Effects of Natural Flavones and Flavonols on the Kinase Activity of Cdk5

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A number of natural and synthetic flavonoids have been assessed previously with regard to their effects on the activity of cyclin-dependent kinases (Cdk1 and -2) related to the inhibition of cell cycle progression. On the other hand, the Cdk5/p35 system is of major importance in neuronal migration phenomena and brain development, and its deregulation is implicated in neurodegenerative diseases, particularly Alzheimer's. Here we show that some natural flavonoids inhibit the activity of the Cdk5/p35 system in the micromolar range, while others are practically inactive. Ring B-unsubstituted and highly methoxylated flavones were inactive or gave irreproducible results, and 6-methoxyapigenin and 6-methoxyluteolin were the most potent Cdk5 complex inhibitors within this series, while the common flavonols kaempferol and quercetin showed intermediate behavior. The reported crystal structure of the Cdk5 complex with its activator p25 was used for docking studies, which also led to the identification of the two 6-methoxyflavones, kaempferol and quercetin, as well as the untested 6-methoxy derivatives of kaempferol and quercetin and the corresponding 6-hydroxy analogues as compounds exhibiting a good fit to the active site of the enzyme.

Flavonoids are prominent plant secondary metabolites that are consumed by humans as dietary constituents in amounts exceeding 0.1 g/day,[1,2] suggesting that their ingestion may play a significant role in health and disease. The antioxidative effects of flavonoids are well documented, and their antiinflammatory activity can also be ascribed in some cases to COX-2 inhibition.[3] Binding to the benzodiazepine site of the $GABA_A$ receptor explains the anxiolytic or sedative effects of a few natural flavones and synthetic analogues.[4,5]

The widespread flavonol quercetin (Figure 2) has long been known as a protein-tyrosine kinase inhibitor,[6] and several studies have uncovered a small number of related natural products with similar activity.[7−9] Tentative structure−activity relationships for the inhibition of other protein kinases by natural flavonoids have also been published.[7,10] Quercetin has very recently been shown to modulate the pro-survival Akt/PKB and ERK1/2 signaling cascades.[11] In the past few years a particular family of protein kinases, the cyclin-dependent kinases (Cdk's), has attracted considerable attention because of the role of several of these enzymes in the regulation of the cell cycle and the potential of Cdk inhibitors for cancer treatment.[12] Indeed, the synthetic flavone derivative flavopiridol (Figure 1) is the first compound with this activity to have entered the clinic as an anticancer drug.[13]

The data regarding flavopiridol and the fact that dietary or other natural flavonoids are known as protein kinase inhibitors would seem to point to these plant metabolites as potential Cdk inhibitors. Nevertheless, we know of only a single, recent precedent with regard to the activity of a small group of natural flavonoids on the cell signaling kinases Cdk1 and Cdk2.[14]

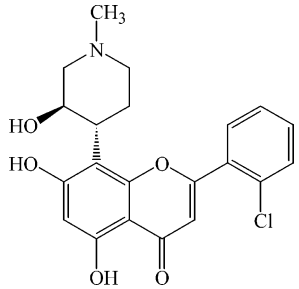

Figure 1. Structure of flavopiridol.

