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Exploiting Chemical Libraries, Structure, and Genomics in the Search for Kinase Inhibitors

Nathanael S. Gray, Lisa Wodicka, Andy-Mark W. H. Thunnissen, Thea C. Norman, Soojin Kwon, F. Hernan Espinoza, David O. Morgan, Georjana Barnes, Sophie LeClerc, Laurent Meijer, Sung-Hou Kim, David J. Lockhart, and Peter G. Schultz

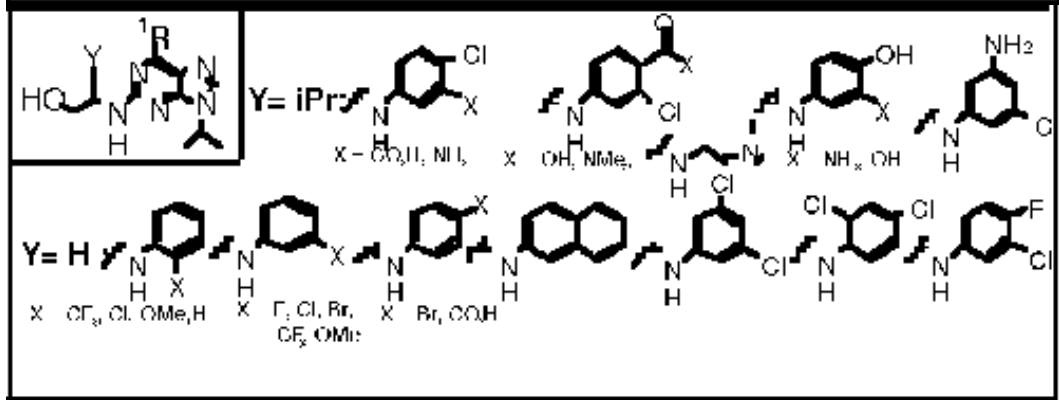
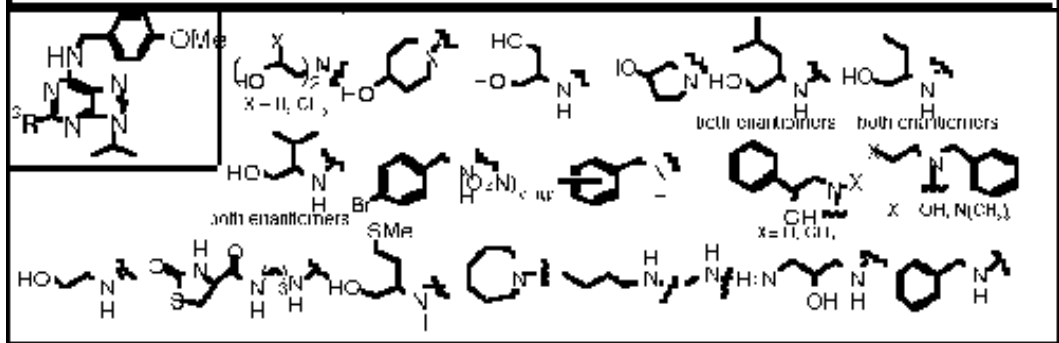
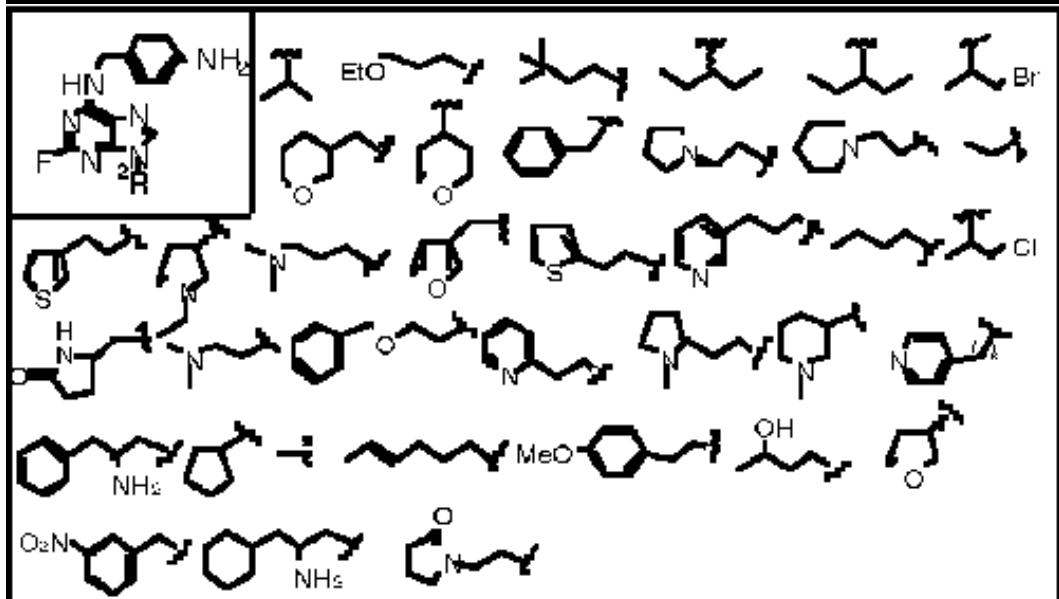
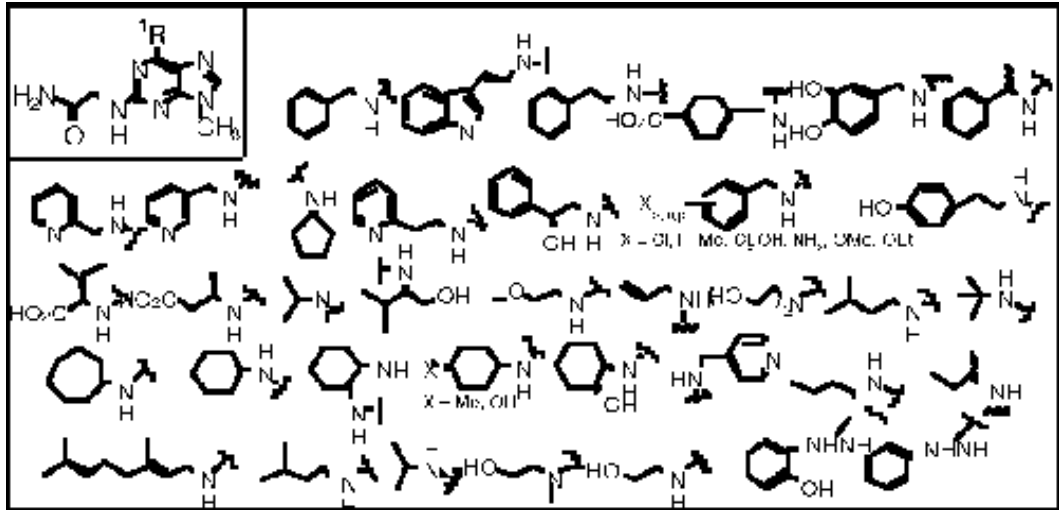
Supplementary Material

