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Inhibition of Protein Kinase CK2 by Flavonoids and Tyrphostins. A Structural Insight

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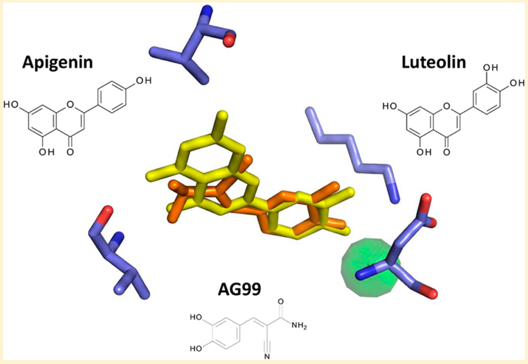
Supporting Information



ABSTRACT: Sixteen flavonoids and related compounds have been tested for their ability to inhibit three acidophilic Ser/Thr protein kinases: the Golgi apparatus casein kinase (G-CK) recently identified with protein FAM20C, protein kinase CK1, and protein kinase CK2. While G-CK is entirely insensitive to all compounds up to 40 μM concentration, consistent with the view that it is not a member of the kinome, and CK1 is variably inhibited in an isoform-dependent manner by fisetin and luteolin, and to a lesser extent by myricetin and quercetin, CK2 is susceptible to drastic inhibition by many flavonoids, displaying with six of them IC$_{50}$ values < 1 μM. A common denominator of these compounds (myricetin, quercetin, fisetin, kaempferol, luteolin, and apigenin) is a flavone scaffold with at least two hydroxyl groups at positions 7 and 4′. Inhibition is competitive with respect to the phospho-donor substrate ATP. The crystal structure of apigenin and luteolin in complex with the catalytic subunit of *Zea mays* CK2 has been solved, revealing their ability to interact with both the hinge region (Val116) and the positive area near Lys68 and the conserved water W1, the two main polar ligand anchoring points in the CK2 active site. Modeling experiments account for the observation that luteolin but not apigenin inhibits also CK1. The observation that luteolin shares its pyrocatechol moiety with tyrphostin AG99 prompted us to solve also the structure of this compound in complex with CK2. AG99 was found inside the ATP pocket, consistent with its mode of inhibition competitive with respect to ATP. As in the case of luteolin, the pyrocatechol group of AG99 is critical for binding, interacting with the positive area in the deepest part of the CK2 active site.

Flavonoids belong to a group of natural substances and are found in fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine. Flavonoids are the most important plant pigments for flower coloration and the most common group of polyphenolic compounds in the human diet.[1] Over 9000 naturally occurring flavonoids have been characterized and their aglyconated forms classified into four main groups, flavones, flavanols, flavanones, and flavanonols, based on differences in molecular backbone structure as outlined in Table 1.[2] Moreover, the position of the benzenoid substituent splits flavonoids into two subclasses: flavonoids (2-position) and isoflavonoids (3-position). Because of the variety of pharmacological activities in the mammalian body, flavonoids are intensively studied for their potentials as therapeutic agents. The best known property of almost every group of flavonoids is their ability to act as antioxidants and metal chelators, protecting against reactive oxygen species (ROS). Free radicals and ROS have been implicated in a large number of human diseases, and many flavonoids, notably, quercetin, kaempferol, myricetin, have been reported to display beneficial effects as anti-inflammatory, anticancer, and cardioprotective compounds.[3−5] This seems to account for the decreased incidence of cardiovascular disease in the Mediterranean population associated with red wine consumption (known as the "French Paradox", i.e., the observation that French people suffer a telatively low incidence of coronary heart disease despite having a diet rich in saturated fats, possibly balanced by red wine rich in flavonoids[6]). Moreover, several other beneficial effects of flavonoids have been reported; for example, flavonoids may display anti-inflammatory and antiulcer activity by inhibiting lipooxygenase (LO) and cyclo-oxygenase (COX).[7,8] An antidiabetic effect has been also reported due to the stimulation by flavonoids of insulin release.[9] Finally antibacterial, antiviral,

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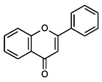
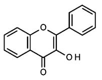
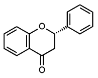
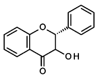
Table 1. Classification of Flavonoids

Group	Name	Formula	Examples
Flavone	2-phenylchromen-4-one		Apigenin
Flavanol	3-hydroxy-2-phenylchromen-4-one		Fisetin
Flavanone	2,3-dihydro-2-phenylchromen-4-one		Naringenin
Flavanonol	3-hydroxy-2,3-dihydro-2 phenylchromen-4-one		Taxifolin

and antifungal activity has been also described in the literature.[10,11]

On the other hand a number of reports support the view that flavonoids may exert chemopreventive effects by impinging on protein kinase signaling pathways, rather than as conventional antioxidants. In particular, recent studies have related the biological effect of flavonoids to the inhibition of both Tyr-kinases (e.g., Fyn and JAK1) and Ser-Thr kinases, such as Raf 1, AKT, MEK1, PI3K, MKK4, and CK2.[12,13]

Casein kinase 2 (CK2) regulates gene expression, protein synthesis and degradation, cell growth, cell cycle, differentiation, and apoptosis. It is overexpressed in several types of cancer, and, as a consequence of its antiapoptotic and pro-survival nature, it establishes favorable conditions for tumorigenesis and for cancer growth and maintenance.[14] CK2 therefore has to be considered a valuable target for cancer therapy,[15] as also highlighted by downregulation experiments leading to apoptosis of cancer cells and to tumor shrinkage in xenograft models.[16] Recently, an ATP site-directed CK2 inhibitor, CX-4945 from Cylene Pharmaceuticals, has entered clinical trials for the treatment of cancer.[17,18]

Here we have assayed a panel of 16 flavonoids and related compounds for their ability to inhibit CK2 and two other classes of acidophilic Ser/Thr protein kinases, the Golgi apparatus casein kinase (G-CK) and CK1, showing that while G-CK and, to a lesser extent, CK1 isoforms are refractory to inhibition, CK2 is more or less inhibited by the majority of the compounds tested, displaying with six of them IC_{50} values in the nanomolar range. We also describe the crystal structures of CK2α from *Zea mays* in complex with the flavones apigenin and luteolin and with the tyrphostin AG-99, which share the pyrocatechol moiety also present in luteolin. All inhibitors are bound to the CK2α ATP pocket mainly through hydrophobic interactions but also with a relevant polar contribution given by interactions with the hinge region and the Lys68 pocket. The simultaneous targeting of these two regions has been proven essential to achieve high inhibitory potency. Tyrphostins and flavones represent two new promising scaffolds for the generation of CK2 inhibitors and in particular AG-99 and luteolin are excellent candidates for the optimization phase aimed at increasing potency and selectivity.

