

Inhibition of *Klebsiella Pneumoniae* DnaB Helicase by the Flavonol Galangin

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Published online: 7 January 2011
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Abstract *Klebsiella pneumoniae* is a ubiquitous opportunistic pathogen that colonizes at the mucosal surfaces in humans and causes severe diseases. Many clinical strains of *K. pneumoniae* are highly resistant to antibiotics. Here, we used fluorescence quenching to show that the flavonols galangin, myricetin, quercetin, and kaempferol, bearing different numbers of hydroxyl substituent on the aromatic rings, may inhibit dNTP binding of the primary replicative DnaB helicase of *K. pneumoniae* (*Kp*DnaB), an essential component of the cellular replication machinery critical for bacterial survival. The binding affinity of *Kp*DnaB to dNTPs varies in the following order: dCTP ~ dGTP > dTTP > dATP. Addition of 10 μ M galangin significantly decreased the binding ability of *Kp*DnaB to dATP, whereas the binding affinity of *Kp*DnaB to dGTP that was almost unaffected. Our analyses suggest that these flavonol compounds may be used in the development of new antibiotics that target *K. pneumoniae* and other bacteria.

Keywords Flavonol · Galangin · DnaB helicase · *Klebsiella pneumoniae*

Abbreviations

Kp *Klebsiella pneumoniae*
 K_d The dissociation constant

dsDNA	Double-stranded DNA
ssDNA	Single-stranded DNA
Myr	Myricetin
Que	Quercetin
Kae	Kaempferol
Gal	Galangin

1 Introduction

DNA helicases are motor proteins essential for DNA replication, repair, and recombination [8, 15, 16, 18]. During DNA replication, the leading strand is directly synthesized by DNA polymerase. On the lagging strand, the primase interacts with the hexameric helicase to synthesize short RNA primers. These primers are used to generate the Okazaki fragments necessary for progression of the replication fork. The most widely studied replicative helicase is *Escherichia coli* DnaB helicase (*Ec*DnaB) [1, 21]. *Ec*DnaB is a multifunctional ATPase that catalyzes the unwinding of double-stranded DNA (dsDNA) into single-stranded DNA (ssDNA) intermediates at the replication fork to provide ssDNA templates for DNA polymerases [21]. Recently, we resolved the three-dimensional structures of the *Geobacillus kaustophilus* DnaB-family protein both in the apo state and complexed it with ssDNA and the non-hydrolysable NTP ATP γ s, and showed that ATP hydrolysis may drive the movement of the helicase toward the 3' end of the lagging strand [13].

Klebsiella pneumoniae (*Kp*) is a ubiquitous opportunistic pathogen that colonizes at the mucosal surfaces in humans and causes severe diseases such as septicemia, pneumonia, urinary tract infections, and soft tissue infections [17].

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Despite advances in treatment and prevention, *K. pneumoniae* still poses a major threat to public health worldwide. Although *K. pneumoniae* strains are generally susceptible to some antibiotics, such as cephalosporins, many clinical strains of *K. pneumoniae* are highly resistant to antibiotics. Currently, few therapies are effective against these extended-spectrum β -lactamase (ESBL)-producing *K. pneumoniae* strains [7, 30]. Discovering virulence factors and identifying novel targets for drug development and new therapies against *K. pneumoniae* are critical for public health [4, 27, 29]. Since DnaB is required for DNA replication, blocking the activity of DnaB would be detrimental to bacterial survival [22]. Because of the distinct differences between eukaryotic and prokaryotic DnaB-like helicases [21, 22], the *K. pneumoniae* DnaB helicase may be a promising target in developing antibiotics.

Flavonoids are the most common group of plant polyphenols, and are responsible for much of the flavor and color of fruits and vegetables [19]. To date, over 5,000 different flavonoids have been described; many of these compounds display structure-dependent biological and pharmacological activities [19, 23, 26]. The 6 major subclasses of flavonoids are: flavonols, flavones, flavanones, catechins (flavanols), anthocyanidins, and isoflavones [19]. Flavonols are polyphenol compounds with in vitro antioxidant and anti-radical activity [3, 26], as well as antibacterial activity [5]. A flavonol is composed of 2 aromatic rings linked by a heterocyclic pyran-4-one ring.

In this study, we have cloned, expressed, and purified *KpDnaB*, and found that the 4 flavonols (Fig. 1) myricetin (Myr), quercetin (Que), kaempferol (Kae), and galangin (Gal) can interact with *KpDnaB* and prevent dNTP binding. These flavonols may be potential leads in anti-*Kp* drug development.

2 Materials and Methods

2.1 Materials

All restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA) unless explicitly stated otherwise. All custom oligonucleotide primers were obtained from Invitrogen Corporation

(Carlsbad, CA, USA). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless explicitly stated otherwise.