Kinase assays. Inhibitor (20 μ l, 15% DMSO in H₂O) was introduced to a solution containing CAK-activated CDK2/cyclinA (20 μ l, 0.3 mg/ml, 80 mM Tris, pH 7.2, 40 mM MgCl₂) in a 96-well microtiter array. The kinase reaction was initiated by the addition of substrate histone H1, ATP mixture (20 μ l, 0.22 mg/mL histone H1, 10 mM HEPES, pH 7.2, 45 μ M ATP, 150 μ g/ml BSA, 1.5 mM DTT, 0.1 vol % [γ -³²P]ATP, 10 μ Ci/mL). After 30 minutes, the reaction mixtures were transferred to a 96-well dot-blot apparatus and quenched by the addition of 35 μ l of 10% TCA. The phosphorylated histone H1 was immobilized onto a nitrocellulose membrane, washed with 10% TCA followed by H₂O, and quantitated by densitometry on a phosphoimager. Starfish *cdc2/cyclinB* (which is the major source for isolating this kinase) was purified by affinity chromatography as described [J. Vesely *et al.*, *Eur. J. Biochem.* **224**, 771 (1994)]. Kinase assays were performed in the presence of 1 mg/ml histone H1 (Sigma type III-S), 15 μ M [γ -³²P]ATP (1 mCi/ml), in a final volume of 30 μ L. After 10 minutes at 30°C, 25- μ l aliquots were spotted onto 2.5 cm x 3 cm pieces of phosphocellulose (Whatman P81), and after 20 s the filters were washed five times with dilute acid (1 ml phosphoric acid/100 ml H₂O). The filters were transferred into 2 ml of ACS (Amersham) scintillation fluid and counted.

