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Discovery of Cyclin-dependent Kinase Inhibitor, CR229, Using Structure-based Drug Screening

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Abstract To generate new scaffold candidates as highly selective and potent cyclin-dependent kinase (CDK) inhibitors, structure-based drug screening was performed utilizing 3D pharmacophore conformations of known potent inhibitors. As a result, CR229 (6-bromo-2,3,4,9-tetrahydro-carbolin-1-one) was generated as the hit-compound. A computational docking study using the X-ray crystallographic structure of CDK2 in complex with CR229 was evaluated. This predicted binding mode study of CR229 with CDK2 demonstrated that CR229 interacted effectively with the Leu83 and Glu81 residues in the ATP-binding pocket of CDK2 for the possible hydrogen bond formation. Furthermore, biochemical studies on inhibitory effects of CR229 on various kinases in the human cervical cancer HeLa cells demonstrated that CR229 was a potent inhibitor of CDK2 (IC_{50}: 3 μM), CDK1 (IC_{50}: 4.9 μM), and CDK4 (IC_{50}: 3 μM), yet had much less inhibitory effect (IC_{50}: >20 μM) on other kinases, such as casein kinase 2-α1 (CK2-α1), protein kinase A (PKA), and protein kinase C (PKC). Accordingly, these data demonstrate that CR229 is a potent CDK inhibitor with anticancer efficacy.

Keywords: CR229, CDK inhibitor, structure-based drug screening

The cell cycle is coordinated by three families of molecules; cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs). CDKs play important roles in the signal transduction pathways that control the proliferation and differentiation of eukaryotic cells [17], and are a group of protein kinases whose products must assemble into a holoenzyme with a cyclin subunit to become catalytically active. The function of cyclin-CDK complexes is regulated by the phosphorylation of CDKs at different positions and proteolysis of cyclins [22]. In mammalian cells, CDK2, 4, and 6 play a role in the G1 and/or S phases, whereas CDK1 appears to be restricted to mitosis [23].

To antagonize the actions of the CDK-cyclin complexes in the cell cycle, mammalian cells also express CDK inhibitors, such as p21 and p27, which bind to the complexes, thereby preventing their activation and inhibiting previously activated complexes. The expression of these CKIs is induced by inhibitory regulators of the cell cycle, and occurs during the normal state of growth arrest [24, 25].

Recent studies have shown that CDK regulation is impaired in various diseases [1, 18], which has led to an intensive search for selective chemical inhibitors of these kinases [5, 9–16, 19]. Consequently, more than 50 chemical CDK inhibitors have been found to inhibit CDK activity *in vitro* or in cell cultures, and are currently being evaluated for therapeutic use with various diseases, including cancer, alopecia, neurodegenerative disorders, cardiovascular disorders, glomerulonephrites, and viral and parasitic protozoal infections [2, 4, 20, 28]. These chemical inhibitors all act by binding to or near the ATP-binding pocket of the CDK catalytic site, yet certain problems still exist, like nonselective inhibitory activity towards CDKs.

During recent years, many novel and potent ATP-site directed inhibitors of CDKs have been discovered and improved using a structure-based rational design. Thus, numerous structurally diverse compounds with remarkable activity and selectivity for individual CDK families are currently available. These discoveries in diverse fields of biomedical research have elucidated the molecular basis of cell cycle control and shown how alterations to this homeostatic mechanism play an important role in cancer development [8, 27].

Accordingly, the current study was designed to find a novel CDK inhibitor with selective inhibitory activity towards CDK2. As such, CR229 was generated from a

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chemical library according to structure-based drug screening against the ATP-binding site of CDK2.

