

Crystallographic Approach to Identification of Cyclin-dependent Kinase 4 (CDK4)-specific Inhibitors by Using CDK4 Mimic CDK2 Protein*

Mari Ikuta, Kenji Kamata, Kazuhiro Fukasawa, Teruki Honma, Takumitsu Machida, Hiroshi Hirai, Ikuko Suzuki-Takahashi, Takashi Hayama, and Susumu Nishimura‡

From the Banyu Tsukuba Research Institute/ Merck Research Laboratories, Okubo 3, Tsukuba, Ibaraki 300–2611, Japan

Genetic alteration of one or more components of the p16^{INK4A}-CDK4,6/cyclin D-retinoblastoma pathway is found in more than half of all human cancers. Therefore, CDK4 is an attractive target for the development of a novel anticancer agent. However, it is difficult to make CDK4-specific inhibitors that do not possess activity for other kinases, especially CDK2, because the CDK family has high structural homology. The three-dimensional structure of CDK2, particularly that bound with the inhibitor, has provided useful information for the synthesis of CDK2-specific inhibitors. The same approach used to make CDK4-specific inhibitors was hindered by the failure to obtain a crystal structure of CDK4. To overcome this problem, we synthesized a CDK4 mimic CDK2 protein in which the ATP binding pocket of CDK2 was replaced with that of CDK4. This CDK4 mimic CDK2 was crystallized both in the free and inhibitor-bound form. The structural information thus obtained was found to be useful for synthesis of a CDK4-specific inhibitor that does not have substantial CDK2 activity. Namely, the data suggest that CDK4 has additional space that will accommodate a large substituent such as the CDK4 selective inhibitor. Inhibitors designed to bind into this large cavity should be selective for CDK4 without having substantial CDK2 activity. This design principle was confirmed in the x-ray crystal structure of the CDK4 mimic CDK2 with a new CDK4 selective inhibitor bound.

The cyclin-dependent protein kinases (CDKs)¹ are regulators of the timing and coordination of eukaryotic cell cycle events (1, 2). CDKs are inactive as monomers, and their activation requires binding to cyclins, a diverse family of proteins whose levels oscillate during the cell cycle, with phosphorylation by CDK-activating kinase on a specific threonine residue (3, 4). In addition to positive regulatory proteins, CDK inhibitors have also been reported such as p16 (5, 6), p21 (7–10), and p28 (11).

There is now strong evidence that CDKs, their regulators, and substrates are the targets of genetic alteration in many human cancers. The best characterized case of such alteration

is the p16-CDK4,6/cyclin D-retinoblastoma pathway (12–17). Under normal conditions, phosphorylation of pRB by the CDK4 or CDK6 in complex with one of the D-type cyclins is required for G₁-S transition. The INK4 family of proteins, such as p16^{INK4A}, specifically inhibit CDK4,6-cyclin D complexes and thus play a key role in inhibiting the G₁ to S cell cycle transition. Alterations in one or more components of the p16-CDK4,6/cyclin D-retinoblastoma pathway are found in more than half of all human cancers. For example, cyclin D1 overexpression, loss of function of p16^{INK4A}, and Rb alterations have all been found in human tumors. The universal involvement of the Rb pathway in human tumors has motivated the development of compounds specific for CDK4,6.

Small molecular CDK inhibitors have already been identified; the purine-based olomoucine (18) and its analogues, butyrolactone (19) and flavopiridol (20). Flavopiridol has been shown to have growth inhibitory activity against a number of tumor types *in vitro* and *in vivo* (21). Flavopiridol is now in clinical trials as an anticancer drug (22, 23). However, flavopiridol is not selective for CDK4; it also inhibits CDK2 to the same extent as CDK4. This low selectivity of flavopiridol may cause serious adverse effects. Thus it is desirable to have more potent and selective CDK4 inhibitors as second generation anticancer agents.

Three-dimensional structural information on the protein-inhibitor complex would be useful for the development of a potent and selective inhibitor. The crystal structures of CDK2 bound with inhibitors have been reported (24–28). S. H. Kim and co-workers (28) previously reported the isolation of potent CDK2 inhibitors by using structural information on CDK2 bound to inhibitors, together with combinatorial chemistry and molecular modeling approaches (28). To obtain CDK4-specific inhibitors, we adopted a similar approach, starting from the lead generation, using the structure of the ATP binding pocket of CDK2 as a guide. Of the many compounds chemically synthesized, some turned out to be potent CDK4 inhibitors, as represented by compound I. However, these compounds showed almost the same inhibitory activity against CDK2, although they showed negligible activity against other protein kinases. Obviously structural information on CDK4 was needed. However, we could not obtain CDK4 crystals for x-ray crystallography despite tremendous efforts. To overcome this problem, we made a mutant of CDK2 that possesses the same amino acid sequence as CDK4 in the region of the ATP binding pocket but contains the same sequence as CDK2 in other regions. This mutant protein, called CDK4 mimic CDK2 gave good crystals for x-ray analysis. The structure of the ATP binding pocket of the CDK4 mimic CDK2 protein showed an important difference from that of wild-type CDK2, which turned out to be very useful for the development of a CDK4-specific inhibitor, compound II.

