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Inhibition of *Staphylococcus aureus* PriA Helicase by Flavonol Kaempferol

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Abstract *Staphylococcus aureus* is an important etiological agent responsible for healthcare-associated infections. In this study, the effect of flavonoids on the inhibition of *S. aureus* PriA (SaPriA), an essential helicase for DNA replication restart, which is critical for bacterial survival, was investigated. Using vanadate-sensitive colorimetric assay, the concentration of phosphate, from ATP hydrolysis by SaPriA, was decreased to 37 and 69 %, respectively, in the presence of 35 μM kaempferol and myricetin. The effect of quercetin, galangin, dihydromyricetin, and myricitrin was insignificant. From titration curve, IC_{50} of kaempferol for SaPriA was determined to be 22 ± 2 μM. Using fluorescence quenching, we identified that kaempferol can bind to SaPriA with K_d of 9.1 ± 3.2 μM. To our knowledge, these preliminary results constituted the first study regarding that naturally occurring product such as flavonols kaempferol and myricetin can be potent inhibitors targeting PriA.

Keywords *Staphylococcus aureus* · PriA helicase · Inhibitor · Flavonol · Kaempferol · Myricetin · Drug development

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Abbreviations

SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
K_d	The apparent dissociation constant
Sa	*Staphylococcus aureus*

1 Introduction

DNA helicases are required in most stages of DNA metabolism, ranging from DNA replication, repair, and recombination [1, 2]. DNA helicases utilize energy via the hydrolysis of nucleoside triphosphate (NTP) to move along nucleic acid filaments with either a 5′–3′ or 3′–5′ direction and separate double-stranded DNA (dsDNA) into the complementary single-stranded DNA (ssDNA). Helicases were grouped into several superfamilies (SF) on the basis of the helicase motifs. Most SF1 and SF2 helicases have low activity in unwinding dsDNA when acting without their partner proteins and might need these accessory proteins to stimulate the helicase activity. In addition, helicases are also needed for the removal of nucleoproteins from DNA in many different stages of DNA metabolism and gene expression [1].

PriA is a SF2 helicase possessing ATPase and 3′–5′ helicase activities and is originally discovered as a major factor essential for phage ϕX174 replication [3]. PriA is a poor helicase when acting alone in vitro and might need other accessory proteins, such as PriB and SSB, to stimulate the helicase activity [4, 5]. The PriA-orientated replication restart primosome is a formidable enzymatic machine used in reactivation of stalled DNA replication [3]. Unlike the DnaA-directed primosome, which is always

initiated at the unique *oriC* site for further assembly, the PriA-directed replication restart primosome does not assembled for specific DNA sequence but preferentially recognizes branched DNA structures [6–8]. The mechanisms of action of replication restart primosome in the Gram-negative *Escherichia coli* have been established [9–11]. In *E. coli*, the replication restart primosome consists of PriA helicase, PriB, PriC, DnaB helicase, DnaC, DnaT, and DnaG primase [12]. In the Gram-positive *Bacillus subtilis*, the DNA replication initiator protein PriA helicase has homolog of *E. coli* [13]. Nevertheless, PriB, PriC, DnaT, and DnaC proteins, essential components of the replication restart primosome, are not found in Gram-positive bacteria. Instead, DnaD, DnaB, and DnaI are found as essential factors for replication restart of the Gram-positive *B. subtilis*. On the basis of the function and activity, DnaI may be the Gram-positive functional counterpart of *E. coli* DnaC, a helicase loader protein [14].

Flavonoids are plant polyphenols [15], and many display unique biological activities which have been used as some pharmaceutical agents [16]. Flavonols belong to one of the six major subclasses of flavonoids, and others are flavones, flavanones, flavanols, anthocyanidins, and isoflavones. Some flavonols are known to have significant antioxidant [17], antiradical [18], and antibacterial activities [19–22]. Recently, few therapies are effective against the antibiotic-resistant pathogens, especially the six antibiotic-resistant ESKAPE pathogens [23, 24]. *S. aureus*, which is a Gram-positive pathogen, has a remarkable ability to develop antibiotic resistance. Although some advances in treatment and prevention are made, *S. aureus* still create serious problems to public health worldwide. It is thought that PriA is an essential initiator protein required for restart of DNA replication in the Gram-positive bacteria, inhibiting the activity of PriA will be detrimental to block bacterial growth and survival.

