A Novel Class of Cyclin-dependent Kinase Inhibitors Identified by Molecular Docking Act through a Unique Mechanism*§

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The cyclin-dependent kinase (Cdk) family is emerging as an important therapeutic target in the treatment of cancer. Cdns 1, 2, 4, and 6 are the key members that regulate the cell cycle, as opposed to Cdns that control processes such as transcription (Cdk7 and Cdk9). For this reason, Cdns 1, 2, 4, and 6 have been the subject of extensive cell cycle-related research, and consequently many inhibitors have been developed to target these proteins. However, the compounds that comprise the current list of Cdk inhibitors are largely ATP-competitive. Here we report the identification of a novel structural pocket on Cdk2 that is conserved on Cdns 1, 4, and 6. Using a screen of this in silico screen of this pocket exhibit cytostatic effects and act by reducing the apparent protein levels of cell cycle Cdns. Drug-induced cell cycle arrest is associated with decreased Rb phosphorylation and decreased expression of E2F-dependent genes. Multiple lines of evidence indicate that the primary mechanism of action of these compounds is the direct induction of Cdk1, Cdk2, and Cdk4 protein aggregation.

Uncontrolled cell proliferation is one of the defining features of cancer. Cdns are serine/threonine protein kinases that play key roles in controlling cell cycle progression (1, 2). The concerted activities of Cdns result in chromatin condensation, nuclear envelope breakdown, and the up-regulation of genes involved in nucleotide synthesis and DNA replication, among other events. Cdns have long been considered ideal targets for anti-cancer drugs, owing to their importance in the cell cycle. As a result, many Cdk inhibitors have been developed, some of which have progressed to clinical trials. Roscovitine (Selicilib) and flavopiridol (Alvocidib) are examples of Cdk inhibitors that have passed Phase I clinical trials (3, 4) and have entered Phase II clinical trials (6, 7).4 However, these drugs, as well as most other Cdk inhibitors, are ATP-competitive. The disadvantage of using a therapeutic strategy involving ATP competition is that all kinases possess an ATP-binding site, leading to the potential for reduced target specificity. Recently, advances have been made in identifying Cdk inhibitors that act through novel mechanisms. One example of this effort is the identification of a series of cyclic peptides designed to mimic the structure of the Cdk inhibitor p27. These compounds bind to the substrate recognition site of Cdk complexes and inhibit their kinase activity in vitro (8). Another example is the use of a small, 39-amino acid peptide that inhibits the kinase activity of Cdk2 by mimicking the inhibitory effects of the pRb2/p130 spacer domain (9). These approaches are promising, but rely on peptide-based inhibitors that have inherent disadvantages for use as therapeutic agents.

The use of knockout mice has recently generated much information about the role of Cdns with respect to cell cycle regulation. For instance, mice lacking Cdk2, Cdk4, or Cdk6 are all viable (10–12). Furthermore, cells lacking both Cdk4 and Cdk6 proliferate almost normally (13). More recently it has been discovered that mouse embryonic fibroblast cells are able to cycle in the absence of Cdk2, Cdk4, and Cdk6, needing only Cdk1 to complete cell division (14). In light of the fact that Cdns are able to functionally replace one another, highly selective Cdk inhibitors that target only one type of cell cycle Cdk may not be as effective as anti-tumor agents that inhibit Cdk1, Cdk2, Cdk4, and Cdk6. An inhibitor that acts on multiple cell cycle Cdns would therefore have a greater probability of inhibiting tumor cell growth by ensuring that the cell cycle is arrested. Here we report the identification of a novel structural pocket present on Cdk2 that is conserved on Cdns 1, 4, and 6. Using a high throughput in silico screening procedure we have identified compounds that decrease the function of Cdns in cells through binding to this site.

