Crystal Structure of Pyridoxal Kinase in Complex with Roscovitine and Derivatives

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Pyridoxal kinase (PDXK) catalyzes the phosphorylation of pyridoxal, pyridoxamine, and pyridoxine in the presence of ATP and Zn²⁺. This constitutes an essential step in the synthesis of pyridoxal 5'-phosphate (PLP), the active form of vitamin B₆, a cofactor for over 140 enzymes. (R)-Roscovitine (CYC202, Seliciclib) is a relatively selective inhibitor of cyclin-dependent kinases (CDKs), currently evaluated for the treatment of cancers, neurodegenerative disorders, renal diseases, and several viral infections. Affinity chromatography investigations have shown that (R)-roscovitine also interacts with PDXK. To understand this interaction, we determined the crystal structure of PDXK in complex with (R)-roscovitine, N⁶-methyl-(R)-roscovitine, and O⁶-(R)-roscovitine, the two latter derivatives being designed to bind to PDXK but not to CDKs. Structural analysis revealed that these three roscovitines bind similarly in the pyridoxal-binding site of PDXK rather than in the anticipated ATP-binding site. The pyridoxal pocket has thus an unexpected ability to accommodate molecules different from and larger than pyridoxal. This work provides detailed structural information on the interactions between PDXK and roscovitine and analogs. It could also aid in the design of roscovitine derivatives displaying strict selectivity for either PDXK or CDKs.

Pyridoxal kinase (PDXK) catalyzes the phosphorylation of the three forms of vitamin B₆ (pyridoxal, pyridoxamine, and pyridoxine) in the presence of ATP and Zn²⁺. This phosphorylation constitutes an essential step in the synthesis of pyridoxal 5'-phosphate (PLP), the intracellular active form of vitamin B₆, a key cofactor for at least 140 enzymes, such as aminotransferases and decarboxylases (1–3). PDXK is connected to nervous system functions, because many neurotransmitters such as dopamine, norepinephrine, serotonin, and γ-aminobutyric acid are synthesized by PLP-dependent enzymes (4). The mechanisms underlying pyridoxal kinase inhibition by several drugs have been studied (5). Recently, the crystal structure of PdxY, a PDXK homology protein of Escherichia coli was reported (6). Concurrently, the three-dimensional structures of sheep brain PDXK alone and in complex with various ligands (PDXK/ATP, PDXK/AMP-PCP/pyridoxamine, PDXK/ADP/PLP, and PDXK/ADP) have been determined, providing a better understanding of the catalysis mechanism of PDXK (7, 8).

Cyclin-dependent kinases (CDKs) play an important role in the regulation of cell division, apoptosis, transcription, neuronal functions, and exocytosis. The frequent deregulation of CDKs and their regulators in human tumors (9–13) and the involvement of CDK5 in Alzheimer (14), Parkinson (15), Nieman-Pick diseases (16), and ischemia (17) have stimulated an active search for chemical CDK inhibitors (12, 18, 19). Among the numerous inhibitors that have been identified, roscovitine, one of the early compounds, appears to be relatively potent and selective. Because of its cell growth inhibiting and neuroprotective activities, this purine is currently considered as a potential drug to treat, respectively, cancers, glomerulonephritis, and various neurodegenerative diseases (20). Roscovitine exists as two stereoisomers, (R)-roscovitine and (S)-roscovitine, the (R)-roscovitine isomer being slightly more active on CDKs (21–23). (R)-Roscovitine has been selected, under the name CYC202 (or Seliciclib), for preclinical and clinical evaluations (24, 25). (R)-Roscovitine has been crystallized with two protein kinases, CDK2 (21) and CDK5 (26). Like most CDK inhibitors it binds to the catalytic site of the kinases and acts as a competitive inhibitor for ATP binding (21, 27).

The selectivity of pharmacological inhibitors of protein kinases is an important issue. Because many inhibitors act by interacting within the ATP-binding pocket of their target kinases, a most conserved domain among the 518+ human kinases, concern about their selectivity is sensible. In the accompanying article (32), we showed that (R)-roscovitine is a relatively selective CDK inhibitor, based on a selectivity study involving 148 kinases (23). This good selectivity was recently confirmed by a new in vitro kinase/ligand competition assay (28). However, affinity chromatography on immobilized (R)-
Roscovitine revealed that (R)-roscovitine interacts with PDXK from various biological sources (23). In addition, this study showed that ATP competes with the binding of ERK1, ERK2, CDK5, and PDXK to roscovitine, whereas pyridoxal only competes with the binding of PDXK, but not of the protein kinases.

In this study we have solved the crystal structure of PDXK in complex with (R)-roscovitine and with two roscovitine derivatives designed not to interact with CDKs. All three purines were found to bind at the pyridoxal-binding site of PDXK, rather than at the expected ATP-binding site. Analysis of the PDXK/roscovitine crystal structure will allow the design of derivatives exclusively interacting with either CDKs or PDXK, thereby leading to new generations of roscovitine-derived pharmacological inhibitors of potential therapeutic interest.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Roscovitine, Roscovitine Derivatives, and Resins Thereof**

NMR spectra were recorded on a Bruker 400 MHz spectrometer. Structural assignments were achieved by one- and two-dimensional methods.

