Kaempferol is a potent inhibitor of recombinant human protein kinase CK2 holoenzyme *in vitro*

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Abstract：**AIM**　In order to search inhibitors of protein kinase CK2, we observed the inhibitory effects of kaempferol on recombinant human protein kinase CK2 holoenzyme and its kinetics *in vitro*. **METHODS** Cloning, prokaryotic expression and purification of human protein kinase CK2 α' and β subunits by gene engineering, the two subunits were mixed at equal molar ratio to reconstitute CK2 holoenzyme and identify its biological properties. The CK2 activity was assayed by detecting incorporation of ^{32}P of $[\gamma\text{-}^{32}P]ATP$ into the substrate. The inhibitory effect of kaempferol on CK2 was assayed in the presence of different concentrations of kaempferol. Kinetic analysis of kaempferol-induced inhibition was carried out in the condition that casein concentration was fixed at 2 $g \cdot L^{-1}$ and ATP was changed at various concentrations (10, 20, 40, 80 $\mu mol \cdot L^{-1}$), or ATP was fixed at 10 $\mu mol \cdot L^{-1}$ and casein was changed at different concentrations (1, 2, 4, 8 $g \cdot L^{-1}$). **RESULTS**　Kaempferol was shown to strongly inhibit the holoenzyme activity of recombinant human protein kinase CK2 with IC_{50} of 1.9 $\mu mol \cdot L^{-1}$, which was more effective than chrysin, morin and genistein which are both known as CK2 special inhibitors. Kinetic studies of kaempferol on recombinant human CK2 showed that kaempferol acted as a noncompetitive inhibitor with substrate ATP (K_i = 1.1 $\mu mol \cdot L^{-1}$) and casein (K_i = 3.1 $\mu mol \cdot L^{-1}$). **CONCLUSION** Kaempferol is a novel potent inhibitor of protein kinase CK2 *in vitro*. Discussions indicate that flavonoid inhibitors of CK2 may adopt different orientations in the active site of CK2 and that these are determined by the number and position of their hydroxyl groups.

Key words：caseins; protein kinases; recombinant proteins; kaempferol; kinetics

Protein kinase CK2 is a ubiquitous and pleiotropic Ser/Thr protein kinase composed of two catalytic（α and/or α'）and two regulatory（β）subunits generally combined to form $\alpha_2\beta_2$, $\alpha'_2\beta_2$ or $\alpha\alpha'\beta_2$ heterotetramers[1-3]. Over 300 well-characterized substrates of CK2 are currently known. Among them are nuclear and cytoplasmic enzymes and structural proteins which play important roles in transcription, translation, signal transduction, cell-cycle regulation, *etc*[2]. CK2 has some features unusual among the eukaryotic protein kinases：① CK2 recognizes phosphoacceptor sites specified by several acidic determinants；② CK2 can use both ATP and GTP as phosphoryl donors；and ③ the regulatory properties of CK2 are poorly understood. It is insensitive to any known second messengers[1-3].

Many reports support the view that CK2 is involved in proliferation and tumorigenesis[4-8]. CK2 α or α' gene is a protooncogene[4]. CK2 activity has been enhanced in transformed cell lines, solid tumors and rapidly proliferated tissues[5,6]. Several studies have shown that CK2 is correlated to a more aggressive behavior such as invasion and metastasis of the tumor cells[5-7]. In addition, CK2 seems to play an essential role in drug-induced apoptosis in cancer cell[8]. On the other hand, CK2 is exploited by viruses to phosphorylate

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proteins essential to their life cycle, and may play a role in viral infections as well[9,10]. Recently, it was pointed out that CK2 is a potential target for anti-neoplastic and anti-infectious compounds[2,3,9,10].

