Inhibition of D-glycero-β-D-manno-heptose 1-phosphate adenylyltransferase from *Burkholderia pseudomallei* by epigallocatechin gallate and myricetin

Suwon Kim¹, Seri Jo¹, Mi-Sun Kim¹, Heejin Kam² and Dong Hae Shin*¹

¹Graduate School of Pharmaceutical Sciences, Ewha Womans University, 52, Ewhayeodae-gil, Seoul, 03760, Korea

²Department of Pharmacy, Ewha Womans University, 52, Ewhayeodae-gil, Seoul, 03760, Korea

**Running title:** Flavonoids as antibacterial agents.

*Co-corresponding authors:

Prof. Dong Hae Shin

Department of Pharmacy, Ewha W. University, Seoul, Republic of Korea 03760

Tel: (82) 2-3277-4502; Fax: (82) 2-3277-2851; E-mail: dhshin55@ewha.ac.kr

ORCID: 0000-0002-2205-1453
Abstract

Flavonoids play beneficial roles in various human diseases. In this study, a flavonoid library was employed to probe inhibitors of d-glycero-β-D-manno-heptose-1-phosphate adenylyltransferase from Burkholderia pseudomallei (BpHldC) and two flavonoids, epigallocatechin gallate (EGCG) and myricetin, have been discovered. BpHldC is one of the essential enzymes in the ADP-L-glycero-β-D-manno-heptose biosynthesis pathway constructing lipopolysaccharide of B. pseudomallei. Enzyme kinetics study showed that two flavonoids work through different mechanisms to block the catalytic activity of BpHldC. Among them, a docking study of EGCG was performed and the binding mode could explain its competitive inhibitory mode for both ATP and βG1P. Analyses with EGCG homologues could reveal the important functional moieties, too. This study is the first example of uncovering the inhibitory activity of flavonoids against the ADP-L-glycero-β-D-manno-heptose biosynthesis pathway and especially targeting HldC. Since there are no therapeutic agents and vaccines available against melioidosis, EGCG and myricetin can be used as templates to develop antibiotics over B. pseudomallei.

Keywords  d-glycero-β-D-manno-heptose-1-phosphate adenylyltransferase; Burkholderia pseudomallei; Epigallocatechin gallate; Myricetin; Antibiotics
Introduction

Flavonoids are polyphenols that perform various biological activities such as anti-inflammation, anti-oxidation, anti-cancer and cardiovascular protective action [1]. They are synthesized from phenylalanine and their scaffolds can be categorized into more than seven subclasses [2]. Many kinds of catalytic enzymes such as hydrolases, oxidoreductases, phosphatases, DNA synthases and RNA polymerases have been known to be inhibited by various flavonoids. They also attract the interests of people due to the increasing evidence of the versatile health benefits as dietary foods [3]. Flavonoids interact well with proteins due to their dual properties of hydrophilicity and hydrophobicity. Therefore, many X-ray crystal structures of flavonoids complexed with enzymes such as ecto-5’-nucleotidase (PDB ID: 4H2B), dihydroflavonol 4-reductase (3C1T) and UDP-glucose flavonoid 3-O glycosyl have been reported. Because of the binding characteristics, flavonoids and their derivatives have been developed as new drug candidates targeting various kinds of illnesses [4].

In drug discovery, flavonoids drew the attention to be utilized as lead compounds to develop new bactericidal drugs. The antibacterial activity of flavonoids may be occurred by three different mechanisms [5]; inhibition of nucleic acid synthesis, inhibition of energy metabolism and cytoplasmic membrane damage. Combined with available antibiotics, flavonoids work synergistically to cope with bacterial infections [6].

*Burkholderia pseudomallei* is a pathogenic bacteria with relatively high mortality causing melioidosis that affects people, especially in the tropics. [7-9]. Death rates are comparable to measles, higher than leptospirosis and dengue fever [10]. From pneumonia and sepsis to skin abscesses [11], various symptoms of melioidosis make their clinical diagnosis difficult. Nevertheless, no vaccines are commercially available to prevent melioidosis infection, despite intensive research efforts [12-14]. As Gram-negative bacteria, *Burkholderia* spp. construct a condensed network of surface-exposed polysaccharides including capsular
polysaccharides (CPS), lipopolysaccharides (LPS), and exopolysaccharides (EPS). They are known as the main virulence factors and their biosynthesis pathways play important roles in the pathogenesis and immunomodulation of melioidosis [15-17]. The LPS consists of lipid A, core and O-antigen domains in most Gram-negative bacteria [18]. The core oligosaccharide is divided into an outer part and an inner part. The inner part is composed of L-glycero-D-manno-heptose (or heptose) and 3-deoxy-D-manno-octulosonic acid. Heptose is a greatly conserved component of the LPS core among some genera of bacteria. The precursors of heptose were synthesized through the ADP-L-glycero-β-D-manno-heptose biosynthesis pathway [19]. This pathway is well conserved in Gram-negative bacteria species [20]. D-glycero-β-D-manno-heptose-1-phosphate adenylyltransferase (HldC) involved in this pathway is one component of HldE from Escherichia coli, a bifunctional protein including both kinase (HldA) and HldC domains [21]. The deletion mutant of the hldE gene exhibited increased sensitivity to some antibiotics like erythromycin, telithromycin, novobicin, rifampicin, etc [22]. It implies that the inhibition of the ADP-L-glycero-β-D-manno-heptose biosynthesis pathway is a potential target to develop antibiotic adjuvants. Therefore, inhibitors targeting HldC from B. pseudomallei (BpHldC) can be good therapeutic adjuvants to shorten the duration of medication to cure melioidosis combined with known antibiotics [23].