■ EXPERIMENTAL PROCEDURES

Chemistry. Flavonoids were purchased from Sigma Aldrich, while tyrphostin was purchased from Calbiochem.

Source and Purification of Protein Kinases. Recombinant CK2 α-subunit from *Z. mays*, used for crystallography, was obtained from *Escherichia coli* cultures and purified as previously described.[19]

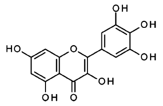
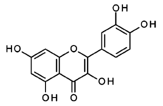
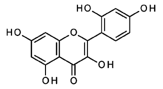
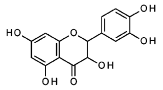
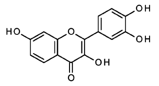
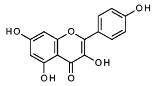
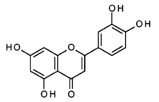
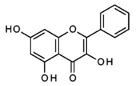
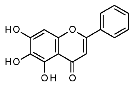
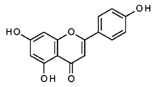
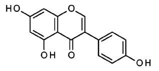
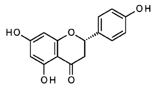
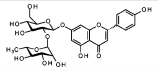
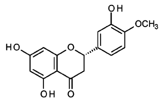
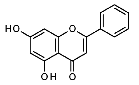
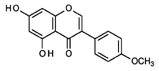
Human recombinant α-subunit and $\alpha_2\beta_2$ holoenzyme of CK2 were expressed in *E. coli* and purified as described elsewhere.[20] Mutants of CK2α subunit were generated as reported in ref 21. Native CK1 purified from rat liver[22] is a mixture of the α (predominant), γ, and δ isoforms.[23] G-CK was purified from the Golgi fraction of lactating mammary gland as previously described.[24] Its activity, detected by in gel assays, displays an apparent molecular weight of around 80 kDa and comigrates with dentin matrix protein 4 (DMP4), also termed FAM20C, a member of the "family with sequence similarity 20" (FAM20) according to the Human Genome Organization Gene Nomenclature Committee[25] (Supporting Information, Figure S1). Zebrafish recombinant isoforms α, γ_1, and δ of CK1 were expressed in *E. coli* and purified as previously described.[26]

Protein Crystallization, and Structure Determination. Prior to cocrystallization experiments, different aliquots of *Z. mays* CK2 α subunit (6 mg/mL protein stock solutions) were incubated for 1 h in ice with the different inhibitors at a concentration of 1 mM. Crystallization drops were prepared by spotting a 1 μL drop of precipitant solution onto 3 μL of the protein–inhibitor solution in a microbridge. The drops were left to equilibrate with 0.1 M Tris-HCl pH = 8, 10–20% PEG 4000, 0.2 M Na-acetate.

Diffraction data were collected at the Elettra synchrotron facility (Trieste). Before mounting, crystals were cryoprotected by a flash soaking in type B immersion oil (Hampton Research). Data were analyzed using the CCP4 software suite (CCP4, 1994) and specifically indexed with MOSFLM[27] and scaled using SCALA. Refinement was carried out using Refmac5.[28]

Phosphorylation Assays. CK2 activity was assayed in a final volume of 25 μL containing 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$, 100 μM synthetic peptide substrate RRRADDSDDDDD, and 0.02 mM [γ-^{33}P]ATP (500–1000 cpm/pmol), unless indicated otherwise, and 20 ng of either the catalytic subunit or the holoenzyme. After 10 min at 37 °C. The reaction was stopped by addition of 5 μL of 0.5 M orthophosphoric acid before spotting 20 μL aliquots on to phosphocellulose filters. Filters were washed in 75 mM phosphoric acid (5–10 mL each) four times and then once in methanol and dried before counting. CK1 activity was

Table 2. IC_{50} (μM) of Flavonoids for Selected Protein Kinases

Compound		Name	CK2 holoenzyme	CK1α	CK1δ	CK1γ1	nCK1	G-CK
Myricetin		3,3',4',5,5',7-hexahydroxy flavone	0.92±0.13	9.15±0.90	2.20±0.21	21.5±2.15	35.0±3.30	>40.0
Quercetin		3, 3',4',5,7-pentahydroxy flavone	0.55±0.08	15.8±1.90	3.40±0.33	10.2±3.20	40.0	>40.0
morin		2',3,4',5,7-pentahydroxy flavone	10.00±1.10	>40.0	>40.0	>40.0	>40.0	>40.0
taxifolin		3, 3',4',5,7-pentahydroxy flavanone	29.00±2.80	>40.0	>40.0	>40.0	>40.0	>40.0
fisetin		3, 3',4',7-tetrahydroxy flavone	0.35±0.05	16.8±2.22	2.47±0.30	3.8±0.37	25.0±3.22	>40.0
kaempferol		3,4',5,7-tetrahydroxy flavone	0.40±0.06	>40.0	27.0±2.21	>40.0	>40.0	>40.0
luteolin		3',4',5,7-tetrahydroxy flavone	0.50±0.08	3.20±.0.32	1.60±0.24	2.00±0.28	23.0±3.40	>40.0
galangin		3, 5, 7-trihydroxy flavone	22.00±3.30	>40.0	>40.0	>40.0	>40.0	>40.0
baicalein		5,6,7-trihydroxy flavone	10.00±0.90	>40.0	16.0±2.2	>40.0	>40.0	>40.0
apigenin		4',5,7-trihydroxy flavone	0.80±0.12	>40.0	18.6±2.51	23.3±3.20	>40.0	>40.0
genistein		4', 5, 7-trihydroxyiso flavone	32.00±3.20	>40.0	>40.0	>40.0	>40.0	>40.0
naringenin		4', 5, 7-trihydroxy flavanone	>40.00	>40.0	>40.0	>40.0	>40.0	>40.0
Naringin		4', 5, 7-trihydroxy flavanone-7-glucoside	>40.00	>40.0	>40.0	>40.0	>40.0	>40.0
hesperetin		3', 5, 7-trihydroxy-4'-metoxyflavan one	>40.00	35.0±2.12	>40.0	>40.0	>40.0	>40.0
chrysin		5,7-dihydroxy flavone	9.00±0.70	>40.0	>40.0	>40.0	>40.0	>40.0
biochanin A		5, 7-dihydroxy-4'-metoxyiso flavone	>40.0	>40.0	>40.0	>40.0	>40.0	>40.0

determined by the same procedure, using as phosphoacceptor substrate the peptide IGDDDDAYSITA (200 μM). G-CK activity was assayed on the specific peptide substrate β^{28-40} (0.5 mM) by incubating the kinase for 10 min at 30 °C in a mixture containing 50 mM imidazole, pH 7.0, 5 mM $MnCl_2$, 25 μM [γ-^{32}P]-ATP (specific activity 1500 cpm/pmol), as detailed in ref 29. For IC_{50} determinations the standard deviations were calculated from at least three independent experiments.