3,4′,5,7-Tetrahydroxyflavone (kaempferol) is a flavonoid extracted from *Kaempferia galanga*, *Diphylleia sinensis*, *Thesium chinense*, *etc*. It has been well established in traditional herbal medicine for a wide range of beneficial effects such as antiphlogistic, cough-relieving and anti-histamine, *etc*[11]. Some investigations showed that kaempferol is a specific inhibitor of cyclin-dependent protein kinase(CDK)[12]. Since CK2 has closely relationship with CDK, the inhibition of CDK may affect the activity of CK2. Thus, we used kaempferol as the subject to investigate whether kaempferol could modulate the activity of CK2 and its kinetics *in vitro*.

1 MATERIALS AND METHODS

1.1 Reagents

Kaempferol, heparin, spermine, and ATP were from Sigma. 5, 6-Dichloro-1-β-*D*-ribofuranosylbenzimidzole (DRB) were from CalBiochem. P81 phosphocellulose filter paper was the product of Whatman. [γ-^{32}P]ATP(370 GBq·L^{-1}, specific activity > 185 PBq·mol^{-1}) were purchased from Furui Biochemical Technology Co. Ltd., Beijing. All other chemicals were of analytical grade.

1.2 Cloning and sequencing of cDNA encoding human protein kinase CK2 α′ and β subunit

Methods were carried out as described previously[13,14].

1.3 Prokaryotic expression, purification and characterization of recombinant human protein kinase CK2 α′ and β subunit

Human CK2 α′ and β subunits were expressed in a bacterial expression system [pT7-7/BL21(DE3)] and purified to homogeneity as described previously[13,15], after equal molar subunit were mixed, CK2 holoenzyme was spontaneously reconstituted.

1.4 Protein quantitation[16]

Protein concentration in the samples was determined by the method of staining with Coomassie Brilliant blue G-250, using bovine serum albumin (BSA) as the standard.

1.5 Protein kinase CK2 activity assay[13,15]

Partially dephosphorylated casein was prepared by incubating 5 g casein in 50 mL of 50 mmol·L^{-1} Tris-HCl(pH 9.5) at 100℃ for 10 min and dialyzing against buffer containing 50 mmol·L^{-1} Tris-HCl (pH 7.5) and 50 mmol·L^{-1} edetic acid(pH 7.5). The standard assay for CK2 activity was conducted in a reaction mixture containing 50 mmol·L^{-1} Tris-HCl (pH 7.2), 150 mmol·L^{-1} KCl, 10 mmol·L^{-1} MgCl$_2$, 50 μmol·L^{-1} ATP, 18.5 kBq[γ-^{32}P]ATP and 2 g·L^{-1} dephosphorylated casein in a total volume of 35 μL at 30℃. Reactions were started by the addition of 15 μL recombinant CK2 holoenzyme and terminated after 10 min by spotting 30 μL of the reaction mixture onto 3 pieces of 2 cm diameter P81 phosphocellulose paper. After the filter papers were washed thoroughly with 85 mmol·L^{-1} phosphoric acid with occasional stirring, washed one time with acetone finally and dried at 80℃, the radioactivity was measured in a LS6000C (Beckman) scintillation counter. Protein kinase CK2 activity is defined as the amount of transferred phosphate from [γ-^{32}P]ATP to casein per min per gram of enzyme protein at 30℃, which is shown as μmol·min^{-1}·g^{-1}.

1.6 Enzyme kinetics

In the condition that casein was fixed at concentration (2 g·L^{-1}) and ATP was changed at various concentrations (10, 20, 40, 80 μmol·L^{-1}), or ATP was fixed at 10 μmol·L^{-1} and casein was changed at different concentrations (1, 2, 4, 8 g·L^{-1}), the kinetics of protein kinase CK2 activity was assayed. Two kaempferol concentrations (1.5, 6 μmol·L^{-1}) were used for inhibitory kinetics experiment. Each sample was simultaneously performed three parallel tubes. The kinetic parameters, apparent K_m and apparent V_{max}, were calculated according to Lineweaver-Burk plot, and the types of inhibitory effect of kaempferol on recombinant human protein kinase

CK2 holoenzyme were identified. Then, inhibitory constant K_i was calculated by using above data, the calculation method may refer the monograph "Essentials of Applied Enzymology" written by Yu[17].