Immunoprecipitations and kinase assays. Cells were grown to mid-log phase in rich media. Frozen cell pellets (~120 OD₆₀₀) were resuspended in lysis buffer (25 mM HEPES-NaOH pH 7.5, 250 mM NaCl, 0.2% Triton X-100, 5 mM β -glycerolphosphate, 5 mM NaF, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 10 mTIU/ml aprotinin, 1 mM PMSF) and lysed by mechanical disruption in a Beadbeater (Biospec). Lysates were clarified by centrifugation for 15 minutes at 14,000g and protein content assessed with the BioRad protein assay method (BioRad). A total of 400 μ l of pA:sepharose (Sigma) and 40 μ g of mAb12CA5 (BAbCo), which binds to the HA epitope present at the COOH-termini of the CDKs, were added to 20 mg of crude lysate and rotated for 1 hour at 4°C. The beads were then washed twice with HBST (10 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 0.2% Triton X-100) and twice with HBS (10 mM HEPES pH 7.5, 150 mM NaCl) and then distributed evenly into 40 tubes. Next, 1 μ l of inhibitor (1 μ M to 10 mM) or DMSO was added to 20 μ l of kinase assay buffer (10 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 20 μ M ATP, 2 μ Ci [γ -³²P]ATP), which included 5 μ g of histone H1 for Cdc28 assays, 2 μ g of Pho4 for PHO85 assays, or 2.5 μ g of GST-CTD for Srb10 and Kin28 assays. This mixture was added to the washed beads, incubated for 15 minutes at room temperature, and stopped by the addition of 4x stop buffer [250 mM Tris pH 6.8, 40% (v/v) glycerol, 20% (v/v) β -mercaptoethanol, 9.2% SDS, 0.04% bromophenol blue]. Reaction products were analyzed by SDS-PAGE. Incorporation of ³²P into substrates was quantified by excising the substrate from dried gels and measuring Cerenkov radiation in a scintillation counter.

Yeast strains. To epitope tag yeast CDKs, a W303 strain (MATa *ura3-52 leu2-3112 trp1-1 his3-11 ade2-1 can1-100*) was transformed with an integrating plasmid [P. Hieter, R.S. Sikorski, *Genetics*, **122**, 19 (1989)] containing a 5' fragment of a yeast CDK fused to a hemagglutinin tag (underlined) followed by the *ACT1* terminator. YlpFHE83 contained a 744-bp fragment of the *CDC28* coding region and altered the COOH-terminus of Cdc28 from QES to QESMAYPYDVPDYASLGPGP. YlpJC01 contained a 866-bp fragment from the *KIN28* coding region and altered the COOH-terminus of Kin28 from IRN to IRTMAYPYDVPDYASLGPGP. YlpFHE48 contained a 667-bp fragment of the *PHO85* coding region and altered the COOH-terminus of Pho85 from HAS to HASMAYPYDVPDYASLGPGP. YlpFHE101 contained a 1097-bp fragment from the *SRB10* coding region and altered the COOH-terminus of Srb10 from NRR to NRTMAYPYDVPDYASLGPGP.