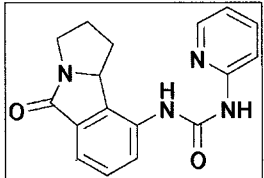
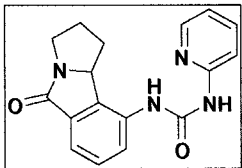
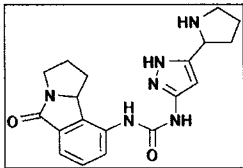
* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1GIH, 1GII, and 1GIJ) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

‡ To whom correspondence should be addressed. Tel.: 81-298-77-2003; Fax: 81-298-77-2034; E-mail nismrasm@banyu.co.jp.

¹ The abbreviations used are: CDK, cyclin-dependent kinase; compound I, N'-(pyrrolidino[2,1-b]isindolin-4-on-8-yl)-N-(pyridin-2-yl)urea; compound II, N'-(pyrrolidino[2,1-b]isindolin-4-on-8-yl)-N-(5-(pyrrolidin-2-yl)pyrazol-3-yl)urea; r.m.s., root mean square.

TABLE I
Summary of X-ray diffraction and refinement data

	compound I/ WT CDK2	compound I/ CDK4 mimic CDK2	compound II/ CDK4 mimic CDK2
			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions	a=73.87, b=72.67, c=53.85	a=73.91, b=72.43, c=53.97	a=74.02, b=72.61, c=53.98
Resolution (Å)	50.0–2.8	50.0–2.0	50.0–2.2
Total number of reflections	38408	68987	58086
Unique number of reflection	7545	19273	14195
Completeness(%)	99.9	94.7	92.3
Average I/σ	9.0	25.2	17.6
Rmerge(%)	7.5	4.8	6.0
X-plor refinement			
Resolution (Å)	8–2.8	8–2.0	8–2.2
Rfactor	16.9	20.2	22.8
Rfree	27.5	25.9	28.6
R.m.s.bonds(Å)	0.010	0.015	0.018
R.m.s.angles	1.677°	1.767	1.946

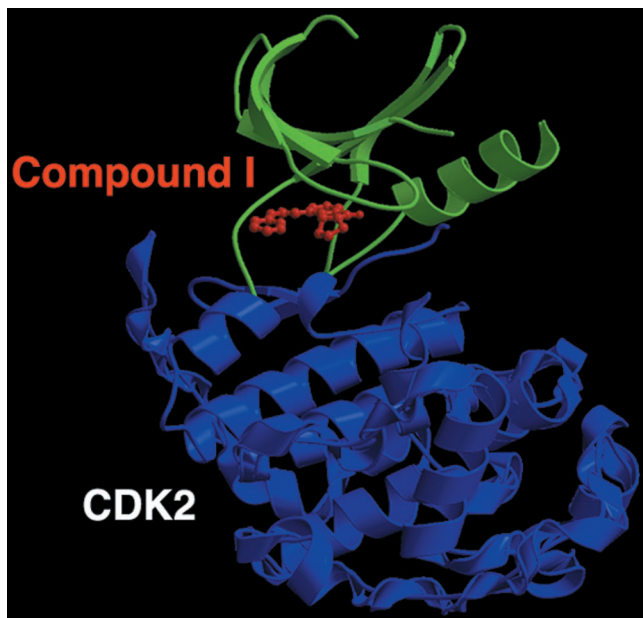


FIG. 1. Schematic drawing of CDK2 with the inhibitor compound I in the ATP binding pocket. This figure was created using the programs Molscript and Raster3D.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Crystallization—CDK2 was purified by a modification of the published method (29). Human CDK2 cDNA was inserted into the baculovirus expression vector pBacPAK9 (CLONTECH). The CDK2 vector was cotransfected with genomic DNA of baculovirus, and recombinant baculovirus was isolated by plaque formation. CDK2 was produced in Sf9 cells infected with baculovirus encoding the human CDK2 gene. The cells were maintained at 28 °C for 72 h and then harvested. A whole cell lysate was prepared by sonication of the cells with lysis buffer (10 mM Tris-HCl pH 7.4, 25 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM dithiothreitol, and a protease inhibitor mixture (Roche Molecular Biochemicals)) and centrifuged at $38,900 \times g$ for 30 min. The supernatant of the cell lysate was loaded on a Source15Q column (Amersham Pharmacia Biotech), previously equi-

brated with the lysis buffer followed by an HiTrapSP column (Amersham Pharmacia Biotech). The flow-through fraction was loaded onto an ATP affinity column (Sigma), previously equilibrated with buffer A (10 mM Hepes-NaOH, pH 7.4, 25 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM dithiothreitol), and the protein was eluted with a linear gradient (25–500 mM in buffer A). Further purification was performed by Superdex200 (Amersham Pharmacia Biotech) gel filtration with buffer B (10 mM Hepes-NaOH, pH 7.4, 250 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM dithiothreitol). Purified protein was dialyzed against buffer C (10 mM Hepes-NaOH, pH 7.4, 15 mM NaCl) and concentrated in a Centricon (Amicon) to 10 mg/ml for crystallization experiments.