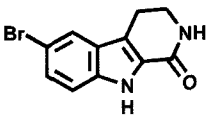
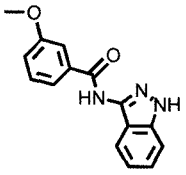
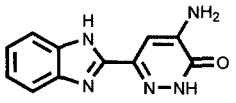
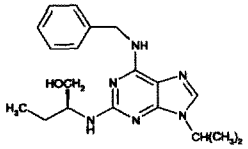
Generation of CR229, a New Scaffold Candidate

To construct a focused-chemical library for a target CDK inhibitor, structure-based drug screening technology was used, along with ligand-based pharmacophoric searches using the ISIS/3D scientific information management system (ISIS is available from MDL Information Systems, Inc., San Leandro, CA, U.S.A. In this research, the ISIS/Draw and ISIS/Base version 2.4, ISIS/Host version 2.5.1 for SGI IRIX was used), MDL Drug Data Report-3D (MDDR-3D), and Available Chemicals Directory-Screening Compounds (ACD-SC) database (MDL Information Systems, Inc., CA, U.S.A.).

A key concept for 3D searching is the pharmacophore. Thus, X-ray crystallographic data of known potent CDK inhibitors, such as roscovitine, oxindole, staurosporin, indirubin, quinazoline, and hymenialdisine were used as the model set, and then the 3D pharmacophore conformations of each known potent inhibitor were used to define the 3D queries and execute the 3D database searches [3].

As a result, 868 compounds with molecular masses of less than 350 Da were generated as novel scaffolds with similar 3D conformations to known potent CDK inhibitors from the ACD-SC database. Among these compounds, 708 compounds with skeletons or functional groups that were unacceptable for the development of lead compounds were omitted. Finally, 160 compounds were selected using this approach. These compounds were then purchased or chemically synthesized for screening in CDK2 assay at concentrations up to 100 μM.

Table 1. The chemical structure of preliminary hit-compounds and R-roscovitine as an authentic reference compound with IC_{50} values under 5 μM on CDK2.

Compound	Structure	CDK2 (IC_{50}, μM)
CR229		2.0
CR63		1.4
CR189		1.7–2.5
R-roscovitine		1.0

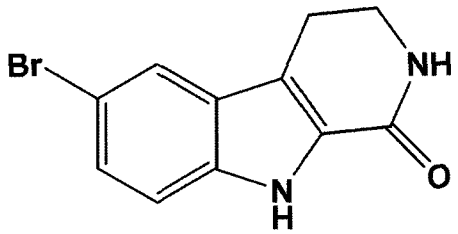


Fig. 1. Chemical structure of CR229.

As shown in Table 1, three compounds, including CR229 (Fig. 1), with IC_{50} values under 5 μM on CDK2 were identified as preliminary hit-compounds.

Generally, the NH group in Leu83 and carbonyl groups in Glu81 and Leu83 of CDK2 are considered as the most significant binding sources for inhibitors, as they serve as a hydrogen-bonding donor and acceptor, respectively, in every structure reported so far. Therefore, the information concerning these structural requirements was considered useful for generating a new class of CDK2 inhibitors. According to the binding mode studies of 3 compounds with CDK2 using the Global Range Molecular Matching (GRAMM) calculation [7, 26], CR229 (6-bromo-2,3,4,9-

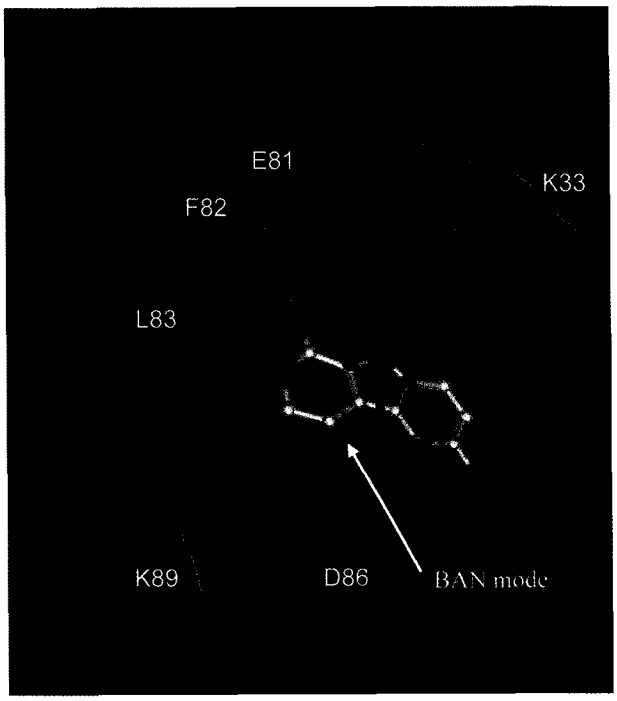


Fig. 2. Two predicted binding modes of CR229 in the CDK2 model.

