Antimicrobial Flavonoid, 3,6-Dihydroxyflavone, Have Dual Inhibitory Activity against KAS III and KAS I

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Three types of β-ketoacyl acyl carrier protein synthase (KAS) are important for overcoming the bacterial resistance problem. Recently, we reported the discovery of a antimicrobial flavonoid, YKAF01 (3,6-dihydroxyflavone), which exhibits antibacterial activity against Gram-positive bacteria through inhibition of β-ketoacyl acyl carrier protein synthase III (KAS III). In this report, we suggested that YKAF01 can be an inhibitor β-ketoacyl acyl carrier protein synthase I (KAS I) with dual inhibitory activity for KAS I as well as KAS III. KAS I is related to the elongation of unsaturated fatty acids in bacterial fatty acid synthesis and can be a good therapeutic target of designing novel antibiotics. We performed docking study of *Escherichia coli* KAS I (ecKAS I) and YKAF01, and determined their binding model. YKAF01 binds to KAS I with high binding affinity (2.12×10^6) and exhibited an antimicrobial activity against the multidrug-resistant *E. coli* with minimal inhibitory concentration (MIC) value of 512 μg/mL. Further optimization of this compound will be carried out to improve its antimicrobial activity and membrane permeability against bacterial cell membrane.

Key Words : FAS, KAS I, Docking, Antibiotics, Flavonoid

Introduction

In bacteria, fatty acid synthesis is controlled by a multi-functional enzyme complex system (fatty acid synthase; FAS) which consists of seven protein domains.[1-3] It is well known that three types of β-ketoacyl acyl carrier protein synthase (KAS) enzymes, KAS I (FabB), KAS II (FabF), and KAS III (FabH), of FAS system are predominant targets for the design of novel antibiotics.[4,5]

KAS I is related on the elongation of an intermediate in unsaturated fatty acid synthesis, whereas KAS II is required to the final step in the unsaturated pathway.[4] In the case of synthesis of saturated fatty acid, an enzyme, thioesterase, plays key role in the final step of its biosynthesis. KAS III, the initiation condensing enzyme, catalyzes the initiation of bacterial fatty acid synthesis.[4,6] The active site of KAS I forms a catalytic triad hole which consist of His-His-Cys, and it is conserved in most bacterial KAS I.[7] The inhibition of the fatty acids elongation by inhibiting KAS I is a prominent target for discovery of novel antibiotics and overcoming the bacterial antibiotics resistance. The whole structure of KAS III displays a five-layered core structure which comprises two α-helices and five-stranded, mixed β-sheet. KAS I adopts the thiolase fold α-β-α-β-α sandwich motif. The active site of KAS I is centered by a cysteine residue which accepts the acyl chain from the substrates. The catalytic triad of KAS III is formed Cys-His-Asn type, while active site of KAS I contains a His-His-Cys catalytic triad hole.

We have successfully discovered natural and synthetic antimicrobial inhibitors of KAS III as well as KAS I with various *in silico* screening techniques in our previous
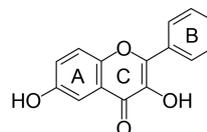
Figure 1. Chemical structure of YKAF01 (3,6-Dihydroxyflavone).

reports.[8-11] In this study, we showed that an antimicrobial flavonoid, YKAF01 (3,6-Dihydroxyflavone) as shown in Figure 1, which has high antimicrobial activities against *S. aureus* and MRSA and good binding affinity for ecKAS III, can be an inhibitor of another FAS enzyme, KAS I. To verify the possibility of YKAF01 as inhibitors of KAS I, we performed docking study and several biological assays.

On the basis of experience for discovery of KAS III or KAS I inhibitors, we performed docking for *E. coli* KAS I (ecKAS I) and YKAF01. Antimicrobial activities were measured against Gram-negative *Escherichia coli* (*E. coli*) and the multidrug-resistant *E. coli* (MDREC1229). Binding affinity of inhibitors to ecKAS I was assessed using fluorescence quenching experiment.

