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A flavonoid gossypin binds to cyclin-dependent kinase 2

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Abstract—Flavonoids have low toxicity and mild activity. In order to find flavonoids showing cyclin-dependent kinase 2 (CDK2) binding effects, 347 flavonoid derivatives were docked into the crystal structure of the CDK2. The docking study showed that gossypin has a good conformational match with CDK2, which was confirmed by the binding affinity assay using NMR experiments.

In order to improve chemotherapeutic agents against cancer, apoptotic inducers are under investigation. The regulation of the cell cycle can affect cancer cells selectively.[1] Cell cycle in eukaryotes is regulated by a precise balance between positive and negative regulatory components that exert their effects during G_1 phase. The most critical positively acting components are cyclin E and cyclin-dependent kinase 2 (CDK2),[2,3] and the G_1-S transition is dependent on activation of CDK2 followed by phosphorylation of retinoblastoma protein.[4] Upon inhibition of CDK2 activity, cell proliferation is blocked[5] and the CDK2 inhibitors such as olomoucine, isopentenyladenine, flavopiridol, roscovitine, and staurosporine have potential as anticancer agents.[6–9]

Flavonoids are secondary metabolites produced by plants, which show many biological activities like estrogenic, antigiogenic, antioxidant, and apoptotic effects.[10] In this study, in order to discover flavonoid CDK2 inhibitors, a QSAR model was constructed by a structure-based 3D-QSAR study of oxindole derivatives. Various flavonoids were then docked into the CDK2 and their antiproliferative activities were predicted based on the constructed QSAR model. Finally, binding assay of the potential CDK2-binding flavonoids was performed by NMR spectroscopy.

The three-dimensional (3D) structures of CDK2 have been determined by many X-ray crystallographers. Of more than 100 structures deposited in protein data bank, an oxindole-bound form (PDB code: 1FVT) was selected, which was used as a target protein for structure-based 3D-QSAR. Before docking, in order for the protein to adapt bulkier ligand in its binding site, the structure of 1FVT was modified: the torsion angle of side chain (CD_1–CG_1–CB–CG_2) of Ile10 was changed from 60.230 to −54.388 using the software package SYBYL v 7.2 (Tripos, St. Louis, MO) running on a Linux workstation under the enterprise operating system. The modified 3D structure of 1FVT was energy minimized.[11] As the binding site of flavonoid derivatives was not clear, binding pocket for calculations was defined as all residues within the volume surrounding oxindole [ø 6.5 Å].[12] For construction of the 3D-QSAR model, 60 oxindole derivatives taken from the literature[13] were randomly divided into a training set and a test set. The training set and the test set were composed with 50 and 10 oxindole derivatives, respectively. A short energy minimization was carried out and the conformer showing the lowest total energy was selected, which was docked into the enzyme structure using the FlexX docking suite of the Sybyl v 7.2. 3D-QSAR was performed using comparative molecular field analysis (CoMFA).[14–16] The steric and electrostatic fields in CoMFA were calculated at each lattice intersection of a regularly spaced grid of 1.0 Å in all three dimensions within the defined region, and the van der Waals potential and Coulombic energy between the probe and the molecule were calculated using a Tripos standard force field. A statistical analysis using partial least-squares (PLS) method was performed to derive relationships between the calculated CoMFA field and biological activity. In order to dock the flavonoids into the

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CDK2, a short energy minimization was also carried out and the conformer showing the lowest total energy was selected. Docking followed by activity prediction based on the oxindole QSAR model provided the predicted binding affinity of the flavonoids. In order to prove the result obtained from in silico experiments, NMR spectroscopy was applied. A large molecule such as protein has faster relaxation in solution and slower diffusion than a small molecule such as a ligand. The NMR signal is broadened by fast relaxation. While a ligand results in sharp NMR signal, the complex of a ligand and a protein makes a broad NMR signal.[17] As a result, the change of NMR signals of a ligand can give information about formation of the complex.[18] The CDK2 enzyme and gossypin (3,3′,4′,5,7,8-hexahydroxyflavone 8-glucoside) were prepared as described in Ref. 19.