Schematic representation of the conserved hydrogen bonds between the backbone atoms of the CDK2 residues Leu83 (L83) and Glu81 (E81), located in the hinge region, and CR229 (Green: Oxindole mode; Gray: Banyu mode). CR229 is rendered as ball and stick, and hydrogen bonds are drawn as yellow dotted lines.

Table 2. The selective inhibitory activity of CR229 on the various cellular kinases.

Kinases	IC_{50} (μM)
CDK1	4.9
CDK2	3.0
CDK4	3.0
GSK3β	>20
CK2-α1	>20
PKA	>20
PKC-α	>20

tetrahydro-carbolin-1-one), which showed the lowest estimated binding energy with CDK2 among the 3 compounds, was selected as the final hit-compound.

As shown in Fig. 2, the possible hydrogen bond formation between CR229 and CDK2 was predicted. According to the Oxindole binding mode [21], the amide group of CR229 interacted with the strand of protein that connects the two domains of CDK2, donating a hydrogen bond to the backbone carbonyl of Glu81 and accepting a hydrogen bond from the backbone NH of Leu83. However, when considering the binding mode according to the Banyu mode [6], the amide group of CR229 donated a hydrogen bond to the carbonyl of Leu83, and the carbonyl group of CR229 accepted a hydrogen bond from the backbone NH of Leu83 (Fig. 2).

Furthermore, to examine the selective inhibitory activity of CR229, simple kinetic assays against several kinases, including CDKs, isolated from HeLa cells were performed. As shown in Table 2, CR229 inhibited the activity of CDK1 (IC_{50}: 4.9 μM), CDK2 (IC_{50}: 3 μM), and CDK4 (IC_{50}: 3 μM), yet not that of other kinases, such as CK2-α1, PKA, and PKC (IC_{50}: >20 μM).

Unfortunately, the resulting compound, CR229, is not a novel compound. However, it is a compact and novel scaffold with many possibilities for further development, such as modification of the structure and the improvement of the pharmacological properties (Fig. 4). Accordingly, such studies are currently under way.

Inhibitory Effect of CR229 on Cell Cycle Regulatory Kinases

To verify the inhibitory activity of CR229 on the cell cycle regulatory kinases in HeLa cells, exogenous kinetic assays were performed using purified CDK1, 2, and 4. As shown in Fig. 3A, the exogenous kinetic assay demonstrated that CR229 inhibited the activity of CDK1, 2, and 4. However, in an endogenous kinetic assay using the cell lysate of HeLa cells pretreated with 1, 5, and 50 μM CR229 for 24 h, interestingly, only the CDK1 activity was reduced and the inhibitory effect on the CDK2- and CDK4-associated kinase activities was of a lower magnitude (Fig. 3B). Next, to confirm the inhibitory activity of CR229 on CDK1 in