Methods

Docking Study. We defined the active site of ecKAS I, based on the binding model of x-ray complex structure of ecKAS I and TLM or CER (1FJ4.pdb and 1FJ8.pdb).[12] YKAF01 was docked to ecKAS I using AutoDock.[13] The Lamarckian Genetic Algorithm (LGA) of the Autodock 3.05 was used for docking experiments. Distance-dependent function of the dielectric constant was used for the calculation of the energetic maps and all other parameters were used by

default value. We carried out 150 and 250 independent docking processes for each complex.

Expression and Purification of ecKAS I. The *fab*B gene encoding KAS I was amplified from *E. coli* K-12 genomic DNA. The sense primer is 5'-catatgaaacgtgcagtgattact-3', and antisense primer is 5'-ctcgagttaatctttcagcttgc-3'. At the 5' end of each primer, a restriction site (*Nde*I for the sense primer and *Xho*I for the antisense primer) was attached to facilitate cloning. PCR was performed under the following conditions: 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 1 min at 72 °C. The resulting product was sequenced and cloned between the *Nde*I and *Xho*I sites of pET-15b vector. The ligation mixture was transformed into *E. coli* DH5α competent cells. The pET-15b/*ec*KAS I plasmid was transformed into the expression host, *E. coli* BL21 (DE3). Transformed cells were grown on Luria-Bertani (LB) agar plates containing 50 mg/L ampicillin and grown overnight in a 37 °C shaking incubator. Fully grown culture (10 mL) was mixed with 1 liter of fresh AMP-LB, and grown at 37 °C until an optical density at 600 nm of 0.5. The culture was induced with 1 mM IPTG and grown for 5 more hours at 18 °C. Cells were harvested, and the pellet stored at −80 °C.

All lysis and purification procedures were performed at 4 °C. The frozen pellet was resuspended and lysed by ultrasonication in buffer A containing 50 mM Tris-HCl and 300 mM NaCl at pH 8.0. The cell lysate was centrifuged and suffered denaturation step in 8 M urea. The supernatant loaded onto a HiTrap chelating column (GE) pre-equilibrated with buffer A. The column was washed with buffer A, and the bound material eluted with a linear gradient from 0 to 300 mM imidazole. SDS-PAGE was applied to identify the ecKAS I-containing fraction at each stage of purification and suffered refolding step with buffer A which added 2 mM DTT.

Fluorescence Quenching. We titrated inhibitor to 10 μM ecKAS I protein solution in 50 mM sodium phosphate buffer containing 100 mM NaCl at pH 8.0, with a final protein-to-inhibitor ratio of 1:10. The sample was placed in a 2 mL thermostatted cuvette, with excitation and emission path lengths of 10 nm. Using tryptophan emission, we determined the fluorescence quantum yields of KAS I and the ligand. The detailed methods are provided in our previous article.[9,10]

MIC Test. *E. coli* and the multidrug-resistant *E. coli* (MDREC-CCARM 1229) were supplied by the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University in Korea. Minimum inhibitory concentrations (MICs) were determined using a broth microdilution assay. Briefly, single colony of bacteria was incubated into LB (Luria-Bertani) and cultured overnight at 37 °C. Two-fold serial dilutions of compound in 1% peptone were prepared. 100 μL of compound were added to 100 μL of cells (2 × 10⁶ CFU/mL) in 96-well microtiter plates and incubated 37 °C for 16 h. MIC was defined as the lowest concentration which completely inhibited cell growth. MIC

