Discovery and Initial Characterization of the Paullones, a Novel Class of Small-Molecule Inhibitors of Cyclin-dependent Kinases

Daniel W. Zaharevitz, Rick Gussio, Maryse Leost, Adrian M. Senderowicz, Tyler Lahnusen, Conrad Kunick, Laurent Meijer, and Edward A. Saussville

Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, Maryland 20892-7444 [D. W. Z., R. G., A. M. S., T. L., E. A. S.]; Cell Cycle Group, Centre National de la Recherche Scientifique, 29680 Roscoff, Bretagne, France [L. M., M. L.]; and Institute für Pharmazie, Universität Hamburg, D20146 Hamburg, Germany [C. K.]

Abstract

Analysis of the National Cancer Institute Human Tumor Cell Line Anti-Cancer Drug Screen data using the COMPARE algorithm to detect similarities in the pattern of compound action to flavopiridol, a known inhibitor of cyclin-dependent kinases (CDKs), has suggested several possible novel CDK inhibitors. 9-Bromo-7,12-dihydro-indolo[3,2-d]benzazepin-6(5H)-one, NSC-664704 (kenpaullone), is reported here to be a potent inhibitor of CDK1/cyclin B (IC50, 0.4 μM). This compound also inhibited CDK2/cyclin A (IC50, 6.8 μM), CDK2/cyclin E (IC50, 7.5 μM), and CDK5/p25 (IC50, 0.85 μM) but had much less effect on other kinases; only c-src (IC50, 15 μM), casein kinase 2 (IC50, 20 μM), erk 1 (IC50, 20 μM), and erk 2 (IC50, 9 μM) were inhibited with IC50s less than 35 μM. Kenpaullone acts by competitive inhibition of ATP binding. Molecular modeling indicates that kenpaullone can bind in the ATP binding site of CDK2 with residue contacts similar to those observed in the crystal structures of other CDK2-bound inhibitors. Analogues of kenpaullone, in particular 10-bromopaullone (NSC-672234), also inhibited various protein kinases including CDKs. Cells exposed to kenpaullone and 10-bromopaullone display delayed cell cycle progression. Kenpaullone represents a novel chemotype for compounds that preferentially inhibit CDKs.

Introduction

The CDKs are a family of protein kinases the activity of which has been shown to be required for initiation and traverse of specific phases of the cell cycle as well as regulation of transcription (reviewed in Refs. 1–3). An active CDK consists of a catalytic subunit (CDK1–CDK9) and a regulatory subunit (cyclin A–cyclin J and cyclin T). The activity of CDKs is regulated by a variety of mechanisms including cyclin expression, phosphorylation, dephosphorylation, binding of negative regulatory proteins (e.g., p16INK4, p21CIp1, and p27KIP1), and necessity for correct alignment with subcellular organelles or proteins. Deregulation of CDK activity has been documented in a number of human primary tumors and tumor cell lines (reviewed in Ref. 4). Inhibition of CDK activity, therefore, represents a logical target for the development of drugs that may be useful in the treatment of cancer and other proliferative diseases. Several compounds that preferentially inhibit CDKs have been identified (reviewed in Refs. 5 and 6), including several purines (7) and flavopiridol, which has entered clinical trials (8), but a thorough evaluation of the biochemical and therapeutic applications of CDK inhibitors would usefully employ a variety of chemotypes.

Previous work has demonstrated that the COMPARE algorithm (9) is a useful computerized pattern recognition tool to define novel chemotypes that can act with a cellular mechanism or biochemical target in a manner similar to that of a reference or “seed” compound. COMPARE examines the pattern of antiproliferative activity in the NCI Human Tumor Cell Line Anti-Cancer Drug Screen of the reference compound and compares it with other compounds tested in the screen. Data from about 35,000 nonproprietary compounds and ~72,000 total compounds form the database for this comparison. This methodology has been successfully used to associate a number of novel chemotypes with important potential antiproliferative targets, including inhibitors of tubulin polymerization (10), inosine monophosphate (IMP) dehydrogenase (11), dihydroororate dehydrogenase (12), and topoisomerase I (13). Flavopiridol (NSC 649890), a known inhibitor of CDKs (14–16), has demonstrated a potent inhibition of cell growth and displays a unique pattern in the NCI Human Tumor Cell Line Anti-Cancer Drug Screen. We have used COMPARE with flavopiridol as a reference to search the database of compounds tested in the NCI Screen, and we report here that this search has yielded a specific inhibitor of CDKs with a novel chemotype.