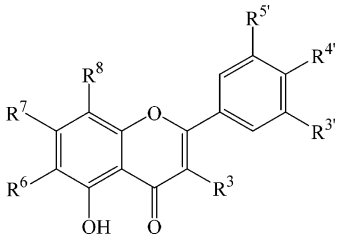

Figure 2. Structures of the natural flavonoids. Chrysin: $R^3 = R^6 = R^8 = R^{3'} = R^{4'} = R^{5'} = H$, $R^7 = OH$. Baicalein: $R^3 = R^8 = R^{3'} = R^{4'} = R^{5'} = H$, $R^6 = R^7 = OH$. Apigenin: $R^3 = R^6 = R^8 = R^{3'} = R^{5'} = H$, $R^7 = R^{4'} = OH$. Luteolin: $R^3 = R^6 = R^8 = R^{5'} = H$, $R^7 = R^{3'} = R^{4'} = OH$. 6-Hydroxyapigenin: $R^3 = R^8 = R^{3'} = R^{5'} = H$, $R^6 = R^7 = R^{4'} = OH$. 6-Hydroxyluteolin: $R^3 = R^8 = R^{5'} = H$, $R^6 = R^7 = R^{3'} = R^{4'} = OH$. 6-Methoxyapigenin: $R^3 = R^8 = R^{3'} = R^{5'} = H$, $R^7 = R^{4'} = OH$, $R^6 = OCH_3$. 6-Methoxyluteolin: $R^3 = R^8 = R^{5'} = H$, $R^7 = R^{3'} = R^{4'} = OH$, $R^6 = OCH_3$. Kaempferol: $R^6 = R^8 = R^{3'} = R^{5'} = H$, $R^3 = R^7 = R^{4'} = OH$. Quercetin: $R^6 = R^8 = R^{5'} = H$, $R^3 = R^7 = R^{3'} = R^{4'} = OH$. Myricetin: $R^6 = R^8 = H$, $R^3 = R^7 = R^{3'} = R^{4'} = R^{5'} = OH$. 6-Hydroxykaempferol: $R^8 = R^{3'} = R^{5'} = H$, $R^3 = R^6 = R^7 = R^{4'} = OH$. 6-Hydroxyquercetin: $R^8 = R^{5'} = H$, $R^3 = R^6 = R^7 = R^{3'} = R^{4'} = OH$. 6-Methoxykaempferol: $R^8 = R^{3'} = R^{5'} = H$, $R^3 = R^7 = R^{4'} = OH$, $R^6 = OCH_3$. 6-Methoxyquercetin: $R^8 = R^{5'} = H$, $R^3 = R^7 = R^{3'} = R^{4'} = OH$, $R^6 = OCH_3$. 5,4′-Dihydroxy-3,6,7,8,3′-pentamethoxyflavone: $R^{5'} = H$, $R^{4'} = OH$, $R^3 = R^6 = R^7 = R^8 = R^{3'} = OCH_3$. 5,4′,5′-Trihydroxy-3,6,7,8-tetramethoxyflavone: $R^{5'} = H$, $R^{3'} = R^{4'} = OH$, $R^3 = R^6 = R^7 = R^8 = OCH_3$.

Cdk5, unlike the other Cdk's, is active mainly in the brain and is not involved in cell cycle progression, but

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rather in neuronal differentiation and the migration of neurons during development of the brain cortex.[15−17] It is also associated with processes involved in neurodegenerative diseases such as Alzheimer's and amyotrophic lateral sclerosis: deregulation of the activity of Cdk5 leads to hyperphosphorylation of the microtubule-associated proteins tau and MAP-1b, the initial events in the formation of the paired helical filaments that are a hallmark of Alzheimer's disease (AD).[18−21] Moreover, inhibition of Cdk5 protects neurons from death induced by β-amyloid peptide,[22,23] which aggregates and forms neuritic plaques to a greater extent in the brains of patients with AD or Down's syndrome than in normal subjects and appears to be another mechanism involved in AD.

In the present work we tested several commonplace flavones (chrysin, baicalein, apigenin, luteolin) and flavonols (kaempferol, quercetin), as well as the more unusual 6-methoxyapigenin and 6-methoxyluteolin,[24] and the highly methoxylated 5,4′-dihydroxy-3,6,7,8,3′-pentamethoxy- and 5,4′,5′-trihydroxy-3,6,7,8-tetramethoxyflavones,[25] as inhibitors of Cdk5 (Figure 2). We also estimated the affinities of these flavonoids for the active site of Cdk5 by docking their structures into a model of the enzyme based on its recently published crystallographic structure,[26] and identified some of the key interactions determining the potency of these compounds.