**EXPERIMENTAL PROCEDURES**

Molecular Docking—The two protein crystal structures used for identification and in silico screening of the structural site

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‖ The abbreviations used are: Cdk, Cyclin-dependent kinase; GFP, green fluorescent protein; NSC, Nomenclature Standards Committee; DTP, Developmental Therapeutics Program; Rb, retinoblastoma; PBS, phosphate-buffered saline; ErK1/2, extracellular signal-regulated kinase 1 and 2.
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were the Cyclin A-Cdk2 and p27kip1. Cyclin A-Cdk2 complexes (RCSB Protein Data Bank codes: 1FIN (15) and 1JSU (16), respectively). A molecular surface of 1JSU was prepared using the MSROLL program, which was then used as input for the sphere-generating program SPHGEN. A cluster of spheres that was shown to be within the pocket of interest was then selected and edited manually to leave a cluster of 21 spheres. The SHOWBOX program was used to construct a three-dimensional rectangle, 4 Å in every direction from the sphere cluster. The program CHIMERA was used to convert the PDB file of 1JSU into the appropriate mol2 format. The box file that was generated was then used as input for the GRID program, which calculates information concerning the steric and electrostatic environment within the box of the 1JSU mol2 file. DOCK was used to screen the entire NCI/Developmental Therapeutics Program (DTP) database of small molecules (which consisted of ~140,000 small molecules at the time of docking) within the 1JSU grid, with the selected spheres as theoretical binding sites. CHIMERA was subsequently used to rank the small molecule output based on predicted energy scores composed of electrostatic interactions and van der Waals’ forces. The top 40 compounds were obtained from the NCI/DTP for cell culture testing. All of the programs listed for this procedure were part of the DOCK5.0 suite developed at University of California, San Francisco (17). Protein structure visualization and image generation were performed using PyMOL software (DeLano Scientific, Palo Alto, CA).

Cell Culture—All experiments involving mammalian cell culture were performed using Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (35-011-CV, Mediatech, Inc., Manassas, VA). BT549 and HCT116 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). QBI-293A kidney cells were obtained from Quantum (Montreal, Canada).

Chemical Synthesis—Chemical reagents and solvents were purchased from Sigma-Aldrich and Acros (Morris Plains, NJ). Both synthesized compounds displayed spectroscopic data consistent with the proposed structures.

NSC43067 (1-(5-methylthiophen-2-yl)-3-phenylpropeneone): solid NaOH (1.78 g, 44.5 mM) was dissolved in 50 ml of water/ethanol (2:1, v/v) with cooling in an ice bath. Sequential addition of 5-methyl-2-acetyl thiophene (5.00 g, 35.6 mM) and benzaldehyde (3.77 g, 35.6 mM) to the cooled solution of NaOH was followed by rapid stirring on ice for 2 h. The mixture was stored overnight at 4 °C, resulting in the formation of an oily solid. The solid was removed by vacuum filtration, and the filtrate was then concentrated in vacuo. The residue was dissolved in hot absolute ethanol and left to cool to provide 4.0 g (50% yield) of NSC43067 as flaky yellow crystals; melting point, 93–95 °C.

NSC63002 (2-(4-methoxy-phenyl)-3-pyridin-2-yl-acrylonitrile): The compound was prepared in 26% yield as described in a previous study (18) (melting point, 69–70 °C (literature: 69.5–70.5).

Cell Cycle and Proliferation Assays—For [3H]thymidine incorporation assays, cells were plated in 24-well plates at a density of 30,000 cells per well and allowed to incubate overnight at 37 °C. Following treatment periods, cells were incubated for a further 2 h at 37 °C with 40 μl per well [3H]thymidine (0.1 mCi per ml diluted in PBS, NET027005MC, PerkinElmer Life Sciences). Cells were then fixed with 10% trichloroacetic acid for 15 min, followed by a further two washes with 10% trichloroacetic acid. The DNA was then dissolved with 300 μl of 0.2 N sodium hydroxide. For each well, 100 μl was counted in a Beckman Coulter LS6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

For cell viability assays, drug activities were determined using a high-throughput CellTiter-Blue assay. Cells (1200–6000; 24-μl volumes) were plated in each well of 384-well plates and incubated overnight at 37 °C, 5% CO2. The next day, the drugs were diluted in media, and 6 μl of these dilutions was added to appropriate wells using an automated pipetting station. Four replicate wells were used for each drug concentration and an additional four control wells received a diluent control without drug. Drug dilutions generally consisted of 1.5-fold dilutions from a maximum final concentration of 100 μM. The cells were incubated with the drug for 72–120 h. At this time, 5 μl of CellTiter-Blue reagent (Promega, Madison, WI) was added to each well. Cell viability was assessed by the ability of the remaining viable cells to bioreduce resazurin to resorufin. Resazurin is dark blue in color and has little intrinsic fluorescence until it is reduced to resorufin (579 nm Ex/584 nm Em). The change in fluorescence was measured with a Synergy HT microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The fluorescence data were transferred to a spreadsheet program to calculate the percent viability relative to the four replicate cell wells that did not receive drug. IC50 values were determined using a sigmoidal equilibrium model regression using XLfit version 4.3.2 (ID Business Solutions Ltd.). The IC50 value was defined as the concentration of drug required for a 50% reduction in growth/viability.