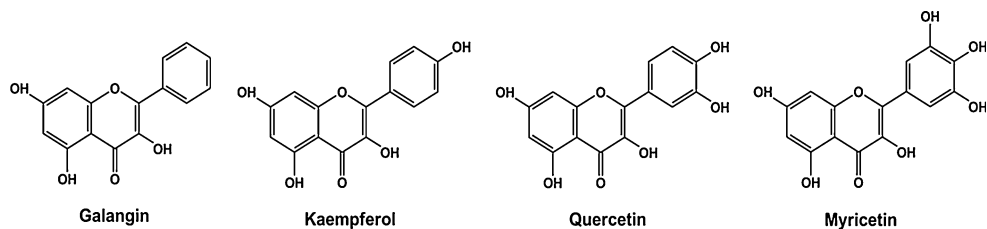
2.2 Construction of the *KpDnaB* Expression Plasmid

KPN04439, the gene encoding the putative *KpDnaB*, was amplified by PCR using genomic DNA of *K. pneumoniae* subsp. *pneumoniae* MGH 78578 as the template. The forward (5'-GGGGAATTCACAGCACAATCCCAGGTAT TGAAA-3') and the reverse (5'-GGGAAGCTTCTCAT CATCATACTGAGGACCGGC-3') primers were designed to introduce unique *EcoRI* and *HindIII* restriction sites (underlined) into *KpDnaB*, permitting the insertion of the amplified gene into the pET21e vector. The pET21e vector was engineered from the pET21b vector (Novagen Inc., Madison, WI, USA), to avoid having the *N*-terminal T7 tag fused with the gene product [24]. Briefly, the region containing the *NheI* site of the pET21b vector (CATAT GGCTAGC) was mutated to introduce a new *EcoRI* site (CATATGGAATTC) by using the primers E1 (5'-AAG GAGATATACATATGGAATTCATGACTGGTGGACA G-3') and E1' (5'-TGCTGTCCACCAGTCATGAATTC-CATATGTATATCTCCT-3'). The resultant plasmid was digested with *EcoRI* to remove the DNA fragment spanning from the original *NheI* site to the *EcoRI* site, and then religated onto itself at the *EcoRI* site to generate the expression vector pET21e. The *KpDnaB* DNA fragment was then inserted into pET21e to produce the plasmid pET21e-*KpDnaB* for *KpDnaB* expression. Therefore, the expected gene product expressed by pET21e-*KpDnaB* will have 2 additional artificial residues, EF, introduced by the *EcoRI* site located at the *N*-terminus, and a C-terminal His tag (KLAAALEHHHHHH), useful for purifying the recombinant protein.

2.3 Protein Expression and Purification

Recombinant *KpDnaB* protein was expressed and purified using the same protocol as described previously for the protein PriB [10], with a minor modification. Briefly, *E. coli* BL21(DE3) cells were transformed with the wild-type pET21e-*KpDnaB* plasmid and grown to 0.9 OD₆₀₀ at 37 °C in Luria–Bertani medium containing 250 μ g/mL

Fig. 1 Molecular structure of Gal, Kae, Que and Myr



ampicillin with rapid shaking. Overexpression of the *KpDnaB* construct was induced by incubating with 1 mM isopropyl thiogalactoside (IPTG) for 3 h at 37 °C. The cells overexpressing the protein were chilled on ice, harvested by centrifugation, resuspended in Buffer A (20 mM Tris-HCl, 5 mM imidazole, 0.5 M NaCl; pH 7.9) and disrupted by sonication with ice cooling between pulses. The *KpDnaB* was purified from the soluble supernatant by Ni²⁺-affinity chromatography (HiTrap HP; GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Protein purity remained greater than 95% as determined by Coomassie-stained SDS-PAGE.

2.4 Dissociation Constant of dNTPs and *KpDnaB* Determined by Fluorescence Spectrophotometer

An aliquot amount of nucleotides was added into the solution containing *KpDnaB* (1 μM), 50 mM HEPES at pH 7.0 with a final volume of 2 mL in a quartz cuvettes of 1 cm square cross-section. The decrease in intrinsic fluorescence of protein was measured at 330 nm upon excitation at 280 nm and 25 °C with a spectrofluorimeter (Hitachi F-2700; Hitachi High-Technologies, Tokyo, Japan). Each data point was duplicated, and the difference was within 10%. An aliquot

amount of nucleotides was added with or without 10 μM flavonol to a predetermined concentration of *KpDnaB* to obtain at least 7 data points for each dissociation constant (K_d). The K_d was obtained by the equation: $\Delta F = \Delta F_{\max} - K_d(\Delta F/[\text{nucleotide}])$ (Enzyme Kinetics module of Sigma-Plot; Systat Software, Chicago, IL, USA).