CDK2 was crystallized under conditions described previously (29). Crystals of CDK2 were obtained from protein solutions at 10 mg/ml in buffer C equilibrated against 800 mM Hepes-NaOH, pH 7.4 at 4 °C. After CDK2 crystals were formed, they were soaked in solution containing the inhibitor in order to obtain CDK2 bound with the inhibitor. Exactly the same procedures were adopted for the expression of CDK4 mimic CDK2 and its crystallization.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using two subsequent polymerase chain reaction amplifications. As a template, we used human CDK2 in pBacPAK9 (CLONTECH). In the first step, the megaprimer was generated using an oligonucleotide priming within the multicloning site of pBacPAK9 and the corresponding mutagenesis primer. This megaprimer was used in a second polymerase chain reaction. CDK4 mimic CDK2 was cloned into pBacPAK9. All constructs were verified by sequencing.

Data Collection and Structure Refinement—X-ray diffraction data for the compound I/wild-type CDK2 complex crystals were collected on an R-axis IIc area detector (Rigaku Co., Tokyo). The diffraction data were reduced using MOSFLM (30). Data for compound I/CDK4-mimic CDK2 and compound II/CDK4 mimic CDK2 were collected at the BL6B beam line at the Photon Factory, KEK. The images were integrated with the DENZO package (31), and reflections were subsequently scaled and merged using SCALEPACK (31). Details of the crystal parameters and statistics of the data sets used in the structure determination are given in Table I.

The structures were solved by the molecular replacement method using the AMORE program (32). The human CDK2 structure (Protein Data Bank identification: 1HCL) was used as a search model. A clear solution was detected with a correlation of 80.3% and an R factor of 31.5 for compound I/wild-type CDK2, a correlation of 82.0% and an R factor of 30.7 for compound I/CDK4-mimic CDK2, and a correlation of 78.3% and an R factor of 33.2 for compound II/CDK4-mimic CDK2 in the 20–3.0 range. These models were refined by rigid body motion, followed by simulated annealing using X-PLOR (33–35) and manual fitting into

FIG. 2. The hydrogen bond and hydrophobic interactions of compound I bound to wild-type CDK2. Compound I is shown in magenta. Wild-type CDK2 is shown in green. The figure was prepared using InsightII (Molecular Simulations, Inc.).

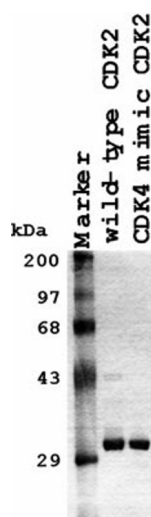
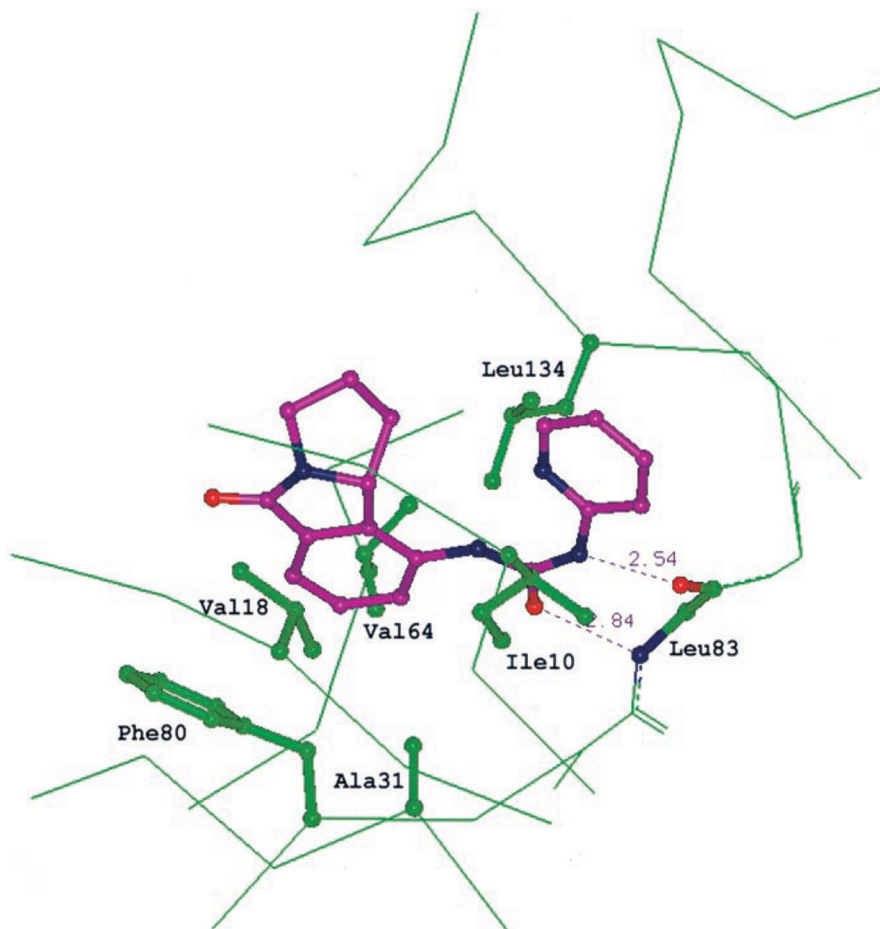