For flow cytometry analysis, cells were plated at a density of 300,000 cells per plate in P100 dishes. Following treatment, cells were removed from the plate by trypsin digestion and suspended in a solution containing 3.4 mM sodium citrate, 75 μM propidium iodide, 0.1% Triton X-100, and 50 μM microcystin, 1 mM sodium orthovanadate, and 40 mM sodium pyrophosphate) followed by sonication. Extracts were cleared by centrifugation at 16,000 × g for 20 min. The cleared supernatant was then analyzed for total protein concentration with Bradford protein assay dye reagent (500-0006, Bio-Rad), and all extracts were normalized to the lowest protein concentration. The extracts were boiled with one-third volume of 4× SDS sample buffer (60 mM Tris, pH 6.7, 2 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol, 5% glycerol, 10 mM microcystin, 1 mM sodium orthovanadate, and 40 mM sodium pyrophosphate) followed by sonication. Extracts were blotted with Bradford protein assay dye reagent (500-0006, Bio-Rad), and all extracts were normalized to the lowest protein concentration. The extracts were blotted with one-third volume of 4× SDS sample buffer (60 mM Tris, pH 6.7, 24 mM EDTA, 200 mM SDS, 40% glycerol, 300 μM bromphenol blue, 0.4% β-mercaptoethanol) for 5–10 min. Samples were resolved on SDS-PAGE gels, and transferred to nitrocellulose membranes. Membranes were immunoblotted using antibodies specific for either actin (sc-1616-P, Santa Cruz Biotechnology, Santa Cruz, CA), Cdk1 (sc-54, Santa Cruz Biotechnology), Cdk2 (sc-163, Santa Cruz Biotechnology), Cdk4 (sc-601, Santa Cruz Biotechnology), E2F1...
in-frame with cDNA encoding Cdk4-His6. The orientation of creating a construct that contains cDNA encoding GFP and insert DNA were ligated for 18 h at room temperature, and E2F1 were described previously (19, 20).

cells (Mv1Lu) stably expressing a Cyclin D1-Cdk2 fusion protein (GFP) fused to Cdk4 was stably expressed in 293A cells. Molecular Probes, Carlsbad, CA), or Caspase 3 (sc-7148, Santa Cruz Biotechnology), p27 (sc-6246, Santa Cruz Biotechnology), OP76, Calbiochem, San Diego, CA), p21 (sc-6246, BD Biosciences, San Jose, CA), or Caspase 3 (sc-7148, Santa Cruz Biotechnology).

In Vitro Kinase Assays—Cyclin E-Cdk2 complexes (#7524, Cell Signaling) were diluted to a concentration of 12 μg/ml and preincubated with either the vehicle or NCI compounds for 24 h at room temperature. The kinase/treatment mixture was then mixed with the substrate solution for a final concentration of 6 μg/ml Cyclin-Cdk, 25 μg/ml GST-Rb (sc-4112, Santa Cruz Biotechnology), 18 μCi/ml [γ-32P]ATP (BLU002A250UC, PerkinElmer Life Sciences), 0.1 μM unlabeled ATP, 50 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM sodium fluoride, and 0.1 mM sodium orthovanadate. Reactions were allowed to incubate for 1 h at 37 °C, followed by quenching with one-third volume 4

High Throughput Screening of a Novel Cdk Drug Binding Site—We examined the differences between several crystal structures of Cdk2 to identify a novel inhibitor binding site on Cdks. A side-by-side comparison of the structures of the catalytically active Cyclin A-Cdk2 complex and the catalytically inactive p27-Cyclin A-Cdk2 complex revealed the formation of a struc-
tural pocket, present only in the inhibited, p27-bound form of Cyclin A-Cdk2 (Fig. 1A). We hypothesized that a small molecule could bind to this pocket and stabilize the Cyclin A-Cdk2 complex in an “open” conformation which would mimic the p27-bound form of Cyclin A-Cdk2, and would thereby inactivate the enzyme catalytically. A sequence alignment of Cdk1, Cdk2, Cdk4, and Cdk6 revealed that the residues that comprise the pocket are relatively well conserved between these Cdkks, indicating that this pocket is likely to be present in the other cell cycle Cdkks.