Materials and Methods

COMPARE. The COMPARE algorithm was used as described (9). Non-confidential compounds tested before December 1996 in the NCI human tumor cell line screen were searched. Both the screening data and structural data for this database (December '98 Release) are available to the public from the DTP web site (http://dtp.nci.nih.gov/). COMPARE is also available on the DTP web site.4

Enzyme Assays. Initial screening was performed using starfish oocyte CDK1/cyclin B purified by affinity on immobilized p9CKShs1 as described previously (7). The kinase assay was run for 10 min at 30°C with 1 mg/ml histone H1 (Sigma type III-S), in the presence of 15 μM [γ-32P]ATP (3000 Ci/mmol; 1 mCi/ml) in a final volume of 30 μl. Purification and assays for inhibition of other kinases were performed as described (7). In kinetic experiments, the histone H1 concentration was lowered to 3.5 mg/ml; the ATP concentration ranged from 50 to 400 μM, and the kenpaullone concentration ranged from 1 to 4 μM.

4 To run the COMPARE calculation described here: (a) point your web browser to http://dtp.nci.nih.gov/; (b) click on “Search” on the menu bar at the top; (c) under “Search Databases of Compounds Tested in the AntiCancer Screen” click on “By NSC Number”; (d) enter “649890” in the text field and click on “Submit Query”; (e) in the row with the Log(High Concentration) of f enter “649890” in the text field and click on “Submit Query.” Note that the results presented here describe the calculation run on the December '98 data release; different data releases may change the results somewhat. Any questions or problems can be emailed to zaharevitz@dtpax2.ncifcrf.gov.
Cell Cycle Analysis. MCF10 A cells (obtained from David Solomon, NIH) were plated in 100-mm dishes after trypsinizing. After allowing the cells to attach for 12 h, they were then treated with varying concentrations of drugs for 24 h. In another set of plates, the medium was removed, and the cells were washed with PBS. Serum-free medium was added for 24 h, and then serum-containing medium and drug were added to the cells for 20 h. The cells were harvested by trypsinizing and washed once with PBS. The cells were then suspended in 1 ml of PBS and fixed with 4 ml of 100% ethanol while vortexing. The cells were then placed at −20°C for 24 h. For cell cycle analysis, the ethanol was removed, and the cells were washed once with PBS. The RNA was digested with 1 μg/ml of RNase, and cells were stained with 50 μg/ml of propidium iodide. Data acquisition and analysis was completed on a Becton Dickinson FACSCalibur using Modfit software.

Molecular Modeling. The structure of kenpaullone was first modeled using semiempirical quantum mechanical calculations using the PM3 Hamiltonian of MND094 in the Unichem software package (Oxford Molecular Group, Inc.). Molecular mechanics potentials were assigned so that geometry optimization in Discover (MSI, Inc.) with molecular mechanics energy minimization resulted in a structure that closely matched the semiempirically optimized structure (RMS deviation, 0.157). The resulting structure was docked into the CDK2 ATP binding site using the Insight II molecular modeling software (MSI, Inc.). Coordinates for the protein were taken from crystal structures published previously (17, 18). Constrained molecular mechanics energy minimization (cf91 force field) of the inhibitor-protein complex was performed, and the minimized structure was subjected to hydroscopic analysis using the program HINT (eduSoft, Richmond, VA). Small adjustments in the sidechain torsion angles and inhibitor positioning were made to resolve unfavorable hydrophobic interactions, and molecular mechanics energy minimization was again performed. The cycle was repeated until molecular mechanics and hydrophobic analysis both indicated good complementarity between inhibitor and protein.