In order to confirm whether our docking protocol works correctly, flavopiridol known as a good inhibitor of CDK2 was docked first. H-bond acceptor (C=O) in flavopiridol was hydrogen bonded to CDK2 backbone NH of Leu83, and H-bond donor (–OH) formed a hydrogen bond with CDK2 backbone CO of Glu81. This docking result was matched with previously reported CDK2 binding site.[8] 3D-QSAR with CoMFA was performed using oxindole derivatives which were known as CDK2 inhibitors. Their biological activities were adapted from the data published previously by Bramson et al.[13] Sixty ligands (50 training set and 10 test set compounds) were aligned on the target protein. Their biological data were inhibitory concentration (IC_{50}), which were logged. The final CoMFA model has 1.0 Å grid spacing and considering a Tripos Standard field. The result exhibited a cross-validated correlation coefficient (q^2) of 0.790, conventional correlation coefficient (r^2) of 0.961, number of components of 5, standard error of estimate of 0.169, and F value of 251.945. The gradient of CoMFA graph was 0.95. That is, the CoMFA model indicates good predictive properties. In order to check the reliability of QSAR equation, a test set composed of ten compounds was used. Ten compounds were randomly selected from the data set published previously by Bramson *et al.*[13] Their predicted activities were calculated in the same way as the training set. The average of residual values (experimental—predicted) is 0.43. As a result, CoMFA model is reliable. The electrostatic contour map for CDK2 CoMFA model is displayed in Figure 1. The blue contours favoring the positive charge are located at 5 position and 1–2 position of indole ring. The red contours favoring the negative charge are at 5–6 position of indole ring and 3 position of benzenesulfonamide.

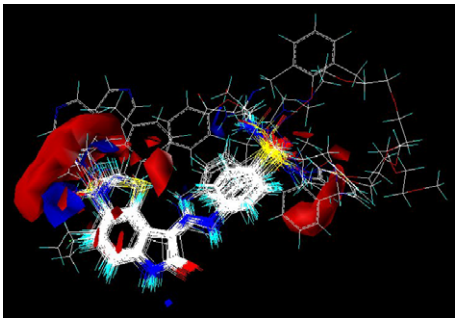

Figure 1. CoMFA contour specification showing electrostatic sites for CoMFA training set in gossypin. The blue region is favored in positive potential, and the red region is favored in negative potential.

Then flavonoids were docked into the same binding pocket of the CDK2 and among 347 compounds tested, 205 compounds were found to be correctly docked. Of them, gossypin showed the best fit. As shown in Figure 2, its

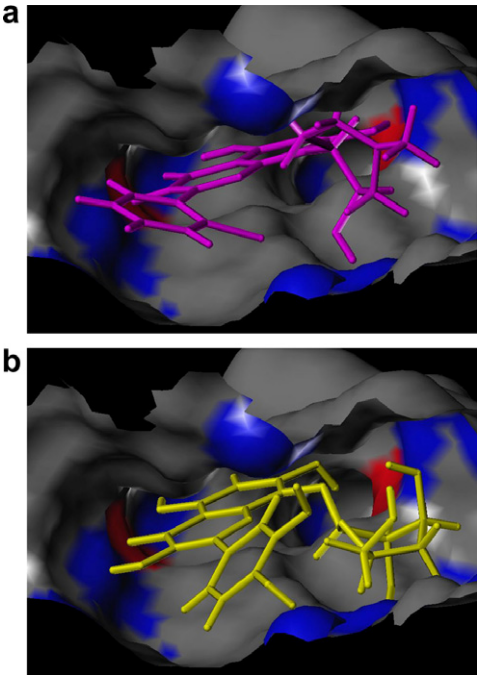

Figure 2. Comparison of (a) flavopiridol and (b) gossypin placed in CDK2 binding site.

Table 1. The predicted IC_{50} of 21 flavonoid derivatives whose IC_{50} values are less than 10 nM

Flavonoid derivatives	Predicted IC_{50} (nM)
2′-Hydroxy-α-naphthoflavone	1.741
4′-Hydroxy-α-naphthoflavone	2.523
4′-Chloro-6,8-dibromoflavone	2.636
7-Hydroxyflavone-β-D-glucoside	2.999
6,7-Dimethoxy-5,3′,4′-trihydroxyflavone	3.184
Karanjin	3.334
3-Hydroxy-7,3′,4′,5′-tetramethoxyflavone	3.801
2′-Methoxy-α-naphthoflavone	3.801
Gossypin	4.436
5-Hydroxy-6,7,8,3′,4′,5′-hexamethoxyflavone	4.875
7,3′-Dimethoxy-3-hydroxyflavone	5.023
Flavopiridol	5.248
3′-Hydroxy-α-Naphthoflavone	5.420
3,7-Dihydroxy-3′,4′,5′-trimethoxyflavone	6.081
3-Hydroxy-7-methoxyflavone	6.576
7,4′-Dimethoxy-3-hydroxyflavone	8.241
5,4′-Dihydroxy-7-methoxyflavone	8.279
5,3′-Dihydroxy-6,7,4′-trimethoxyflavone	9.120
3′,5′-Dimethoxy-3,5,7,4′-tetrahydroxyyflavone	9.268
3′,4′-Dihydroxy-α-naphthoflavone	9.527
7,2′,4′-Trimethoxyflavone	9.908