3 Results

The purpose of this study was to identify some naturally occurring compounds that can inhibit the activity of bacterial replicative helicase(s). The results presented here show that 4 flavonol compounds may be useful in developing anti-*K. pneumoniae* antibiotics.

3.1 Fluorescence Quenching of *KpDnaB* by dATP, dTTP, dCTP, or dGTP

The fluorescence emission spectra of *KpDnaB* quenched with dATP, dTTP, dCTP, and dGTP are shown in Fig. 2a–d. Fluorescence intensity of *KpDnaB* decreased remarkably with increasing concentrations of dNTP (0–10⁻³ M). At 10⁻³ M, dCTP (Fig. 2c) and dGTP (Fig. 2d) quenched

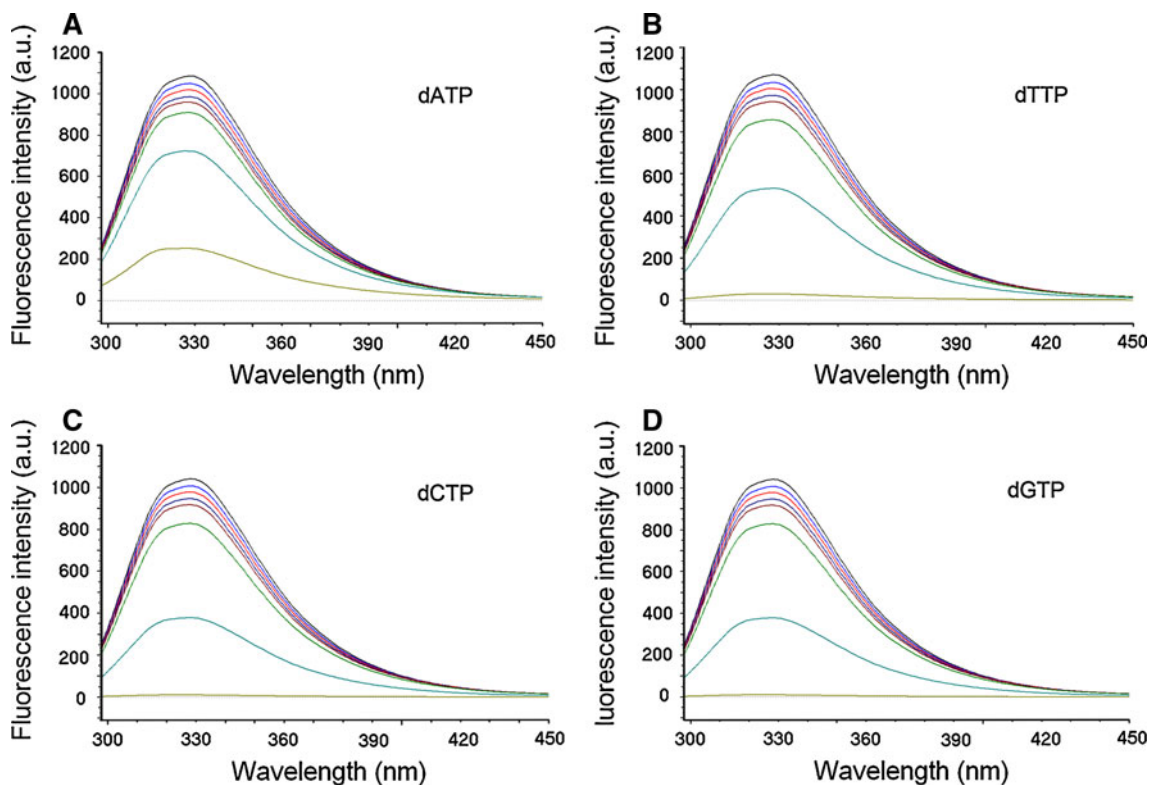


Fig. 2 The fluorescence quenching of *KpDnaB* by **a** dATP, **b** dTTP, **c** dCTP or **d** dGTP. dNTP, from the top down, are as follows: 0, 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵, 10⁻⁴, and 10⁻³ M. An aliquot amount of the nucleotide was added into the solution containing *KpDnaB* (1 μM),

50 mM HEPES at pH 7.0 and 25 °C. After the addition of the nucleotide, the reaction solution was equilibrated for 10 min until no fluorescence change could be observed