The X-ray crystal structure of BpHldC revealed that it forms a homotetrameric structure with a flexible C-terminal helix presumed to be crucial for its enzymatic function. A MES buffer molecule occupies a predicted catalytic site in two subunits only indicating that BpHldC may act in an allosteric mode. Highly conserved residues, His40 and Lys69, interact with the MES molecule. In this study, a flavonoid library (Figure S1)(Table S1) has been applied to search flavonoids blocking the catalytic activity of BpHldC. Furthermore, their inhibitory modes were analyzed based on the X-ray crystal structure of BpHldC. Since there
is no antagonist against HldC has been reported, the flavonoids found in this study are the first examples targeting this enzyme.

Materials and Methods

Preparation of the protein

The BphldC gene (NCBI Reference Sequence: WP_004189202.1) coding for BpHldC protein was amplified with a pair of primers. The PCR product was ligated into the amplified expression vector pB2 via the ligation-independent cloning (LIC) method. The transformant was acquired by the use of Escherichia coli (E. coli) DH5a, amplified and sequenced for identifying. Transformation into E. coli BL21 (DE3), large-scale cultivation for protein overexpression and purification of BpHldC were performed with the method previously reported [24]. The expressed proteins contained non-cleavage N-terminal His6-tags followed by five glycines (MHHHHHH GGGGG). The protein was purified by Ni2+-affinity chromatography. The ion-exchange chromatography using a 5 ml Hi-Trap Q column followed for the secondary purification. The purity of the protein was at least 95% as considered by SDS/PAGE (Figure S2). The pooled proteins were concentrated to the proper concentration for the enzyme assay.

Chemical screening with a malachite green assay method

One of the colorimetric assays well known as a malachite green assay was used for a screening of flavonoids (Supplementary Table 1) [25]. The reaction mechanism of BpHldC is to transfer adenosine-monophosphate (AMP) moiety from adenosine 5′-triphosphate (ATP) (SIGMA) to d-glycero-β-d-manno-heptose-1-phosphate. d-glycero-β-d-manno-heptose-1-phosphate was surrogated β-glucose-1-phosphate (βG1P) (Tokyo Chemical Industry, TCI)
which could get commercially available substrates. As a result of the reaction, the precursor which become LPS components and pyrophosphate (PPI) are made. To detect PPI using the malachite green reagent, PPIs were degraded by IPP (Inorganic pyrophosphatase) and turned into phosphates. The malachite green reagent was mixed with ammonium molybdate ((NH₄)₆Mo₇O₂₄), malachite green solution and Tween 20 in the ratio 1 : 3 : 0.1. A 0.20 μm PVDF syringe filter (AFrontier Inc.) was used for filtering the mixture and let it stand at room temperature (RT) for two hours before use. 40 μl of reaction mixtures were mixed with 160 μl of the malachite reagent. The mixtures were left for 10 min to develop the color.

**Enzyme kinetics of the protein**

The malachite green assay method [25] was used to get the steady-state kinetics of the enzyme. For the kinetic studies, components of the reaction mixture (40 μl) were the same as for the chemical screening. BpHldC was incubated with ATP at 0.0078 – 1.5 mM in the presence of 1 mM βG1P and with βG1P at 0.0078 – 2.5 mM in the presence of 0.5 mM ATP. The reaction was incubated at RT for two hours. The steady-state curve was fitted using GraphPad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). The standard graph was plotted from the results of IPP reaction with various concentrations of PPI (0.01–0.16 mM).

The mixture for the standard plots contained 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and 0.04 unit IPP

**IC₅₀ values of flavonoids**

The malachite green assay method was also used to study the dose-dependent inhibitory effect of epigallocatechin gallate (EGCG) ([(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-chromen-3-yl] 3,4,5-trihydroxybenzoate) and myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one). The dose-dependent inhibitory
effect of gallocatechin gallate (GCG, \((2S,3R)\)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2\(H\)-chromen-3-yl] 3,4,5-trihydroxybenzoate) and catechin gallate (CG, \((2S,3R)\)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2\(H\)-chromen-3-yl]3,4,5-trihydroxybenzoate) was also measured, which are derivatives of EGCG. The reaction mixture with different concentrations of EGCG (0.0245 - 250 μM), myricetin (0.0487 - 250 μM), GCG (0.0487 - 250 μM) and CG (0.0975 - 250 μM) was incubated at RT for two hours. For blanks, an enzyme-free reaction mixture was made and also incubated with the same range of concentrations of flavonoids. The reaction was initiated by adding saturating two substrates and stood for two hours. After the reaction, 160 μl of the malachite reagent was treated and the absorbance was measured at 620 nm using the microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA, USA). Wavelengths of maximum absorption \((\lambda_{\text{max}})\) of EGCG was 274 nm [26] (All catechin derivatives, gallic acid were reported that they have \(\lambda_{\text{max}}\) at 210 nm and from 275–280 nm [27]). \(\lambda_{\text{max}}\) of myricetin was 328 nm and 369nm [28]. The difference in absorbance with and without enzyme in the reaction mixture was managed into the percentage reactivity (%Reactivity). The IC\(_{50}\) curves of three flavonoids against BpHldC were fitted with nonlinear regression analysis using GraphPad software.