**(R)- and (S)-Roscovitine**

Roscovitine enantiomers were prepared according to Wang et al. (22) by a slightly modified procedure. Briefly, to a solution of benzyl-(2-chloro-9-isopropyl-9H-purin-6-yl)-methyl-amine (1) (1.2 g, 4 mmol) in 5 ml of dimethyl sulfoxide (Me₃SO) was added (R)-2-amino-1-butanol (1 ml, 5 mmol) in ethanol (150 ml) and extracted with CH₂Cl₂ (3 × 50 ml). The organic layer was washed twice with brine, dried over MgSO₄, and evaporated. The residue was chromatographed on silica gel using CH₂Cl₂/ethanol/NEt₃ (95:4.5:0.5) as eluent to afford (R)- and (S)-roscovitine in 70–76% yield.

**(R)-Roscovitine**—(R)-Roscovitine was used m.p. 104–107°C. The spectra used was ¹H NMR (CDCl₃), δ 1.04 (t, 3H, J = 7.5 Hz), 1.50 (dd, 6H), 1.60 (m, 2H), 3.60 (m, 1H), 3.80 (m, 1H), 4.60 (septet, 1H, J = 7.5 Hz), 4.80 (2H, s), 4.95 (broad s, 1H), 5.10 (s, 1H), 6.05 (s, 1H), 7.25 (m, 5H), 7.45 (s, 1H).

**(S)-Roscovitine**—(S)-Roscovitine was used m.p. 103–106°C. ¹H NMR spectra was identical to the ¹H NMR spectra of (R)-roscovitine.

R-2-[6-(Benzyl-methyl-amino)-9-isopropyl-9H-purin-2-ylamino]-butan-1-ol ("N6-methyl-(R)-roscovitine") (2)

A solution of 2,6-dichloro-9-isopropyl-9H-purine (50 mg, 0.22 mmol), N-benzylmethylamine (26 mg, 0.22 mmol), and triethylamine (44 mg, 0.43 mmol) in ethanol was stirred at 60°C for 1 h. The solvent was removed under vacuum, and the residue was purified by HPLC (C₁₈ column, eluted with CH₃CN/H₂O with 0.05% trifluoroacetic acid) to afford (S)-2-(6-benzyloxy-9-isopropyl-9H-purin-2-ylamino)-butan-1-ol ("N6-methyl-(R)-roscovitine") (2).

R-2-[6-(Benzyl-methyl-amino)-9-isopropyl-9H-purin-2-ylamino]-butan-1-ol ("N6-methyl-(R)-roscovitine")

A solution of 2,6-dichloro-9-isopropyl-9H-purine (50 mg, 0.22 mmol), N-benzylmethylamine (26 mg, 0.22 mmol), and triethylamine (44 mg, 0.43 mmol) in ethanol was stirred at 120°C for 30 min. After cooling to room temperature, the solution was treated with 1.5 ml of dimethyl sulfoxide (Me₂SO) and stirred using microwave irradiation. Water (15 ml) was then added to the mixture and it was extracted with ethyl acetate (3 × 10 ml). The organic layers were combined, washed with brine (10 ml), and dried with Na₂SO₄. The drying agent was removed, and the solvent was evaporated. The resulting residue was purified by HPLC (C₁₈ column, eluted with CH₃CN/H₂O with 0.05% trifluoroacetic acid) to afford the title compound as a hygroscopic white solid (31 mg, 80%). The spectra used was: ¹H NMR (methanol-d₄), δ 0.99 (t, 3H, J = 7.2 Hz), 1.59 (d, 6H, J = 7.2 Hz), 1.60–1.66 (m, 1H), 1.68–1.76 (m, 1H), 3.38 (b, 3H), 3.63–3.71 (m, 2H), 3.98–4.06 (m, 1H), 4.65–4.75 (m, 1H), 5.50 (b, 2H), 7.28–7.39 (m, 5H), 8.03 (s, 1H); m/z [M⁺] + 1 369.2.

R-2-[6-(Benzyloxy)-9-isopropyl-9H-purin-2-ylamino]-butan-1-ol ("O6-Benzyl-(R)-roscovitine")