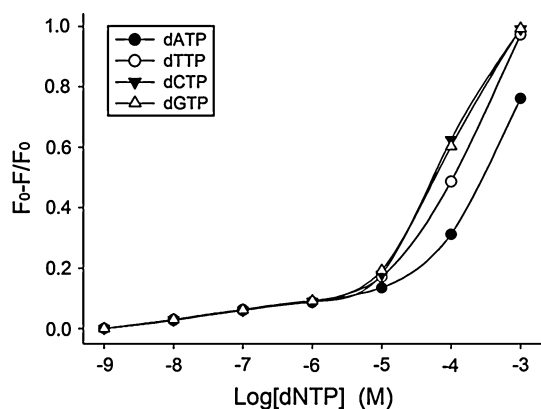


Fig. 3 The fluorescence quenching of *KpDnaB* plotted as relative fraction of $(F_0 - F)/F_0$ against the concentrations of the dNTP

the fluorescence intensity of *KpDnaB* almost completely, whereas dATP (Fig. 2a) only quenches approximately 80% of the *KpDnaB* fluorescence intensity. Adding dNTPs resulted in a significant blue shift (~ 10 nm) of the emission wavelength (λ_{em}) of *KpDnaB*, indicating an interaction between dNTP and *KpDnaB* and suggesting formation of a dNTP-*KpDnaB* complex. The dissociation constant

(K_d) values of *KpDnaB* bound to dATP, dTTP, dCTP, and dGTP determined from the titration curves (Fig. 3) were 159.8 ± 60 , 106.0 ± 30 , 60.3 ± 13 , and 63.2 ± 16 μM , respectively, meaning that *KpDnaB* exhibited the strongest binding activity towards dCTP.

3.2 Fluorescence Quenching of *KpDnaB* by Flavonols

The fluorescence emission spectra of *KpDnaB* quenched by Gal, Kae, Que, and Myr are shown in Fig. 4a–d. Due to the compounds' solubility, 10 μM of each flavonol was used. *KpDnaB* fluorescence intensity decreased significantly with increasing concentrations of flavonols (0–10 μM). The maximum λ_{em} of these flavonols alone in response to excitation at 280 nm was approximately 520 nm (data not shown). In addition, these 4 flavonols resulted in a slight blue shift (~ 1 nm) of the maximum λ_{em} of *KpDnaB*. Thus, the quenching of *KpDnaB* fluorescence mainly depended on the formation of a complex between the flavonol and *KpDnaB* (Fig. 4). The titration curves shown in Fig. 5 suggest that *KpDnaB* binds most strongly to Myr, and, in the order of decreasing affinity, to the other flavonols as follows: Myr > Kae > Gal > Que.