**Inhibitory enzyme kinetics**

ATP concentrations were varied at the range of 0.0078 – 1.5 mM and 1 mM βG1P, the saturated concentration, was mixed with the reaction mixture with 10 mM Tris buffer (pH 7.5) at room temperature in a 96-well microplate. On the other hand, when βG1P concentrations were varied at 0.0078 – 2.5 mM, the ATP concentration was constant at 0.5mM. Components of the reaction mixture were the same as those of enzyme kinetic assay but flavonoids were also added with 0 μM (DMSO), 10 μM, 20 μM and 40 μM, respectively. The data obtained
from the spectrophotometer were used for fitting steady-state kinetic graphs and secondary plots using the GraphPad Prism program.

**Ligand preparation, target preparation, and induced-fit docking**

All the docking and scoring calculations were performed using the Schrödinger software suite (Maestro, version 11.8.012). The SDF file of EGCG was got from the PubChem database. The file was imported into Maestro and prepared for docking using Ligand Preparation. The atomic coordinates of the crystal structure of BpHldC (PDB ID: 5X9Q) were saved from the Protein Data Bank and prepared by removing all solvent and adding hydrogens and minimal minimization using Protein Preparation Wizard. Ionizer was used to generate an ionized state of all compounds at the target pH 7.0 ± 2.0. The input for an induced-fit docking is the prepared low-energy ligand forms. The induced-fit docking protocol [29] was worked on the graphical user interface, Maestro 11.8.012 linked with the Schrödinger software. Receptor sampling and refinement were conducted for residues within 5.0 Å of each ligand for each ligand-protein complex. With Prime [30], energy minimizing with a side-chain sampling, prediction module, and the backbone of BpHldC, were carried out. A total of induced-fit receptor conformations with EGCG were generated and were scored using a combination of Prime and Glide Score scoring functions [31].

**Results**

**Chemical screening with a malachite green assay method**

A malachite green test that can easily detect free phosphate was performed to find inhibitory compounds for BpHldC. The adenylyltransferase catalyzes the transfer of adenylyl groups to heptose and produced byproduct, pyrophosphates. Pyrophosphatase turns a pyrophosphate into two phosphates and the malachite green agent detects phosphates. An in-house
flavonoids library (Table S1) which consists of about 70 flavonoids has been examined against BpHldC. Comparing the amount of phosphate after the reaction of BpHldC with flavonoids, it turned out that EGCG and myricetin have inhibitory activity against the enzyme (Figure S3).

**Enzyme kinetics of the protein**

Enzyme kinetic assays of BpHldC were performed with various concentrations of ATP at a fixed concentration of 1 mM βG1P and the kinetic parameters were determined. Enzyme assays with various concentrations of βG1P at a fixed concentration of 0.5 mM ATP were also carried out. Both ATP and βG1P versus velocity plots of BpHldC were well fitted by the allosteric sigmoidal model (Figure 1). The steady-state kinetic data of BpHldC was obtained through a nonlinear regression analysis by using GraphPad Prism 8.4.3 and organized in Table 1.

**IC$_{50}$ values of flavonoids**

To measure the dose-dependent inhibitory activities of EGCG and myricetin on BpHldC, a malachite green assay method was used at the saturated substrate concentrations of 0.5 mM ATP and 1 mM βG1P. The data were plotted as log inhibitor concentration versus percentage reactivity based on absorbance obtained from the spectrophotometer (Figure 2) (Figure S4). The 50% chemical inhibitory concentration (IC$_{50}$) was determined from the dose-dependent inhibitory curve of BpHldC for each concentration using GraphPad Prism and was 18.61 µM with EGCG and 18.32 µM with Myricetin. In addition, IC$_{50}$ values of GCG and CG which were similar to EGCG structurally were 18.44 µM and 487.5 µM, respectively.

**Inhibitory enzyme kinetics and K$_t$$^*$, K$_i$ values**
A steady-state inhibitory enzyme kinetics data were fitted by the allosteric sigmoidal model (Figure 3). The inhibitory kinetic assay was performed at varying concentrations of one substrate and at saturating another substrate with different concentrations of EGCG and myricetin. The two substrates (ATP and βG1P) were carried out alternately and the data was obtained from the spectrophotometer.

The inhibitory analysis of BpHldC was determined from secondary plots. The inhibition constants ($K_i'$ and $K_i$) were determined from $1/V_{max}$ vs $[I]$ for $K_i'$ and slope ($K_m/V_{max} \approx K_{prime}/V_{max}$) vs $[I]$ for $K_i$ (Figure 4 and 5). The alpha value (the ratio of $K_i'/K_i$) reveals the binding modality. Alpha values of the non-competitive inhibitor statistically equal to 1.0, values of the uncompetitive inhibitor are statistically less than 1.0, and the values of competitive or mixed are statistically greater than 1.0 [32]. The inhibition type and constants ($K_i'$ and $K_i$) of EGCG and myricetin on BpHldC are shown in Table 2 and Figure 3-5. The inhibition type of EGCG was presented in competitive inhibition for ATP and βG1P. The inhibition type of myricetin revealed an uncompetitive mechanism for ATP and a mixed-type inhibition for βG1P. All parameters were calculated using GraphPad Prism program.