docking conformation is very close to that of flavopiridol. Even though the plain structures of flavopiridol and gossypin are similar to each other, their 3D structures are not. As shown in Figure 2, however, their conformations embedded in the binding pocket of CDK2 are similar.

The biological activities of 205 flavonoid derivatives which were docked into CDK2 were predicted, and the top ranked 21 compounds were listed in Table 1. Of them, gossypin is glycosylated in position C-8 of gossypetin which is pentahydroxylated flavonol. Glucose group enhances the hydrophilicity and give the electrostatic potential. This is agreed with the interpretation of CoMFA contour maps. That is, the hydroxyl groups and oxygen atoms contained in glucose have electrostatically negative potential because of their lone paired electrons. The electrostatic contour using MOLCAD provided by Tripos is shown in Figure 3.

In order to confirm whether the result obtained from in silico experiments is correct, in vitro binding assay using

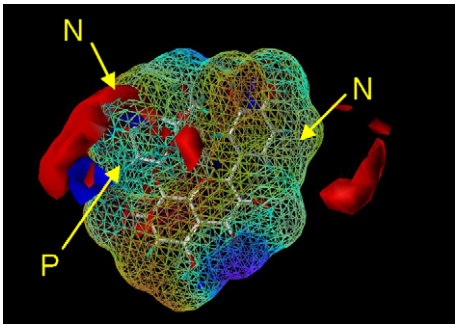

Figure 3. CoMFA contour specification for electrostatic MOLCAD mapping in gossypin. The blue region is favored in positive potential, and the red region is favored in negative potential. (N: electrostatic negative, P: electrostatic positive).

NMR spectroscopy was performed. Since NMR can distinguish the signals caused by a small molecule alone and those caused by a complex of a small molecule and a protein,[17] it is possible to decide whether the compound is bound to the CDK2 or not. Thus, ATP was dissolved in a mixture of glutathione *S*-transferase (GST) elution buffer in D_2O solution, because ATP is known to be bound to CDK2. The concentration of ATP was adjusted to 100 μM and its volume was 500 μL. The protein solution containing CDK2 was prepared and its concentration was adjusted to 100 μM too. The protein solution was added into the ATP solution in the NMR sample tube from 20 μL to 100 μL. The ^{1}H NMR data of the mixture were collected at every 20 μL addition. In the stacked plot of the NMR spectra, drastic changes of the NMR signals of ATP are observed. Like ATP, the solution containing gossypin was prepared and the same experiments were carried out. Here, since the NMR signals of gossypin are changed too, it is considered for gossypin to bind to CDK2. In order to know whether gossypin resides in the ATP binding site of CDK2 or not, the mixed solution of ATP and gossypin was prepared, where each concentration was adjusted to 100 μM. When the protein solution was added into the mixed solution, the NMR signals of ATP and gossypin were changed together as shown in Figure 4. Therefore, it can be said that gossypin and ATP bind to the different binding sites of CDK2. In these experiments, the addition of the protein solution into the ligand solution results in the dilution from 100 μM to 83.33 μM. In order to clarify whether the drastic changes mentioned above are caused by the dilution or not, only buffer solution was added into the ligand solution containing ATP and gossypin. Their NMR signals were not changed. Therefore, it can be concluded that the changes of the NMR signals by the addition of the protein solution into the ligand solution were caused by the protein–ligand complex.