values were determined as the average triplicate measurements.
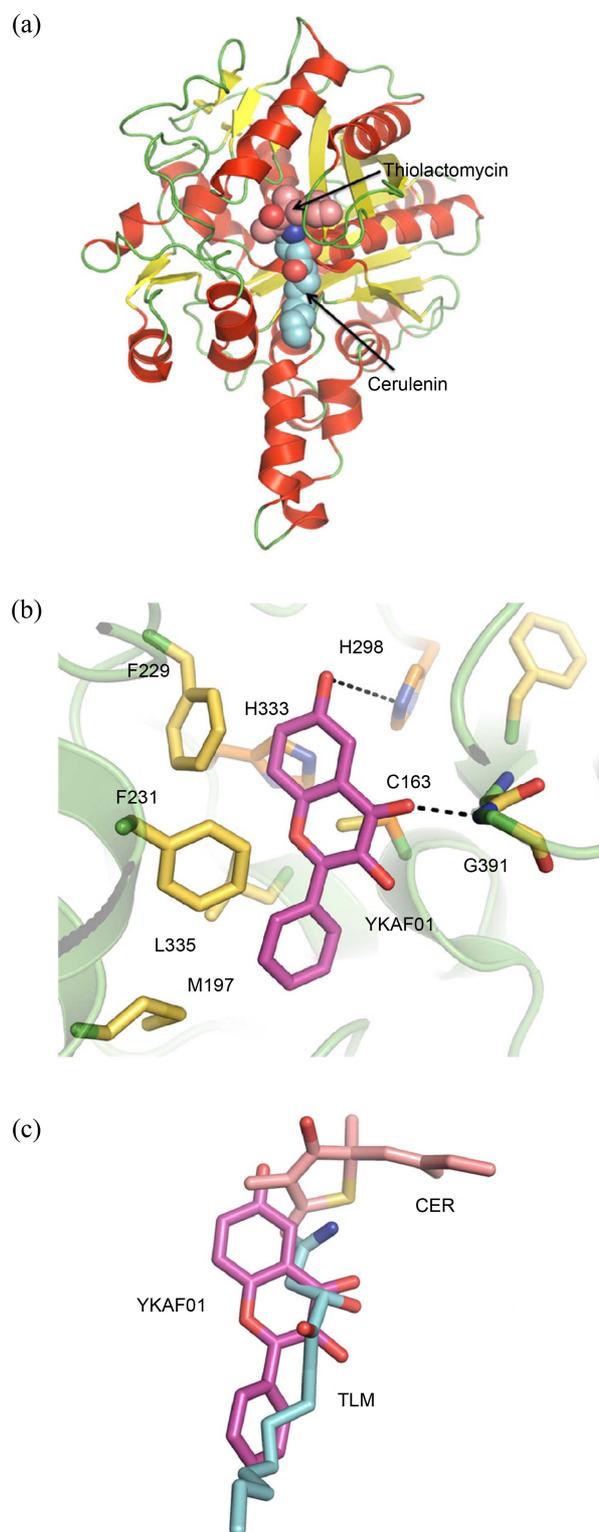
Figure 2. Comparative binding model of ecKAS I and YKAF01. (a) 3D structure of ecKAS I with inhibitors, TLM and CER. (b) Docking model of ecKAS I and YKAF01. Hydrogen bonding interactions are denoted by black dashed line. (c) Superimposed model of two known KAS I inhibitors and YKAF01.

Results and Discussion

To estimate the possibility of YKAF01 as an inhibitor of ecKAS I, we performed docking study. In our latest report, we discovered a synthetic antimicrobial inhibitor of ecKAS I, YKAe1008, N-(3-pyridinyl) hexanamide using receptor-oriented pharmacophore based *in silico* screening.[11] Thiolactomycin (TLM) and Cerulenin (CER) are known inhibitors of KAS.[12] Based on the complex structures of TLM-ecKAS I and CER-ecKAS I (1FJ4.pdb and 1FJ8.pdb) as shown in Figure 2(a),[12] we defined the proper interactions between inhibitors and ecKAS I and confirmed that a new KAS I inhibitor, YKAe1008, formed two hydrogen bonding interactions with side chain of H333 and backbone amide hydrogen of G391, and a hydrophobic interaction with several residues, such as M197, F229, and F231.