A solution of benzyl-(2-chloro-9-isopropyl-9H-purin-6-yl)-methyl-amine (25 mg, 0.079 mmol) in (R)-2-amino-1-butanol (1.5 ml) in a Smith Process vial was sealed and heated at 180°C for 40 min using microwave irradiation. Water (15 ml) was then added to the mixture and it was extracted with ethyl acetate (3 × 10 ml). The organic layers were combined, washed with brine (10 ml), and dried with Na₂SO₄. The drying agent was removed and the solvent was evaporated. The resulting residue was purified by HPLC (C₁₈ column, eluted with CH₃CN/H₂O with 0.05% trifluoroacetic acid) to afford the title compound as a hygroscopic white solid (31 mg, 80%). The spectra used was: ¹H NMR (methanol-d₄), δ 1.30 (t, 3H, J = 7.2 Hz), 1.53–1.61 (m, 1H), 2.65–2.71 (m, 2H), 3.69 (m, 2H), 4.85 (septet, 1H, J = 7.2 Hz), 8.11 (s, 1H); m/z [M⁺] + 1 303.1. A solution of benzyl-(2-chloro-9-isopropyl-9H-purin-6-yl)-methyl-amine (1) (25 mg, 0.079 mmol) in (R)-2-amino-1-butanol (1.5 ml) in a Smith Process vial was sealed and heated at 120°C for 30 min using microwave irradiation. Water (15 ml) was then added to the mixture and it was extracted with ethyl acetate (3 × 10 ml). The organic layers were combined, washed with brine (10 ml), and dried with Na₂SO₄. The drying agent was then removed, and the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, eluted with EtOAc-hexanes) to afford 6-benzyl-oxy-2-chloro-9-isopropyl-9H-purine (20.2 mg, 0.88 mmol) in tetrahydrofuran (10 ml) at 0°C. The reaction was stirred for 1 h and quenched with saturated NH₄Cl (15 ml). It was extracted with ethyl acetate (3 × 10 ml). The organic layers were combined, washed with brine (10 ml), and dried with Na₂SO₄. The drying agent was removed, and the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, eluted with EtOAc-hexanes) to afford 6-benzyl-oxy-2-chloro-9-isopropyl-9H-purine (3) (0.20 g, 76%). The spectra was: ¹H NMR (methanol-d₄), δ 1.62 (d, 6H, J = 6.8 Hz), 4.86 (septet, 1H, J = 7.2 Hz), 5.84 (s, 2H), 7.34–7.41 (m, 2H), 7.35 (d, 2H, J = 6.8 Hz), 8.39 (s, 1H); m/z [M⁺] + 1 303.1. A solution of 6-benzyl-oxy-2-chloro-9-isopropyl-9H-purine (3) (0.10 g, 0.33 mmol) in (R)-2-aminobutan-1-ol (1.5 ml) in a Smith Process vial was sealed and heated at 120°C for 30 min using microwave irradiation. Water (15 ml) was then added to the mixture and it was extracted with ethyl acetate (3 × 10 ml). The organic layers were combined, washed with brine (10 ml), and dried with Na₂SO₄. The drying agent was removed, and the solvent was evaporated. The residue was purified by flash column chromatography (silica gel, eluted with EtOAc-hexanes) and followed by HPLC (C₁₈ column, eluted with CH₃CN/H₂O with 0.05% trifluoroacetic acid) to afford the title compound as a hygroscopic white solid (23.4 mg, 15%). The spectra was: ¹H NMR (methanol-d₄), δ 0.99 (t, 3H, J = 7.2 Hz), 1.35–1.61 (m, 1H), 1.63 (d, 6H, J = 7.2 Hz), 1.74–1.80 (m, 1H), 3.59–3.69 (m, 2H), 4.00–4.08 (m, 1H), 4.85 (septet, 1H, J = 7.2 Hz), 5.60 (s,
layers were combined and dried with Na$_2$SO$_4$. The drying agent was removed and the solvent was evaporated.

The (R)-rosocvitine matrix was prepared as described in the accompanying article (23). Purvalanol (NG-97) matrix was synthesized as previously described (29–31).

**Synthesis of Linker-attached N$_6$-methyl-(R)-rosocvitine**

A solution of 2,6-dichloro-9-isopropyl-9H-purine (0.45 g, 1.9 mmol), 4-methyaminomethyl-benzonic acid (0.30 g, 1.8 mmol), and triethylamine (0.5 g, 5.0 mmol) in ethanol was stirred at 60 °C for 1 h. The solvent was removed under vacuum, and the residue was purified by column chromatography (silica gel, eluted with 10% CH$_3$CN/H$_2$O to afford benzyl-(2-chloro-9-isopropyl-9H-purin-6-yl)-methyl-amine (0.5 g, 5.0 mmol) in ethanol was stirred at 60 °C for 1 h. The reaction mixture was diluted with water (10 ml) and extracted with ethyl acetate (3 ml) in a sealed Smith Process vial was heated at 180 °C for 30 min. After a brief spin at 10,000 g and the resulting residue was purified by HPLC (C$_{18}$ column, eluted with CH$_3$CN/H$_2$O with 0.035% trifluoroacetic acid) to afford the Compound as a colorless oil (0.22 g, 65%). The spectra was $^1$H NMR (Me$_2$SO-d$_6$, $^1$H NMR, J = 6.8 Hz), 7.32–7.41 (m, 3 H), 7.81 (d, 2 H, J = 8.0 Hz), 7.30 (d, 2 H, J = 8.0 Hz), 7.91 (s, 1 H), 8.45 (s, 1 H); m/z [M$^+$ + 1] 675.1.

**Affinity Chromatography on Immobilized Rosocvitine Buffers**

**Homogenization Buffer**—The homogenization buffer was 80 mM β-glycerophosphate, 15 mM p-nitrophenyl phosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM MgCl$_2$, 1 mM diithiothreitol, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10 μg of leupeptin/ml, 10 μg aprotinin/ml, 10 μg soybean trypsin inhibitor/ml, and 100 μg benzamidine.

