new antimalarial drug.[6] In this paper, we report the *pf*GLOI inhibitory activity of puberulic acid and its related compounds.

Puberulic acid, stipitatic acid and viticolins A, B and C were obtained from a culture broth of FKI-4410.[1] Tropone (252832, Aldrich, St Louis, MO, USA), tropolone (T89702, Aldrich), hinokitiol (H0142, TCI, Tokyo, Japan), curcumin (038-04921, Wako, Osaka, Japan) and other chemical reagents were commercially obtained. Evaluation of antimalarial activity has been described previously.[7] Recombinant *Pf*GLOI was prepared according to Iozef *et al.*[8] and Deponte *et al.*[9] with a slight modification. In brief, complementary DNA was obtained from cultured *P. falciparum* FCR3 strain. The plasmid pGEM were freshly transformed into *Escherichia coli* strain DH5α for blue-white selection, recloned into the expression plasmid pTrcHisA and transformed into *E. coli* strain DH5α. Bacteria, precultured for 12 h at 37 °C in Luria-Bertani (LB) medium supplemented with 0.02 mg ml^{-1} ampicillin, were inoculated into 1 l of LB medium at 0.2% (v/v) supplemented with 0.02 mg ml^{-1} ampicillin. The culture flask was incubated (140 r.p.m. at 37 °C for 10 h) to an absorbance at 600 nm of 0.2–0.3. Subsequently, 286 μl of 1 g ml^{-1} $ZnSO_4 \cdot 7H_2O$ aqueous solution was added to the culture flask before 30 min of induction with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside. After 14 h incubation (140 r.p.m. at 25 °C), bacterial cells were collected by centrifugation and then resuspended with 10 mM MOPS/NaOH buffer (pH 7.8), followed by sonication on ice. After centrifugation, supernatant containing recombinant protein was purified with *S*-hexylglutathione-agarose column eluted with 10 mM MOPS/NaOH buffer (pH 7.8) containing 200 mM NaCl and 5 mM *S*-hexylglutathione, after washing with 10 mM MOPS/NaOH buffer (pH 7.8) containing 200 mM NaCl. Finally, *pf*GLOI was purified via a His-Trap column eluted with 50–125 mM imidazole gradient. The purified protein showed a single band at ~50 kDa on 12.5% SDS-polyacrylamide gel electrophoresis. The band was analyzed by matrix-assisted laser desorption/ionization time-of-flight MS after in-gel digestion.[10] From the result of peptide matching and protein database searches, the purified protein was confirmed as *Pf*GLOI. *Pf*GLOI inhibitory assay was modified into the method with 96-well microtiter plate format from methods of Oray and Norton[11] and Deponte *et al.*[9] Each stock solution of 155 mM MG and 6.0 mM GSH were freshly prepared. To obtain the desired substrate concentration, stock solution was appropriately diluted. The substrate mixture was prepared with 2000 μl of MilliQ water (Millipore, Billerica, MA, USA), 1000 μl of 500 mM MOPS/NaOH buffer (pH 7.0), 1000 μl of MG and 1000 μl of GSH. Substrate concentration was calculated according to following equation: $K_d = 3\ \text{mM} = ([\text{MG}][\text{GSH}]/[\text{hemithioacetal}])$; free GSH was 0.1 mM. The substrate mixture was incubated for over 15 min for 25 °C. Ten microliters of compound solution (25% methanol or 5% dimethyl sulfoxide) and 40 μl of enzyme solution (2.33 nM) diluted with 100 mM MOPS/NaOH buffer (pH 7.0) containing 0.1% bovine

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serum albumin were added to each well in the microtiter plates (UV Flat Bottom Microtiter Plate, Thermo, Waltham, MA, USA). The enzyme reaction was started by adding 50 µl of substrate mixture solution. Formation of *S*-D-lactoylglutathione was monitored by the increase in absorbance at 240 nm to obtain Δ240 nm in kinetic mode (SH-9000Lab, CORONA ELECTRIC, Ibaraki, Japan). Compound stock solution was prepared with methanol or dimethyl sulfoxide. Solvent did not affect the enzymatic reaction under the experimental condition. Percentage inhibition was calculated using the following formula.

$$\text{Inhibition } \% = (\Delta 240\,\text{nm of compound } (-) - \Delta 240\,\text{nm of compound } (+)) / \Delta 240\,\text{nm of compound } (-) \times 100$$

*Pf*GLOI inhibitory activity and *in vitro* antimalarial activity against *P. falciparum* K1 strain are shown in Table 1 and structures of compounds are exhibited in Figure 1. *Pf*GLOI activity was determined with low (5 µM) or high (500 µM) substrate concentration because *pf*GLOI exists in a high-affinity conformation at low substrate concentration and in a high-activity conformation at high substrate concentration, respectively.[6] Curcumin was reported as a *pf*GLOI inhibitor only at low substrate concentration;[12] thus, it was used as a positive control in this assay. The *pf*GLOI inhibitory assay was repeated at least three independent times or more and an average IC_{50} value and s.d. were calculated.