We performed a high-throughput in silico molecular docking screen on the p27-Cyclin A-Cdk2 crystal structure using the University of California, San Francisco program suite DOCK5.0. Approximately 140,000 small molecules from the NCI/DTP data base were docked into the pocket, designated by the appropriate spheres and scoring grid, and ranked according to their predicted binding energies. The top 40 compounds were ordered from the NCI/DTP and tested in cell culture. Fig. 1C shows a selection of the most active compounds as determined by assays described in later sections, with their predicted energy scores.

The NSC Compounds Inhibit the Proliferation of Cells in Culture—[3H]Thymidine incorporation assays were performed to examine the influence of the compounds on cell proliferation. Initially, all 40 compounds ordered from the NCI/DTP were tested in cell culture at a concentration of 100 nM to determine if any cytostatic effect could be measured (data not shown). The most promising compounds were subsequently used in more detailed dose-response [3H]thymidine incorporation assays, as well as in further mechanistic studies. The BT549 human breast cancer cell line and the HCT116 human colon cancer cell line exhibited decreased cell proliferation as measured by [3H]thymidine incorporation as measured by [3H]thymidine incorporation after treatment with compounds NSC43067, NSC43042, and NSC63002 for 24 h (Fig. 2A). Cell cycle analyses of each of these two cell lines were performed by flow cytometry of propidium iodide stained cells after treatment with the same compounds for 24 h. In both cell lines, and for both compounds, an arrest at the G0/G1 phase of the cell cycle is observed at lower concentrations, but an accumulation of cells in the G2/M cell cycle phase is apparent at higher concentrations (Fig. 2, B and C). Importantly, the profiles from these experiments reveal a lack of a sub-G1 population, indicating that apoptosis is not a significant contributing factor to the inhibition of cell proliferation by these compounds.

![FIGURE 1. Identification of a novel Cdk2 binding pocket and interacting molecules by high-throughput in silico screening.](image-url)
observed decrease in cell proliferation. This assay confirms that the decrease in DNA synthesis as determined by \[^{3}H\]thymidine incorporation is associated with cell cycle arrest. IC\textsubscript{50} values were also determined for compounds NSC63002 and NSC117024 by Cell Titer-Blue assay in several other breast and lung cancer cell lines (supplemental Fig. S1).

**The Cytostatic Effects of the Compounds Are a Result of an Apparent Reduction Both in Cellular Levels and Activity of Cdks** —Total levels of several Cdks were analyzed by immunoblot to determine whether the compounds affect Cdk abundance. BT549 cells were treated with compounds NSC43067, NSC269621, and NSC63002 at 100 \(\mu M\) and 200 \(\mu M\) for 24 or 48 h. All three compounds significantly reduced the levels of soluble Cdk1, Cdk2, and Cdk4, especially at the highest concentrations and longest time points. The levels of several Cyclins were also affected, but to a lesser extent. This result was not due to a universal effect on all cellular proteins, because the levels of the protein phosphatase PP2A and the structural protein actin did not change appreciably (Fig. 3A). Additionally, no cleaved Caspase 3 bands were observed, indicating that apoptotic cell death did not play a major role in the effects of the NSC compounds on the cells, consistent with the flow cytometry results in Fig. 2.