**Bead Buffer**—The bead buffer was 50 mM Tris (pH 7.4), 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% Nonidet P-40, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, and 100 μg benzamidine.

**Preparation of Extracts**

Pork brains were obtained from a local slaughterhouse and directly homogenized and processed for affinity chromatography or stored at -80 °C prior to use. Tissues were weighed, homogenized, and sonicated in homogenization buffer (2 ml/g of material). Homogenates were centrifuged for 10 min at 14,000 $\times$ g at 4 °C. The supernatant was recovered, assayed for protein content (Bio-Rad protein assay), and immediately loaded batchwise on the affinity matrix.

**Affinity Chromatography of Rosocvitine Interacting Proteins**

Just before use, 10 μl of packed roscvitine beads were washed with 1 ml of bead buffer and resuspended in 600 μl of this buffer. The tissue extract supernatant (3 mg of total protein) was then added; the tubes were rotated at 4 °C for 30 min. After a brief spin at 10,000 $\times$ g and removal of the supernatant, the beads were washed 4 times with bead buffer before addition of 60 μl of 2× Laemmli sample buffer. Following heat denaturation for 3 min, the bound proteins were analyzed by SDS-PAGE and Western blotting or silver staining as described below.

**Electrophoresis and Western Blotting Antibodies**

Some antibodies were obtained from commercial sources: anti-CDK5 C-8 (Santa Cruz, Sc-173, 1:500, 1 h), anti-Erk1/2 (Sigma, M7927, 1:4000, 1 h). Anti-PDXK was generated by Eurogentec Europe (Double XP program). Two rabbits were immunized with a mixture of two human PDXK internal peptides: LLAWTHKHPNNLK (amino acids 241–253) and LRMVQSKRDIEDPEI (amino acids 291–305). The resulting antiserum (1:500, 1 h) cross-reacts with PDXK from a variety of species including mouse, rat, porcine, monkey, and human.

Following heat denaturation for 3 min, the proteins bound to the beads were eluted with 30 μl of elution buffer (20 mM Tris, 50 mM NaCl, 5 mM EDTA, 100 μg aprotinin/ml, 50 mM NaF), and assayed for protein content (Bio-Rad protein assay) and immediately loaded batchwise on the affinity matrix.
roscovitine matrix were separated by 10% SDS-PAGE (0.7 mm thick gels) followed by immunoblotting analysis or silver staining using an Amersham SDS-PAGE silver staining kit. For immunoblotting, protein/EK2—EK5 was obtained by 40 μg/liter EK5ase (Schleicher and Schuell). These were blocked with 5% low fat milk in Tris-buffered saline/Tween 20, incubated for 1 h with antibodies, and analyzed by Enhanced Chemiluminescence (ECL, Amersham Biosciences).

Protein Kinase Assays

Buffers

Homogenization Buffer—Homogenization buffer was 60 mM β-glycerophosphate, 15 mM β-nitrophenyl phosphate, 25 mM Mops (pH 7.2), 15 mM EGTA, 15 mM MgCl2, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenylphosphate, 10 μg/ml pepstatin/ml, 10 μg/ml soybean trypsin inhibitor/ml, and 100 μg/ml benzamidine.

Buffer C—Homogenization buffer but 5 mM EGTA, no NaF, and no protease inhibitors.

Kinase Preparations and Assays

Kinase activities were assayed in buffer C, at 30 °C, at a final ATP concentration of 15 μM. Blank values were subtracted and activities were calculated as micromoles of phosphate incorporated during a 10-min incubation. The activities were expressed in % of the maximal activity, i.e. in the absence of inhibitors. Controls were performed with appropriate dilutions of dimethyl sulfoxide. Unless otherwise stated, the P81 phosphocellulose assay was used.

CDK1/Cyclin B—CDK1/cyclin B was extracted in homogenization buffer from M phase starfish (Marthasterias glacialis) oocytes and its derivatives were obtained by co-crystallization under conditions similar to those used for the PDXK/AMP-PCP/pyridoxamine crystallization (8). For the PDKX(R)-rosco-vitine and PDXKN5-methyl(-R)-rosco-vitine complexes, a protein solution was prepared containing 10 mg/ml PDKX and 3 mM rosvitine in 50 mM KH2PO4/K2HPO4 (pH 8.2). Crystals of PDKX complexes with these two purines were grown using the hanging drop vapor diffusion technique with a reservoir solution of 1.4 M ammonium sulfate and 100 mM KH2PO4/K2HPO4 (pH 8.2), in a drop obtained by mixing 1 μl of protein solution and 1 μl of reservoir solution. Crystals appeared after about 1 month at a constant 17 °C. Similar conditions were used for the crystallization of the PDKXO6(R)-rosco-vitine complex, except that pH was 6.4.