Library design. The following table indicates the structures of representative compounds used in this study. These compounds were synthesized on solid support (using either polystyrene resin or Chiron pins [H.M. Geysen *et al.*, *Immunol. Methods* **102**, 259 (1987)] or in solution. The solid phase chemistry has been described [N. S. Gray *et al.*, *Tetrahedron Lett.* **38**, 1161 (1997); T. C. Norman *et al.*, *J. Am. Chem. Soc.* **118**, 7430 (1996)] and the solution phase chemistry will be described elsewhere.



Crystallization and structure determination. Human CDK2 was purified and crystallized as previously described [J. Rosenblatt *et al.*, *J. Mol. Biol.* **230**, 1317 (1993)]. Crystals were soaked with purvalanol B in a solution containing 1% DMSO and 5% ethyleneglycol, necessary to solubilize the compound. A procedure involving chemical crosslinking was employed to prevent crystals from cracking. The crystals were first soaked in a solution containing 0.5 mM ATP, 1 mM MgCl₂ for 2 hours, then crosslinked with 0.1% glutaraldehyde for 1 hour at 4°C. After extensive washing the crystals were transferred to an inhibitor solution in 200 mM HEPES, 5% ethyleneglycol and 1% DMSO. This procedure allowed crystals to be soaked at inhibitor concentrations up to 0.2 mM for several days without showing any damage. X-ray data collection was carried out on an R-Axis II image plate detection system, mounted on a Rigaku rotation-anode generator. Data were collected under a stream of cold nitrogen (100 K) from a single crystal in 25% ethyleneglycol. The CDK2-purvalanol B crystals diffracted as well as native, although freezing altered slightly the unit cell dimensions and increased the mosaic spread from 0.2° to 0.6°. The crosslinking itself did not alter the diffraction characteristics significantly. Intensity data were processed with the programs DENZO and SCALEPACK [Z. Otwinowski and W. Minor, *Methods Enzymol.* **276**, 307 (1997)]. The program TRUNCATE, as implemented in the CCP4 suite [*Acta Crystallogr.* **D50**, 760 (1994)], was used to obtain the final set of structure factor amplitudes. Refinement of the CDK2-purvalanol B complex was started from the coordinates of the highly refined CDK2-ATP model. All refinement steps were carried out with the program X-PLOR (A. T. Brünger, Yale Univ. Press, Version 3.0, 1991). Molecular replacement followed by rigid body refinement was necessary to successfully reorient and reposition the CDK2 molecule in the unit cell of the frozen crystal. The CDK2 model was further refined by several rounds of conjugated-gradient energy minimization. At this stage the electron density corresponding to purvalanol B was clearly visible from $2F_o - F_c$ and $F_o - F_c$ Fourier maps and the inhibitor could be added to the model. Several rounds of both x-ray restrained energy minimization and molecular dynamics in the resolution range 7 to 2.05 Å, alternated with model building with the program O [T. A. Jones and M. Kjeldgaard, *Acta Crystallogr.* **A47**, 110 (1991)], were necessary to improve the model. In the last rounds of refinement low resolution data were included by applying a bulk solvent correction [J. S. Jiang and A. T. Brünger, *J. Mol. Biol.* **243**, 100 (1994)]. At this point a simulated annealing omit map [A. Hodel *et al.*, *Acta Crystallogr.* **A48**, 851 (1992)] of the inhibitor binding site indicated that a minor portion of the purvalanol B molecules was bound with its aniline ring flipped ~160° (based on electron density for the 3-chloroanilino atom). The double conformation of purvalanol B was included in the refinement, lowering the R_{free} by 0.5%. The final model includes 279 residues of CDK2 (residues 36-43 and 153-163 are not included because of weak or missing electron density), purvalanol B, 91 water molecules, and one molecule of ethyleneglycol. Contact surfaces were calculated with program MS [*J. Appl. Crystallogr.* **16**, 548 (1983)] by using standard VDW's radii and a probe radius of 1.7 Å.