Results and Discussion

Although flavonoids are best known as antioxidants, a variety of other potentially useful activities have been described, ranging from the inhibition of many enzymes, including protein kinases,[6−10,27,28] to the modulation of neurotransmitter receptors.[4] In particular, the widespread natural flavonoids apigenin and kaempferol have been shown to inhibit the cell signaling kinase Cdk1, while luteolin and quercetin inhibit Cdk2.[14]

Cdk5 Inhibition. As an experimental strategy for the analysis of the effect of a set of natural flavonoids on the activity of the neuron-specific Cdk5, we used immunoprecipitation of fetal brain extracts, and the kinase activity of the enzyme was determined in the presence 1−250 μM flavonoid. Using a densitometric method we analyzed the level of histone H1 phosphorylation. Figure 3A shows a representative assay of 6-methoxyapigenin in the 0−250 μM range. We also determined the kinase activity index (eq 1, Experimental Section), plotted this index as a function of the concentration of inhibitor (Figure 3B), and estimated the IC_{50} of each compound. Negative controls of the kinase reaction were assays performed without histone H1 or Cdk5 immunoprecipitate. DMSO used at concentrations of up to 4% to dissolve the flavonoids in the reaction medium had no effect on the basal activity of Cdk5.

Several of the 12 compounds tested showed no significant Cdk5 inhibitory activity (IC_{50} > 250 μM), but some proved to be active in the low micromolar range (Table 1). In particular, the flavonols kaempferol and quercetin were significantly more potent than the corresponding flavones apigenin and luteolin. The most potent compounds tested, however, were 6-methoxyapigenin and 6-methoxyluteolin. The highest potencies found by us are a couple of orders of magnitude lower than that of flavopiridol (0.17 μM), but are in the same range as some other compounds described in the literature.[12] The spread of the potencies of these natural flavonoids suggests that their structure is critical for measurable inhibition to be observed. A tentative structure−activity relationship suggests that Cdk5 inhibition by the more abundant natural flavones requires a hydroxylated 2-phenyl ring (ring B), that a hydroxyl group at C-3 (as in flavonols) contributes to this activity, and that a methoxyl group at C-6 is a particularly favorable modification.

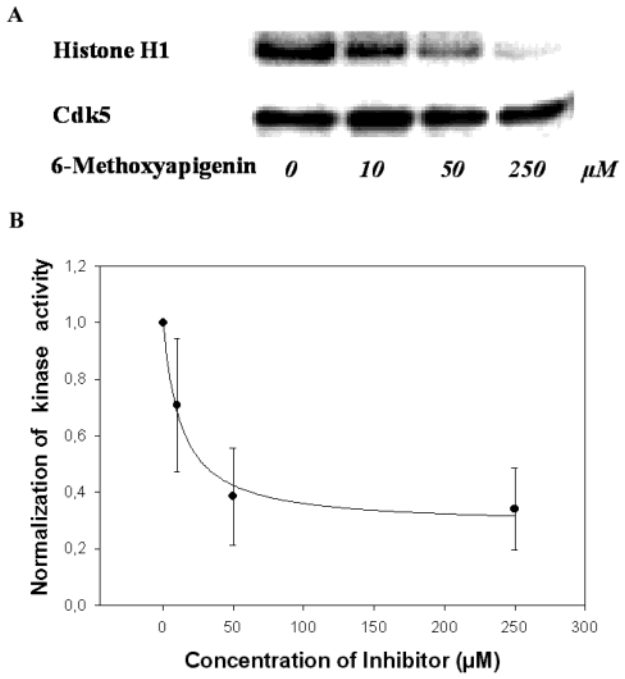

Figure 3. Effect of 6-methoxyapigenin on kinase activity of Cdk5. (A) Cdk5 immunoprecipitate was treated with 6-methoxyapigenin (range 0−250 μM) and subjected to a histone H1 kinase assay. Then, the proteins were fractionated in SDS/PAGE and transferred to nitrocellulose membranes. Autoradiograms of the phosphorylated histone H1 band are shown. Cdk5 immunodetection was used as load control. (B) Normalization of kinase activity of Cdk5 as a function of the concentration of 6-methoxyapigenin (n = 5). The concentration of histone H1 was kept constant at 0.08 μg/μL. Cdk5 kinase activity was normalized against the total mass of Cdk5 (eq 1).