Structure Determination and Refinement

X-ray diffraction data for PDKX(R)-rosco-vitine complexes were collected using a Mar345 image plate in the Synchrotron Radiation Laboratory, Institute of High Energy Physics (Beijing, China). Data for PDXKN5-O6(R)-rosco-vitine and PDXKN5-methyl(-I)-rosco-vitine crystals were collected using a Rigaku R-Axis IP IV+ imaging plate area detector in the Institute of Physics, Chinese Academy of Sciences (Beijing, China). Before data collection, the crystals were flash frozen in liquid N2 after being immersed in Paratone-N oil, used as a cryoprotectant. The data were processed with the HKL suite of programs (32) and on-axis data processing software.

The three complex structures were solved by molecular replacement using AMORE (33), and solutions were obtained using the unliganded structure (Protein Data Bank code 1LHP) as search model. After rigid body refinement, the F̅2-F̅1 maps showed clear electron density representing the three roscovitines. Models of the inhibitors were made using (R)-rosco-vitine from the PDKX2 from roscovitine crystal structure as the initial model. Parameter and topology files for the three roscovitines were made using XPLOR20 (Uppsala Software Factory). Crystallography and NMR System software (34) was used for refinement of coordinates and B-factors. Final models were obtained after several cycles of positional refinement and manual rebuilding using program O (35), and addition of water molecules. The quality of the final structures was verified using PROCHECK (36). The Ramachandran plot for the three inhibitors showed that the three structures had good stereochemistry. Structural statistics are shown in Table I. Coordinates and structure factors have been deposited in the Protein Data Bank (codes 1YKG, 1YGJ, and 1YHJ) (Table I).

RESULTS AND DISCUSSION

Crystal Structure of PDKX-Rosco-vitine Complex: Rosco-vitine Binds in the Pyridoxal Binding Site—We were able to obtain crystals of PDKX in complex with (R)-rosco-vitine at pH 8.2. Similar to the PDXK/AMP-PCP/pyridoxamine crystal, the PDKX(R)-rosco-vitine complex structure belongs to the P321 space group, with the complex having one monomer in the asymmetric unit. The overall structure of PDKX contains 9 α-helices, 10 β-strands, and 3 segments of 3I0 helices. The 10 β-strands form a central β-sheet flanked by α2, α3, α4, α5, and α6 on one side and α1, α7, α8, and α9 on the other side (Fig. 1A). (R)-Rosco-vitine binds in the groove formed by the ends of β-strands on the enzyme surface. The electron density for all atoms of the inhibitor (R)-rosco-vitine (and the two related purines, see below) was clear and strong (Fig. 1B). Surprisingly, (R)-rosco-vitine, as well as its inactive analogs, binds in the pyridoxal-binding site rather than at the ATP-binding site as initially expected (Figs. 1A and 2A). In the PDKX(R)-rosco-vitine complex, PDKX interactions with (R)-rosco-vitine are characterized by predominantly hydrophobic and van der Waals interactions and hydrogen bonds (Fig. 2B). The purine ring of rosvitine is enclosed in a hydrophobic pocket formed by Tyr4, Val15, Val115, and Gly326. The benzyl ring has hydrophobic interactions with Val43 and Arg296. Three direct hydrogen bonds are formed between (R)-rosco-vitine and the surrounding amino acid residues. One bond is formed between N7 of (R)-rosco-vitine and the hydroxyl group of the Ser12 carbonyl. The second bond is formed between N3 of (R)-rosco-vitine and the hydroxyl group of Thr47. The third bond is formed between N2 of (R)-rosco-vitine and the same Thr 47 hydroxyl group. In contrast, in the PDKX-AMP-PCP/pyridoxamine complex, PDKX interacts with pyridoxamine through three hydrogen bonds formed between the N1, O3, and O5 atoms of pyridoxamine and the side chains of Ser12, Thr47, and Asp235 of PDKX, respectively.

Comparison of the rosvitine-binding site in the Cdk-roscovitine complexes (21, 26) and in the PDKX(R)-rosco-vitine complex revealed that the atoms of roscovitine involved...
Roscovitine/Pyridoxal Kinase Co-crystal Structure

TABLE I
Structure determination and refinement of PDXK in complex with (R)-roscovitine, N6-methyl-(R)-roscovitine (2), and O6-(R)-roscovitine (4)

<table>
<thead>
<tr>
<th>Protein Data Bank file</th>
<th>PDXK/(R)-roscovitine</th>
<th>PDXK/N6-methyl-(R)-roscovitine</th>
<th>PDXK/O6-(R)-roscovitine</th>
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<tr>
<td>Space group</td>
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<tr>
<td>Cell dimension (Å)</td>
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<td>a = b = 100.9, c = 55.7</td>
<td>a = b = 103.2, c = 58.5</td>
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<tr>
<td>Resolution (Å)</td>
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<td>2.7</td>
<td>2.8</td>
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<tr>
<td>Unique reflections</td>
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<td>9,190</td>
<td>9,063</td>
</tr>
<tr>
<td>Completeness, %</td>
<td>99.0 (99.3)</td>
<td>99.6 (99.1)</td>
<td>99.6 (100)</td>
</tr>
<tr>
<td>Rmerge *</td>
<td>0.047 (0.353)</td>
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<td>0.105 (0.267)</td>
</tr>
<tr>
<td>Rwork b</td>
<td>23.48</td>
<td>21.68</td>
<td>22.56</td>
</tr>
<tr>
<td>Root mean square deviation from ideal geometry of final models</td>
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<tr>
<td>Root mean square deviation bonds (Å)</td>
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<td>0.015</td>
<td>0.012</td>
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<tr>
<td>Root mean square deviation angles (°)</td>
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<td>1.71</td>
<td>2.03</td>
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<tr>
<td>Ramachandran</td>
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<td></td>
<td>Additional allowed</td>
<td>18.2%</td>
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<td></td>
<td>Generously allowed</td>
<td>2.9%</td>
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</table>