Modeling. The human CK1δ catalytic subunit was built using an homology modeling approach described in ref 30 with δ isoform from *Schizosaccharomyces pombe* as template (PDB code: 2CSN and 1EH4).[31] To minimize contacts between hydrogens, the structures were subjected to Amber99 force field minimization until the rms of conjugate gradient was <0.05 kcal mol^{-1} $Å^{-1}$ keeping all heavy atoms fixed. To strictly validate the generated models and to calibrate our docking protocol, a small database of known CK1δ inhibitors was built and sets of docking runs were performed using the program GOLD.[32]

Kinetic Determination. Initial velocities were determined at each of the tested substrate concentrations. K_m values were calculated either in the absence or in the presence of increasing concentrations of inhibitor, from Lineweaver−Burk double-reciprocal plots of the data. Inhibition constants were then calculated by linear regression analysis of K_m/V_{max} against inhibitor concentration plots.

■ RESULTS

Variable Inhibition of "Casein Kinases" by a Panel of Flavonoids. In Table 2 the structure of 16 flavonoids and related compounds is shown. All these compounds were assayed for their ability to inhibit three classes of Ser/Thr protein kinases sharing the ability to phosphorylate in vitro casein and thereafter also commonly termed "casein kinases" though only in the case of G-CK (Golgi apparatus casein kinase) casein represents a physiological target.

As summarized in Table 2, G-CK proved totally refractory to all compounds up to 40 μM concentration. By sharp contrast CK2 is more or less drastically inhibited by the majority of the flavonoids tested, displaying with six of these (myrcetin, quercetin, fisetin, kaempferol, luteolin, and apigenin) IC_{50} values in the sub-micromolar range.

These six flavonoids all belong to the subclass of hydroxyflavones and they share two hydroxyl groups at positions 7 and 4′. The crucial importance of the latter is highlighted by comparing galangin with kaempherol (IC_{50} 22.00 vs 0.40 μM). As shown below this acidic hydroxyl group makes polar interactions with Lys68 and water molecule W1, two contacts that are observed in all inhibitors carrying acidic functions (either acidic OH or carboxylate) in similar position and that are fundamental for high affinity binding.[33] The structures of apigenin and luteolin show that the inhibitors are able to interact both with the positive area near Lys68 and the hinge region, that is, the two main polar interaction sites in the CK2 cavity. The major contribution for the binding to the hinge region comes from the interaction between the flavon carbonyls and the amide NH of Val116. The interaction between the OH at position 5 and the amide carbonyl of Val116 seems less relevant for the binding, given the acid nature of the hydroxyl function.

The hydroxyl groups at positions 3′ and 5′ present in the most hydroxylated of these hydroxy-flavones, myricetin, are dispensable, as they are individually or collectively lacking in one or more of the other five hydroxyflavones inhibiting CK2 with IC50 ≤ 1 μM (e.g., kaempferol and apigenin). In particular from the crystal structures described in this work, it turns out that the 5′ position (myricetin) and the 2′ position (morin) would be close to the hydrophobic residues Phe 113, Val 95, Ile 66, and Ile 174 (Figure 3B); this may explain why these hydroxyl groups are not tightening the binding, which, in the case of morin is conversely drastically weakened (IC50 = 10 μM). The OH in the 3′ position is pointing toward the bulk solvent, with no relevant interaction with the protein.

In accordance with the importance of planarity in the structure of inhibitors to efficiently target the narrow CK2 binding site,[33] planar flavones are superior to nonplanar flavanones, as seen by comparing quercetin (IC_{50} = 0.55 μM) with its counterpart, taxifolin (IC_{50} = 29 μM). Accordingly, naringenin and hesperetin display IC_{50} > 40 μM. Furthermore, isoflavones do not have the correct geometry to simultaneously target the two CK2 polar binding sites, as flavones can do, and hence apigenin (IC_{50} = 0.80 μM) is much more potent than its counterpart, genistein (IC_{50} = 32 μM), this latter being instead a powerful inhibitor of some receptor protein tyrosine kinases.[34]

In the case of CK1 the inhibition assays were run using its individual isoforms, α, $γ_1$, and δ as well as native rat liver CK1 which is a mixture of different isoforms with the α isoform being predominant over the others.[23] As also shown in Table 2 none of the flavonoids examined is able to inhibit any of the CK1 isoforms as drastically as CK2. The strongest inhibition is observed with luteolin, displaying IC_{50} values ranging between 1.60 and 3.20 μM depending on the CK1 isoforms considered. By sharp contrast kaempherol, which is as potent as luteolin toward CK2 (IC_{50} 0.40 vs 0.50 μM), fails to inhibit CK1 isoforms to any appreciable extent, highlighting the relevance of the hydroxyl group at position 3′, present in luteolin but lacking in kaempherol. Indeed the 3′ hydroxyl is also found in myricetin and quercetin, both fairly good inhibitors of CK1 isoforms with special reference to CK1δ, while it lacks in apigenin, a powerful inhibitor of CK2 (IC_{50} = 0.80 μM) almost ineffective, however, on all CK1 isoforms. The data in Table 2 are worthy to note in two other respects. First they show that, as a general rule, the δ isoform of CK1 is more prone to inhibition by flavonoids than the α and $γ_1$ isoforms, although in a manner which is variable depending on the compound considered: while, in fact, luteolin inhibits the α and $γ_1$ isoforms almost as effectively as the δ one, fisetin is equally potent on the δ and $γ_1$ isoforms but much less on CK1α, and myricetin is a fairly good inhibitor of CK1δ alone. Second, the data in Table 2 disclose a discrepancy between the fairly good inhibition of the individual CK1 isoforms by some flavonoids and the refractoriness to the same compounds of native CK1 enzyme purified from rat liver, whose IC_{50} values are never lower than 20 μM with any of the flavonoids tested. In some cases, for example fisetin and apigenin, this may reflect the composition of native CK1 enzyme whose predominant component, α,[23] is the one less susceptible to inhibition. In the case of luteolin, however, this explanation does not hold, given that all the IC_{50} values calculated for the individual isoforms (3.20, 1.50, and 2.00 μM, respectively) are far below the IC_{50} value of the native enzyme (23.0 μM). It is very unlikely that such an incongruity is due to the presence in native CK1 of additional isoforms of CK1 particularly refractory to inhibition, considering the very close relatedness of CK1 isoforms β, ε, and $γ_2/γ_3$ (not tested by us) to CK1α, δ, and $γ_1$, respectively, included in our assays. On the other hand, the usage for our assays of a phosphoacceptor peptide substrate displaying an absolute selectivity for CK1[35] rules out any major contribution of protein kinases other than CK1 to our native CK1 assay. The most likely explanation therefore seems to be that some of the post-translational modifications which CK1 isoforms are known to undergo[36] and which are obviously lacking in bacterially expressed recombinant isoforms are conferring to the native enzyme a reduced sensitivity to inhibition by luteolin and possibly by other flavonoids.