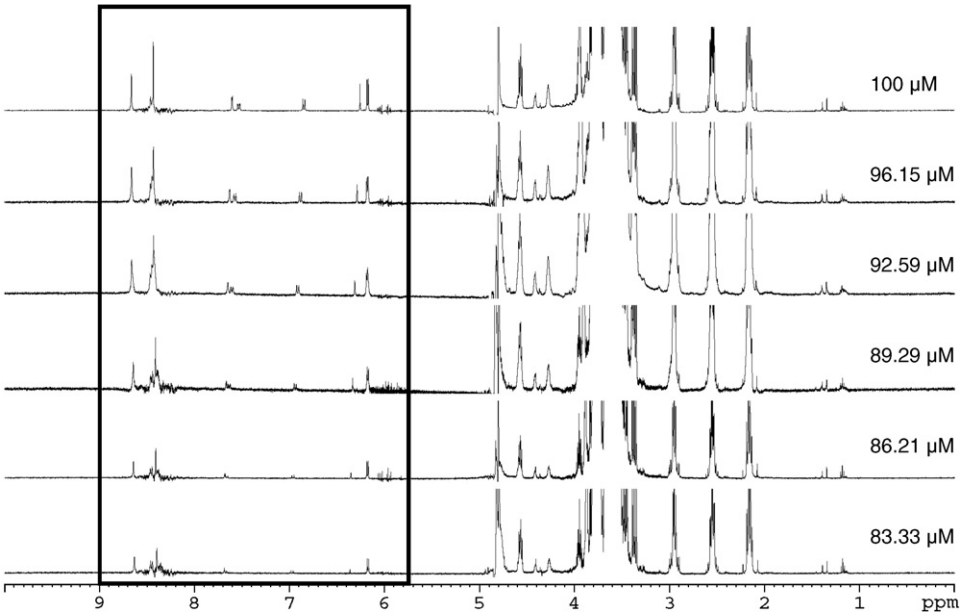

Figure 4. The stacked plot of the ^{1}H NMR spectra obtained from the addition of the protein solution containing CDK2 into the mixed solution of ATP and gossypin. The box indicates the ^{1}H NMR signals by ATP and gossypin. The numbers denote the concentrations of the ligands.

As a result, it was proved that NMR spectroscopy can be used for binding study between the protein and its ligands. A candidate obtained from in silico screening, gossypin, binds to CDK2, and it resides in the different binding site with ATP. Based on in silico docking and NMR experiments, flavopiridol which is known as a potential CDK2 inhibitor and gossypin show the same pattern in binding with CDK2. Even though the inhibitory activity of gossypin was not measured directly in this experiment, our evidence strongly suggests it is a CDK2 inhibitor with low toxicity and mild activity.