* Rmerge = \( \frac{\sum_{h} |I(h)| - \langle I(h) \rangle}{\sum_{h} |I(h)|} \) where \( I(h) \) is the ith integrated intensity of a given reflection and \( \langle I(h) \rangle \) is the weighted mean of all measurements of \( I(h) \).

** Rwork = \( \frac{\sum_{h} |I(h)| - \langle I(h) \rangle}{\sum_{h} |I(h)|} \) for the 90% of reflection data used in refinement.

*** Rfree = \( \frac{\sum_{h} |I(h)| - \langle I(h) \rangle}{\sum_{h} |I(h)|} \) for the 10% of reflection data excluded from refinement.

in the interactions with each target are not the same (Fig. 2, C and D). In the CDK2-roscovitine (21) complex, the purine ring binds in the ATP-binding pocket. Leu83 interacts with roscovitine through two hydrogen bonds, involving N7 and N6 of the purine. In addition, a third weak hydrogen bond is formed between O1 of the purine and a water molecule. Ile10 and Leu134 have hydrophobic interactions with roscovitine from two sides. A very similar binding mode was described in the CDK5-roscovitine complex (26).

PDXK Interacts with N6-Methyl-roscovitine, a Protein Kinase Inactive Derivative—Roscovitine interactions with CDKs and PDXK are thus quite different. In the PDXK/(R)-roscovitine complex, N6 of roscovitine does not make any contribution to its binding in the active site, whereas in the CDK-roscovitine complex N6 acts as a hydrogen donor in a hydrogen bond with Leu83 of CDK2 (21) or Cys83 of CDK5 (26). The N6 position acts as a H-bond donor that is essential for the inhibitor/protein kinase interaction. We therefore synthesized two roscovitine analogs with modifications designed so that the H-bonding possibility at N6 would be prevented (Table II). Either methylation on N6 or replacement of N6 by an oxygen, leading, respectively, to N6-methyl-(R)-roscovitine (2) and O6-(R)-roscovitine (4), converted roscovitine to derivatives devoid of protein kinase inhibitory activity (Table II).

We next immobilized (R)-roscovitine, N6-methyl-(R)-roscovitine or purvalanol on Sepharose beads. Porcine brain extracts were loaded on these matrices, and the bound proteins were analyzed by SDS-PAGE after extensive washing (Fig. 3). Silver staining and Western blotting revealed the expected ERK1, ERK2, PDXK, and CDK5 proteins that specifically bind to (R)-roscovitine-Sepharose. No PDXK was detectable on purvalanol Sepharose beads. In contrast, ERK1, ERK2, nor CDK5 were found on N6-methyl-(R)-roscovitine-Sepharose beads while PDXK was present. This direct approach thus confirmed that N6-methyl-(R)-roscovitine has lost the capacity to interact with its protein kinase targets, whereas its ability to interact with PDXK remains intact. Interaction of these two protein kinase inactive compounds with PDXK were further confirmed by the co-crystallization experiments (Fig. 4).

Crystallization of PDXK-selective Roscovitine Analogs with PDXK—The crystal of the PDXK-N6-methyl-(R)-roscovitine complex was obtained at pH 8.2 and its structure was solved (Fig. 4, A and C). Superposition of the N6-methyl-(R)-roscovitine and (R)-roscovitine complexes indicates that their overall
structures are almost identical, that they form the same hydrogen bonds with PDXK (Fig. 5A), and that their binding in the pyridoxal-binding pocket involves very similar hydrophobic interactions. In the N\textsuperscript{6}-methyl-(R)-ros covitine-PDXK complex structure, the benzyl ring moved toward Phe\textsuperscript{43}, resulting in a slight conformational change of Trp\textsuperscript{52}, which is maintained in a position located adjacent to Phe\textsuperscript{43} (Fig. 5A).

Our initial attempts to obtain a PDXK/O\textsuperscript{6}-(R)-ros covitine crystal at pH 8.2 were unsuccessful. However, we obtained the PDXK/H\textsubscript{18528}O\textsuperscript{6}-(R)-ros covitine complex crystal at pH 6.4 and determined its structure (Fig. 4, B and D). Previous studies indicate that the optimum pH for PDXK activity is 6.5 (37), suggesting that this pH might provide a slight conformation change favorable for interaction with specific ligands. Comparison of PDXK in complex with O\textsuperscript{6}-(R)-ros covitine, (R)-ros covitine, and N\textsuperscript{6}-methyl-(R)-ros covitine indicates that they had a similar overall structure (Fig. 5A).