E2F-1 is a transcription factor that plays a crucial role in mediating Cdk-initiated cell cycle progression. Early cell cycle (G\textsubscript{1} to S phase) Cdk activity leads to the phosphorylation of the E2F inhibitor Rb, resulting in its release from E2F family proteins and increased E2F-dependent transcription (for review see Ref. 21). Activation of E2F-dependent transcription is thought to be one of the primary cell cycle-related functions of the G\textsubscript{1}/S Cdks. Therefore, E2F-1 overexpression would be expected to result in the partial reversion of the Cdk-reducing effects of the compounds. To examine this possibility, we used a mink lung epithelial cell line (Mv1Lu) engineered to overexpress E2F-1 (Mv1Lu-E2F1–11) (20). Treatment of the parental Mv1Lu cells with 200 \(\mu M\) NSC63002 resulted in an almost complete reduction of phosphorylation of Rb at serine 780, as well as a decrease in the levels of several E2F-1 dependent gene products including Cyclin A, B-Myb, and E2F-1 itself (Fig. 3B). Levels of Cdk1 and Cdk4 were also found to decrease. E2F1 overexpression resulted in a diminished response to compound NSC63002 compared with the effect observed in the parental cells. A decrease in E2F dependent gene products, as well as serine 780 phosphorylation of Rb, was observed, albeit the inhibition was weaker than that observed in the parental cells. The effect of NSC63002 on Rb phosphorylation...
tion and E2F-dependent transcription was also dampened in an Mv1Lu cell line that expresses a constitutively active CyclinD1-Cdk2 fusion protein (19). Although the levels of the CyclinD1-Cdk2 fusion protein decreased upon drug treatment, Cyclin D1 alone did not. This would indicate that the effects of the compounds are Cdk-dependent. In all of the cell lines, there was no observable change in Erk1/2, and p38, two kinases closely related to the Cdks, or in the loading control, actin. Finally, there was no change in levels of the heat shock proteins Hsc70 or Hsp70, indicating that the compound did not simply induce a general stress response in the cells.

In vitro kinase assays were performed using purified CyclinE-Cdk2 complexes to determine whether the compounds directly inhibit Cdk activity. After a 24-h preincubation with the NSC compounds, Cdk-dependent phosphorylation of Rb was dramatically reduced as compared with the vehicle-treated samples (Fig. 3C). Together, these data indicate that the NSC compounds decrease Cdk abundance in cultured cells and directly inhibit Cdk activity in in vitro kinase assays.

The Decrease in Cdk Levels Occurs on the Same Time Scale as the Inhibition of Cell Proliferation—A decrease in Cdks 1, 2, and 4 was observed as early as 4 h after treatment (Fig. 4A). Time-course experiments were performed to ensure that Cdk down-regulation by the compounds occurred in parallel with cell cycle arrest. BT549 cells treated with 200 μM NSC43042 or NSC63002 were incubated for 4, 8, 12, or 24 h before measuring cell proliferation by [3H]thymidine incorporation (Fig. 4B). The cytostatic effects of both compounds were observed as early as 4 h after treatment, in accord with the apparent decrease in Cdk levels. Taken together, these data suggest that the cytostatic effects of the compounds are mediated through a decrease in soluble Cdk levels.

The Decrease in Cdk Levels Is a Result of Protein Aggregation—Initially, we examined the possibility that the compounds increase protein degradation of the Cdks through proteasomal degradation. Co-treatment of cells with proteasome inhibitors, such as lactacystin or 4-hydroxy-5-iodo-3-nitrophénylacetyl-Leu-Leu-leucinal-vinylsulfone, did not affect compound-induced Cdk ablation, indicating that the effect was not induced by proteasomal degradation of the Cdks (data not shown). Consistent with this result, transfection of a degradation-resistant mutant of ubiquitin did not reduce the effect of the compounds, also suggesting that the effects of the compounds were not due to ubiquitin-dependent proteasomal degradation (data not shown). Overexposure of the higher molecular weight region of a Cdk4 immunoblot of 293A cell lysates after treatment with 200 μM NSC63002 revealed the presence of Cdk4 immunoreactive bands exhibiting decreased electrophoretic mobility (Fig. 5A). Overexpression of Cdk4 by transfection intensified this effect. This result is suggestive of an aggregation event involving Cdk4, occurring in a compound-dependent manner.
The decrease in Cdk levels occurs on the same time frame as the decrease in cell proliferation. A. BT549 cells were treated with 0.2% DMSO or 200 μM NSC43042 or NSC63002 for 1, 2, 4, or 24 h. Immunoblot analysis reveals a decrease in the levels of Cdk1 as early as 4 h, with Cdk2 and Cdk4 decreasing by 24 h. B. BT549 cells were treated with 0.2% DMSO or 200 μM NSC43042 or NSC63002 for 4, 8, 12, and 24 h. [3H]Thymidine incorporation analysis reveals that the decrease in Cdk levels occurs on the same time scale as the inhibition of cell proliferation.