Table 1. Estimated EC_{50} Values and GOLD Fitness Scores for Flavonoids

compound	EC_{50} (μM)	GOLD fitness score
Flavones		
chrysin	N.R.[a]	44.51
baicalein	N.R.	44.50
apigenin	>250	50.94
luteolin	>250	49.84
6-hydroxyapigenin	N.D.[b]	51.95
6-hydroxyluteolin	N.D.	49.86
6-methoxyapigenin	19.1 ± 3.7	51.40
6-methoxyluteolin	40.0 ± 25.1	53.23
6-hydroxy-5,7-dimethoxyflavone	>250	48.43
Flavonols		
kaempferol	66.1 ± 25.1	51.39
quercetin	63.4 ± 29.4	51.77
6-hydroxykaempferol	N.D.	51.05
6-hydroxyquercetin	N.D.	51.13
6-methoxykaempferol	N.D.	54.21
6-methoxyquercetin	N.D.	53.75
myricetin	71.1 ± 19.3	47.39
Polymethoxylated Analogues		
5,4′-dihydroxy-3,6,7,8,3′-pentamethoxyflavone	>25[c]	47.02
5,4′,5′-trihydroxy-3,6,7,8-tetramethoxyflavone	N.R.	48.21

[a] N.R.: Not reproducible. [b] N.D.: Not determined. [c] Too insoluble to test at higher concentration.

Molecular Modeling of Cdk5. To interpret these experimental results, a structural alignment was done between the crystal structures of Cdk2 (Protein Data Bank

entry 1FIN.pdb) and Cdk5 (1H4L.pdb). This alignment consisted of the superimposition of the backbone atoms of both structures and contained 1052 atoms with a root-mean-square deviation (rmsd) of 0.81 Å, indicating that the overall geometry is highly conserved. A sphere ($r = 10$ Å) was created around a carboxylate oxygen atom of Asp144 in Cdk5 (this residue is conserved in position but it corresponds to Asp145 in the Cdk2 sequence, and has been shown to be important for ATP binding).[29] Within this radius we found only one difference corresponding to Cys83 in Cdk5 (Leu83 in Cdk2). A published sequence alignment for the human Cdk's shows that the amino acid residues surrounding the residues involved in the binding of ATP and several inhibitors are also highly conserved.[29] This high degree of similarity between both active sites presumably explains the lack of selectivity of all known Cdk inhibitors toward these two enzymes and suggests that the pursuit of drugs with good selectivity for Cdk5 over the other Cdk's, based on binding to the ATP site, may not be worth the effort.

Docking Studies. The three-dimensional structure of the Cdk5-p25-ATP complex has not yet been experimentally determined. Therefore, to elucidate the structural basis for Cdk5 inhibition and taking advantage of the high degree of identity existing between the primary sequences of Cdk5 and Cdk2, the crystallographically solved structure of Cdk2-cyclin A cocrystallized with ATP was used as a guide for docking this ligand into its binding site in the crystallographic structure of apo-Cdk5.[30] We oriented the ATP molecule in such a way that hydrogen bond interactions were created with Glu81 and Cys83. The Cdk5-p25-ATP complex was thus built and then minimized, with the result that it faithfully reproduces the Cdk2-Cyclin A-ATP interactions. This description was used to compare the positions of the flavonoids using Jones et al.'s genetic optimization for ligand docking (GOLD).[31]