CK2 Inhibition by Hydroxyflavones Is Competitive with Respect to ATP. To assess the mechanism by which CK2 is drastically inhibited by hydroxyflavones, inhibition kinetics were run with three of them, namely, luteolin,

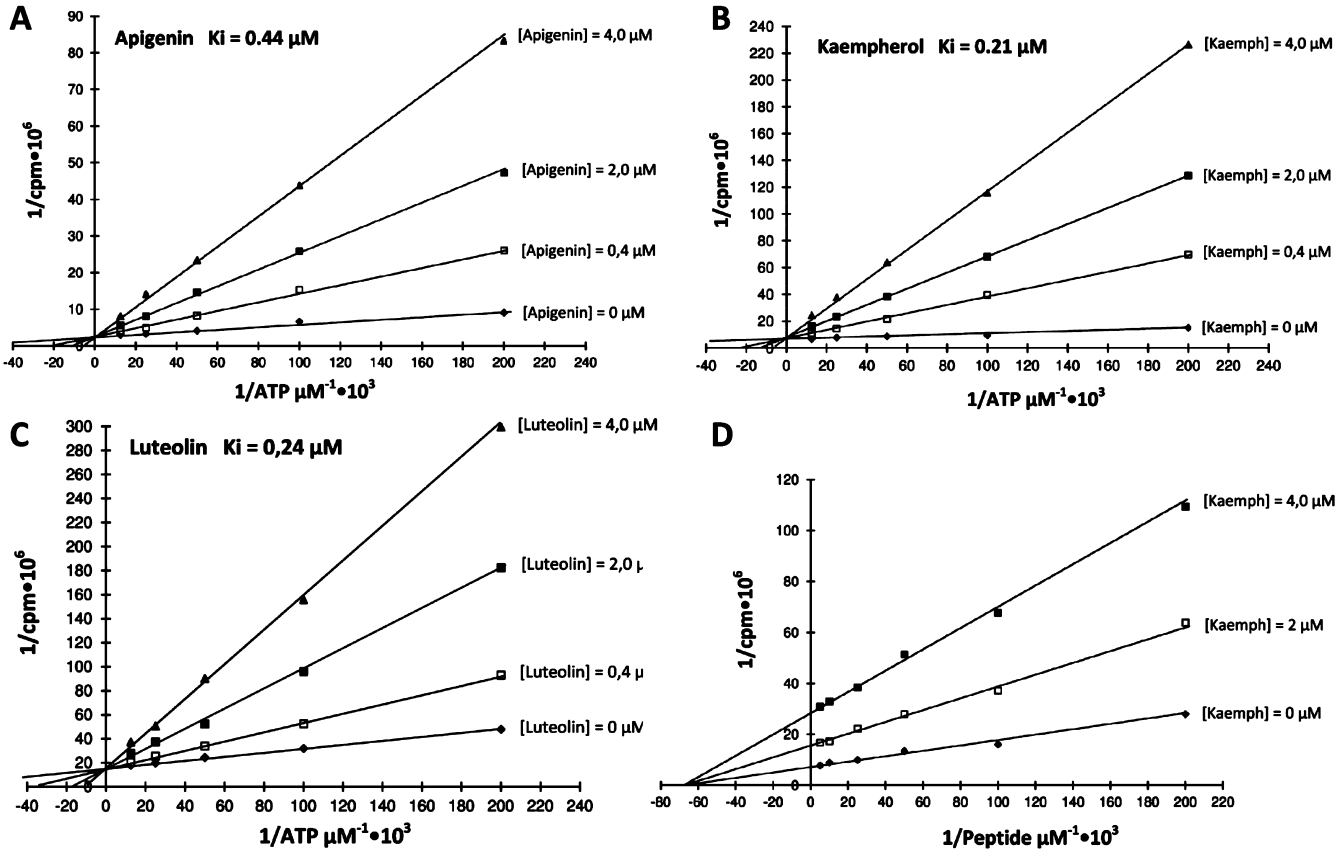


Figure 1. Kinetic analysis of CK2 inhibition by apigenin, kaempherol (B, D) and luteolin are consistent with a competitive mechanism with respect to ATP (A, B, C), and a noncompetitive mechanism with respect to the peptide substrate RRRAADDSDDDDD (D). The results reported represent means of at least three independent experiments with SEM never exceeding 15%.

kaempferol, and apigenin. As shown in Figure 1, where the double reciprocal plots are reported, these compounds are purely competitive inhibitors with respect to ATP. One of them (kaempferol) was also tested at increasing concentrations of the phosphoacceptor peptide subtrate, revealing in this case a purely noncompetitive mode of inhibition. It has to be concluded therefore that hydroxyflavones are typical ATP site-directed CK2 inhibitors. Since luteolin is a fairly potent inhibitor of CK1 isoforms as well, its mode of inhibition of CK1δ has been also kinetically analyzed, reaching the conclusion that, as in the case of CK2 inhibition, it is competitive with respect to ATP (data not shown).