1.7　Statistics analysis

All values were expressed as $\bar{x} \pm s$. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett's t test for multiple comparisons. All statistics were performed using the SPSS 11.0 software.

2　RESULTS

2.1　Characterization of recombinant human CK2 holoenzyme

The results (Tab 1) indicated that reconstituted CK2 holoenzyme possessed the same properties as natural CK2: casein was its substrate. If basic protein histon Ⅲ S or poly (Glu∶Tyr) 4∶1 (the substrate of PTK) was used to replace casein as its substrate, its activity was much lower than casein itself as substrate. CK2 activity was depressed with heparin and its specific inhibitor DRB, stimulated by spermine. The second messenger molecules cAMP, cGMP and Ca^{2+} had no effect on its activity.

Tab 1.　Characterization of recombinant human CK2 holoenzyme

Substrate	CK2 activity/ $\mu mol \cdot min^{-1} \cdot g^{-1}$ protein
Casein	2.99 ± 0.15
Histone Ⅲ S (0.5 $g \cdot L^{-1}$)	0.34 ± 0.01 * *
Poly(Glu∶Tyr)4∶1 (0.4 $g \cdot L^{-1}$)	0.05 ± 0.03 * *
Casein + heparin (8 $mg \cdot L^{-1}$)	1.36 ± 0.13 * *
Casein + DRB (40 $\mu mol \cdot L^{-1}$)	1.26 ± 0.04 * *
Casein + spermine (2.5 $\mu mol \cdot L^{-1}$)	4.22 ± 0.13 * *
Casein + Ca^{2+} (5 $\mu mol \cdot L^{-1}$)	2.91 ± 0.14
Casein + cAMP (10 $\mu mol \cdot L^{-1}$)	3.15 ± 0.09
Casein + cGMP (10 $\mu mol \cdot L^{-1}$)	2.98 ± 0.18

DRB: 5, 6-dichloro-1-β-D-ribofuranosylbenzimidzole. Assay of CK2 holoenzyme activity was described in materials and methods using equimolar amounts (14 pmol) of CK2 α′ and β subunits. $\bar{x} \pm s$, $n = 3$. * * $P < 0.01$, compared with casein group.

2.2　Direct effect of kaempferol on recombinant human CK2 holoenzyme

The inhibitory effect of kaempferol on CK2 was assayed in the presence of different concentrations of kaempferol (0, 1, 2, 4, 8, 16 $\mu mol \cdot L^{-1}$). It was found that kaempferol had stronger inhibition on recombinant human CK2 holoenzyme in a concentration-dependent manner (Tab 2). Calculation of IC_{50} was performed according to semi-effect-probit method[18]. Using logarithm of kaempferol concentration as horizontal coordinate, probit of inhibitory rate of corresponding kaempferol concentration as vertical coordinate, the equation of linear regression was elicited, which was $Y = 2.1169 X - 1.8613$, r^2 was 0.9435, IC_{50} was 1.9 $\mu mol \cdot L^{-1}$.

Tab 2.　Effect of kaempferol on recombinant human CK2 holoenzyme

Kaempferol		CK2 activity/ $\mu mol \cdot min^{-1} \cdot g^{-1}$ protein	Inhibition rate	
$\mu mol \cdot L^{-1}$	log value		%	Probit
0		4.43 ± 0.21		
1	3.000	3.24 ± 0.39 * *	26.7	4.3567
2	3.301	2.19 ± 0.06 * *	50.5	5.0251
4	3.602	0.68 ± 0.17 * *	84.7	6.0364
8	3.903	0.22 ± 0.04 * *	95.1	6.6449
16	4.204	0.17 ± 0.02 * *	96.2	6.7507

$\bar{x} \pm s$, $n = 3$. * * $P < 0.01$, compared with 0 $\mu mol \cdot L^{-1}$ group.