We hypothesized that if aggregated Cdks were being formed in cells treated with the NSC compounds, and that if they were of higher molecular weight than free, soluble Cdks, this could be indicative of protein aggregation and that these aggregates could be pelleted by ultracentrifugation. To test this hypothesis, BT549 cells were treated with either DMSO or 200 μM NSC63002 for 24 h. After harvesting and sonicating the cells, the cellular particulates were removed by centrifugation at either 16,000 x g or 150,000 x g for 20 min or 1 h, respectively. The supernatants and the pellets from both centrifugation runs were collected and analyzed by immunoblot (Fig. 5B). Although a dramatic decrease in the levels of Cdks 1, 2, and 4 was observed in the supernatant, a corresponding increase in Cdk levels was observed in the pellet, particularly after ultracentrifugation. Although Erk1/2 was also increased in the pellet, the relative ratio between the decrease in the supernatant levels of Erk1/2 and the increase in the pellet levels was much less than that observed for the Cdks. The levels of the Cdk inhibitory proteins p21 and p27 also changed upon treatment, with an overall increase of p21 levels, and an overall decrease in p27 levels. Drug treatment decreased the solubility of Cyclins A, D1, and E, but not to the extent that was observed with the Cdks, as expected based on immunoblot analysis of whole cell lysates (Fig. 3). This might be due to the presence of both Cdk-associated and Cdk-unassociated pools of the Cyclins.

Immunofluorescence microscopy was performed to visualize possible Cdk aggregates in the cells. BT549 cells were treated with DMSO vehicle or 200 μM NSC63002 for 24 h. Cells were subsequently fixed and stained with an antibody for Cdk4. Cells treated with the vehicle exhibited a Cdk4 staining pattern that was mostly uniform and homogenous, while NSC63002 treatment caused the formation of concentrated Cdk4 staining at intense foci (Fig. 5C). The presence of these foci is consistent with Cdk aggregation, and although these are seen to some extent in vehicle-treated cells, they become much more prevalent and much larger upon compound treatment. We generated a cell line that stably expressed GFP fused to Cdk4 (293A/GFP-Cdk4) to ensure that the apparent aggregation observed was not due to an artifact of the immunofluorescence staining procedure. These cells were treated in the same manner as in the previous experiment, except that after fixation the cells were mounted and viewed directly. A similar result was observed in the 293A/GFP-Cdk4 cells as in the BT549 cells, where treatment with the NSC compounds induced the formation of aggregate foci of Cdk4 (Fig. 5D). To confirm that the GFP fluorescence observed was from full-length Cdk4, we analyzed the 293A/GFP-Cdk4 cell extract by immunoblot. We observed no significant bands of a lower molecular weight, indicating that the foci viewed by microscopy were not degradation products of full-length GFP-Cdk4 (Fig. 5E). These results indicate that the apparent decrease in Cdk levels in cells is due to the formation of insoluble protein aggregates that would presumably not have access to cellular substrates, and would be functionally inactive.

The Effects of the Compounds in Cell Culture Are Reversible—We monitored the formation of the Cdk aggregates by time-lapse confocal microscopy to better understand their nature and to follow their formation and turnover in real time. 293A/GFP-Cdk4 cells were treated with 200 μM NSC63002 and visualized by a confocal microscopy for 18 h, with images captured every 30 min. As early as approximately three hours, aggregates are observed in some of the cells. By 18 h, the final image showed that more than 50% of the cells had developed aggregates (Fig. 6A and supplemental Fig. S3).

We performed the reverse of the preceding experiment to determine whether the aggregates, once formed, could be eliminated from the cells. 293A/GFP-Cdk4 cells were treated with 200 μM NSC63002 for 12 h and subsequently re-fed with 10% fetal bovine serum-Dulbecco’s modified Eagle’s medium for 16 h, with images taken every hour. Cells were visualized by time-lapse confocal microscopy for the final 16 h. While in the first several hours multiple cells are shown to contain aggregates, no aggregates remain by the final image at 16 h after re-feeding (Fig. 6B and supplemental Fig. S4).

We next examined the ability of compound-treated cells to recover with respect to cell proliferation. In Fig. 6C, BT549 cells were treated with 100 μM NSC43042 or NSC63002 for 2, 4, 8, 12, or 24 h before the addition of [3H]thymidine to measure cell proliferation. In Fig. 6D, cells were treated in the same manner, except that their media was replaced with 10% fetal bovine serum-Dulbecco’s modified Eagle’s medium for a further 24 h before the addition of [3H]thymidine. By comparing the results of Figs. 6C and 6D, it is clear that cells treated with the NSC compounds recover their proliferative capacity after removal of...
the compounds. This also indicates that the compound effects are largely reversible under these conditions.