2 Materials and Methods

The gene *SAAV1184*, encoding SaPriA, was amplified by PCR using genomic DNA of *Staphylococcus aureus subsp. aureus* ED98 as template. The forward (5′-GGGAAGGATCCATGATAGCGAAAGTCA-3′) and the reverse (5′-CCATTCTCGAGCATCATCATCTGTGGA-3′) primers were designed, and BamHI and XhoI restriction sites into SaPriA were introduced. The PCR product was then ligated to the pET21b vector. SaPriA was purified by Ni^{2+}-affinity chromatography (GE Healthcare Bio-Sciences) eluted with Buffer A (20 mM Tris–HCl, 250 mM imidazole, and 0.5 M NaCl, pH 7.9). After dialysis against Buffer B (20 mM HEPES and 100 mM NaCl, pH 7.0), the SaPriA solution was further purified by the Heparin HP column (GE Healthcare Bio-Sciences), eluted with a linear NaCl gradient from 0.1 to 1.0 M with Buffer B using the AKTA-FPLC system (GE Healthcare Bio-Sciences). SDS-PAGE was used to show the protein purity (Fig. 1). By the vanadate-sensitive colorimetric assay, the ATPase activity of SaPriA was detected: more the concentration of inorganic phosphate released by ATP hydrolysis, more the OD_{610} intensity. The binding of kaempferol to SaPriA was analyzed by the fluorescence emission spectra of SaPriA quenched by kaempferol.

3 Results

Myricetin is a flavonol, and has three hydroxyl substituents on the aromatic ring (Fig. 2). The inhibitory effect of myricetin on several helicases, such as the SARS coronavirus helicase [25], the replicative DnaB helicase [21, 26, 27], and RSF1010 RepA helicase [28], has been established. Given that the inhibitory effect of myricetin on PriA helicase is not known yet, we used myricetin as a potential inhibitor on SaPriA. Other flavonoids, namely, dihydromyricetin, myricitrin, quercetin, galangin, and kaempferol, were further used to test the structure–inhibition relationship for SaPriA. We analyzed the ATPase activity of SaPriA by detecting the concentration of inorganic phosphate released by ATP hydrolysis [29]. The concentration of phosphate from ATP hydrolysis by SaPriA was decreased to 37 and 69 %, respectively, in the presence of 35 μM kaempferol and myricetin (Fig. 3). The effect of quercetin, galangin, dihydromyricetin, and myricitrin was insignificant (Fig. 3). From titration curve,

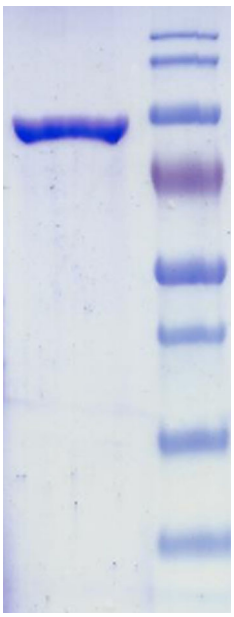
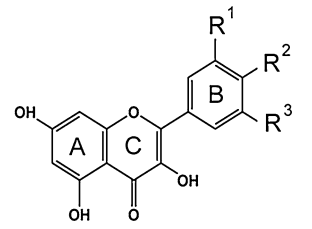


Fig. 1 SDS-PAGE of the purified SaPriA and molecular mass standards



	R^1	R^2	R^3
Galangin	H	H	H
Kaempferol	H	OH	H
Quercetin	OH	OH	H
Myricetin	OH	OH	OH

Fig. 2 Molecular structure of myricetin, quercetin, kaempferol, and galangin

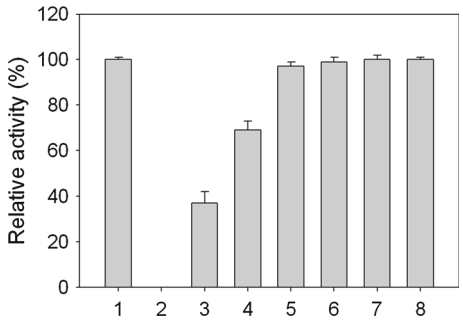


Fig. 3 Inhibition of the ATPase activity of SaPriA. The ATPase activity of purified SaPriA was analyzed using the vanadate-sensitive colorimetric assay. The ATPase activity assay (2 mL of reaction volume) for SaPriA (2 μM) was analyzed in 20 mM HEPES (pH 7.0), 5 mM of $MgCl_2$, 1 mM of ATP, and 35 μM of flavonoid for 2 h, and then the OD_{610} was analyzed. The reaction mixture with (*lane 1*) or without SaPriA (*lane 2*) was analyzed. The reaction mixture with SaPriA was analyzed in the presence of kaempferol (*lane 3*), myricetin (*lane 4*), dihydromyricetin (*lane 5*), galangin (*lane 6*), quercetin (*lane 7*), and myricitrin (*lane 8*)