FIG. 3. SDS-polyacrylamide gel showing the purified wild-type CDK2 and CDK4 mimic CDK2. CDK4 mimic CDK2 was purified by the same procedures as wild-type CDK2.

electron density maps with the graphics program O (36). Successive rounds of rebuilding and simulated annealing refinements provided electron density maps that allowed complete interpretation of both the CDK2 and the inhibitor structures. X-PLOR omit maps from the final model were used to check every part of CDK2 and the inhibitor molecules. The final model has an R factor of 16.9% and free R factor of 27.5% for compound I/wild-type CDK2, an R factor of 20.2% and free R factor of 25.9% for compound I/CDK4 mimic CDK2, and an R factor of 22.8% and free R factor of 28.6% for compound II/CDK4 mimic CDK2.

Enzyme Assays—CDK2 and CDK4 kinases were assayed as previously reported (37–39). CDK2-cyclin A and CDK4-cyclin D complexes

TABLE II
Comparison of kinase activity

Enzyme	CDK4 mimic CDK2-CyclinA	Wild-type CDK2-CyclinA
	unit ^a /mg CDK2	
Specific activity	5.7×10^4	6.1×10^4

^a One unit of CDK2-CyclinA is defined as 1 pmol P incorporated into the S1 peptide over 1 min at 30 °C.

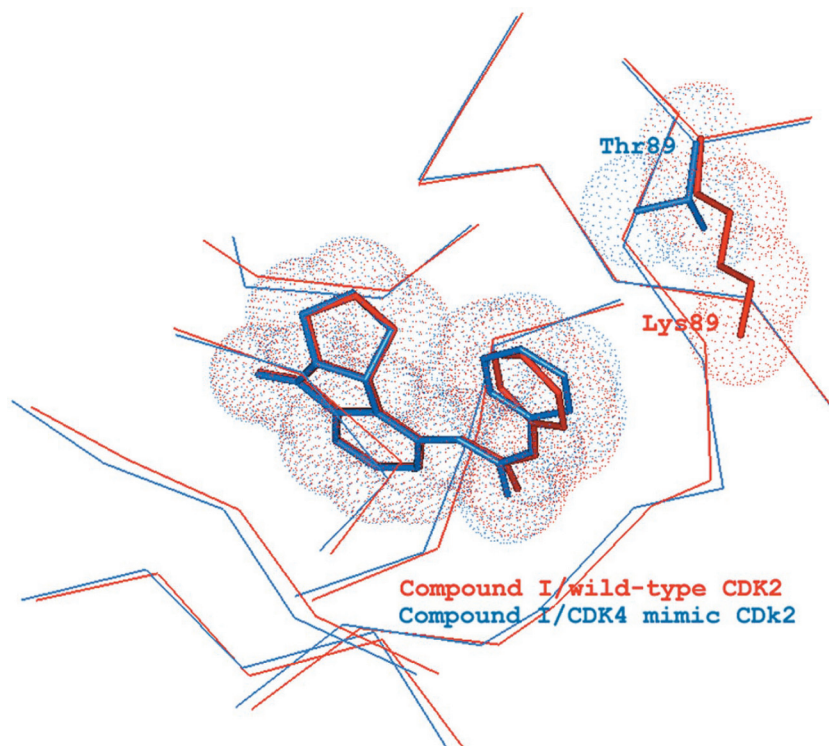
were added to a reaction mixture that contained 10 μ g/ml S1 peptide (AKAKKTPKKAKK) as a specific substrate for CDK2 kinase or G1 peptide (RPPTLSPIPHIPR) as a specific substrate for CDK4 kinase, R buffer (20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 4.5 mM 2-mercaptoethanol, 1 mM EGTA), 50 μ M ATP, and 0.5 μ Ci of [³²P]ATP (1 μ Ci of [³²P]ATP for CDK4-cyclin D) in a final volume of 20 μ liters. Assay mixtures were incubated at 30 °C for 45 min, terminated by addition of 10 μ liters of 350 mM H₃PO₄, trapped on P81 papers, washed six times with 75 mM H₃PO₄, and then counted for radioactivity.

RESULTS

X-ray Crystal Structure of the Compound I/Wild-type CDK2 Complex—The CDK2 portion of the compound I/CDK2 complex structure is almost identical to the CDK2 structure found in the CDK2 apoenzyme, the ATP complex, and moreover is very similar to that of the CDK2-cyclin A complex (40, 41). As observed in several CDK2 structures, electron density is weak in two regions in the enzyme, spanning residues 37–44 and 150–163. These segments have been omitted in the compound I/CDK2 structure because of very weak density.