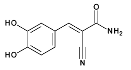
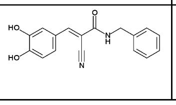
The efficacy of many ATP site-directed CK2 inhibitors has been shown to critically rely on a number of hydrophobic side chains, two of which, Val 66 and Ile 174 are replaced by alanine or anyway by smaller residues in the great majority of other kinases.[37,38] To check if this also applies to flavonoids, two mutants in which either Val 66 or Ile 174 were replaced by alanine were compared to wild type for their susceptibility to inhibition by some hydroxy-flavones. The IC$_{50}$ values are reported in Table 3 together with those of DMAT, a typical representative of CK2 inhibitors whose efficacy depends on the presence of Val 66 and Ile 174.[38] It can be seen that while the IC$_{50}$ value of DMAT is increased 12- and >20-fold by the V66A and I174A mutations, respectively, the IC$_{50}$ values of flavonoids, with special reference to fisetin and luteolin and with the notable exception of crysin (lacking hydroxyl groups on its phenyl ring) are only marginally affected by the two mutations. This suggests that the shape complementary between inhibitors and the CK2 binding site, that is, the importance of the van der Waals interactions, is less relevant for flavonoids as well as for another polyphenolic inhibitor, quinalizarin.[39]

Table 3. IC$_{50}$ (μM) Values for the Inhibition of CK2 Wild Type and Mutants Val66Ala and Ile174Ala by Flavonoids

compound	CK2αWT	CK2α Val66Ala	CK2α Ile174Ala
quercetin	0.85 ± 0.12	1.10 ± 0.14	3.20 ± 0.31
fisetin	0.60 ± 0.08	0.70 ± 0.10	0.50 ± 0.05
luteolin	0.35 ± 0.04	0.80 ± 0.10	0.56 ± 0.08
apigenin	1.20 ± 0.15	10.00 ± 0.90	4.29 ± 0.60
crysin	7.00 ± 0.8	43.00 ± 5.50	75.89 ± 8.90
morin	14.00 ± 1.6	23.00 ± 3.10	17.71 ± 2.65
DMAT	0.14 ± 0.01	1.78 ± 0.21	3.30 ± 0.30

Tyrphostins AG490 and AG99 Are ATP Site-Directed CK2 Inhibitors. Since the pyrocatechol moiety of luteolin is also found in tyrphostins AG490 and AG99 (see Table 4), we wanted to check if these compounds, originally developed as inhibitors of protein tyrosine kinases owing to their ability to mimic tyrosine[40] might also inhibit CK2, and eventually by which mechanism. As shown in Table 4, AG99 and to a lesser extent AG490 are both fairly good inhibitors of the CK2 catalytic subunit (α) (IC$_{50}$ 0.57 and 1.6 μM, respectively). Interestingly their inhibitory potency is significantly reduced with CK2 holoenzyme, where two catalytic subunits are assembled with a dimer of the regulatory β subunit. Note that this property is not shared by flavonoids whose IC$_{50}$ values with the holoenzyme and with the catalytic subunit are quite

Table 4. IC_{50} (μM) for Inhibition of CK2 and CK1 Isoforms by Tyrphostins AG99 and AG490

Compound		Name	CK2 α-subunit	CK2 holoenzyme	nCK1	CK1δ	CK1α	CK1γ_1
AG99		alpha-cyano-(3,4-dihydroxy) cinnamide	0.57±0.08	4.7±0.60	>40.0	15.0±1.25	>40.0	>40.0
AG490		alpha-cyano-(3,4-dihydroxy)-N-benzylcinnamide	1.6±0.24	15.8±1.37	6.5±0.80	40.0	7.5±0.90	>40.0

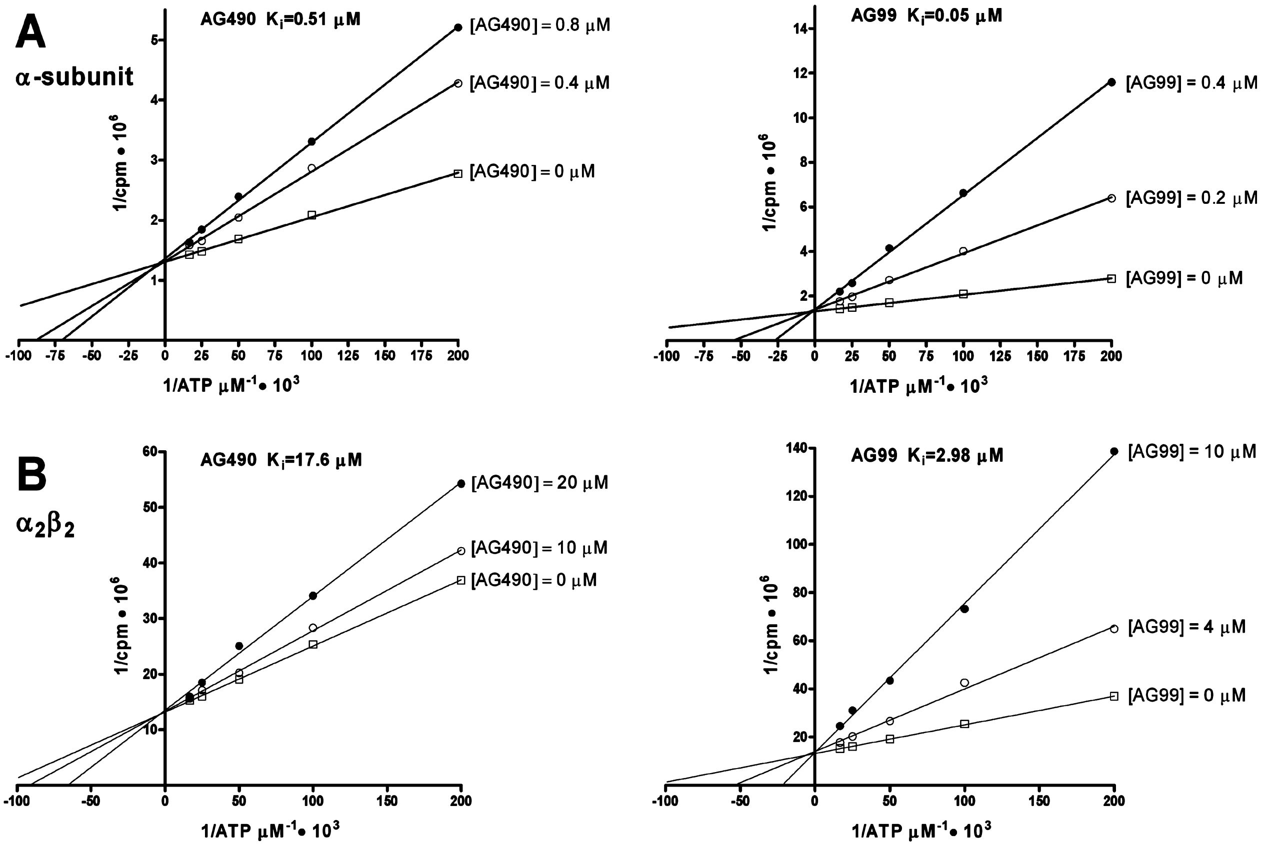


Figure 2. (A, B) Inhibition of CK2 by tyrphostins is competitive with respect to ATP. The results reported represent means of at least three independent experiments with SEM never exceeding 15%.