**Docking**

To deduce the binding modes of EGCG and GCG with BpHldC at the atomic level, an in-depth theoretical investigation with an induced-fit docking study using the Schrödinger program was carried out (Figure 6). The crystal structures of BpHldC deposited in the Protein Data Bank was retrieved and docked with EGCG and GCG to predict its binding mode. Top-ranked structures with the highest Glide g-scores from the induced-fit docking results were surveyed. The top model has a Glide g-score of -9.53 and -9.19 with respect to EGCG and GCG, respectively and docked reasonably in the active site pocket. The predicted complex structure and 2D schematic representation are illustrated in Figure 6(c) and 6(d).
Discussion

Melioidosis causing 90,000 deaths across the tropics is a pathogenic disease triggered by the intracellular Gram-negative bacterium *B. pseudomallei* [10]. In poorly resourced regions, the overall mortality rate increases by up to 50% [10][33]. However, if patients are properly administered with antimicrobials, the rate decreases to 10% [34]. The symptoms become severe if patients have underlying comorbidities such as diabetes [35]. Until now no vaccine is available. Therefore, the development of anti-melioidosis agents is required. In our previous study, 3-deoxy-d-manno-oct-2-ulosonic acid cytidylyltransferases (KdsBs) from two pathogenic microbes, *B. pseudomallei* and *Pseudomonas aeruginosa*, have been studied and proven to be directly inhibited by Rose Bengal. Since KdsB is one of the key enzymes in the CMP-3-deoxy-d-manno-oct-2-ulosonic acid biosynthesis pathway of *B. pseudomallei*, the scaffold of Rose Bengal can be used as a template to develop anti-melioidosis agents.

In this study, we have studied *BpHldC* involved in the ADP-β-D-manno-heptose pathways. Since there is no antibiotic agent currently available against these biosynthesis pathways, the enzymes playing here are emerging targets to develop multi-drug resistant microbes. To assay with *BpHldC*, a surrogate of D-glycero-β-D-manno-heptose-1-phosphate, βG1P was employed as a substrate. An in-house flavonoid library (Supplementary Table 1) was built and used to probe inhibitory compounds. Enzyme kinetics of *BpHldC* (Figure 1) indicated that the substrate was enough to detect their catalytic activity and could be applied to perform inhibitory assays. Two flavonoids, EGCG and myricetin, were found to effectively blocking *BpHldC*. Their IC$_{50}$ values against *BpHldC* were obtained (Figure 2) and were 18.61 µM with EGCG and 18.32 µM with myricetin. The values indicate a potent inhibitory activity of the two flavonoids against *BpHldC*. 
The inhibitory enzyme kinetics of the two flavonoids were tried to survey its inhibitory mechanism. According to the steady-state inhibitory enzyme kinetic analysis (Figure 3), EGCG tends to act as a competitive inhibitor with respect to βG1P and ATP. The secondary plot (Figure 4 and 5) was depicted and the $K'_i$ and $K_i$ values were calculated to predict the inhibition models. The $K'_i$ and $K_i$ values of EGCG were 150.6 µM and 0.39 µM for βG1P, respectively. Those values are 99.63 µM and 1.818 µM for ATP. EGCG can be considered as a tight-binding inhibitor in that $K_i$ values of EGCG are within 10 fold of the enzyme molar concentration (1.3 µM) [32]. In both cases, the ratio of $K'_i$ over $K_i$ was much larger than 1 confirming the competitive inhibitory mode of action of EGCG [36]. The $K'_i$ and $K_i$ of myricetin were 4.99 µM and 3.75 µM for βG1P and the ratio of $K'_i$ over $K_i$ was larger than 1 implying the mixed model. The $K'_i$ and $K_i$ of myricetin were 1.44 µM and 5.89 µM for ATP and $K'_i$ over $K_i$ was smaller than 1. Since the ratio is around 0.25, its mode of action may be not competitive. Therefore, two flavonoids act differently on their inhibition over BpHldC.

BpHldC is a tetrameric enzyme with a large C-terminal helix domain connected to a hinge loop [19]. For the proper catalytic action of BpHldC, its C-terminal domain should experience a conformational change. In addition, it requires two substrates. The uncompetitive inhibitory mode of myricetin over ATP and the mixed-mode over βG1P could be related to the complicated characteristics of BpHldC. However, EGCG showed the competitive inhibitory mode for both ATP and βG1P. In order to elucidate the inhibitory mode of EGCG at the molecular level, induced-fit molecular docking trials have been performed. In the previous X-ray crystal structure study [19], the nucleotide-binding site was predicted from the comparison of BpHldC with the nucleotidylytransferase α/β phosphodiesterase superfamily members. Interestingly, the MES buffer molecule occupied the nucleotide-binding site of BpHldC. The substrate-binding site was assumed from the structural alignment between BpHldC and phosphopantetheine adenylyltransferase with 4-
phosphopantetheine (PDB ID: 1H1T). Applying Glide scoring and docking methodology implemented in the Schrödinger software, the substrate and nucleotide-binding sites in the catalytic cavity of BpHldC have been surveyed and docked with EGCG. The best Glide g-score calculated from the docking on the catalytic cavity was -9.53. In the top model, EGCG is well docked in the catalytic pocket of BpHldC. There are three major interactions between EGCG and BpHldC (Figure 6). At first, there are two hydrogen bonds formed by Asn30 with 1-oxygen of the chromene moiety and the 3-hydroxyl group of the phenyl moiety, respectively. Thr101 also forms a hydrogen bond with the 7-hydroxyl group of the chromene moiety. Asn30 and Thr101 of BpHldC are predicted to comprise the substrate-binding site. Second, Gly68 and Asp71 arisen from the flap domain of BpHldC make hydrogen bonds with the carbonyl oxygen and the 3-hydroxyl group of the trihydroxybenzoate moiety, respectively. Third, two residues in the C-terminal domain, Arg147 and Lys154, interact with the 4-oxide ion and 3-hydroxyl group of the trihydroxybenzoate moiety, respectively. The former forms an ionic bond and the latter a hydrogen bond. It is worthwhile to note that the flap domain and the C-terminal domain participate in the interaction with ATP. Therefore, the docking study of EGCG with BpHldC revealed that EGCG occupies some parts of the substrate and nucleotide-binding sites together. Since its binding has been turned out to be tight according to conditions of the tight-binding inhibition [32], the docking result is well-matched with the kinetic study. The predicted occupation of EGCG in the catalytic cavity explains its competitive mode of inhibitory action for both substrate and nucleotide.