Acknowledgments

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References and notes

1. Lee, J. M.; Bernstein, A. *Cancer. Metast. Rev.* **1995**, *14*, 149.
2. Desai, D.; Gu, Y.; Morgan, D. O. *Mol. Biol. Cell* **1992**, *3*, 571.
3. Hunt, T. *Curr. Opin. Cell. Biol.* **1989**, *1*, 274.
4. Zhou, W.; Takuwa, N.; Kumada, M.; Takuwa, Y. *J. Biol. Chem.* **1993**, *268*, 23041.
5. Tian, J. Q.; Quaroni, A. *Am. J. Physiol.-Cell. Ph.* **1999**, *276*, C1245.
6. Schulze-Gahmen, U.; Brandsen, J.; Jones, H. D.; Morgan, D. O.; Meijer, L.; Vesely, J.; Kim, S. H. *Proteins: Structure, Function, Genetics* **1995**, *22*, 378.
7. de Azecedo, W. F.; Mueller-Dieckmann, H. J.; Schulze-Gahmen, U.; Worland, P. J.; Sausville, E.; Kim, S. H. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2735.
8. Lawrie, A. M.; Noble, M. E. M.; Tunnah, P.; Brown, N. R.; Johnson, L. N.; Endicott, J. A. *Nat. Struct. Mol. Biol.* **1997**, *4*, 796.
9. Bohm, B. A.. In *Flavonoid Functions in Nature: Introduction to Flavonoids, Chemistry and Biochemistry of Organic Natural Product*; Academic Publishers: Amsterdam, 1998; Vol. 2, pp.339.
10. Richardson, H. E.; Stueland, C. S.; Thomas, J.; Russel, P.; Reed, S. I. *Gene. Dev.* **1990**, *4*, 1332.
11. Gästeiger-Hückel charge was given to the protein, and minimization was performed by the steepest decent followed by conjugate gradient algorithm using Tripos Force field. The minimization process was forced to stop either when the iteration number reached 1000 or when the convergence criteria were met (maximum RMS gradient, 0.05 kcal/molÅ). The 3-D structures of 347 flavonoids tested as lignads were built up using Sybyl 7.25 (Tripos, St. Louis, MO) on a Pentium IV 3.2 GHz Linux PC. The derivatives were subjected to energy minimization. Conjugate gradients were carried out using the same method as the protein.
12. The residues contained in the binding pocket are as follows: I10–G11–G13–T14–Y15–G16–V18–K20–V30–A31–K33–V64–K65–L66–L78–V79–F80–E81–F82–L83–H84–Q85–D86–L87–K88–K89–F90–Q131–N132–L134–I135–L143–A144–D145–F146–L148–L298. The docking was carried out on a Pentium 3.2 GHz PC with a Linux OS (Red Hat Enterprise WS) using FlexX (Tripos). In order to select the most biologically active conformation of ligand, the maximum number of poses per ligands was set to five.
13. Bramson, H. N.; Corona, J.; Davis, S. T.; Dickerson, S. H.; Edelstein, M.; Frye, S. V.; Gampe, R. T., Jr.; Harris, P. A.; Hassell, A.; Holmes, W. D.; Hunter, R. N.; Lackey, K. E.; Lovejoy, B.; Luzzio, M. J.; Montana, V.; Rocque, W. J.; Rusnak, D.; Shewchuk, L.; Veal, J. M.; Walker, D. H.; Kuyper, L. F. *J. Med. Chem.* **2001**, *44*, 4339.
14. Cramer, R. D.; Patterson, D. E.; Bunce, J. D. *J. Am. Chem. Soc.* **1988**, *110*, 5959.
15. Calder, J. A.; Wyatt, J. A.; Frenkel, D. A.; Casida, J. E. *J. Comput. Aided Mol. Des.* **1993**, *7*, 45.
16. Klebe, G.; Abraham, U. *J. Med. Chem.* **1993**, *36*, 70.
17. Lee, Y.; Kim, K.; Suh, J. W.; Rhee, S.; Lim, Y. *FEMS. Microbiol. Lett.* **2006**, *266*, 236.
18. All NMR measurements were performed on a Bruker Avance 400 spectrometer system (9.4 T, Karlsruhe, Germany) at 298 K. The ^{1}H NMR spectra were collected in D_2O elution buffer solution. For the ^{1}H NMR analyses, 64 transients were acquired with a 1-s relaxation delay, using 32 K data points. The 90° pulse duration was of 10.2 μs and the spectral width was 5000 Hz. Prior to Fourier transformation, 2 K zero filling and sine-squared bell window functions were applied using XWIN NMR (Bruker).
19. For preparation of the CDK2 enzyme, polymerase chain reaction was carried out with Hot start Taq DNA polymerase (Qiagen, Germany).[20] The full length CDK2 cDNA was subcloned into pGEX 5X-1 which is a glutathione *S*-transferase (GST) gene fusion system.[21] The expressed protein was purified with GST-affinity chromatography. The supernatant was used for analysis by SDS–PAGE. The final concentration of CDK2 was 1.56 mg/ml. It was concentrated to 100 μM using Centriplus (Millipore, MA). Gossypin was purchased from INDOFINE chemical company, Inc. (Hillsborough, NJ). The chemical was supplied from the company at the purity of 98% and used for the NMR experiments [22] without further purification. The flavonoids were dissolved in D_2O elution buffer (10 mM Glutathione, 50 mM Tris–HCl [pH 8.0], 10% Glycerol) and concentrations of the samples were 100 μM.
20. Forty cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C, and 1-min amplification at 72 °C. Primers which were designed based on CDK2 of human were ATGAATTCATGGAGAACTTCCAAAAGGT as a forward primer and ATGTCGACCTATCAGAGTAGAAGATGGGGT as a reverse primer. The PCR product was subcloned into pGEM-T easy vector (Promega, Madison, WI, USA) and the resulting plasmid was sequenced.
21. The transformant was grown for the seed culture in LB medium containing 50 μg/ml ampicilin. The culture grew until absorbance at 600 nm reached to 0.7 ~ 0.8. At this point, IPTG was added at the final concentration of 0.1 mM, and the transformant was grown for 5 more hours at 30 °C. The cell was harvested, washed with PBS buffer (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2), resuspended in the same buffer, and then lysed by sonication.
22. Data not to be shown in the text can be found in supporting information for details.