Comparison with PDXK in Complex with Other Ligands—Structural analysis of our previous PDXK-ATP (structure determined at pH 6.4) (7) and the PDXK-ADP-PLP (determined at pH 6.4) (8) complexes reveals that there is a 12-residue loop (117–128) connecting \( \beta \)5 and \( \alpha \)4, which presents different conformations in the catalytic process carried out by PDXK (Fig. 6). There are two identical monomers (A and B) assembled by a non-crystallographic 2-fold axis in the asymmetric unit of PDXK/ATP. Loop 117–128 occupies a different position in monomers A and B. In monomer A the loop is restricted by the other symmetrical molecule in the crystal and occupies the pyridoxal binding pocket. This loop is then referred to as “loop of state A.” In monomer B, the loop is in contact with the solvent region of the crystal without any lattice limitation and completely sways to another direction, partially covering the ATP-binding site. This loop is then referred to as “loop of state B.” PDXK can bind vitamin B\textsubscript{6} before ATP, suggesting that, under these conditions, this loop neither occupies the pyridoxal-binding site nor covers the ATP-binding site. In this situation the loop is referred to as “loop of state C.” Based on this classification, loop 117–128 in the PDXK-O\textsuperscript{6}-(R)-ros covitine complex displays a loop of state C conformation in which PDXK contains ros covitine in the pyridoxal-binding site without ATP.
Roscovitine/Pyridoxal Kinase Co-crystal Structure

Three purines were synthesized and tested on various kinases: \((R)-\text{ros covitine}\) and the kinase inactive \(\text{O}^6\)-methyl-\((R)-\text{ros covitine}\) \(\left(R-2-\left\{6-\text{(benzyl-methyl-amino)}-9\text{-isopropyl-9H-purin-2-ylamino)}-\text{butan-1-ol}\right\}\) (2) and \(\text{O}^6\)-\((R)-\text{ros covitine}\) \(\left(R-2-\left\{6\text{-benzyl氧-9-isopropyl-9H-purin-2-ylamino))\text{-butan-1-ol}\right\}\) (4). \(\text{IC}_{50}\) values for several kinases are indicated under each structure. The purified protein kinases were assayed in the presence of 15 \(\mu\text{M}\) ATP and increasing concentrations of each purine. \(\text{IC}_{50}\) values, evaluated graphically, are in \(\mu\text{M}\).

### Table II: Structure and kinase inhibitory properties of ros covitine and derivatives

<table>
<thead>
<tr>
<th>Kinase</th>
<th>((R)-\text{ros covitine})</th>
<th>N6-methyl-((R)-\text{ros covitine}) (2)</th>
<th>O6-((R)-\text{ros covitine}) (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK1/cyclin B</td>
<td>0.35</td>
<td>45</td>
<td>100</td>
</tr>
<tr>
<td>CDK2/cyclin E</td>
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<td>250</td>
<td>250</td>
</tr>
<tr>
<td>CDK5/p25</td>
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<td>45</td>
<td>200</td>
</tr>
<tr>
<td>Erk2</td>
<td>22</td>
<td>&gt;1000</td>
<td>700</td>
</tr>
<tr>
<td>CK1</td>
<td>100</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>DYRK1A</td>
<td>11</td>
<td>&gt;1000</td>
<td>250</td>
</tr>
</tbody>
</table>

**Fig. 3.** PDXK binds to immobilized \(\text{N}^6\)-methyl-\((R)-\text{ros covitine}\), an inactive derivative of ros covitine. A, \((R)-\text{ros covitine}\) and \(\text{N}^6\)-methyl-\((R)-\text{ros covitine}\) were immobilized to agarose beads through a polyethylene glycol linker attached on the benzyl ring. A arrow indicates the \(\text{N}^6\) position that provides an essential hydrogen bond between ros covitine and its CDK targets. B, a porcine brain extract was loaded on these beads, as well as control and purvalanol beads. Beads were extensively washed and the bound proteins were analyzed by SDS-PAGE followed by silver staining (left) and Western blotting (right) with antibodies directed against ERK1, ERK2, PDXK, and CDK5.

The conformation of PDXK-ADP-PLP complex is believed to be very close to the reaction transition state. Structural comparison reveals that the substrate binding pocket of PDXK-ADP-PLP is more compact than that of PDXK-\(\text{O}^6\)-(\(R\))-ros covitine. Compared with PDXK-\(\text{O}^6\)-(\(R\))-ros covitine, the loop connecting \(\beta2\) and \(\alpha2\) in PDXK-ADP-PLP moved \(\sim2\) \(\text{Å}\) toward the substrate-binding site. Concurrently, the distance between two reaction group of substrates in PDXK-ADP-PLP is about 2 \(\text{Å}\) closer than that in PDXK-AMP-PCP-pyridoxamine (Fig. 6B). Based on this analysis, the following conclusion can be reached. At the optimum \(\text{pH}\) (6.4), when PDXK only contains one substrate (ATP or pyridoxal), substrate binding does not induce any dramatic conformational change in PDXK (as observed, for example, in the PDXK-ATP and the PDXK-\(\text{O}^6\)-(\(R\))-ros covitine structures). At \(\text{pH}\) 8.2, which is less favorable for enzyme activity, no conformational changes occur either when two substrates can bind simultaneously (as observed, for example, in the PDXK-AMP-PCP-pyridoxamine complex structure).