**Cdk Aggregates Co-localize with Markers of Aggresomes**—To better characterize the nature of the aggregates observed in cells, we performed additional immunofluorescence experiments to determine what other proteins localized with the Cdk aggregates.

Given the nature of the hypothesis that the Cdks aggregate in response to compound treatment, they would likely be associated with chaperone proteins. In 293A/GFP-Cdk4 cells, Hsp70 co-localized with Cdk4 aggregates in samples treated with 200 µM NSC63002 and NSC43042, but not in vehicle control samples (Fig. 7A).

Furthermore, immunoblots carried out on soluble or particulate fractions generated from ultracentrifuged samples revealed an increase in several chaperone proteins in the pelleted fraction. This is consistent with their association with the insoluble Cdks (Fig. 7B).

The nature and intracellular localization of many of the Cdk4 aggregates suggested that the Cdks were being incorporated into aggresomes. To test this hypothesis, 293A/GFP-Cdk4 cells were incubated with an antibody for γ-tubulin, a marker for centrosomes and aggresomes (22). In cells treated with 200 µM NSC63002, γ-tubulin clearly co-localized with Cdk4 aggregates (Fig. 7C). These data further support the hypothesis that the NSC compounds affect Cdk solubility and strongly suggest that the method used by the cells to manage the aggregates is their incorporation into aggresomes.

**DISCUSSION**

Cyclin D1 is overexpressed in ~50% of human primary breast cancers (23). Cdk4 is also up-regulated by gene amplification in several types of cancers (24, 25) and subject to mutational activation (26). The prevalence of overactive Cdk activity in many cancers highlights the need for therapeutics that target Cdks. Cdk inhibitors that do not act through ATP competition have the potential to be more selective drugs. Recent evidence also suggests that ATP-competitive inhibitors bind preferably to kinases in their active conformation, inadvertently causing their phosphorylation on activating sites (27). We have identified a novel site on Cdk2 that can be exploited for drug targeting. Moreover, this site is relatively well conserved among cell cycle Cdks and is therefore ideal for decreasing overall Cdk activity, because there is significant functional redundancy among cell cycle Cdks.

The pocket is generated by p27 binding to Cdk2, and p27 is a selective inhibitor of cell cycle Cdks (28). The compounds identified from a screen of this pocket potently inhibit cell proliferation in a mechanism distinct from ATP competition. Flow cytometric analysis of cells treated with increasing doses of the compounds indicate that a G1 arrest occurred at lower concentrations, but a G2 arrest resulted after treatment with higher doses. This may be due to the fact that the compounds affect the activity of Cdks 1, 2, and 4; most likely with different IC50 values for each kinase. Possibly, the G1 arrest observed was due to the compounds displaying a higher affinity for the G1-S phase regulators, Cdk2 and -4, and therefore affecting them at lower doses, while the G2 arrest observed at high doses was due to inhibition of Cdk1 activity. Alternatively, the presence of the...
aggregates themselves may be responsible for the G2/M phase arrest at higher concentrations. Aggregates are processed in cells by machinery that transports them along microtubules to the microtubule-organizing center (29). If the aggregates become too great in number or in size, the microtubule machinery may become overwhelmed and be unable to properly execute mitosis.

Our studies suggest that the NSC compounds bind directly to Cdns 1, 2, and 4 and result in a decrease in the levels of soluble Cdk proteins in intact cells. The mechanism through which these compounds act in a cell culture system is, to the best of our knowledge, novel for a small molecular agent. A possible explanation for the ability of the compounds to reduce the solubility of the Cdk4 to the point of aggregation could be the properties of the residues that line the structural pocket in question. The crystal structure of Cdk2 used to screen for interacting molecules includes p27 in the structural complex, however we have no evidence to indicate that p27 is a necessary prerequisite for compound binding. Artificial removal of p27 from the crystal structure reveals that a series of hydrophobic residues are exposed to the solvent. If the compounds bind to this pocket in the absence of p27, it is possible that these residues would be exposed to the intracellular milieu resulting in aggregation. Another potential explanation for the induced aggregation is that the Cdk4 may be inherently prone to aggregation themselves. Cdk1-Cyclin B complexes exist in aggregates in Xenopus oocytes before resolubilizing and becoming active for the initiation of mitosis (30, 31). Moreover, smaller and less frequent Cdk aggregates appear to be naturally present in some cell types, as they can be observed in vehicle-treated cells. If Cdk4s are inherently prone to aggregation, the stabilization by a small molecule of an alternate conformation in which hydrophobic residues become more exposed may be sufficient to trigger Cdk precipitation.