Results and Discussion

Using the pattern of activity of flavopiridol (tested at a high concentration of 1 μM) as a seed, the COMPARE algorithm finds 11 compounds6 with a correlation of >0.60 in the database examined. In past work (9–13), a correlation of 0.6 has been found to be a useful cutoff for distinguishing compounds that have some likelihood of sharing the mechanism or biochemical target of the reference compound from compounds that are less likely to act by that mechanism. The highest correlation in the flavopiridol COMPARE was 0.67 for olomoucine (NSC 666096), a purine analogue that has been reported to be a specific inhibitor of CDKs (7). Five of the other compounds with a correlation coefficient >0.60 (NSC 63191, 658082, 658086, 673347, and 685828) did not have sufficient sample available for testing. Five compounds (NSC 85561, 649311, 651704, 657589, and 664704) were tested for their ability to inhibit CDK1/cyclin B. NSC 649311 was inactive up to 1 mM, NSC 85561 was inactive at 10 μM, whereas NSC 651704 (IC50 120 μM) and NSC 657589 (IC50 180 μM) were weak inhibitors. By far the most potent inhibitor was NSC-664704 (Fig. 1) with an IC50 of 0.4 μM. This compound, 9-bromo-7,12-dihydro-indolo[3,2-b][1]benzazepin-6(5H)-one, has been synthesized previously (19), and we now name it kenpaullone.6 The IC50 for CDK1/cyclin B inhibition can be compared with IC50S reported in this system for flavopiridol (NSC-649890; 0.3 μM), olomoucine (NSC-666096; 7.0 μM), roscovitine (0.65 μM), butyrolactone I (0.6 μM); (Refs. 5 and 6), and purvalol A (0.004 μM; Ref. 20). Several other analogues were available and tested (Figs. 1 and 2A), and all showed at least some ability to inhibit CDK1/cyclin B. Kinetic studies showed that kenpaullone acts by competitive inhibition of ATP binding (Fig. 2B). The apparent KI was 2.5 μM. The ability of kenpaullone and the 10-bromo analogue to inhibit a broader range of protein kinases was evaluated (Table 1). Various Ser/Thr and Tyr kinases were expressed and/or purified and assayed with appropriate substrates as described previously (7) in the presence of 15 μM ATP and increasing concentrations of the paullones. IC50S are presented in Table 1. Most of the 25 kinases tested were not inhibited. A strong preference for CDK1/CDK2/CDK5 over CDK4 was observed, unlike flavopiridol, which is equipotent for all CDKs tested (14–16). This specificity is similar to olomoucine and roscovitine (7). The change from 9-bromo to kenpaullone (NSC-664704) to 10-bromo (NSC-672234) leads to a reduction in kinase specificity, with a potent inhibition now observed in several protein kinase C isozymes and casein kinase 2.

Kenpaullone (mean GI50, 42 μM), NSC-672234 (mean GI50, 14 μM), and NSC-641166 (mean GI50, 33 μM) has easily measured antiproliferative activity in the human tumor cell line screen. Although NSC-672234 was somewhat more potent overall than kenpaullone, its pattern of activity was substantially different (correlation coefficients of 0.33 with kenpaullone, 0.16 with flavopiridol, and 0.13 with olomoucine). Whether the change in pattern is related to the change in kinase specificity remains to be investigated. Despite relatively strong inhibition of CDK1/cyclin B, NSC-672232 only inhibited the growth of two cell lines (HCT-116 and LOX IMVI). NSC-672233 and NSC-641167 were inactive in the cell screen at 100 μM. To clarify the potential of NSCs 664704 and 672234 to alter cell cycle expression, exponentially growing MCF10A cells were exposed to 30 μM of each compound for 24 h. NSC 664704 caused at best a slight increase in S-phase fraction (25–32%), whereas NSC 672234 showed a clear decrease in S-phase fractions (25–11%; Fig. 3A). After serum starvation, which results in virtually complete loss of S+G2 phase cells (Fig. 3B, top left), addition of serum results after 20 h in the predominant fraction of the cell population to be in S or G2 (Fig. 3B, top right); however, in the presence of serum plus NSC 664704 or

---

6 Structural and screening data for all compounds listed by NSC number can be obtained on the DTP web site. See the instructions in footnote 4 and substitute the NSC(s) of interest for 649890 in step d.

6 We propose the name paullone for the unsubstituted compound and kenpaullone for the 9-bromo analogue to honor the memory of Dr. Kenneth Paull, inventor of the COMPARE algorithm, whose insight, wisdom, and generosity greatly influenced not only this particular work but the whole field of cancer drug discovery.
NSC 672234, there is substantial retardation in progression through S phase (Fig. 3B, bottom left and right).