The enzyme is folded into the typical bilobal structure, with the smaller N-terminal domain consisting predominantly of the β -sheet structure and the larger C-terminal domain consisting primarily of α -helices. There are no significant differences in the domain orientations between the inhibitor-enzyme com-

FIG. 4. Superimposed binding pocket of compound I/wild-type CDK2 complex and compound I/CDK4 mimic CDK2 complex. Compound I/wild-type CDK2 is shown in red, compound I/CDK4 mimic CDK2 complex is shown in blue.



plexes and the ATP-enzyme complex. The inhibitor binds, as seen for ATP and the other CDK inhibitors, in the deep cleft between the two domains (Fig. 1). A comparison of all backbone atoms for the compound I-CDK2 complex, and the ATP-CDK2 complex shows an r.m.s. derivation of 0.517.

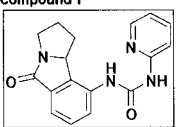
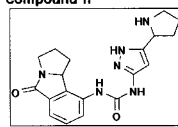
The hydrogen-bonding pattern and hydrophobic interactions of compound I are shown in Fig. 2. A pair of H-bonds are present between the O-17 oxygen and the backbone NH of Leu-83, and between the N-16 nitrogen and the backbone carbonyl of Leu-83. This hydrogen-bonding pattern is conserved in the CDK2 binding of the inhibitors olomoucine (25), roscovitine (26), and purvalanol B (28). The tricyclic amine makes mostly hydrophobic and van der Waals contacts with CDK2 residues.

There is one chiral center in compound I. The electron density indicates that the bound inhibitor is the (*R*)-stereoisomer of compound I, which is more potent than the (*S*)-isomer (data not shown).

Construction of CDK4 Mimic CDK2—The overall homology between CDK2 and CDK4 is 45%, suggesting that both enzymes are folded in a similar fashion. It is likely that sequence differences in the ATP binding pocket could affect inhibitor binding directly through different side chain contacts. The amino acid sequence of this region of CDK2 is FEFLHQDLKK, whereas the amino acid sequence of CDK4 in the corresponding region is FEHVDQLRT, indicating three nonconservative sequence differences at residues 82, 83, and 89. Thus the gene for CDK4 mimic CDK2 was synthesized by site-directed mutagenesis. The CDK4 mimic CDK2 gene possesses the nucleotide sequence of CDK4 in the ATP binding pocket, but the sequences of other regions are the same as those of CDK2. CDK4 mimic CDK2 was expressed well in Sf9 cells, producing a soluble protein as in the case of wild-type CDK2 (Fig. 3). We evaluated the kinase activity of CDK4 mimic CDK2. As shown in Table II, CDK4 mimic CDK2-cyclin A shows almost the same kinase activity as wild-type CDK2-cyclin A. This CDK4 mimic CDK2 produced good crystals for x-ray crystallography under the same conditions used for crystallization of wild-type CDK2.

X-ray Crystal Structure of CDK4 Mimic CDK2 Complexed

TABLE III
Properties of CDK4 inhibitors

	compound I	compound II
<div style="display: flex; justify-content: space-around;"> <div style="text-align: center;">  </div> <div style="text-align: center;">  </div> </div>		
Inhibitor data		
IC50(μ M)		
CDK4-cyclinD	0.051	0.21
CDK6-cyclinD	0.071	0.33
CDK2-cyclinA	0.096	25
cdc2-cyclinB	0.28	45
Ratio(CDK2/CDK4)	1.9	120
CDK4 mimic CDK2-cyclinA	0.25	1.6

with Compound I—Although there may be many notable similarities between the ATP binding site of CDK2 and CDK4, it is important to identify and characterize differences between the two sites if structural information is to be used to aid drug design. To identify structural differences of the inhibitor binding site of CDK4 and CDK2, we determined the crystal structure of CDK4 mimic CDK2 bound to compound I and compared it with the structure of the compound I/wild-type CDK2 complex (Fig. 4).

Comparison of these structures showed that the most striking difference between the inhibitor binding sites of CDK4 mimic CDK2 and wild-type CDK2 is that CDK4 mimic CDK2 has additional space caused by differences in the size of the side chain of residue 89 (CDK2,Lys; CDK4,Thr). According to these x-ray structures, it is likely that the CDK4 structure has additional space to accommodate the large substituents on the inhibitors. Inhibitors designed to bind into this additional space should be selective for CDK4 without exhibiting substantial CDK2 activity. Based on this design, we introduced bulky groups into compound I and synthesized inhibitors represented by compound II.

CDK4 Specificity of Compound II—Based on differences in the inhibitor binding site between CDK4 mimic CDK2 and

FIG. 5. The hydrogen bond and hydrophobic interactions of compound II bound to CDK4 mimic CDK2. Compound II is shown in magenta. CDK4 mimic CDK2 is shown in green.