similar (compare data in Tables 2 and 3). Also of interest is the observation that, at variance with CK2, CK1 isoforms, with the partial exception of CK1*δ*, are nearly unaffected by AG99, while AG490 displays a marked selectivity toward CK1*α* (IC_{50} = 7.5 μM) as compared to the *δ* and *γ* isoforms (IC_{50} > 40 μM). To note that with tyrphostins a good correlation between inhibition of the native CK1 enzyme and of CK1*α* is observed, consistent with the concept that the *α* isoform is the major component of the native enzyme purified from rat liver.[23]

Protection by the *β* subunit might suggest that tyrphostins hit CK2 at site(s) different from the ATP binding one since this latter is not shielded by the *α*−*β* interactions. However the kinetics reported in Figure 2 show that also with tyrphostins CK2 inhibition is competitive with respect to ATP.

To get a clear-cut insight into the mode of binding of hydroxyflavones and tyrphostins to CK2 advantage has been taken of X-ray crystallography of complexes of luteolin, apigenin, and of the tyrphostin AG99, with the catalytic subunit of *Z. mays* CK2 whose catalytic core is almost identical to that of the human enzyme.

Structural Analysis. As shown above, the two flavones apigenin and luteolin and tyrphostin AG-99 inhibit CK2*α* activity with low micromolar to high nanomolar IC_{50}'s. Diffraction data were obtained from inhibitor-soaked *Z. mays* CK2*α* crystals and their structures solved to high resolution (Table 5). It has been shown that ligands that do not use the low-hinge/*α*D region (residues 118−127) to anchor to the protein active site will bind in a similar way to the maize and the human enzyme.[41] The inhibitors here described belong to this class of ligands, establishing direct polar interactions only with a limited structurally conserved portion at the N-terminal of the hinge region (i.e., backbone of residues 114 and 116) and/or the deepest part of the cavity, principally with the conserved water molecules and Lys68. For inhibitors of this type, structural information obtained with the *maize* enzyme are comparable with those obtained with the human enzyme.[41,42]

Each inhibitor could be easily located inside the CK2*α* ATP pocket (Figure 3). Many crystal structures of the CK2*α* catalytic subunits are available, from maize and from human,

Table 5. Data Collections and Final Models Statistics[a]

	apigenin	luteolin	Ag-99
		Data Collection	
space group	C2	C2	C2
unit cell	142.36, 59.87, 45.93 β = 103.40	142.86, 59.37, 45.48 β = 103.47	142.97, 59.55, 46.35 β = 103.81
resolution (Å)	69.2−1.65 (1.74−1.65)	36.5−1.75 (1.84−1.75)	54.7−1.96 (2.07−1.96)
total observations	179329	109972	94953
unique observations	45035	36948	27064
R_{sym}	0.062 (0.446)	0.054 (0.419)	0.070 (0.384)
R_{meas}	0.072 (0.515)	0.067 (0.514)	0.083 (0.456)
R_{pim}	0.036 (0.254)	0.039 (0.295)	0.044 (0.243)
completeness (%)	99.5 (100)	98.8 (98.6)	99.2 (99.5)
redundancy	4.0 (4.0)	3.0 (2.9)	3.5 (3.5)
$I/\sigma I$	11.7 (2.3)	10.4 (2.0)	8.7 (2.1)
B_{wilson} (Å^2)	24.9	25.7	28.1
		Refinement	
R	0.212 (0.359)	0.195 (0.352)	0.201 (0.329)
R_{free}	0.252 (0.342)	0.214 (0.380)	0.230 (0.357)
completeness (%)	99.3 (98.7)	98.6 (96.0)	99.0 (97.3)
rmsd bond (Å^2)	0.009	0.012	0.009
rmsd angle (°)	1.362	1.474	1.280

[a] Numbers in parentheses refer to the highest resolution shell.

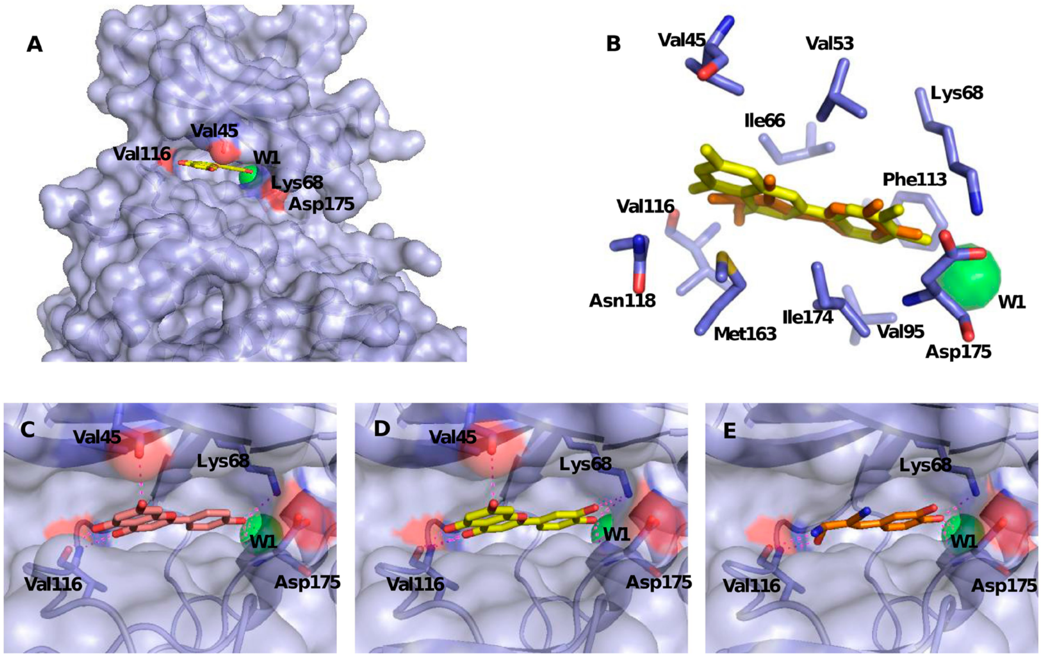


Figure 3. (A) Inhibitors described in this work bind deeply inside CK2α ATP pocket. Luteolin is in yellow, H-bonding amino acids are labeled and colored by atom type, and W1 is shown as a green sphere. (B) Luteolin (yellow) and AG-99 (orange) adopt different orientations in CK2α ATP pocket. Apigenin is not displayed as it orients identically to luteolin. Amino acids contributing to the binding are shown. (C−E) Details of apigenin (salmon) and of the other inhibitors (color code same as above) bound to the CK2α ATP pocket. H-bonds are shown as magenta dotted lines.