Kenpaullone is clearly structurally different than previously described CDK inhibitors (5, 6). Like previous inhibitors, it is competitive with respect to ATP binding, and it does have features (hydrogen bonding atoms and aromatic rings) that suggest it could bind in the CDK ATP binding site. Using coordinates from crystal structures published previously of CDK2 with bound inhibitor (17, 18), we have developed a molecular model of kenpaullone binding to the ATP site of CDK2 (Fig. 4). The model shows a binding mode for kenpaullone that is broadly similar to the binding mode observed in crystal structures of other CDK inhibitors. Major contacts include hydrogen bonds to both the backbone carbonyl and amide of Leu-83 and positioning of ring atoms between the Leu-134 and Ile-10 hydrophobic sidechains, features observed in the crystal structures of olomoucine (17), roscovitine (18), and purvalanol B (20). The D ring in kenpaullone occupies a hydrophobic pocket formed by mainly by Phe-80, Val-18, Ala-144, and the hydrocarbon part of Lys-33 and thus serves a function similar to the N9 isopropyl group in roscovitine (18) and purvalanol B (20).

Kenpaullone, according to this model, occupies very little of the pocket volume where the ribose and phosphate groups of ATP would be, suggesting that it may be useful to design analogues that can extend into these regions. A more complete description of this model, as well as coordinates, is available online at http://dtp.nci/nih.gov/Docs/Branches/ltb/tsddg/paullones/paullone.html.

Kenpaullone represents a novel chemotype for compounds that preferentially inhibit CDKs. Like olomoucine (7) and roscovitine (7), kenpaullone can inhibit CDK1, CDK2, and CDK5 but has little effect on CDK4. Kenpaullone can inhibit the growth of tumor cells in culture (mean GI50, 4 ± 3 μM) and causes altered cell cycle progression most clearly revealed under conditions of recovery from serum starvation. Like olomoucine (mean GI50, 51 μM) and roscovitine (mean GI50, 18 μM), it is substantially less potent than flavopiridol (mean

---

**Table 1** Inhibition of selected protein kinases by kenpaullone and 10-bromo-paullone

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>Kenpaullone (NSC 664704)</th>
<th>10-Bromo-paullone (NSC 672234)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdk1/cyclin B</td>
<td>0.4 ± 0.4 μM</td>
<td>1.3 μM</td>
</tr>
<tr>
<td>cdk2/cyclin A</td>
<td>0.68 ± 0.1 μM</td>
<td>3.0 μM</td>
</tr>
<tr>
<td>cdk2/cyclin E</td>
<td>7.5 ± 0.1 μM</td>
<td>4.0 μM</td>
</tr>
<tr>
<td>cdk4/cyclin D1</td>
<td>&gt;100 ± 0.1 μM</td>
<td>n.t.</td>
</tr>
<tr>
<td>cdk5p35</td>
<td>0.85 ± 0.1 μM</td>
<td>2.7 μM</td>
</tr>
<tr>
<td>erk1</td>
<td>20 ± 0.1 μM</td>
<td>88 μM</td>
</tr>
<tr>
<td>erk2</td>
<td>9 ± 0.1 μM</td>
<td>100 μM</td>
</tr>
<tr>
<td>c-ras</td>
<td>38 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>MAPKK</td>
<td>&gt;100 ± 0.1 μM</td>
<td>n.t.</td>
</tr>
<tr>
<td>c-Jun NH2-terminal kinase</td>
<td>&gt;100 ± 0.1 μM</td>
<td>n.t.</td>
</tr>
<tr>
<td>Protein kinase C α</td>
<td>&gt;100 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>Protein kinase C β1</td>
<td>&gt;100 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>Protein kinase C β2</td>
<td>&gt;100 ± 0.1 μM</td>
<td>0.8 μM</td>
</tr>
<tr>
<td>Protein kinase C γ</td>
<td>&gt;100 ± 0.1 μM</td>
<td>4.4 μM</td>
</tr>
<tr>
<td>Protein kinase C δ</td>
<td>&gt;100 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>Protein kinase C ε</td>
<td>&gt;100 ± 0.1 μM</td>
<td>0.75 μM</td>
</tr>
<tr>
<td>Protein kinase C η</td>
<td>&gt;100 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>Protein kinase C ι</td>
<td>&gt;100 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>Protein kinase C τ</td>
<td>&gt;100 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>cAMP-dependent PK</td>
<td>&gt;1000 ± 0.1 μM</td>
<td>&gt;1000 μM</td>
</tr>
<tr>
<td>cGMP-dependent PK</td>
<td>&gt;1000 ± 0.1 μM</td>
<td>&gt;1000 μM</td>
</tr>
<tr>
<td>Caspase kinase 1</td>
<td>&gt;100 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>Caspase kinase 2</td>
<td>20 ± 0.1 μM</td>
<td>0.8 μM</td>
</tr>
<tr>
<td>Insulin receptor tyrosine kinase</td>
<td>&gt;1000 ± 0.1 μM</td>
<td>&gt;100 μM</td>
</tr>
<tr>
<td>c-src</td>
<td>15 ± 0.1 μM</td>
<td>n.t.</td>
</tr>
<tr>
<td>v-abl</td>
<td>350 ± 0.1 μM</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