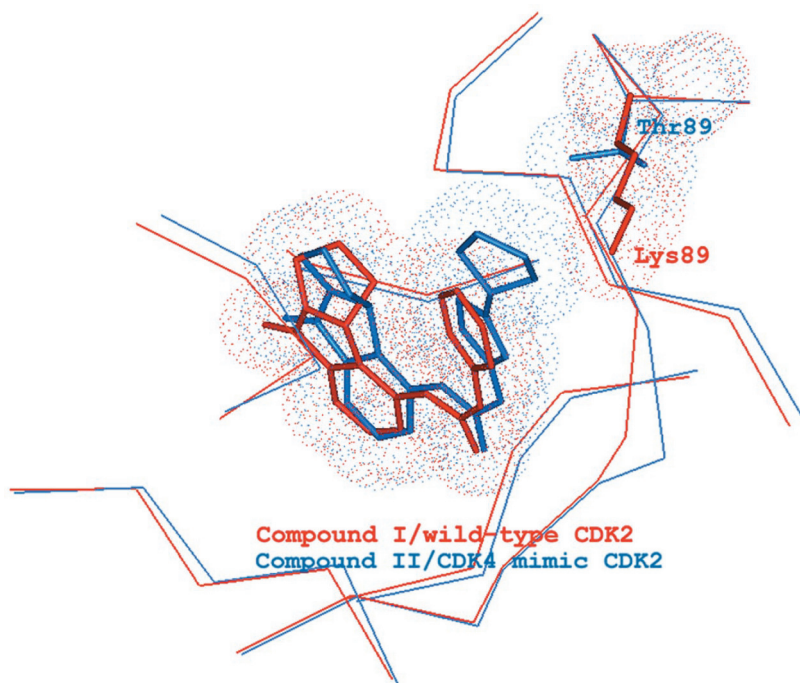
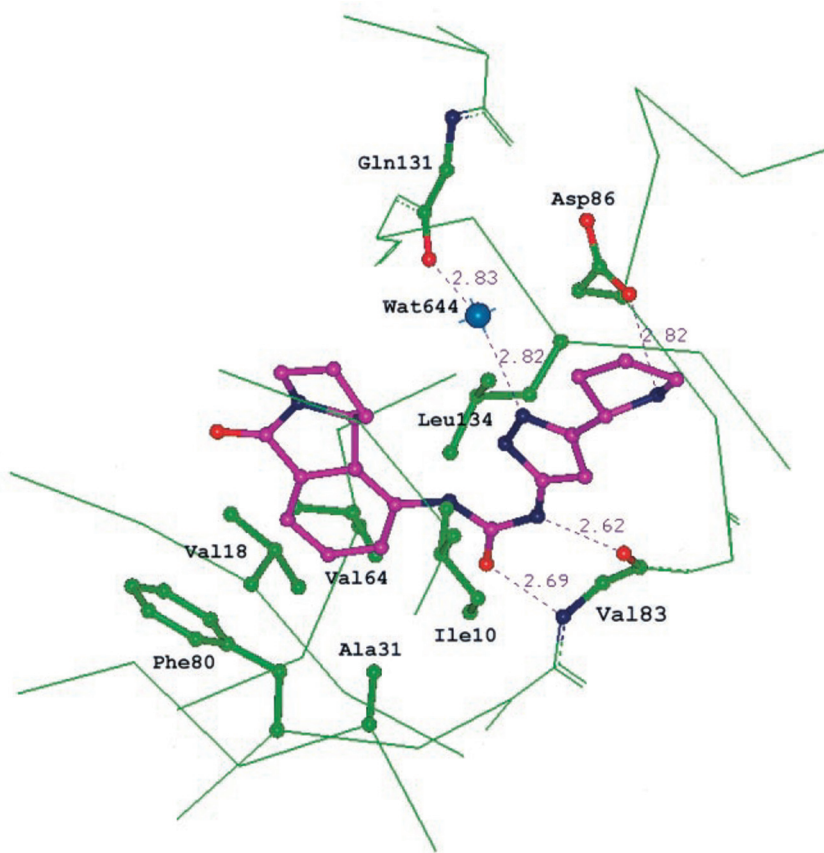


FIG. 6. Superimposed binding pocket of compound I/wild-type CDK2 complex and compound II/CDK4 mimic CDK2 complex. Compound I/wild-type CDK2 is shown in red, compound II/CDK4 mimic CDK2 complex is shown in blue.

wild-type CDK2, we introduced bulky groups into compound I and synthesized inhibitors represented by compound II. According to this design principle, compound II should have CDK4 selectivity. We purified various cyclin/CDK complexes from Sf9 cells expressed from recombinant baculovirus systems and determined selectivity of compounds with *in vitro* kinase assays. IC_{50} values of both compounds on each cyclin/CDK activity are presented in Table III.

Compound I inhibits CDK4, CDK6, CDK2, and cdc2 nearly equally at 0.05–0.28 μM . On the other hand, compound II specifically inhibits CDK4 and 6, and has more than ~ 100 -fold selectivity against CDK2 and cdc2. These results indicate that compound I is a broad CDK inhibitor, and introduction of bulky groups make compound I more selective for CDK4 and CDK6. We also evaluated the inhibitory activity of compounds on other serine/threonine or tyrosine kinases. Our results indicate

that compound I and compound II do not show significant inhibitory activity on other kinases (data not shown).

We also examined the ability of compounds to inhibit CDK4 mimic CDK2 (Table III). The inhibitory activity of compound II is about 10-fold more potent on CDK4 mimic CDK2 compared with wild-type CDK2, but less potent compared with wild-type CDK4. These results suggest that the inhibitor binding pocket of CDK4 mimic CDK2 should, at least in part, represent that of wild-type CDK4, and thus facilitate an estimation of the binding mode of CDK4 with CDK4 selective inhibitors.