some in an apo-form (without any ligand in the ATP-binding site) and many in complex with ligands, particularly inhibitors, prepared either by soaking or by cocrystallization. When compared with all these structures, the three new complexes do not show any indication of an unusual or altered conformation of the protein matrix, in particular, as far as the ATP-binding site is concerned. Hence, the binding mode of inhibitors in the soaked crystals here described can be considered fully representative of that in solution. Luteolin differs from apigenin only for the presence of an additional hydroxyl group. The two inhibitors bind CK2α in an identical fashion. The greatest contribution to the binding energy is given by hydrophobic and van der Waals interactions involving Val45, Val53, Ile66, Lys68, Val95, Phe113, Val116, Asn118, Met163, and Ile174. The dihydroxy-chromone moiety interacts principally with CK2a hinge region via two H-bonds with main chain atoms from Val116 (Figure 3C). An additional H-bond is formed with the carbonyl of Val45 from the CK2α β1 strand. The phenolic group of apigenin is located opposite to the CK2α hinge region well inside the ATP pocket. Its position is locked by polar interactions with the Lys68 side chain, Asp175 main chain, and the W1 water molecule (highly conserved in all CK2α

structures). In luteolin, an additional H-bond is formed between Lys68 and its extra hydroxyl, which is also involved in van der Waals contacts with Asp175 (Figure 3D).

The pyrocatechol moiety of luteolin is present also in the tyrphostin inhibitor AG-99. In this case it is slightly translated with respect to apigenin and luteolin (Figure 3B). The three H-bonds between the *para* hydroxyl group and Lys68, Asp175, and W1 are maintained, while the *meta* hydroxyl moves away from Lys68 and it is only in van der Waals contact with it (Figure 3E). This slight translation is driven by the optimal arrangement of the opposite amide moiety which contributes to inhibitor binding via two H-bonds with Val116 main chain. As already observed with luteolin, the pyrocatechol group makes AG-99 a better inhibitor than apigenin. However its suboptimal interaction with the Lys68 pocket determines the observed reduction in potency with respect to luteolin.

While the binding mode of apigenin and luteolin accounts for their similar efficacy as inhibitors of CK2, we wanted to gain information about the structural features underlying the lower and different efficacy of these two flavonoids on CK1 isoforms, with special reference to CK1δ, whose susceptibility to luteolin is 10-fold higher than that observed with apigenin (see Table 2). To this aim a model of human CK1δ was built as described in the Experimental section and a series of docking experiments were performed using the program GOLD. As shown in Figure 4, both luteolin and apigenin share a binding mode to CK1δ similar to that seen inside the CK2 binding cleft (Figure 3). Note that this kind of binding mode is consistent with other structures of flavonoids cocrystallized with protein kinases, notably CDK6 and PIM1 (1XO2, 2O3P). From the crystal structures presented in this work (Figure 3), it is possible to note that all the hydroxyl groups of both apigenin and luteolin interact with CK2 residues, in particular the ones at position 5 and 7 with the hinge region and the glycin rich loop, while the ones at position 3′and 4′ with Lys 68 and a conserved water molecule. On the contrary, from the docking results performed inside the CK1δ binding cleft, apigenin (IC_{50} = 18.6 μM) can interact only with the hinge region (Leu 85, Gly 86) and only weakly with Lys 38 (Figure 4A). In the case of luteolin (IC_{50} = 1.60 μM), instead, the presence of the hydroxyl group at position 4′ interacting with Lys 38 and Asp 149 critically enhances the inhibitor's efficacy (Figure 4B). The same may apply to fisetin (IC_{50} = 2.47 μM) and myricetin (IC_{50} = 2.20 μM) which share with luteolin both the hydroxyl group at position 3′ and a fairly good inhibitory efficacy toward CK1δ.

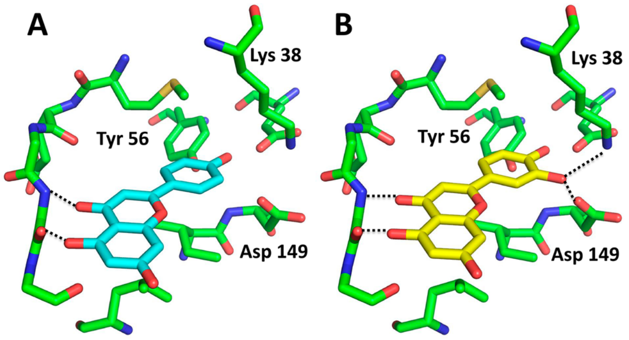


Figure 4. Molecular docking of apigenin (A-cyan) and luteolin (B-yellow) bound to the active site of the human CK1δ model (green); the interactions with the most crucial amino acids are highlighted.

DISCUSSION

Many CK2 inhibitors have been described, which fall into a few classes.[15,33] Anthraquinones, xanthenones, indoloquinazolines, and coumarins bind deeply inside the ATP pocket interacting with W1 and Lys68 but not with the hinge region. By contrast DAA (1,4-diamino-5,8-dihydroxy-anthracene-9,10-dione) prefers a position closer to the hinge region and does not make contacts with W1 and Lys68, a binding mode which is also typical of tetralogenobenzo-imidazoles.[15]

Other inhibitors are able to interact with both the hinge region and the Lys68 pocket, such as, for instance, pyrazolo-triazines derivatives developed by Polaris Pharmaceuticals Inc., among the most potent CK2 inhibitors known,[43] highlighting the importance of simultaneously targeting both key regions. This was confirmed by the recent crystal structures of human CK2 in complex with CX-4945 and some derivatives,[44,45] where inhibitors target the Lys68 pocket through the carboxylic group and the hinge region through the chloroaniline moiety (located opposite to the acidic function).

Here we show that flavones and tyrphostins also have the ability to target simultaneously the CK2 hinge and Lys68 regions. These two scaffolds could then be further modified in order to gain potency and specificity for CK2 starting from the lead structures of luteolin and AG-99. Functional groups could be added to extend interactions with the hinge region (aa. 113–120) and/or with β1 strand (aa. 45–47) as observed with the pyrazolo-triazines compounds.