2.3　Inhibitory kinetics of kaempferol in the presence of different concentrations of ATP

In the condition of fixed casein concentration 2 $g \cdot L^{-1}$ and various ATP concentrations (10, 20, 40, 80 $\mu mol \cdot L^{-1}$), protein kinase CK2 kinetics was studied. In three kaempferol concentrations (0, 1.5, 6 $\mu mol \cdot L^{-1}$), the regression equation of dual-reciprocal plot was $Y = 2.5484X + 0.105$, $Y = 5.8403X + 0.23$ and $Y = 16.338X + 0.596$, respectively. The corresponding apparent K_m was 24.3, 25.4 and 27.4 $\mu mol \cdot L^{-1}$; apparent V_{max} was 9.5, 4.4, 1.7 $\mu mol \cdot min^{-1} \cdot g^{-1}$, respectively. It indicated that with increasing concentration of kaempferol, the K_m value of CK2 was unchanged fundamentally, the V_{max} value was diminished increasingly and these straight lines

intersected on the negative abscissa. All these showed that the inhibitory effect of kaempferol on recombinant human CK2 holoenzyme was noncompetitive with ATP (Fig 1). According to the kinetic parameters, an inhibitory constant K_i of 1.1 $\mu\text{mol} \cdot \text{L}^{-1}$ could be calculated for kaempferol.

○ Kaempferol 0 μmol·L⁻¹
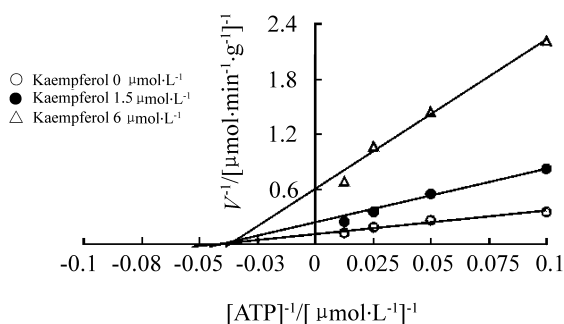● Kaempferol 1.5 μmol·L⁻¹
△ Kaempferol 6 μmol·L⁻¹

Fig 1. Lineweaver-Burk plot for kinetic analysis of kaempferol inhibitory effect on recombinant human CK2 holoenzyme in the presence of different concentrations of ATP. Casein was at fixed concentration (2 g·L⁻¹), ATP was at indicated concentrations. Each value represented the mean of the three separate tubes. $\bar{x} \pm s$, $n = 3$.

2.4   Inhibitory kinetics of kaempferol in the presence of different concentrations of casein

In the condition of fixed ATP 10 $\mu\text{mol} \cdot \text{L}^{-1}$ and different casein concentrations (1, 2, 4, 8 g·L⁻¹), protein kinase CK2 kinetics was studied. In three kaempferol concentrations, the regression equation of dual-reciprocal plot was $Y = 0.2162X + 0.205$ (0 $\mu\text{mol} \cdot \text{L}^{-1}$ kaempferol), $Y = 0.4513X + 0.385$ (1.5 $\mu\text{mol} \cdot \text{L}^{-1}$ kaempferol) and $Y = 2.1056X + 1.7985$ (6 $\mu\text{mol} \cdot \text{L}^{-1}$ kaempferol), respectively. The corresponding apparent K_m was 1.1, 1.2 and 1.2 g·L⁻¹; apparent V_{max} was 4.9, 2.6, 0.6 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, respectively. It indicated that with increasing concentration of kaempferol, the K_m value of CK2 was unchanged fundamentally, the V_{max} value was diminished increasingly and these straight lines intersected on the negative abscissa. Thus, the result of enzyme kinetics of kaempferol on recombinant human CK2 holoenzyme showed that the inhibition was also noncompetitive with casein (Fig 2). Using these kinetic parameters, an inhibitory constant K_i value of CK2 was calculated to be 3.1 $\mu\text{mol} \cdot \text{L}^{-1}$.