Since some flavonoids are displaying the structural similarity with EGCG, two flavonoids, GCG and CG, were analyzed further to investigate their structure and function relationship. GCG is the C-2 epimeric isomer of EGCG. Interestingly, its IC₅₀ value, 18.44 µM, is comparable with that of EGCG. Therefore, its docking mode was searched and analyzed compared with that of EGCG. At first, the best Glide g-score of GCG was -9.19. Intriguingly,
the gallocatechin moiety is inverted at the active site pocket. As a result, the 5- and 7-
hydroxyl groups of the chromene moiety form hydrogen bonds with Ser63 and Val64, respectively. Lys69 also binds with the trihydroxyphenyl ring through π-cation interaction. The three residues, Ser63, Val64 and Lys69 are on the flap domain of BpHldC. The 3- and 5-
hydroxyl groups of the trihydroxyphenyl moiety make hydrogen bonds with Tyr120 and Asn30, respectively. The carboxyl oxygen and the 5-hydroxyl group of the trihydroxybenzoate moiety also build hydrogen bonds with Asn30 and Glu127, respectively. Accordingly, GCG may interact tightly in the active site pocket of BpHldC and thus displays inhibitory activity. The importance of the gallate moiety of GCG has been also proven with Gallic acid. It does not have gallate attached and its inhibitory activity was severely reduced.

On the contrary, CG did not show inhibitory activity against BpHldC. In the chemical structure, it lacks only one hydroxyl (5-hydroxyl) group at the phenyl ring moiety compared with GCG. It implies that one of the key interactions is the hydrogen bond formed between Asn30 and the 5-hydroxyl group of the trihydroxyphenyl moiety of GCG. Actually, the IC50 value of CG is 487.5 μM and is 26-fold higher than that of GCG. In summary, the 5-hydroxyl group of the phenyl ring moiety and the gallate moiety are two key factors of EGCG homologues to interact with BpHldC.

EGCG exhibits antibacterial activity through inhibition of efflux pumps in A. baumannii [37]. Acinetobacter species have been alerted due to their implicated in hospital-acquired and healthcare-associated infections. Ventilator-associated pneumonia is the frequent life-threatening hospital-acquired infection caused by P. aeruginosa and A. baumannii [38]. ECGC together with GCG show antibacterial activity through inhibiting FabG/FabI reductases from E. coli [39]. Myricetin displayed antibacterial activity against several antibiotic-resistant pathogens including multidrug-resistant Burkholderia cepacia [40]. At the
molecular level, myricetin has been found to suppress *E. coli* DnaB helicase with an IC\textsubscript{50} value of 11.3 µM [41]. The compound displayed poor activity against *Klebsiella pneumonia* (MIC\textsubscript{50} 128 mg/mL), but strong synergy was observed at a concentration of 32 µg/mL together with amoxicillin/clavulanate, ampicillin/sulbactam and cefoxitin [42]. Therefore, this study finds other potential antibiotic mechanisms of myricetin and EGCG together with its epimer GCG through targeting *BpHldC*. Since these flavonoids are the first known inhibitors against *BpHldC*, they can be good templates to develop anti-melioidosis agents. Among the five enzymes in the ADP-1-glycero-β-d-manno-heptose biosynthesis pathway, heptose-7-phosphate analogues targeting GmhA [43] and triazine derivatives targeting the kinase domain of HldE from *E. coli* [22] have been reported. The triazine derivatives actually attenuated the Gram-negative bacterial virulence. Therefore, including the flavonoids found in this study, various scaffolds are being used to develop antibiotics targeting this pathway. A further study of these antibiotic candidates could provide new antibiotics working independently or synergistically.

**Conflict of interest statement**

The authors declare that there is no conflict of interest.

**Acknowledgments**

This work was supported by the Basic Science Research Programs, 2018R1D1A1B07050781 to DHS and 2018R1D1A1B07050942 to M-SK, funded by the National Research Foundation of Korea grant granted by the Ministry of Education, Science and Technology, Republic of Korea (MEST).

**References**
[1] Xiao, J., Kai, G. (2012). A review of dietary polyphenol-plasma protein interactions: characterization, influence on the bioactivity, and structure-affinity relationship. *Crit Rev Food Sci Nutr*. 52(1), 85–101. https://doi.org/10.1080/10408398.2010.499017

[2] Falcone, M.L., Rius, S.P., Casati, P. (2012) Flavonoids: Biosynthesis. Biological Functions and Biotechnology applications. Frontiers in Plant Science 3, 222-237.

[3] Aherne, S.A., O'Brien, N.M. (2002). Dietary flavonols: chemistry, food content, and metabolism. *Nutrition (Burbank, Los Angeles County, Calif.)*, 18(1), 75–81. https://doi.org/10.1016/s0899-9007(01)00695-5

[4] Havsteen, B.H. (2002) The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics*, 96, 67-202.

[5] Cushnie, T.P.T., Lamb, A.J. (2005) Antimicrobial activity of flavonoids. *Int J Antimicrob Agents*. 26, 343–56.

[6] Lechner, D., Gibbons, S., Bucar, F. (2008) Modulation of isoniazid susceptibility by flavonoids in Mycobacterium. *Phytochem Lett*. 1, 71–5. https://doi.org/10.1016/j.phytol.2008.01.002.