Tyrphostins are kinase inhibitors developed from the basic structure of the natural product erbstatin. The ability of AG-99 to induce cell death in human bladder carcinoma cells was ascribed to its inhibitory effect on EGFR.[46] In that work however, some of the growth-inhibitory effects of AG-99 could not be entirely explained by inhibition of EGFR, but instead of a different, unidentified, AG-99 target. Considering that the IC_{50} of AG-99 for EGFR has been estimated between 4 to 12.5 μM, depending on the assay conditions,[47] while ranging between 0.57 and 4.7 μM with CK2, it is possible that the off-EGFR effects of AG-99 observed in cultured cells is due to CK2 inhibition. AG-490, an EGFR and JAKs inhibitor, blocks growth and/or induces apoptosis in different types of cancer. Likewise, the pro-apototic effects of AG-490, generally ascribed to its inhibition of EGFR and of the JAK/STAT pathway,[48−53] could be reinforced by inhibition of CK2 and of its pro-survival role. Pertinent to this are the observations that, on one side the unbalanced expression of CK2 subunits[54] may give rise to intracellular catalytic subunits not combined with β, while, on the other tyrphostins AG-99 and AG-490 inhibit with higher efficacy the catalytic subunit of CK2 as compared to the holoenzyme (see Table 4 and Figure 2). Tyrphostins therefore could represent valuable tools for dissecting cellular effects which are specifically promoted by an unbalanced over-expression of CK2 α subunit, which is suspected to potentiate its antiapoptotic and oncogenic potential.[55] While the structural features underlying the marked preference of AG-99 and AG-490 for the not combined CK2 catalytic subunits are unclear, it is tempting to speculate that, given the unequivocal ATP site directed mode of action of tyrphostins, such a behavior reflects a supramolecular organization of the tetrameric holoenzyme[56−58] where the access into the active site of one catalytic subunit could be hampered by the β-subunit of another tetramer. To note in this respect that tyrphostin AG490 also neatly discriminates among CK1 isoforms, displaying its inhibitory potency only with the α isoform, whose 3D structure has not been solved yet. This observation is of special interest from a practical standpoint, since all CK1 inhibitors available to date, including some flavonoids described here, tend instead to be much more effective on the δ/ε isoforms.[30,59] Also of interest may be the observation that native CK1 purified from rat live, a mixture of α (predominant), γ, and δ/ε isoforms[23] displays toward luteolin and fisetin a sensitivity significantly below those observed with any of the recombinant isoforms individually tested (see Table 2). Since it is very unlikely that other isoforms of CK1 besides the ones tested may account for a relevant proportion of nCK1 activity, our data suggest that native rat liver CK1 has undergone post-translational modifications rendering it less susceptible to inhibition. This observation may also be of general interest warning against the expectation that inhibition of recombinant kinase invariably reflects the behavior of the native enzyme.

The antiproliferative and/or pro-apoptotic effects of the flavone apigenin have been ascribed to CK2 inhibition in pancreatic cancer cells,[60] in acute myeloid leukemia cells,[61] and in prostate cancer cells.[62,63] However given the promiscuity of apigenin,[21] a synergistic contribution deriving from inhibition of other kinases (PI3K, PKC, STAT3, IGFR, HER2, and other tyrosine protein kinases) cannot be excluded.[21,64−67]

A part from the special case of apigenin, often used as CK2 inhibitor despite its promiscuity, our data show that CK2 is particularly susceptible to inhibition by a wide variety of flavonoid compounds, many of which either suppress or reduce its activity in their low micromolar range. This does not apply to CK1 nor to a number of other protein kinases, notably CDK1 and the tyrosine kinases cFgr, Lyn, Syk and Csk, none of which are drastically inhibited by numerous flavonoids as observed with CK2 (unpublished data). This makes CK2 a first choice putative mediator of common biological effects of flavonoids, with special reference to those relatable to alterations in signal transduction pathways, such as stress responsive signaling, wnt signaling, and PI3K kinase/Akt pathway, all of which are under the control of CK2.

By sharp contrast G-CK proved totally refractory to all flavonoids tested. Such unique behavior in conjunction with insensitivity to any other known protein kinase inhibitors including the most promiscuous one, staurosporine,[29] supports the view that G-CK may be not a bona fide protein kinase. Intriguingly, in fact, despite the ubiquity of G-CK[24] and its implication in the phosphorylation of many extracellular proteins (ref 68 and references therein), the identity of its gene(s) is still a matter of conjecture. Pertinent to this may be our observation that the catalytic activity of G-CK does not copurify with any detectable bona fide protein kinase,[29] while, as mentioned in the Experimental Procedures (see also Figure S1) it comigrates in PAGE/SDS with dentin matrix protein 4 (DMP4) also termed FAM20C, which was identified by MS analysis of proteins that copurify with G-CK (unpublished data). Interestingly, although the function of FAM20 proteins is as yet unknown, they are reported to have a weak similarity to “four jointed”, a kinase that phosphorylates cadherin,[69] and another member of the family, FAM20B has been shown to phosphorylate xylose in the glycosaminoglycan−protein linkage region.[70] This raises the possibility that the phosphotransferase activity of G-CK is due to DMP4/FAM20C. Indeed a recent report published during the reviewing process of this paper[71] has provided the unambiguous demonstration that FAM20C is the bona fide casein kinase that phosphorylates secretory pathway proteins within S-X-E motif specifically recognized by G-CK.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

In gel casein kinase assays of G-CK was provided (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

Coordinates and structure factors have been deposited in the Protein Data Bank as entries 4DGM (apigenin), 4DGN (luteolin), 4DGO (ag99).

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATION USED

CK2, casein kinase 2; CK1, casein kinase 1; GCK, Golgi apparatus casein kinase; Fyn, tyrosine-protein kinase Fyn; JAK1, Janus kinase 1; Raf1, rapidly accelerated fibrosarcoma 1; AKT, protein kinase B; MEK1, mitogen-activated protein kinase 1; PI3K, phosphoinositide 3-kinase; MKK4, mitogen-activated protein kinase kinase 4; PKC, protein kinase C; STAT3, signal transducer and activator of transcription 3; IGFR, insulin-like growth factor receptor; HER2, human epidermal growth factor receptor 2; EGFR, epidermal growth factor receptor; DMP4, dentri matrix protein 4; FAM20 C, family with sequence similarity 20 protein C

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