X-ray Crystal Structure of CDK4 Mimic CDK2 Complexed with Compound II—With a structure-based approach, we have identified a CDK4-specific inhibitor, represented by compound II. To confirm that compound II fits into the larger binding pocket of CDK4, we determined the x-ray structure of compound II bound to CDK4 mimic CDK2.

The binding mode of compound II is shown in Fig. 5. Compound II also binds in the deep cleft between the two domains of CDK2. There are no significant differences in the domain orientations between the inhibitor-enzyme complexes and the ATP-enzyme complex. A comparison of the backbone atoms for the compound II-CDK4 mimic CDK2 complex and ATP-CDK2 complex shows an r.m.s. deviation of 0.623.

A pair of H-bonds are also present between the O-17 oxygen of the compound and the backbone NH of Leu-83 and between the N-16 nitrogen and the backbone carbonyl of Leu-83. This hydrogen bonding pattern is conserved and is important for this class of inhibitors. In addition, hydrogen bonding interaction is present between the N-25 nitrogen of the compound and the side chain of Asp-86. The N-22 nitrogen of compound II makes hydrogen bond interaction with water 644, which interacts with the carbonyl of Gln-131.

To confirm the design principle of CDK4 selective inhibitors, we compared the structure of the compound II/CDK4 mimic CDK2 complex with that of the compound I/wild-type CDK2 complex (Fig. 6). A comparison of all the backbone atoms in the 2 structures shows an r.m.s. deviation of 0.432.

In fact, compound II is well fitted in the additional space of the CDK4 mimic CDK2 binding cavity. However, the side chain of Lys-89 should interfere with the interaction of compound II and wild-type CDK2, accounting for its CDK4 specificity without substantial CDK2 activity.

DISCUSSION

CDK4 mimic CDK2:CDK2 substitution of the amino acid sequence in the ATP binding pocket with that of CDK4 has been quite useful for structural determination of the protein in order to elucidate its mechanism of kinase specificity. This mutant CDK2, called CDK4 mimic CDK2, was well expressed in Sf9 cells similar to wild-type CDK2, was subsequently purified to homogeneity, and gave good crystals for x-ray crystallographic analysis. Structural comparisons of CDK2 with that of CDK4 mimic CDK2 complexed with a non-selective CDK inhibitor suggested that CDK4 has additional space that will accommodate large substituents on the inhibitors. Based on these findings, we were able to synthesize CDK4 selective inhibitors represented by compound II. In fact, subsequent crystallographic analysis of the CDK4 mimic CDK2 complexed with compound II revealed that compound II fits well into the ATP binding pocket. The additional side chain, however, interferes with the interaction of compound II in the ATP binding pocket of CDK2. This very likely accounts for compound II-CDK4 inhibitory selectivity. In conclusion, comparison of the structures of CDK2 and CDK4 mimic CDK2 has proven to be effective for the development of a CDK4-specific inhibitor. This approach should be useful for the development of a next generation CDK4-specific inhibitor with much improved properties.

For development of an anticancer agent, inhibitors should exhibit inhibitory effects both *in vitro* and *in vivo*. We tested the growth inhibitory activity of compound II against tumor-derived cell lines *in vitro*. Unfortunately, this compound did not inhibit cell growth at 25 μ M concentration (data not shown). It is likely that compound II did not penetrate into the cells. Therefore, further chemical modification of compound II is necessary to obtain CDK4 inhibitors that have activity in tissue culture cells as well as in animal models. For such an approach, structural information of CDK4 mimic CDK2 should be very useful, and a study along this line is now in progress.

Finally, it should be mentioned that a similar approach, namely construction of a mutant protein, can be pursued for proteins that have not been crystallized for structural determination in order to elucidate the binding modes of ligands, agonists, and antagonists.

Acknowledgments—Diffraction data for this study were collected at PF beamline BL6B of the KEK, which was supported by the Foundation for Advancement of International Science. We thank Dr. Kim for his support of this work, for helpful discussions, and for critically reading the manuscript.