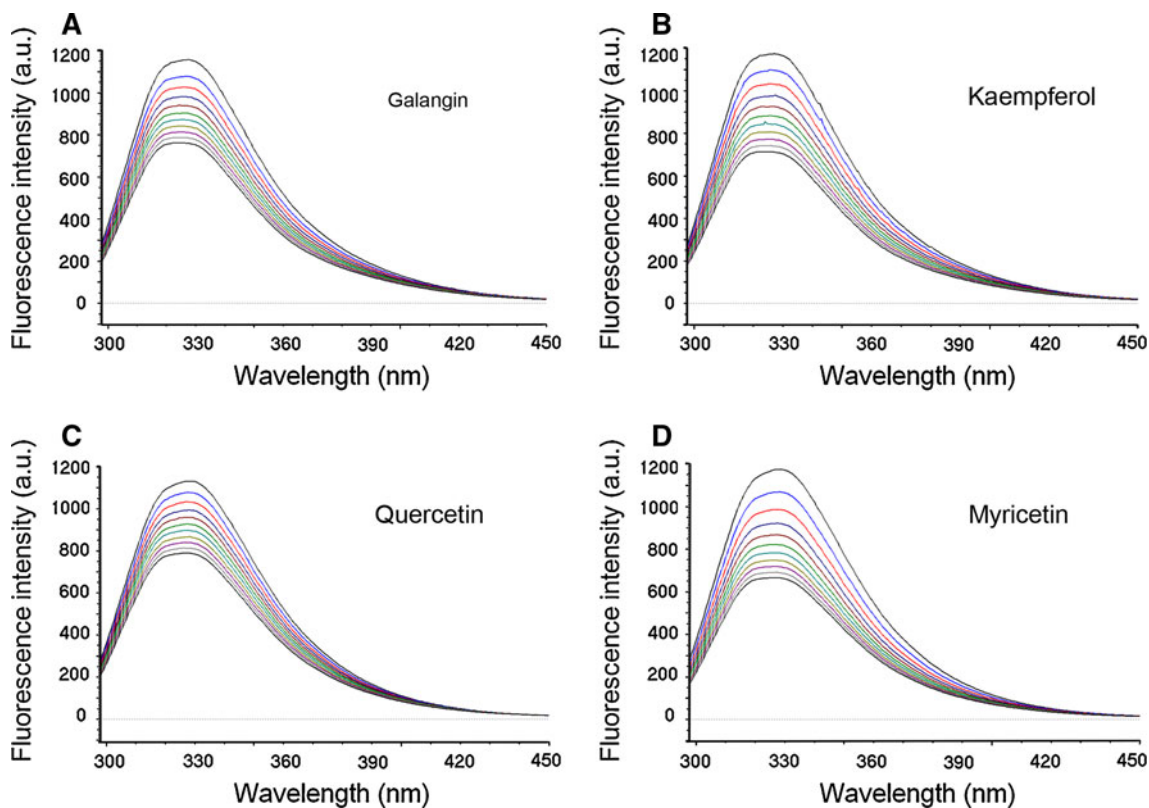


Fig. 4 The fluorescence quenching of *KpDnaB* by a Gal, b Kae, c Que or d Myr. Flavonol, from the top down, are as follows: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μM . An aliquot amount of the flavonol was added into the solution containing *KpDnaB* (1 μM), 50 mM HEPES

at pH 7.0 and 25 $^{\circ}\text{C}$. After the addition of the flavonol, the reaction solution was equilibrated for 10 min until no fluorescence change could be observed

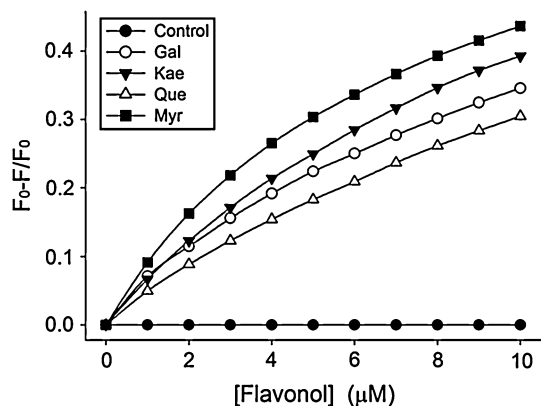


Fig. 5 The fluorescence quenching of *KpDnaB* plotted as relative fraction of $(F_0 - F)/F_0$ against the concentrations of the flavonol

3.3 Fluorescence Quenching of *KpDnaB* by dATP, dTTP, dCTP and dGTP in the Presence of Myr, Kae, Gal, or Que

To investigate whether the flavonols can inhibit the binding of *KpDnaB* to dNTP, we analyzed *KpDnaB* fluorescence quenching by dATP in the presence of Myr, Kae, Gal, or Que (Fig. 6a). The interaction of *KpDnaB* with dATP was much more sensitive to Gal than to Que. Fluorescence quenching of *KpDnaB* was not obvious until the concentration of dATP increased to 10^{-4} M, indicating that the

binding affinity between *KpDnaB* and dATP was decreased by the presence of Gal, Kae, Que, or Myr in the reaction solution.

To test whether the flavonols inhibit the binding of *KpDnaB* to other dNTPs, we also analyzed *KpDnaB* fluorescence quenching by dTTP (Fig. 6b), dCTP (Fig. 6c), and dGTP (Fig. 6d) in the presence of Myr, Kae, Gal, or Que. However, the results of these experiments were quite different from those of the experiment with dATP. dTTP, dCTP, and dGTP quenched *KpDnaB* fluorescence by almost 50% even at 10^{-4} M of the flavonols, suggesting that Myr, Kae, Gal, and Que specifically disrupt the *KpDnaB*-dATP interaction (Fig. 6).

3.4 Inhibition of *KpDnaB*-dNTP Interaction by Gal was the Strongest

Figure 7 shows the effects of flavonol on the interaction between *KpDnaB* and dATP, dTTP, dCTP, or dGTP. *KpDnaB*-dATP binding was inhibited by flavonoids as follows, in the order of decreasing efficiency: Gal > Myr > Kae > Que (Fig. 7a). None of the 4 flavonols disrupted dGTP binding to *KpDnaB* (Fig. 7d). Among the flavonols, Gal inhibited *KpDnaB*-dNTP interaction most strongly, although Myr showed the strongest binding to *KpDnaB* (Fig. 5). Thus, the ability of flavonols to inhibit *KpDnaB* action in general may be dNTP-dependent.