○ Kaempferol 0 μmol·L⁻¹
● Kaempferol 1.5 μmol·L⁻¹
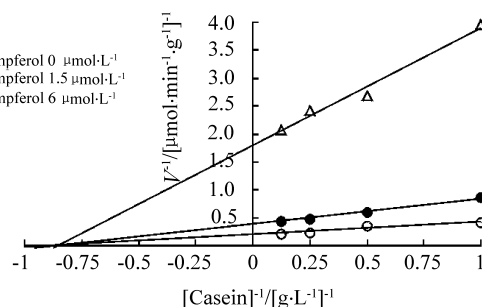△ Kaempferol 6 μmol·L⁻¹

Fig 2. Lineweaver-Burk plot for kinetic analysis of kaempferol inhibitory effect on recombinant human CK2 holoenzyme in the presence of different concentrations of casein. ATP was at fixed concentration (10 $\mu\text{mol} \cdot \text{L}^{-1}$), but casein was changed at indicated concentrations. Each value represented the mean of the three separate tubes. $\bar{x} \pm s$, $n = 3$.

3   DISCUSSION

Protein kinase CK2 is a ubiquitous Ser/Thr protein kinase. Unlike the great majority of protein kinases, CK2 exists at low concentration in tissue and cell, and it is very difficult to gain enough natural human CK2 holoenzyme for laboratory research *in vitro*. Moreover, CK2 is a tetramer in eukaryotic cells and holoenzyme can only be disssociated into its subunit under denaturing conditions. Thus, through cloning, prokaryotic expression and purification of human protein kinase CK2 α' and β subunits by gene engineering, then mixed at equal molar ratio with these two subunits, we can get the same biological properties of human CK2 as the natural holoenzyme (Tab 1).

In contrast to most other kinases, CK2 is characterized by an exceptional ability to utilize as phosphate donors both ATP and GTP[1-3]. The ATP-binding site of CK2 is a site for ribose and triphosphate binding and for the generation of a hydrophobic adenine-binding pocket[3]. The most promising inhibitors of CK2 are acted on ATP-binding site, despite the fact that this site, which is highly conserved among protein kinase, displays in CK2 much fewer distinctive features than the GTP-binding site[9]. So we use ATP as phosphate donors to assay the activity of CK2.

It is proposed that CK2 is an attractive target molecule for tumor and acquired immune deficien-

cy syndrome（AIDS）therapy，the inhibitors of CK2 have anti-neoplasic and anti-immunodeficiency virus（HIV-1）clinical therapeutic potential[5,9,19,20]. Several flavonoid compounds such as quercetin，myricetin，morin，apigenin，chrysin and fisetin，*etc* have been reported to act as inhibitors of CK2. Among these compounds，the most effective inhibitors of CK2 are fisetin（IC_{50} = 0.35 μmol·L^{-1}）and quercetin（IC_{50} = 0.55 μmol·L^{-1}），whose difference is only the hydroxyl group at position 5 of quercetin. While apigenin，myricetin and 4′，7，8-trihydroxyisoflavone which IC_{50} was 0.80，0.92 and 1.5 μmol·L^{-1}，respectively，were more potent CK2 inhibitors. However，chrysin（IC_{50} = 9.0 μmol·L^{-1}），morin（IC_{50} = 10.0 μmol·L^{-1}）and genistein（IC_{50} = 20.0 μmol·L^{-1}）undergo a drop in inhibitory efficiency[9,21].