REFERENCES

- Norbury, C., and Nurse, P. (1992) *Annu. Rev. Biochem.* **61**, 441–470
- Sherr, C. J. (1996) *Science* **274**, 1672–1677
- Desai, D., Gu, Y., and Morgan, D. O. (1992) *Mol. Biol. Cell* **5**, 571–582
- Gu, Y., Rosenblatt, J., and Morgan, D. O. (1992) *EMBO J.* **11**, 3995–4005
- Serrano, M., Hannon, G. J., and Beach, D. (1993) *Nature* **366**, 704–707
- Peter, M., and Herskowitz, I. (1994) *Cell* **79**, 181–184
- Gu, Y., Turck, C. W., and Morgan, D. O. (1993) *Nature* **366**, 707–710
- Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993) *Nature* **366**, 701–704
- Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993) *Cell* **75**, 805–816
- Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J., and Reed, S. I. (1994) *Cell* **76**, 1013–1023
- Hengst, L., Dulic, V., Slingerland, J. M., Lees, E., and Reed, S. I. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5291–5295
- Kamb, A., Gruis, N. A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S. V., Stockert, E., Day, R. S., III, Johnson, B. E., and Skolnick, M. H. (1994) *Science* **264**, 436–440
- Nobori, T., Miura, K., Wu, D. J., Lois, A., Takabayashi, K., and Carson, D. A. (1994) *Nature* **368**, 753–756
- Spruck, C. H. 3rd, Gonzalez-Zulueta, M., Shibata, A., Simoneau, A. R., Lin, M. F., Gonzales, F., Tsai, Y. C., and Jones, P. A. (1994) *Nature* **370**, 183–184
- Hunter, T., and Pines, J. (1991) *Cell* **66**, 1071–1074
- Keyomarsi, K., and Pardee, A. B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 1112–1116
- Wang, T. C., Cardiff, R. D., Zukerberg, L., Lees, E., Arnold, A., and Schmidt, E. V. (1994) *Nature* **369**, 669–671
- Glab, N., Labidi, B., Qin, L. X., Trehin, C., Bergounioux, C., and Meijer, L. (1994) *FEBS Lett.* **353**, 207–211
- Kitagawa, M., Okabe, T., Ogino, H., Matsumoto, H., Suzuki-Takahashi, I., Kokubo, T., Higashi, H., Saitoh, S., Taya, Y., Yasuda, H., Ohba, Y., Nishimura, S., Tanaka, N., and Okuyama, A. (1993) *Oncogene* **8**, 2425–2432
- Losiewicz, M. D., Carlson, B. A., Kaur, G., Sausville, E. A., and Worland, P. J. (1994) *Biochem. Biophys. Res. Commun.* **201**, 589–595
- Carlson, B. A., Dubay, M. M., Sausville, E. A., Brizuela, L., and Worland, P. J. (1996) *Cancer Res.* **56**, 2973–2978
- Kelland, L. R. (2000) *Expert Opin. Invest. Drugs* **9**, 2903–2911
- Senderowicz, A. M. (1999) *Invest. New Drugs* **17**, 313–320
- De Azevedo, W. F., Jr., Mueller-Dieckmann, H. J., Schulze-Gahmen, U., Worland, P. J., Sausville, E., and Kim, S. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 2735–2740
- Schulze-Gahmen, U., Brandsen, J., Jones, H. D., Morgan, D. O., Meijer, L., Vesely, J., and Kim, S. H. (1995) *Proteins* **22**, 378–391
- De Azevedo, W. F., Leclerc, S., Meijer, L., Havlicek, L., Strnad, M., and Kim, S. H. (1997) *Eur. J. Biochem.* **243**, 518–526
- Lawrie, A. M., Noble, M. E., Tunnah, P., Brown, N. R., Johnson, L. N., and Endicott, J. A. (1997) *Nat. Struct. Biol.* **10**, 796–801
- Gray, N. S., Wodicka, L., Thunnissen, A. M., Norman, T. C., Kwon, S., Espinoza, F. H., Morgan, D. O., Barnes, G., LeClerc, S., Meijer, L., Kim, S. H., Lockhart, D. J., and Schultz, P. G. (1998) *Science* **281**, 533–538
- Rosenblatt, J., De Bondt, H., Jancarik, J., Morgan, D. O., and Kim, S. H. (1993) *J. Mol. Biol.* **230**, 1317–1319
- Leslie, A. G. W. (1990) in *Crystallographic Computing 5, From Chemistry to Biology* (Moras, D., Podjarny, A. D., and Thierry, J. C., eds) pp. 50–61, Oxford Univ. Press, Oxford
- Otwinowski, Z. (1993) in *Proceedings of the CCP 4 Study Weekend* (Sawyer, L., Isaacs, N., and Bailey, S., eds) pp. 56–62, Science and Engineering Research Council, Daresbury Laboratory, Washington, UK
- Navaza, J. (1994) *Acta Crystallogr. Sect. A* **50**, 157
- Brunker, A. T. (1992) *Nature* **355**, 472–474

34. Brunger, A. T., Kuriyan, J., and Karplus, M. (1987) *Science* **235**, 458–460
35. Brunger, A. T., Krukowski, A., and Erickson, J. (1990) *Acta Crystallogr. Sect. A* **46**, 585–593
36. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) *Acta Crystallogr. Sect. A* **47**, 110–119
37. Taya, Y., Yasuda, H., Kamijo, M., Nakaya, K., Nakamura, Y., Ohba, Y., and Nishimura, S. (1989) *Biochem. Biophys. Res. Commun.* **164**, 580–586
38. Kitagawa, M., Saitoh, S., Ogino, H., Okabe, T., Matsumoto, H., Okuyama, A., Tamai, K., Ohba, Y., Yasuda, H., and Nishimura, S. (1992) *Oncogene* **6**, 1067–1074
39. Higashi, H., Suzuki-Takahashi, I., Taya, Y., Segawa, K., Nishimura, S., and Kitagawa, M. (1995) *Biochem. Biophys. Res. Commun.* **216**, 520–525
40. De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., and Kim, S. H. (1993) *Nature* **363**, 595–602
41. Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995) *Nature* **376**, 313–320