\(\text{Asp}^{235}\) is believed to be involved in initiating the phosphorylation reaction by abstracting a proton from the hydroxyl group of the vitamin substrate so that the hydroxyl oxygen atom can spontaneously attack the \(\gamma\)-phosphate of ATP. In the structure of PDXK-AMP-PCP-pyridoxamine (\(\text{pH}\) 8.2), \(\text{Asp}^{235}\) is forming a hydrogen bond (2.85 \(\text{Å}\)) with the \(\text{O}^\gamma\) hydroxyl group of pyridoxamine. In contrast, a longer distance (3.4 \(\text{Å}\)) between \(\text{Asp}^{235}\) and the \(\text{O}^\gamma\) atom of PLP is observed in PDXK/ADP/PLP.
**FIG. 4.** Interactions of N<sup>6</sup>-methyl-(R)-ros covitine (2) and O<sup>6</sup>-(R)-ros covitine (4) with PDXK. A and B, electron densities of N<sup>6</sup>-methyl-(R)-ros covitine (2) and O<sup>6</sup>-(R)-ros covitine (4) co-crystallized with PDXK were calculated after rigid body refinement. The maps (contoured at 1.0 σ level) were generated using MOLSCRIPT (38) and Raster3D (39). C, schematic atomic interaction map of N<sup>6</sup>-methyl-(R)-ros covitine (2) with PDXK amino acids. D, atomic interaction map of O<sup>6</sup>-(R)-ros covitine (4) with PDXK amino acids.

**FIG. 5.** A, superposition of (R)-ros covitine, N<sup>6</sup>-methyl-(R)-ros covitine (2), and O<sup>6</sup>-(R)-ros covitine (4) at the active site of PDXK. (R)-Roscovitine and the surrounding PDXK residues are shown in green, whereas N<sup>6</sup>-methyl-(R)-ros covitine and O<sup>6</sup>-(R)-ros covitine with the surrounding PDXK residues are shown in orange and slate, respectively. B, superposition of PDXK/O<sup>6</sup>-(R)-ros covitine and PDXK/ATP. O<sup>6</sup>-(R)-ros covitine is shown in lime and ATP in yellow. The backbone of PDXK/O<sup>6</sup>-(R)-ros covitine and PDXK/ATP are shown in red and blue, respectively.
(pH 6.4), indicating the protonation state of Asp235 (Fig. 6B). It is suggested that the protonation of Asp235 plays an important role in triggering the conformation switch on pH change, and the binding of substrates to the active site would induce further conformational change.

So far we have been unable to obtain any PDXK/ATP/roscovitine co-crystal structure, as if the two ligands were mutually exclusive. However, structural superimposition reveals that when O6-(R)-roscovitine is modeled into the substrate binding pocket of PDXK/ADP/PLP, its benzyl ring will have close contacts with Arg86, Phe43, and Tyr127 (Fig. 6B). Therefore, it is reasonable to postulate that binding of ATP to PDXK/roscovitine may trigger a modest yet significant conformation change that influences the PDXK-roscovitine interaction. This hypothesis is substantiated by the “competing” effect of increasing concentrations of ATP on the binding of PDXK to immobilized roscovitine (22). Further studies will be required to understand this point fully.

In vivo, roscovitine may thus compete with pyridoxal binding to PDXK. However, the presence of ATP negatively influences the binding of roscovitine to PDXK, resulting in a very limited effect of roscovitine on PDXK catalytic activity. This effect of ATP might provide a safety mechanism for mammalian cells to prevent molecules other than vitamin B6 from binding to the active site under the pre-reaction state, thus blocking their effects on PDXK. Our results revealed that the pyridoxal binding pocket has a surprising ability to accommodate large molecules such as roscovitine that are different from vitamin B6, and roscovitine analogs with N6 modifications show promise as specific inhibitors for PDXK.

Conclusion—This work constitutes the first report of a kinase inhibitor being crystallized with both its protein kinase targets (CDK2 and CDK5) and with a non-protein kinase, “off-target” enzyme (PDXK). Structural analysis reveals notable differences between the interactions of roscovitine with PDXK and CDK2/CDK5. Comparison of these structures...
should allow us to design two different classes of roscovitine derivatives, the first one specifically targeting PDXK, whereas being devoid of inhibitory activity toward protein kinases, the second one specifically targeting protein kinases while no longer interacting with PDXK. N6-Methyl-(R)-roscovitine (2) and O6-(R)-roscovitine (4) represent two examples of the first class of PDXK-selective compounds. We are currently synthesizing roscovitine analogs belonging to the second class. The cellular effects and pharmacology of both classes of roscovitine analogs will be investigated in detail.

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