Docking model of YKAF01 and ecKAS I was similar to the model of YKAe1008 and ecKAS I. YKAF01 also formed two hydrogen bonds. Carbonyl oxygen of C-ring of YKAF01 formed hydrogen bond with backbone amide proton of G391 and hydroxyl group of A-ring participated in hydrogen bonding interaction with side chain of H298 as shown in Figure 2(b). H298 is one of the histidine residues which consist of triad hole at the active site of KAS I. Known inhibitors, such as TLM and CER, and our new inhibitor YKAe1008, formed hydrogen bonding interaction with H333 which is another catalytic triad histidine residue, while YKAF01 interacted with H298. As mentioned in our latest report, another hydrogen bond with G391 was found in the binding model of CER and ecKAS I. Hydrophobic interaction of this model was formed with M197, F229, F231, and L335 with B-ring of YKAF01. As shown in Figure 2(c), YKAF01 was well docked with same binding site of TLM and CER and the active site of YKAF01 was merged with two known inhibitors.

We determined binding constants of YKAF01 and TLM to ecKAS I using fluorescence quenching experiments. Fluorescence titration curve of YKAF01 is presented in Figure 3 and the binding constants of compounds are listed in Table 1.[10] YKAF01 bind to ecKAS I with a good binding
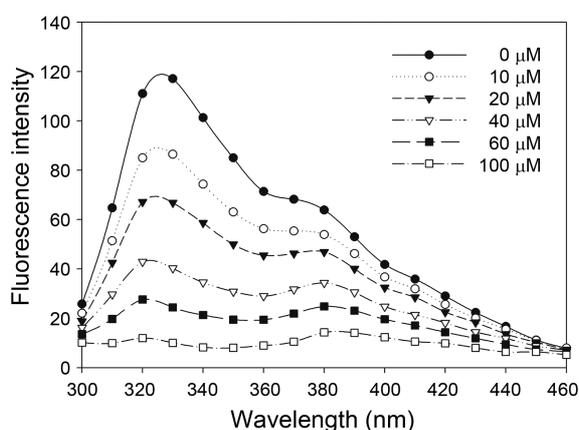
Table 1. MIC (μg/mL) values of YKAF01 and TLM against *E. coli* and the multidrug-resistant *E. coli* (MDDREC1229), and binding affinities (K_d) for ecKAS III and ecKAS I

Compound	MIC (μg/mL)		K_d (M^{-1})	
	E. coli	MDREC1229	ecKAS III[a]	ecKAS I
TLM	128	256	1.18×10^4	3.16×10^5
YKAF01	> 1024	512	1.32×10^4	2.12×10^6

[a]This data is referenced from our previous report (Lee *et al*., 2009).[10]

affinity, 2.12×10^6 M^{-1} compared with that of known inhibitor TLM, 3.16×10^5.

The K_d value of YKAF01 was estimated 2.12×10^6 M^{-1} for ecKAS I and 1.32×10^4 for ecKAS III as shown in Table 1. Therefore, YKAF01 binds to ecKAS I stronger than ecKAS III more than two orders of magnitude binding constant. From this result, we confirmed that an antimicrobial flavonoid YKAF01 targets both KAS III and KAS I enzymes. A natural antimicrobial inhibitor Platencin is also a dual inhibitor of KAS III and KAS I.[14,15] As mentioned before, since three types of KAS proteins are important targets to design of new antibiotics, multi target antibiotics can be expected to have a synergistic effect.

Additionally, we estimated the antimicrobial activities of YKAF01 against one gram-negative bacterium *E. coli* and the multidrug-resistant *E. coli* (MDDREC1229). Multidrug-resistant is a condition enabling a disease-causing organism to resist distinct drugs or chemicals and multidrug-resistant *E. coli* (MDDREC1229) is one of the bacterial resistance *E. coli* strains to antibiotics. As listed in Table 1, YKAF01 showed antibacterial activity against MDDREC1229 with MIC of 512 μg/mL. Even though it is somewhat lower than that of TLM, YKAF01 can be a good candidate of antibiotics as a natural product.

In this study, we successfully found a dual antimicrobial inhibitor of KAS I and KAS III. The further research will be focused on the optimization of YKAF01 to improve its antimicrobial activity and membrane permeability against gram-negative bacteria.

Figure 3. Fluorescence spectra of ecKAS I in the presence of YKAF01.

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