Current work is focused on optimizing the selectivity of the compounds for cancer cells over normal cells. An initial study (supplemental Fig. S2) indicated that NSC117024 has more potent anti-proliferative effects against the BT549 human carcinoma cell line than the MCF10A and NMuMG non-transformed mammary epithelial cell lines or the MDA-MB-231 human carcinoma cell line. The reasons for this difference in selectivity are unclear, although it could relate to the fact that BT549 cells, but not the other cell lines, lack functional Rb. Further studies are required to identify the molecular alterations that sensitize cells to the Cdk inhib-

FIGURE 6. The effects of the compounds are reversible. A, 293A/GFP-Cdk4 cells were treated with 200 μM NSC63002 for 18 h, and visualized by time-lapse microscopy with images taken every 30 min. Aggregates appear to form between 6 and 9 h (earlier in other cells, see supplemental Fig. S3) with most cells containing some degree of Cdk4 aggregation by 18 h. B, 293A/GFP-Cdk4 cells pre-treated for 12 h with 200 μM NSC63002 were re-fed with fresh media and followed by time-lapse microscopy for a further 16 h with images taken every 60 min. By the final image at 15 h, every aggregate appears to have disappeared. C, BT549 cells were treated with 100 μM NSC43042 or NSC63002 for the 2, 4, 8, 12, or 24 h. Cell proliferation was measured by [3H]thymidine incorporation. D, similar to part C, cells were treated for 2, 4, 8, 12, or 24 h, except they were re-fed with fresh media for a further 24 h, before measuring cell proliferation by [3H]thymidine incorporation. Compared with part C part D reveals that cells re-fed after compound treatment recover their proliferative capacity.

There has been much research conducted on the practicality of high throughput molecular screens, including the prevalence of false positives from screens of large collections of molecules. With regards to the mechanism of action of the compounds presented in this report, several studies have addressed the issue of promiscuous inhibitors that act through nonspecific aggregation (32–34). Small molecules identified in high throughput in vitro screens may exhibit apparent inhibition by decreasing enzymatic activity, but only due to the formation of small molecular colloidal aggregates, which adsorb proteins, inhibiting enzymes nonspecifically. The addition of non-ionic detergents such as Triton X-100 at a concentration of 0.01–0.1% to the assay buffers inhibits the formation of these aggregates or even disrupts them after their formation (32–34). In all of our biochemical studies examining the aggregation of Cdk4s, Triton X-100 was present at a concentration of either 0.1% or 1.0%. Furthermore, our screening procedure was based on in silico docking rather than high throughput in vitro kinase assays, in which nonspecific small molecular aggregates might have presented themselves as false positives more frequently. The Cdk aggregates observed in this study were formed in live cells in culture, indicating that the compounds had to be both soluble in the aqueous cell culture medium and able to cross the cell membrane. All of this suggests that the Cdk aggregates reported in this study are not due to nonspecific small molecu-
lar colloidal aggregates, but rather due to specific small molecule-induced protein aggregation.

There is precedent for Cdk-reducing agents, although not for those that induce aggregation. The natural product Silibinin inhibits the progression of prostate cancer in mouse models concomitantly with the decrease of levels of Cdk1, 2, 4, and 6 in prostate tissue (5, 35). These studies contribute to the idea that decreasing Cdk levels can be effective in anti-cancer therapies.

The novel concept of a small molecule being able to decrease total protein levels in live cells can potentially open the door for other targets as well. Through in silico screening, we were able to take advantage of the fact that the RCSB protein structure data base contains crystal structures of Cdk2 in an active and an inactive conformation. By examining key differences between two conformations of the same protein, it may be possible in other circumstances to stabilize proteins in inactive conformations, or to encourage protein aggregation.

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