[7] Wiersinga, W.J., van der Poll, T., White, N.J., Day, N.P., Peacock, S.J. (2006) Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. *Nat Rev Microbiol*. 4(4), 272–282. https://doi.org/10.1038/nrmicro1385

[8] Wiersinga, W.J., Virk, H.S., Torres, A.G., Currie, B.J., Peacock, S.J., Dance, D., Limmmathurotsakul, D. (2018) Melioidosis. *Nat Rev Dis Primers*. 4, 17107. https://doi.org/10.1038/nrdp.2017.107

[9] Currie B.J. (2015) Melioidosis: evolving concepts in epidemiology, pathogenesis, and treatment. *Semin Respir Crit Care Med*. 36(1), 111–125. https://doi.org/10.1055/s-0034-1398389

[10] Limmmathurotsakul, D., Golding, N., Dance, D.A., Messina, J.P., Pigott, D.M., Moyes,
C.L., Rolim, D.B., Bertherat, E., Day, N.P., Peacock, S.J., Hay, S.I. (2016) Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nat Microbiol.* 1, 15008. https://doi.org/10.1038/nmicrobiol.2015.8

[11] Keluangkhot, V., Pethsouvanh, R., Strobel, M. (2005) Mélioidose [Melioidosis]. *Med Mal Infect.* 35(10), 469–475. https://doi.org/10.1016/j.medmal.2005.08.001

[12] Bondi, S.K., Goldberg, J.B. (2008) Strategies toward vaccines against *Burkholderia mallei* and *Burkholderia pseudomallei*. *Expert Rev Vaccines*, 7(9), 1357–1365. https://doi.org/10.1586/14760584.7.9.1357

[13] Sarkar-Tyson, M., Titball, R.W. (2010) Progress toward development of vaccines against melioidosis: A review. *Clin Ther.* 32(8), 1437–1445. https://doi.org/10.1016/j.clinthera.2010.07.020

[14] Titball, R.W., Burtnick, M.N., Bancroft, G.J., Brett, P. (2017) *Burkholderia pseudomallei* and *Burkholderia mallei* vaccines: Are we close to clinical trials?. *Vaccine*, 35(44), 5981–5989. https://doi.org/10.1016/j.vaccine.2017.03.022

[15] Mangalea, M.R., Borlee, G.I., Borlee, B.R. (2017) The Current Status of Extracellular Polymeric Substances Produced by *Burkholderia pseudomallei*. *Curr Trop Med Rep.* 4, 117–126. https://doi.org/10.1007/s40475-017-0118-2

[16] Vinion-Dubiel, A.D., Goldberg, J.B. (2003) Lipopolysaccharide of *Burkholderia cepacia* complex. *J Endotoxin Res.* 9(4), 201–213. https://doi.org/10.1179/096805103225001404

[17] Zamyatina A. (2018) Aminosugar-based immunomodulator lipid A: synthetic approaches. *Beilstein J Org Chem.* 14, 25–53. https://doi.org/10.3762/bjoc.14.3

[18] Adams, G.A., Quadling C., Perry, M.B. (1967) D-glycero-D-manno-heptose as a component of lipopolysaccharides from gram-negative bacteria. *Can J Microbiol.* 13, 1605-1613, https://doi.org/10.1139/m67-210

[19] Park, J., Kim, H., Kim, S., Lee, D., Kim, M.S., Shin, D.H. (2018) Crystal structure of D-
glycero-\(\beta\)-D-manno-heptose-1-phosphate adenylyltransferase from *Burkholderia pseudomallei*. *Proteins*, 86(1), 124–131. https://doi.org/10.1002/prot.25398

[20] Kneidinger, B., Marolda, C., Graninger, M., Zamyatina, A., McArthur, F.; Kosma, P., Valvano, M. A., Messner, P. (2002) Biosynthesis pathway of ADP-L-glycero-\(\beta\)-D-manno-heptose in *Escherichia coli*. *J. Bacteriol*. 184, 363–369.

[21] McArthur, F., Andersson, C. E., Loutet, S., Mowbray, S. L., Valvano, M. A. (2005) Functional analysis of the glycero-manno-heptose 7-phosphate kinase domain from the bifunctional HldE protein, which is involved in ADP-L-glycero-D-manno-heptose biosynthesis. *J. Bacteriol*. 187, 5292–5300.

[22] Desroy, N., Denis, A., Oliveira, C., Atamanyuk, D., Briet, S., Faivre, F., LeFralliec, G., Bonvin, Y., Oxoby, M., Escaich, S., Floquet, S., Drocourt, E., Vongsouthi, V., Durant, L., Moreau, F., Verhey, T.B., Lee, T.W., Junop, M.S., Gerusz, V. (2013) Novel HldE-K inhibitors leading to attenuated Gram negative bacterial virulence. *J Med Chem*. 56(4), 1418-30. doi: 10.1021/jm301499r. Epub 2013 Feb 14. PMID: 23409840.