After docking, all the flavonoids were bound to Cdk5 in the ATP binding pocket, where they occupy the same regions as the ATP adenine and ribose rings. Quite unexpectedly, however, the chromone rings of all the ligands bearing hydroxyl groups on ring B, when bound to Cdk5, are rotated around the axis running through atoms C-4 and O-1 (see Figure 4B) by ca. 180° with regard to the structures of the experimental Cdk2/flavopiridol and *des*-chloroflavopiridol complexes (see Figure 4A),[32,33] and the similar modeled complexes of Cdk5 with chrysin and baicalein. In flavopiridol the chromone oxygen binds to the NH group of Cys83 and the C-5 hydroxyl hydrogen bonds to the carbonyl group of Glu81. Chrysin and baicalein, which have an unsubstituted ring B like *des*-chloroflavopiridol, bind in the same orientation. In all the other flavones and flavonols, the ligands are rotated and the chromone oxygen and the C-5 hydroxyl bind to the NH and C═O groups of Cys83, respectively.

A conserved pair of hydrogen bond interactions between the chromone carbonyl oxygen and the backbone NH of Cys83 and between the C-5 hydroxyl group and the backbone carbonyl oxygen of the same amino acid residue can be seen in the models of the Cdk5 complexes with apigenin, luteolin, kaempferol, quercetin, and their 6-hydroxy or methoxy analogues. Such a pair of hydrogen bonds is believed to be important for good binding to the ATP site of Cdk's.[29] Nevertheless, to explain the spread of the activities of natural flavones and flavonols, additional interactions with the enzyme must be identified. A third hydrogen bond can be observed between the C-7 hydroxyl and the carboxylate moiety of Asp86 for all our compounds

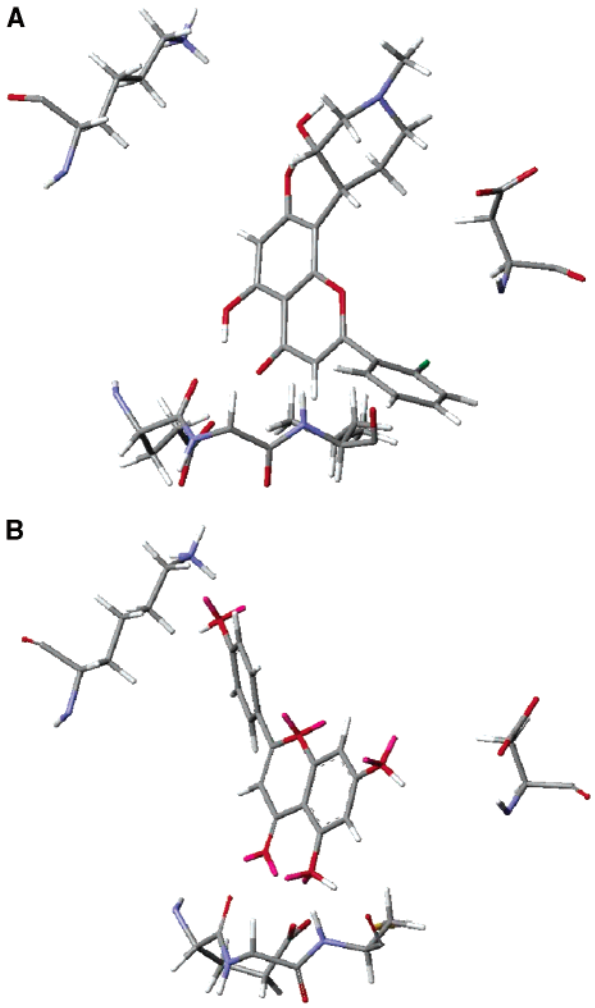

Figure 4. Binding orientation of flavone derivatives in the ATP binding site of Cdk5. (A) Flavopiridol. (B) Apigenin.

with the exception of chrysin and baicalein. In the Cdk5/flavonol complexes an additional hydrogen bond is formed between the C-3 hydroxyl and the carbonyl oxygen of Glu81 mimicking the interaction between the NH_2 group of ATP and the same backbone atom, which does not change the overall orientation of the ligand in the binding site with regard to the flavones lacking a hydroxyl group at C-3. The ring B hydroxyl groups of all these flavonoids form hydrogen bonds with Lys33 and Glu51, and, in the case of the ring B trihydroxylated myricetin, also with Asp144, whose polar side chains line an open pocket in the active site of the enzyme. These interactions, as pointed out below, increase the GOLD scores of these flavonoids and seem to be crucial to determine their reversed orientation in the ATP binding site with respect to the compounds lacking hydroxyl groups on ring B.