Fig. 6 The fluorescence quenching of *KpDnaB* in the presence of 10 μ M flavonol plotted as relative fraction of $(F_0 - F)/F_0$ against the concentrations of **a** dATP, **b** dTTP, **c** dCTP or **d** dGTP. The reaction solution (1 μ M *KpDnaB*, 50 mM HEPES at pH 7.0) was pre-incubated with 10 μ M flavonol for 10 min, and then an aliquot amount of the nucleotide was added to the *KpDnaB* solution. After the addition of the nucleotide, the reaction solution was equilibrated for further 10 min until no fluorescence change could be observed

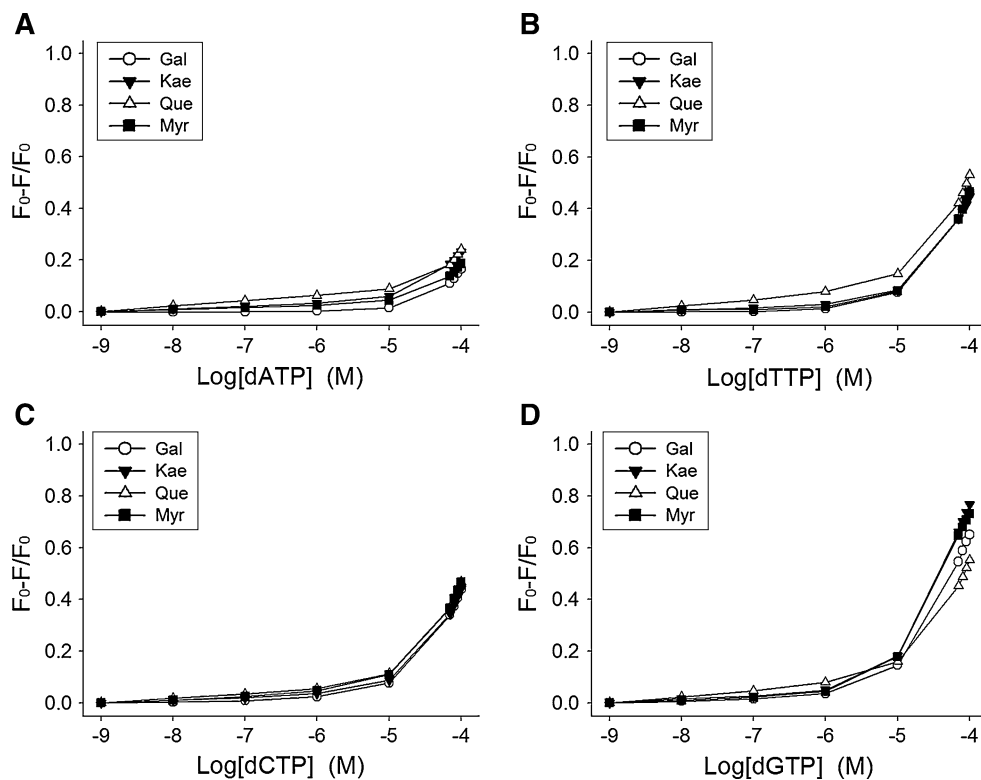
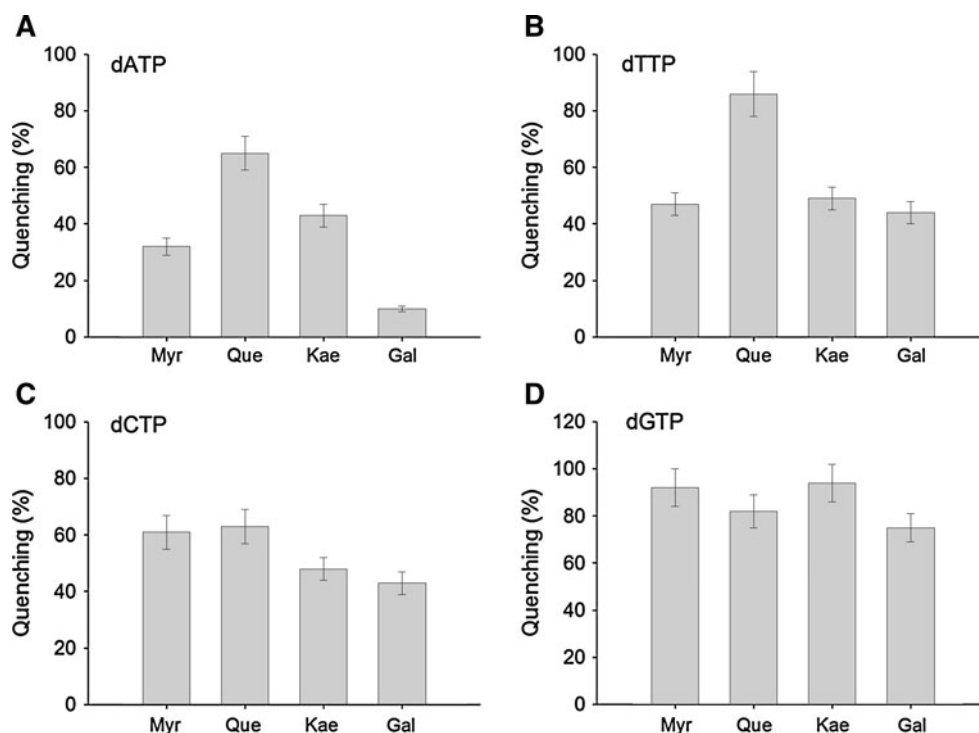