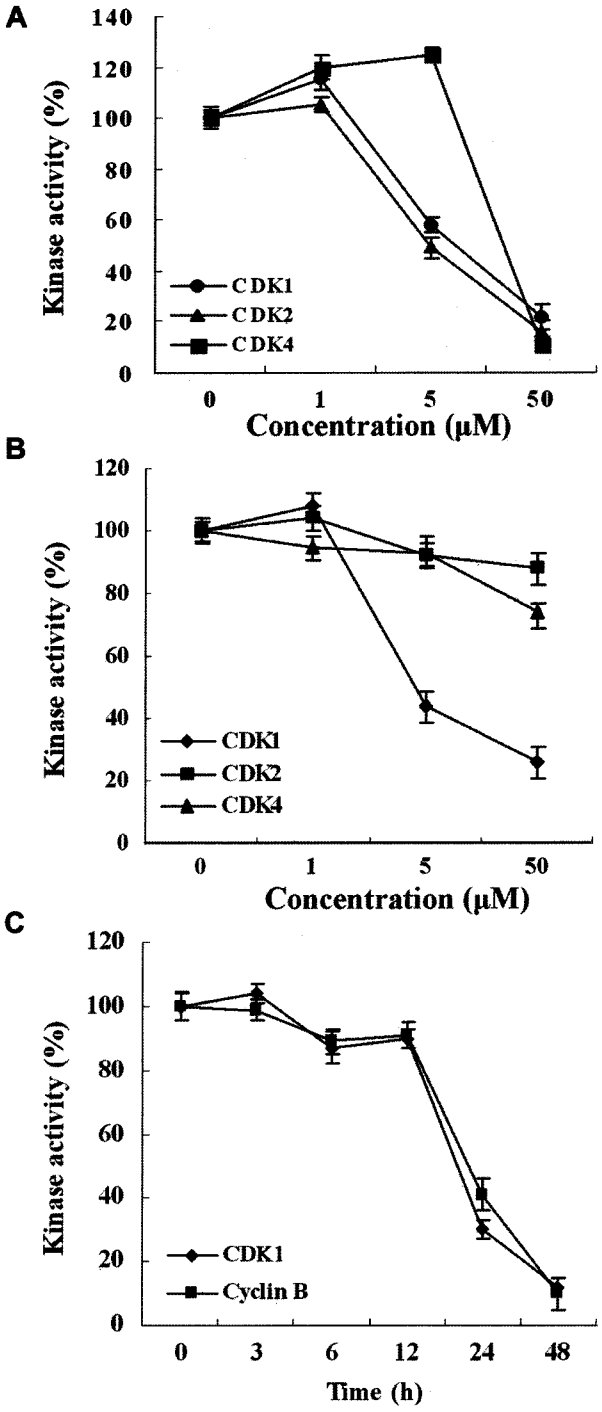


Fig. 3. Inhibitory effect of CR229 on exogenous and endogenous CDKs.

A. Exogenous kinetic assay: CDK immuno-complexes were prepared from HeLa cells and then incubated with varying doses of CR229. **B.** Endogenous kinetic assay: HeLa cells were treated with varying doses of CR229 and then CDK immuno-complexes were prepared to verify the inhibitory activity of CR229. **C.** CDK1 and cyclin B1 kinase activities after treatment with 2.5 μM CR229 for the indicated times. All the reaction mixtures were resolved by 12% SDS-PAGE and visualized by autoradiography. A densitometric analysis showed that CR229 inhibited the CDK activity in a dose-dependent manner. The data are reported as the mean±standard deviation of three independent experiments.

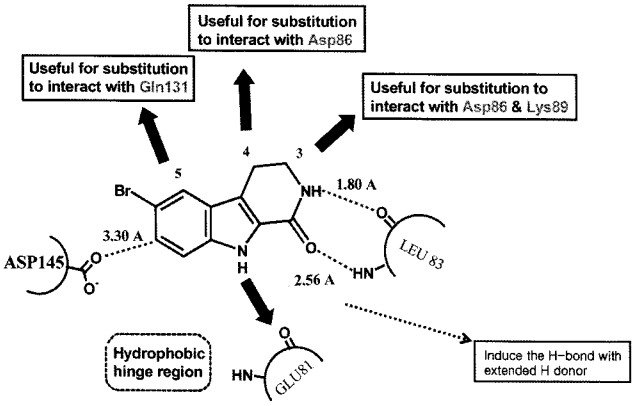


Fig. 4. The modification strategy of CR229.
Additional hydrogen bonds are needed to improve the *in vitro* potency of CR229.

detail, the cell lysate of HeLa cells treated with 2.5 μM CR229 for various incubation times was examined for CDK1 and cyclin B1 kinase activities. As shown in Fig. 3C, at 48 h after treatment, the CDK1 and cyclin B1 kinase activities were almost completely inhibited, showing that CR229 is a potent inhibitor of CDK1 at the cellular level, with an IC_{50} value of 4.8 μM.

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