Goodness of Fit. The GOLD fitness scores (Table 1) reflect the experimental results quite well for the flavones and flavonols. Chrysin and baicalein, which did not give reproducible inhibition of the Cdk5 active complex, had the lowest scores. Within the group of flavones lacking a 3-hydroxy group, the relatively potent 6-methoxyapigenin and 6-methoxyluteolin have better scores than their inactive counterparts apigenin and luteolin. Also, 6-hydroxy-5,7-dimethoxyflavone, the only more highly methylated analogue that could reliably be shown to be inactive, had an even lower score than apigenin or luteolin, while the more active, corresponding flavonols kaempferol and quercetin had somewhat higher scores intermediate between

those of the parent compounds and their 6-methoxy derivatives, which may be attributed to their additional hydrogen bond to Glu81.

For the sake of comparison, models of the 6-hydroxy counterparts of apigenin and luteolin were built and docked into the enzyme model, with the result that these untested compounds also gave higher scores. Finally, models of the untested 6-hydroxy- and 6-methoxyflavonols kaempferol and quercetin were built, docked, and scored. In this subset of compounds, the 6-hydroxy compounds had slightly lower scores than the parent flavonols, but the 6-methoxy analogues showed the highest scores of the whole collection. The higher potencies and GOLD scores of 6-methoxyapigenin and -luteolin versus their unmethoxylated congeners and the high GOLD scores of the 6-methoxyflavonols suggest that testing the latter compounds may be an attractive goal.

In these compounds ring B bears hydrogen-bonding hydroxyl groups that can interact with the same amino acid residues as the *N*-methylpiperidin-3-ol ring of flavopiridol, i.e., Lys33 and Asp144, which line an open pocket in the active site,[29,33] as well as Glu51, in the case of the ring B trihydroxylated myricetin. Most of these flavonoids, not surprisingly, are better inhibitors of the enzyme than chrysin or baicalein, which are unable to form analogous hydrogen bonds. The unhydroxylated ring B of chrysin and baicalein, on the other hand, juts out of the conserved ATP binding site through a hydrophobic channel formed by Ile10 and the backbones of Leu83, His84, and Gln85, with a very clear-cut stacking interaction with the aromatic ring of Phe82. The same interactions are also seen in the crystal structures of the Cdk2/flavopiridol and *des*-chloroflavopyridol complexes. In the case of flavopiridol, the C−Cl bond may also have a dipole−dipole interaction with the Glu81 carboxyl group.

Conclusions. In conclusion, out of a set of 12 naturally occurring flavones and flavonols, 6-methoxyapigenin, 6-methoxyluteolin, kaempferol, quercetin, and myricetin inhibited the kinase activity of a Cdk5/p35 immunoprecipitate with IC_{50} values in the 20−70 μM range, concentrations that might be attained in plasma under normal circumstances by certain dietary flavonoids. Theoretical docking studies indicated that the active compounds bind in such a way that the ring B hydroxyl groups can form hydrogen bonds with the polar side chains of the enzyme, which are expected to interact with the hydroxyl groups at C-7. Within this set of compounds, activity is associated with the presence of one or more hydroxyl groups on ring B and is maximal with a 6-methoxy group between the usual phenolic functions at C-5 and C-7. Our molecular modeling results predict that 6-methoxykaempferol and 6-methoxyquercetin should be particularly potent Cdk5 inhibitors.