Fig. 7 The quantitative analysis of fluorescence quenching of *KpDnaB* plotted as relative fraction of $(F_0 - F)/F_0$ in the presence of 10 μM flavonol and 10 μM of **a** dATP, **b** dTTP, **c** dCTP or **d** dGTP



3.5 The Flavonoids Inhibited Growth of *K. pneumoniae*

To test whether these flavonoids can inhibit growth of *K. pneumoniae*, *K. pneumoniae* cells were grown to 0.5 OD₆₀₀ at 37 °C, and then added 10 μM of Gal, Kae, Que, or Myr into the medium. Figure 8 shows that these 4 flavonoids inhibited growth of *K. pneumoniae*; Gal had a greater inhibitory effect than the others.

4 Discussion

DNA replication is one of the most basic biological functions and should be a prime target in antibiotic development. In fact, it is the target of the bactericidal fluoroquinolone class of antibiotics that interferes with DNA gyrase [9] and topoisomerase [2]. Since DNA helicases are important components of the cellular replication machinery in all organisms, inhibition of helicase activity would be detrimental to bacterial survival as well [1, 6, 20, 21, 28]. In this study, we used fluorescence quenching to analyze the interaction of *KpDnaB* with 4 flavonols, Gal, Kae, Que, and Myr (Fig. 1), which contain different numbers of hydroxyl substituent on the aromatic rings. Our results demonstrated that these flavonols were capable of inhibiting the interaction of *KpDnaB* with dNTPs. The extent of *KpDnaB* fluorescence quenching induced by dATP in the presence of a flavonol was much smaller than

that of dATP alone, indicating inhibition of *KpDnaB*-dATP binding by the flavonol (Figs. 3, 6). In addition, the inhibition depended not only on the flavonol (with Gal displaying the strongest inhibition), but also on the dNTP used (the inhibition was most specific to dATP binding).

Binding and hydrolysis of NTP cofactors by the DnaB helicase before association with ssDNA are essential processes that induce and modulate a high affinity conformation of the enzyme that can bind to ssDNA [14, 21, 25]. Although the primary replicative helicase can hydrolyze all NTPs [20], our studies indicate that *KpDnaB* has a preference for dCTP over other nucleotides (Fig. 3). Thus, other compounds similar to dCTP may be useful in inhibiting *KpDnaB*.

Other studies have shown that Myr non-competitively inhibits *E. coli* DnaB helicase [6] and RSF1010 RepA helicase [28], with IC₅₀ of approximately 10 and 50 μM , respectively. In this study, we found that 10 μM of Gal, Kae, Que, or Myr can inhibit dNTP binding to *KpDnaB*. Although it is well established that flavonoids have several hydroxyl groups and thus have marked potentials to bind (any) proteins, the strength of the inhibition in dNTP binding of *KpDnaB* was not correlated with the number of hydroxyl substituent on the aromatic rings of the flavonols (Fig. 7).

Although Myr binds to *KpDnaB* with the highest affinity among the flavonols (Fig. 5), it did not display the highest inhibition of dNTP-*KpDnaB* binding (Fig. 7). Based on these results, we propose that these flavonols may inhibit

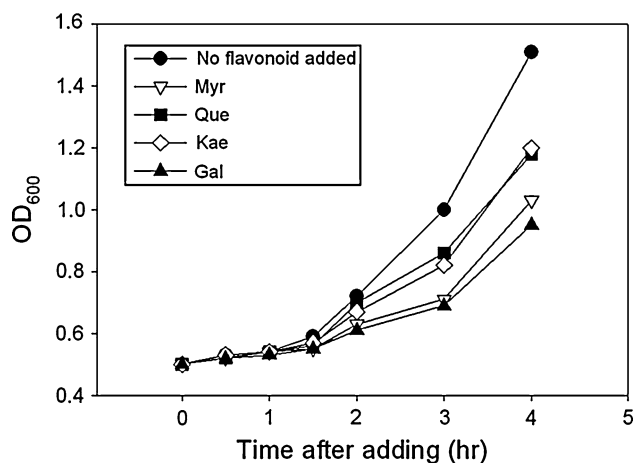


Fig. 8 Growth of *K. pneumoniae* cells in Luria–Bertani medium supplemented with 10 μ M of the flavonoids (Myr, Que, Kae, or Gal)

dNTP binding to *KpDnaB* in 2 possible ways. First, these 4 flavonols may not bind to the active site of *KpDnaB*, or only partially occupy the active site. Second, since DnaB helicase binding to dNTP causes a large conformational change [11, 12, 20], these flavonols may inhibit the conformational change itself, thereby causing varying degree of inhibition. The inhibition of *KpDnaB* by these flavonols appeared to be dNTP-dependent, and, thus, neither of these possibilities can be dismissed.