In our preliminary study，we first demonstrated that kaempferol was able to inhibit more strongly the holoenzyme activity of recombinant human protein kinase CK2 with IC_{50} of 1.9 μmol·L^{-1}（Tab 2）. The results of enzyme kinetics clarified the action mechanism of kaempferol on CK2，displayed that inhibition of kaempferol on CK2 was noncompetition with ATP（Fig 1）which K_i is 1.1 μmol·L^{-1}. Since the K_m of recombinant human CK2 holoenzyme for ATP had been previously determined to be about 4.7 μmol·L^{-1}[13]，it might be inferred that kaempferol exhibited an affinity for CK2 holoenzyme is higher than that of ATP. Also，kaempferol displayed a noncompetitive inhibition with casein（Fig 2），the inhibitory constant K_i was calculated to be 3.1 μmol·L^{-1}. These results clewed that the affinity of kaempferol on CK2 in presence of ATP was about 2.8 times higher than that of casein. In contrast to the other flavonoid inhibitors of CK2，kaempferol is appreciably less effective than myricetin，apigenin and 4′，7，8-trihydroxyisoflavone and more potent than chrysin，morin and genistein. So，kaempferol is a novel potent inhibitor of protein kinase CK2 *in vitro*.

Through a preliminary comparison of compound structure，we found that kaempferol（3，4′，5，7-tetrahydroxyflavone）undergoes a substantial drop in inhibitory efficiency，if 1 hydroxyl group is added at position 2′ to give the pentahydroxyl flavone morin（2′，3，4′，5，7-pentahydroxyflavone）. In addition，The modification of apigenin（4′，5，7-trihydroxyflavone）by addition of 1 hydroxyl group at position 3 to give kaempferol is also effective. So it is possible that a hydroxyl group at the 2′ and 3 positions is detrimental *per se* for their inhibitory effect on CK2. This conclusion is also in accord with the observation that addition of 2 hydroxy groups at position 2′ and 3 to give morin reduce the inhibitory effect of apigenin[9]. On the other hand，when apigenin′s 4′ hydroxyl group is eliminated to give the dihydroxyflavone chrysin（5，7-dihydroxyflavone），the IC_{50} value increases about 10-fold[9]. These results suggest that flavonoid inhibitors adopt different orientations in the active site of CK2 and that these be determined by the number and position of their hydroxyl groups.

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山柰黄素是一种有效的体外重组人蛋白激酶 CK2 的抑制剂

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摘要：目的　为了筛选蛋白激酶 CK2 的抑制剂，观察山柰黄素对重组人蛋白激酶 CK2 的抑制效果及其酶动力学机制。**方法**　利用基因工程技术进行克隆、表达和纯化，获得重组人 CK2 的 α′ 及 β 亚基在体外等摩尔混合构成 CK2 全酶，通过测定转移到 CK2 底物上的［γ-^{32}P］ATP 的 ^{32}P 的放射性活性来检测 CK2 的活性。向反应体系中加入不同浓度的山柰黄素，观察其对 CK2 的抑制效果；通过固定酪蛋白浓度为 2 g·L^{-1}，ATP 浓度为 10，20，40 和 80 μmol·L^{-1} 或固定 ATP 的浓度为 10 μmol·L^{-1}，改变酪蛋白浓度（1，2，4 和 8 g·L^{-1}），观察其酶动力学机制。**结果**　山柰黄素能显著抑制重组人蛋白激酶 CK2 的活性（IC$_{50}$ = 1.9 μmol·L^{-1}）。抑制作用强于已知的 CK2 抑制剂白杨素、桑色素和金雀异黄素。酶动力学分析表明，山柰黄素与 ATP（K_i = 1.1 μmol·L^{-1}）

及酪蛋白（K_i = 3.1 μmol·L^{-1}）均呈非竞争性抑制 CK2 的活性。初步的化合物结构分析表明，2′ 和 3 位上的羟基对山柰黄素及芹黄素发挥其抑制效果产生实质性的负面影响。**结论**　山柰黄素是一种新的体外蛋白激酶 CK2 的有效抑制剂。黄酮类 CK2 的抑制剂可能通过不同的位点作用于 CK2，这种作用主要取决于其羟基的数目和位置。
关键词：酪蛋白类；蛋白激酶类；重组蛋白质类；山柰黄素；动力学

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