Table 1 *Pf*GLOI inhibitory activity of puberulic acid and related tropones

	IC_{50} µM		
	PfGLOI inhibitory activity		*Anti-P. falciparum K1 strain*[a]
Compounds	*Substrate 500 µM*	*Substrate 5 µM*	
Puberulic acid	60.43 ± 19.41	84.13 ± 47.71	0.051
Hinokitiol	16.25 ± 2.75	3.48 ± 1.41	29.96
Tropolone	12.05 ± 3.77	3.42 ± 0.48	22.45[b]
Tropone	> 100.00	> 100.00	> 125.00[b]
Stipitatic acid	> 100.00	> 100.00	38.85
Viticolin A	> 100.00	> 100.00	46.79
Viticolin B	> 100.00	> 100.00	3.72
Viticolin C	> 100.00	> 100.00	> 60.10
Curcumin[c]	> 100.00	43.47 ± 27.39	12.95

Abbreviations: BSA, bovine serum albumin; *Pf*GLOI, *P. falciparum* glyoxalase I.
[a]Data from reference.[1]
[b]Re-assay in this experiment.
[c]*Pf*GLOI inhibitory assay was carried out without BSA and enzyme concentration was 9.32 nM.

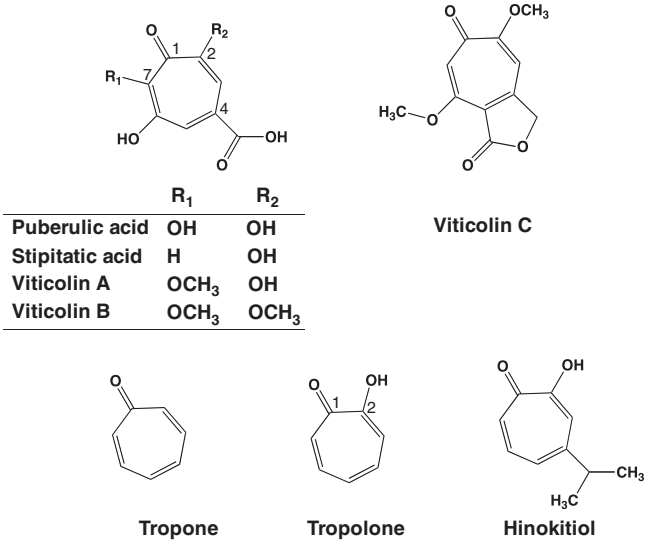

Figure 1 Structures of puberulic acid and related tropones.

In the 500 µM of high substrate concentration, tropolone showed the most potent inhibitory activity against *pf*GLOI, with an IC_{50} value of 12.05 ± 3.77 µM. Hinokitiol showed a little weaker *pf*GLOI inhibitory activity than tropolone, with the IC_{50} value of 16.25 ± 2.75 µM. Puberulic acid showed moderate activity, with an IC_{50} value of 60.43 ± 19.41 µM. The IC_{50} values of tropone, stipitatic acid and viticolins A, B and C were more than 100 µM. In the 5 µM of low substrate concentration, hinokitiol and tropolone showed the most potent inhibitory activity against *pf*GLOI, displaying IC_{50} values of 3.48 ± 1.41 µM and 3.42 ± 0.48 µM, respectively. Puberulic acid showed moderate activity, with an IC_{50} value of 84.13 ± 47.71 µM. The IC_{50} values of tropone, stipitatic acid and viticolins A, B and C were more than 100 µM. *Pf*GLOI inhibitory activity of curcumin was observed only at low substrate condition, with an IC_{50} value of 43.47 ± 27.39 µM, the same as reported in the literature.[12]

Although several tropone derivatives were evaluated for *y*GLOI inhibitory activity, antimalarial and antiprotozoal activity in the past,[2–5] *pf*GLOI inhibitory activity had not been reported until this paper. In the correlation of *y*GLOI inhibitory activity and antimalarial activity, Barnard *et al.*[3] predicted that a free C-2 hydroxyl group was necessary for both properties. In our experiment, hinokitiol and tropolone, each having a free hydroxyl group only at C-2, showed *pf*GLOI inhibitory activity, whereas tropone lacking the free C-2 hydroxyl group did not show *pf*GLOI inhibitory activity at 100 µM. These data denoted the same tendency of *y*GLOI inhibitory activity. We confirmed that an antimalarial mode of action of hinokitol and tropolone might be via *pf*GLOI inhibition. Barnard *et al.*[3] described that the resonance forms of a tropolone could resemble the enediol intermediate formed by GLOI; hence, tropolones could serve as transition state or reactive intermediate analog inhibitors in GLOI inhibition.

On another front, the most potent antimalarial we tested, puberulic acid, showed moderate inhibitory activity against *pf*GLOI. Moreover, stipitatic acid and viticolins A, B and C did not show *pf*GLOI inhibitory activity, even at 100 µM. These results indicate that the antimalarial mode of action of puberulic acid analogs was not via *pf*GLOI inhibition. It was predicted the electric state of the tropone ring of puberulic acid analogs that have C-2 hydroxyl group might differ from that of enediol intermediates formed by *pf*GLOI, depending on the presence or absence of functional groups at C-4, C-6 and C-7 with regard to the *pf*GLOI inhibition.

According to the effect of substrate concentration, the *pf*GLOI inhibitory activity of both hinokitiol and tropolone was enhanced under low substrate condition. It was thought that tropolone and hinokitiol were acting in a substrate mimicry role, better in high-affinity conformation of *pf*GLOI at low substrate concentration than in high-activity conformation at high substrate concentration.

Curcumin, methylgerfelin and synthetic analogs of GSH[12,13] have also been reported as *pf*GLOI inhibitors. However, these compounds were also reported as inhibitors of either or human GLOI and *y*GLOI;[14–16] *pf*GLOI-specific inhibitor of non-GSH

compounds has not been reported. Further investigation is needed to discover specific *pf*GLOI inhibitors having potent antimalarial activity.

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