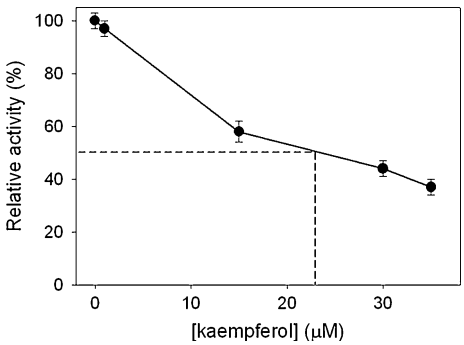


Fig. 4 The titration curve of kaempferol for SaPriA. IC_{50} of kaempferol was determined to be 22 ± 2 μM using graphic analysis

IC_{50} of kaempferol for SaPriA was determined to be 22 ± 2 μM (Fig. 4). To our knowledge, this is the first study regarding that naturally occurring product such as flavonols kaempferol and myricetin can be potent inhibitors targeting PriA.

To determine whether kaempferol can bind to SaPriA, the fluorescence emission spectra of SaPriA quenched by kaempferol are shown in Fig. 5. Quenching refers to the complex formation process that decreases the fluorescence intensity of the protein. As kaempferol (0–10 μM) was added into the SaPriA solution, the intrinsic fluorescence intensity of the protein was progressively decreased. The maximum λ_{em} of SaPriA was slightly shifted (~1.5 nm). Therefore, these results from the quenching of SaPriA fluorescence indicated the formation of a complex between kaempferol and SaPriA. In addition, the intrinsic fluorescence of SaPriA at 330 nm, which is excited at 280 nm, was significantly quenched by 55 % in the presence of 10 μM kaempferol. K_d value of SaPriA bound to kaempferol determined from the titration curve (not shown) were 9.1 ± 3.2 μM. Thus, kaempferol can bind to SaPriA and then inhibit its ATPase activity.

4 Discussion

S. aureus, a Gram-positive pathogen, exhibits a remarkable ability to develop antibiotic resistance [30]. Development of clinically useful small molecule antibiotics is highly needed to target *S. aureus* and other infections [30]. Considering that PriA-directed primosomes are required for

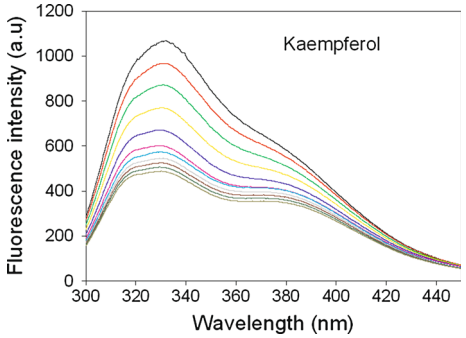


Fig. 5 The fluorescence quenching of SaPriA by kaempferol. Compound concentrations, from the top down, are 0 to 10 μM. Fluorescence titration was performed using a spectrofluorimeter (Hitachi F-2700). An aliquot amount of kaempferol was added into the solution containing SaPriA (0.5 μM), 20 mM HEPES, and 100 mM NaCl at pH 7.0. The K_d value was obtained by the equation: $\Delta F = \Delta F_{max} - K_d(\Delta F/[\text{compound}])$ (Enzyme Kinetics module of Sigma-Plot)

bacterial DNA replication restart processes, PriA may be a suitable target for antibiotic development. In addition, PriA is not found in humans; hence, inhibitors based on PriA inhibition are potentially safe for human use. Flavonoids are plant polyphenols, and many are known to have antiradical, antiviral, antioxidant, and antibacterial activities. Some flavonoids are also served as the ATPase-inhibiting agents for competition with ATP binding of the protein, and thus, these flavonoid derivatives may be the lead compounds for developing therapeutic agents for fighting cancer [31]. Our laboratory is also currently screening some helicases from *Hepatitis* viruses using these flavonoids and the derivatives.

5 Conclusion

In this study, we analyzed the effects of the flavonoids, namely, myricetin, dihydromyricetin, myricitrin, quercetin, galangin, and kaempferol, on ATP hydrolysis ability of SaPriA. For the first time, our results demonstrated that naturally occurring products such as flavonols kaempferol and myricetin were capable of inhibiting the PriA activity. These flavonol compounds as well as their derivatives may be used as lead compounds in the development of new antibiotics that target *S. aureus* and other bacteria.

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