Our crystal structure of *Geobacillus kaustophilus* helicase in complex with ssDNA and the non-hydrolysable NTP analogue ATP γ s [13] revealed that ATP hydrolysis may drive the movement of the helicase toward the 3' end of the lagging strand. In addition, the dNTP-binding site of the helicase at loop I, part of the Walker B motif, is adjacent to the DNA interaction site. From these results, we speculate here that 1 flavonol molecule is enough to bind to the empty active sites of 6 *KpDnaB* subunits to shut down and lock the enzyme in the dNTP-unbound state. We have prepared a crystal of *KpDnaB* in complex with Gal to further investigate this hypothesis, and the resulting information may be useful in designing compounds that fit more precisely into helicase active sites.

Acknowledgments We thank Mr. Shun-Chuan Yang for constructing the pET21e-*KpDnaB* plasmid. This research was supported a grant from the National Research Program for Genome Medicine (NSC 99-3112-B-040-001 to C.Y. Huang).

References

- Baker TA, Bell SP (1998) *Cell* 92:295–305
- Black MT, Coleman K (2009) *Curr Opin Investig Drugs* 10:804–810
- Burda S, Oleszek W (2001) *J Agric Food Chem* 49:2774–2779
- Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, Jin Q (2005) *Nucleic Acids Res* 33:D325–D328
- Cushnie TP, Lamb AJ (2005) *Int J Antimicrob Agents* 26: 343–356
- Griep MA, Blood S, Larson MA, Koepsell SA, Hinrichs SH (2007) *Bioorg Med Chem* 15:7203–7208
- Gupta A, Ampofo K, Rubenstein D, Saiman L (2003) *J Perinatol* 23:439–443
- Heller RC, Mariani KJ (2006) *Nat Rev Mol Cell Biol* 7:932–943
- Hopkins KL, Davies RH, Threlfall EJ (2005) *Int J Antimicrob Agents* 25:358–373
- Huang CY, Hsu CH, Sun YJ, Wu HN, Hsiao CD (2006) *Nucleic Acids Res* 34:3878–3886
- Jezevska MJ, Bujalowski W (1996) *J Biol Chem* 271:4261–4265
- Jezevska MJ, Kim US, Bujalowski W (1996) *Biophys J* 71:2075–2086
- Lo YH, Tsai KL, Sun YJ, Chen WT, Huang CY, Hsiao CD (2009) *Nucleic Acids Res* 37:804–814
- Lohman TM, Bjornson KP (1996) *Annu Rev Biochem* 65: 169–214
- McGlynn P, Lloyd RG (2002) *Nat Rev Mol Cell Biol* 3:859–870
- Mott ML, Berger JM (2007) *Nat Rev Microbiol* 5:343–354
- Podschun R, Ullmann U (1998) *Clin Microbiol Rev* 11:589–603
- Reyes-Lamothe R, Sherratt DJ, Leake MC (2010) *Science* 328:498–501
- Ross JA, Kasum CM (2002) *Annu Rev Nutr* 22:19–34
- Roychowdhury A, Szymanski MR, Jezevska MJ, Bujalowski W (2009) *Biochemistry* 48:6730–6746
- Singleton MR, Dillingham MS, Wigley DB (2007) *Annu Rev Biochem* 76:23–50
- Soultanas P (2005) *Structure* 13:839–844
- Teillet F, Boumendjel A, Boutonnat J, Ronot X (2008) *Med Res Rev* 28:715–745
- Wang CC, Tsau HW, Chen WT, Huang CY (2010) *Protein J* 29:445–452
- West SC (1996) *Cell* 86:177–180
- Wolfe KL, Liu RH (2008) *J Agric Food Chem* 56:8404–8411
- Wu HJ, Wang AH, Jennings MP (2008) *Curr Opin Chem Biol* 12:93–101
- Xu H, Ziegelin G, Schroder W, Frank J, Ayora S, Alonso JC, Lanka E, Saenger W (2001) *Nucleic Acids Res* 29:5058–5066
- Yang J, Chen L, Sun L, Yu J, Jin Q (2008) *Nucleic Acids Res* 36:D539–D542
- Yu WL, Chuang YC, Walther-Rasmussen J (2006) *J Microbiol Immunol Infect* 39:264–277