[23] Lipsitz, R., Garges, S., Aurigemma, R., Baccam, P., Blaney, D. D., Cheng, A. C., Currie, B. J., Dance, D., Gee, J. E., Larsen, J., Limmathurotsakul, D., Morrow, M. G., Norton, R., O’Mara, E., Peacock, S. J., Pesik, N., Rogers, L. P., Schweizer, H. P., Steinmetz, I., Tan, G., Tan, P., Wiersinga, W.J., Wuthiekanun, V., Smith, T. L. (2012). Workshop on treatment of and postexposure prophylaxis for *Burkholderia pseudomallei* and *B. mallei* Infection, 2010. *Emerging infectious diseases*, 18(12), e2. https://doi.org/10.3201/eid1812.120638

[24] Park, J., Lee, D., Seo, E.K., Ryu, J.S., Shin, D.H. (2017) General assay for enzymes in the heptose biosynthesis pathways using electrospray ionization mass spectrometry. *Appl Microbiol Biotechnol*. 101(11), 4521-4532. doi:10.1007/s00253-017-8148-1
Sha, S., Zhou, Y., Xin, Y., Ma, Y. (2012) Development of a colorimetric assay and kinetic analysis for Mycobacterium tuberculosis d-glucose-1-phosphate thymidylyltransferase. *J Biomol Screen.* 17(2), 252-257. doi:10.1177/1087057111421373

Ibrahim, Y.M., Musa, A., Yakasai, I.A. (2017) Spectrophotometric method for determination of catechins in green tea and herbal formulations. 16, 25-30.

Fernando, C. D., Soysa, P. (2016) Simple isocratic method for simultaneous determination of caffeine and catechins in tea products by HPLC. *SpringerPlus*, 5(1), 970. https://doi.org/10.1186/s40064-016-2672-9

Yao, Y., Lin, G., Xie, Y., Ma, P., Li, G., Meng, Q., Wu, T. (2014) Preformulation studies of myricetin: a natural antioxidant flavonoid. *Pharmazie*. 69(1), 19-26. PMID: 24601218.

Sherman, W., Day, T., Jacobson, M.P., Friesner, R.A., Farid, R. (2006) Novel procedure for modeling ligand/receptor induced fit effects. *J Med Chem.* 49(2), 534–553. https://doi.org/10.1021/jm050540c

Jacobson, M. P., Pincus, D. L., Rapp, C. S., Day, T. J., Honig, B., Shaw, D. E., Friesner, R. A. (2004) A hierarchical approach to all-atom protein loop prediction. *Proteins.* 55(2), 351–67. https://doi.org/10.1002/prot.10613

Friesner, R. A., Murphy, R. B., Repasky, M. P., Frye, L. L., Greenwood, J. R., Halgren, T. A., Sanschagrin, P. C., & Mainz, D. T. (2006). Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J. Med. Chem.* 49(21), 6177–6196. https://doi.org/10.1021/jm051256o

Strelow, J., Dewe, W., Iversen, P. W., Brooks, H. B., Radding, J. A., McGee, J., Weidner, J. (2012) Mechanism of Action Assays for Enzymes. In S. Markossian (Eds.) *et al., Assay Guidance Manual.* Eli Lilly & Company and the National Center for Advancing Translational Sciences.

Currie, B.J., Ward, L., Cheng, A.C. (2010) The epidemiology and clinical spectrum of
melioidosis: 540 cases from the 20 year Darwin prospective study. *PLoS Negl Trop Dis.* 4(11), e900.

[34] Virk, H.S., Mukhopadhyay, C., Wiersinga, W.J. (2020) Melioidosis: A Neglected Cause of Community-Acquired Pneumonia. *Semin Respir Crit Care Med.* 41(4), 496-508. doi:10.1055/s-0040-1710570

[35] Birnie, E., Savelkoel, J., Reubsaet, F., Roelofs, J.J.T.H., Soetekouw, R., Kolkman, S., Cremers, A.L., Grobusch, M.P., Notermans, D.W., Wiersinga, W.J.; Dutch Melioidosis Study Group. (2019) Melioidosis in travelers: An analysis of Dutch melioidosis registry data 1985-2018. *Travel Med Infect Dis.* 101461. doi: 10.1016/j.tmaid.2019.07.017.

[36] Yu, M., Magalhães, M. L., Cook, P. F., Blanchard, J. S. (2006) Bisubstrate inhibition: Theory and application to N-acetyltransferases. *Biochemistry,* 45(49), 14788–14794. https://doi.org/10.1021/bi061621t

[37] Lee, S., Razqan, G. S., Kwon, D. H. (2017) Antibacterial activity of epigallocatechin-3-gallate (EGCG) and its synergism with β-lactam antibiotics sensitizing carbapenem-associated multidrug resistant clinical isolates of *Acinetobacter baumannii.* *Phytomedicine.* 24, 49–55. https://doi.org/10.1016/j.phymed.2016.11.007

[38] Peleg, A. Y., Hooper, D. C. (2010) Hospital-acquired infections due to gram-negative bacteria. *N Engl J Med.* 362(19), 1804–1813. https://doi.org/10.1056/NEJMra0904124

[39] Zhang, Y. M., Rock, C. O. (2004). Evaluation of epigallocatechin gallate and related plant polyphenols as inhibitors of the FabG and FabI reductases of bacterial type II fatty-acid synthase. *J Biol Chem.* 279(30), 30994–31001. https://doi.org/10.1074/jbc.M403697200

[40] Xu, H. X., Lee, S. F. (2001) Activity of plant flavonoids against antibiotic-resistant bacteria. *Phytother Res.* 15(1), 39–43. https://doi.org/10.1002/1099-1573(200102)15:1<39::aid.ptr684>3.0.co;2-r

[41] Grieb, M. A., Blood, S., Larson, M. A., Koepsell, S. A., Hinrichs, S. H. (2007) Myricetin
inhibits *Escherichia coli* DnaB helicase but not primase. *Bioorg Med Chem.* 15(22), 7203–7208. [https://doi.org/10.1016/j.bmc.2007.07.057](https://doi.org/10.1016/j.bmc.2007.07.057)

[42] Lin, R.D., Chin, Y.P., Lee, M.H. (2005) Antimicrobial activity of antibiotics in combination with natural flavonoids against clinical extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumonia*. *Phytother Res.* 19, 612–617.