*a* Value reported is IC50 (μM).

*b* n.t., not tested.

---

Fig. 2. Inhibition of CDK1/cyclin B by paullones. **A,** dose-response curves for several paullones. **B,** kinetic analysis of inhibition by kenpaullone.

Fig. 3. Effect of kenpaullone and 10-bromo-paullone on cell cycle progression. **A,** exponentially growing MCF10A cells were exposed to 30 μM each of vehicle control, kenpaullone, or 10-bromo-paullone for 24 h, and cell cycle distribution was determined. **B,** MCF10A cells were serum starved for 24 h, and cell cycle distribution was obtained (top left), or refed with medium containing serum (top right) or serum plus kenpaullone (bottom left) or 10-bromo-paullone (bottom right) for 20 h before determining cell cycle distribution.

Kenpaullone, according to this model, occupies very little of the pocket volume where the ribose and phosphate groups of ATP would be, suggesting that it may be useful to design analogues that can extend into these regions. A more complete description of this model, as well as coordinates, is available online at http://dtp.nci/nih.gov/Docs/Branches/ltb/tsddg/paullones/paullone.html.

Kenpaullone represents a novel chemotype for compounds that preferentially inhibit CDKs. Like olomoucine (7) and roscovitine (7), kenpaullone can inhibit CDK1, CDK2, and CDK5 but has little effect on CDK4. Kenpaullone can inhibit the growth of tumor cells in culture (mean GI50, 43 μM) and causes altered cell cycle progression most clearly revealed under conditions of recovery from serum starvation. Like olomoucine (mean GI50, 51 μM) and roscovitine (mean GI50, 18 μM), it is substantially less potent than flavopiridol (mean...
Acknowledgments

We thank the fishermen of the “Station Biologique de Roscoff” for collecting starfish. We thank A. Link for resynthesis of several compounds. We are grateful to our following colleagues for providing reagents and purified enzymes: D. Alessi, M. Cobb, W. Harper, F. Hoffmann, S. Lohman, H. Mett, D. Morgan, and L. Pina.

GL50 0.066 μM in inhibiting tumor cell growth in culture. Whether this difference in potency is due to the difference in the ability of the compounds to inhibit CDK4 or differences in other factors such as cellular uptake remains to be investigated. Kenpaullone can clearly serve as a lead structure for building molecules that might have more potent activity as antiproliferative agents while retaining this spectrum of kinase activity. The fact that a simple change of the 9-bromo to a 10-bromo results in a different kinase specificity suggests that the paullone skeleton will be useful not only as a starting point for new CDK inhibitors but also as a tool for exploring the structural basis and pharmacological significance of various kinase specificities.

Acknowledgments

We thank the fishermen of the “Station Biologique de Roscoff” for collecting starfish. We thank A. Link for resynthesis of several compounds. We are grateful to our following colleagues for providing reagents and purified enzymes: D. Alessi, M. Cobb, W. Harper, F. Hoffmann, S. Lohman, H. Mett, D. Morgan, and L. Pina.

References

Discovery and Initial Characterization of the Paullones, a Novel Class of Small-Molecule Inhibitors of Cyclin-dependent Kinases


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/11/2566

Cited articles
This article cites 18 articles, 5 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/11/2566.full.html#ref-list-1

Citing articles
This article has been cited by 17 HighWire-hosted articles. Access the articles at:
/content/59/11/2566.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.