Experimental Section

Reagents. All fine chemicals were purchased from Sigma (St. Louis, MO) or Merck (Darmstadt). [γ-^{32}P]ATP was from Perkin-Elmer (Boston). Kaempferol and luteolin were purchased from Sigma (St. Louis, MO), and apigenin, baicalein, chrysin, and myricetin were from Aldrich Chem. Co. (Milwaukee, WI). Quercetin, 6-methoxyapigenin, and 6-methoxyluteolin were isolated from the aerial parts of *Centaurea chilensis* in the laboratory of SSB.[24] The tetra- and pentamethoxyflavones were gifts from Dr. Magalis Bittner (University of Concepción, Chile).[25] Stock solutions of all compounds were prepared at 100 mM in dimethyl sulfoxide with the exception of 5,4′-dihydroxy-3,6,7,8,3′-pentamethoxyflavone, which was insufficiently soluble (25 mM).

Protein Extraction for Kinase Assay and Immunoblot Analysis. The whole brain tissue of 18 day old rat embryos was homogenized in ice-cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/mL PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 μg/mL pepstatin), the homogenate was centrifuged at 15 000 rpm at 4 °C for 1 h, and the soluble fraction was used for kinase activity assay.

Immunoprecipitate Protein Kinase Cdk5 Assays. One milligram of brain protein extract was precleared with 2 μL of A-sepharose CL-4B beads for 1 h at 4 °C. Antibody specific for Cdk5 (C-8, Santa Cruz Biotechnology) was added, and the extract was incubated overnight at 4 °C, followed by an additional incubation for 2 h with protein A-sepharose CL-4B beads, and then centrifuged at 500*g* for 10 min. The immunoprecipitate was washed three times with RIPA extraction buffer and once with kinase buffer (50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DDT, 100 μM orthovanadate, 1 mM NaF, and 5 μM ATP), centrifuging each time for 10 min at 500*g*. The washed immunoprecipitate was incubated with 25 μL of kinase buffer containing histone H1 (2 μg), [γ-^{32}P]-ATP (2 μCi per reaction), and the respective inhibitor (final concentration 0−250 μM), for 30 min at 30 °C. The phosphorylation reactions were terminated by adding 8 μL of 4X Laemmli sample buffer. The total sample was resolved on SDS/12% PAGE. After transferring the proteins to nitrocellulose membranes, immunoblot analysis was performed. The phosphorylated [^{32}P]-histone H1 was quantified by molecular imaging, using the FX program (Bio-Rad).

Cdk5 Immunodetection. The nitrocellulose membranes were blocked with PBS-milk 3%, followed by incubation with antibody specific for Cdk5 (J-3, Santa Cruz Biotechnology) and HRP-labeled monoclonal secondary antibodies. The immunodetection was visualized by using the ECL detection system (Perkin-Elmer). The Cdk5 band was quantified by molecular imaging, using the FX program (Bio-Rad).

Data Analysis. Kinase activity was normalized (*N*) using the following equation:

$$N = \frac{\text{Phosphorylated H1/Cdk5 (flavonoid)}}{\text{Phosphorylated H1/Cdk5 (control)}} \quad (1)$$

where Phosphorylated H1 is the mass of phosphorylated histone H1 (with or without added flavonoid) and Cdk5 is the total mass of Cdk5 in the immunoprecipitate.

Computational Details. Cdk2 and Cdk5 X-ray structures were retrieved from the Protein Data Bank (PDB: entries 1QMZ and 1H4L, respectively).[34] All molecules were built using InsightII,[35] running on an SGI Octane workstation, starting from the fragment library and geometry optimized using the consistent valence force field (CVFF),[36] implemented in Discover.[35] ATP and 20 flavonoid structures were docked into the energy-minimized X-ray structure of Cdk5. The docking program GOLD, version 3.0, was used to position the ligands in the active site of the protein.[31] The active site into which the flavonoids were docked was defined as a sphere (r = 15 Å) around the nitrogen atom of Cys83. The default setup was used with no constraints on coordinates or hydrogen bonds. Early termination was allowed if the top five solutions were within a root-mean-square deviation (rmsd) of 1.5 Å, and a maximum of 10 solutions were retrieved from each docking procedure.

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