[43] Durka, M., Tikad, A., Périon, R., Bosco, M., Andaloussi, M., Floquet, S., Malacain, E., Moreau, F., Oxoby, M., Gerusz, V., Vincent, S. P. (2011). Systematic synthesis of inhibitors of the two first enzymes of the bacterial heptose biosynthetic pathway: towards antivirulence molecules targeting lipopolysaccharide biosynthesis. *Chemistry (Weinheim an der Bergstrasse, Germany)*, 17(40), 11305–11313. [https://doi.org/10.1002/chem.201100396](https://doi.org/10.1002/chem.201100396)
### Tables

**Table 1.** The kinetic parameter data of *BpHldC*

| Proteins | ATP | βG1P |
|----------|-----|------|
|          | $V_{\text{max}}$ (mM/min) | $K_{\text{half}}$ (mM) | $K_m$ or $K_{\text{prime}}^{**}$ (mM) | $V_{\text{max}}$ (mM/min) | $K_{\text{half}}$ (mM) | $K_m$ or $K_{\text{prime}}^{**}$ (mM) |
| *BpHldC* | 0.0007 | 0.228 ± 0.015 | 0.047 ± 0.020 | 0.0006 | 0.683 ± 0.069 | 0.450 ± 0.138 |

*$K_{\text{half}}$ is the concentration of substrate that produces a half-maximal enzyme velocity in the allosteric sigmoidal model. It is the EC$_{50}$.

**$K_{\text{prime}}$** According to GraphPad Prism guide, $K_{\text{prime}}$ in the allosteric sigmoidal model is related to the $K_m$

**Table 2.** Inhibitory properties of flavonoids with *BpHldC*

| Flavonoid | ATP | βG1P | IC$_{50}$ |
|-----------|-----|------|---------|

Downloaded from http://portlandpress.com/biochemj/article-pdf/doi/10.1042/BCJ20200677/900588/bcj-2020-0677.pdf by guest on 24 December 2020
|                   | $K_i$ (µM) | $K_i'$ (µM) | Type       | $K_i$ (µM) | $K_i'$ (µM) | Type       | values (µM) |
|-------------------|-----------|-------------|------------|-----------|-------------|------------|-------------|
| Epigallocatechin  | 99.63     | 1.818       | Competitive| 150.6     | 0.3943      | Competitive| 18.75       |
| gallate           |           |             |            |           |             |            |             |
| Myricetin         | 1.440     | 5.886       | Uncompetitive| 4.994     | 3.747       | Mixed type | 18.29       |

Figures

Figure 1. Curves of steady-state kinetics of BpHldC. (a) Steady-state enzyme kinetics with respect to the ATP at a fixed concentration of 1mM βG1P. (b) Enzyme activity at varying βG1P concentrations with a constant concentration of 0.5mM ATP is presented. The data of enzyme kinetic assays were well fitted by the allosteric sigmoidal model (solid line) rather than the hyperbolic model (Michaelis-Mentens model) (dotted line). Each data point represents the dose-dependent response with BpHldC.
Figure 2. Dose-dependent inhibitory curves of epigallocatechin gallate (a) and myricetin (b). Each data point represents the dose-dependent response against BpHldC.
Figure 3. Inhibitory enzyme kinetics of BpHldC with epigallocatechin gallate ((a) and (b)) and myricetin ((c) and (d)). (a) and (c) Steady-state inhibitory kinetics with respect to the ATP at a fixed concentration of 1mM βG1P. (b) and (d) Inhibition of BpHldC by compounds at varying βG1P concentrations with a constant concentration of 0.5mM ATP is presented. Flavonoids were treated to each point with four concentrations (0, 10, 20, 40 µM).
Figure 4. Determination of $K_i$ with respect to ATP. The secondary plot of slope ($K_{\text{prime}}/V_{\text{max}}$) vs [I] for determination of $K_i$ and secondary plot of $1/V_{\text{max}}$ vs [I] for determination of $K_i'$ of epigallocatechin gallate (EGCG) ((a) and (b)), myricetin ((c) and (d)) with respect to ATP for $Bp$HldC inhibitory activity.
Figure 5. Determination of $K_i$ with respect to $\beta G1P$. The secondary plot of slope ($K_{\text{prime}}/V_{\text{max}}$) vs [I] for determination of $K_i$ and secondary plot of $1/V_{\text{max}}$ vs [I] for determination of $K_i'$ of epigallocatechin gallate (EGCG) ((a) and (b)), myricetin ((c) and (d)) with respect to $\beta G1P$ for $BpHldC$ inhibitory activity.
Figure 6. Predicted docking modes of epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG) in the catalytic cavity of BpHldC. (a) The docking poses of EGCG (light green) and GCG (yellow) were depicted on the electrostatic surface potential of BpHldC (red, negative; blue, positive; white, uncharged). (b) The enlarged view of the active site docked with EGCG and GCG was depicted with interactive residues. Asn30, Gly31, Val32, Phe33, Asp34 and Thr101 belong to the substrate pocket (green letters) and two residues, Arg147 and Thr154, located in the nucleotide-binding pocket (light blue letters). Lys69 is placed in between pockets. In addition, 2D schematic figures of the docked EGCG (c) and GCG (d) with BpHldC were drawn. Figures were created with Maestro v11.5.011. Pink arrows represent hydrogen bond interactions by Asn30, Gly68, Asp71, Thr101, and Lys154 and the blue line represents the ionic interaction with Asp147. The red line represents the \( \pi \)-cation interaction with Lys69.
