Dual G1 and G2 Phase Inhibition by a Novel, Selective Cdc25 Inhibitor 7-Chloro-6-(2-morpholin-4-ylethylamino)-quinoline-5,8-dione*

Lixia Pu‡, Andrew A. Amoscato§, Mark E. Bier¶, and John S. Lazo‡‡

From the ‡Department of Pharmacology and the §Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261 and the ¶Department of Chemistry, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

Received for publication, August 2, 2002, and in revised form, September 25, 2002
Published, JBC Papers in Press, September 27, 2002, DOI 10.1074/jbc.M207902200

The Cdc25 dual specificity phosphatases coordinate cell cycle progression, but potent and selective inhibitors have generally been unavailable. In the present study, we have examined one potential inhibitor, 7-chloro-6-(2-morpholin-4-ylethylamino)-quinoline-5,8-dione (NSC 663284), that was identified in the compound library of the National Cancer Institute. We found that NSC 663284 arrested synchronized cells at both G1 and G2/M phases and blocked dephosphorylation and activation of Cdk2 and Cdk1 in vivo, as predicted for a Cdc25 inhibitor. Using the natural Cdc25A substrate, Tyr15-phosphorylated Cdk2/cyclin A, we demonstrated that NSC 663284 blocked reactivation of Cdk2/cyclin A kinase by Cdc25A catalytic domain in vitro. In-gel trypsin digestion followed by capillary liquid chromatography-electrospray ionization mass spectrometry and tandem mass spectrometry revealed the direct binding of NSC 663284 to one of the two serine residues in the active site loop of the Cdc25A catalytic domain. Cdc25 binding and inhibition could contribute to the anti-proliferative activity of NSC 663284 and its ability to arrest cell cycle progression. Moreover, NSC 663284 should be a valuable reagent to probe the actions of Cdc25 phosphatases within cells and may also be useful structure for the design of more potent and selective antiproliferative agents.

The mammalian cell cycle is temporally controlled by the synthesis and degradation of cell cycle-specific proteins, such as cyclins, by the activation or inactivation of members of a conserved family of serine/threonine protein kinases known as the cyclin-dependent kinases (Cdks)† (1). The activity of Cdks is regulated on four levels. First, Cdk activation requires binding to a cyclin partner. Second, the activity of Cdk/cyclin complexes is negatively regulated by several families of specific Cdk/cyclin inhibitors (the Kip/Cip proteins including p21, p27, and p57, and the Ink4 proteins including p15, p16, p18, and p19) (2). Third, Cdks must be phosphorylated on a threonine residue (Thr160 on Cdk2, Thr161 on Cdk1) located in the “T-loop” to fully open its catalytic cleft (3–5). Finally, the Cdk/cyclin complex is kept inactive by phosphorylation on Tyr15, or sometimes Thr14 and Tyr15 residues in the ATP-binding site of Cdk. The conserved Tyr15 is phosphorylated by Wee1/Myt1 or Myt1. Cdc25 dual specificity phosphatases play key roles in cell proliferation by removing the inhibitory phosphates from the ATP-binding site Tyr15 and/or Thr14 of the Cdk, thus activating cell cycle-specific Cdk/cyclin complexes (1, 3, 4, 8, 9).

Three Cdc25 genes have been found in humans: those for Cdc25A, Cdc25B, and Cdc25C. Both Cdc25B and Cdc25C appear to regulate the G2/M transition, whereas Cdc25A is required for G1/S transition (10–13). Microinjection of anti-Cdc25A antibodies into cells effectively blocks their cell cycle progression from G1 into S phase (10, 11). Ectopic expression of Cdc25A accelerates the G1/S transition (14, 15). Moreover, Cdc25A is a transcriptional target of oncogenes c-myc and E2F and has oncogenic properties (13, 16, 17). In rodent cells, human Cdc25A cooperates with either Ha-RASG12V or loss of RB1 in oncogenic focus formation. Such transfectants are highly aneuploid, grow in soft agar, and form high grade tumors in nude mice (17). Furthermore, overexpression of Cdc25A has been found in a number of human cancers (18, 19). Therefore, Cdc25A is an attractive molecular target for rational antiproliferative drug design. Recently, we identified the most potent in vitro inhibitor of Cdc25A reported to date: 7-chloro-6-(2-morpholin-4-ylethylamino)-quinoline-5,8-dione (NSC 663284), using an artificial substrate O-methyl fluorescein monophosphate (20). Moreover, using this same substrate, we found NSC 663284 was 20- and 450-fold more selective against the Cdc25 phosphatase family compared with VHR or PTP1B, and exhibited mixed inhibition kinetics against Cdc25A, Cdc25B, and Cdc25C with Ki values of 29, 95, and 89 nM, respectively. Although NSC 663284 had marked antiproliferative activity against human MCF-7 breast cancer cells (20), its effects on cell cycle progression, Cdc25-mediated dephosphorylation of Cdk/cyclin biological substrates, and the specific binding sites on Cdc25A have not been examined. Thus, the present study was initiated to delineate the cellular and molecular antiproliferative mechanisms of NSC 663284. We now demonstrated that NSC 663284 arrested cells at both the G1 and G2/M phase and inhibited the dephosphorylation and activation of Cdks in vitro and in vivo. Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and capil-

---

*a This work was supported by National Institutes of Health Grants CA 78039 and CA 52995 and by the Fiske Drug Discovery Fund. The MALDI-TOF MS was supported in part by National Science Foundation Grant CHE-9808188. The Michrom LC and Finnigan LCQ were supported in part by National Science Foundation Grant DMR-9729351. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, University of Pittsburgh, E-1340 Biomedical Science Tower, Pittsburgh, PA 15261. Tel.: 412-648-8319; Fax: 412-648-2229; E-mail: lazoj@pitt.edu.

§ The abbreviations used are: Cdk, cyclin-dependent kinase; Cpd5, compound 5, 2-(2-mercaptoethanol)-3-methyl-1,4-naphthoquinone; BSA, bovine serum albumin; MS, mass spectrometry; LC-ESI, liquid chromatography-electrospray ionization; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MSMS, tandem mass spectrometry; NSC 663284, 7-chloro-6-(2-morpholin-4-ylethylamino)-quinoline-5,8-dione; HPLC, high performance liquid chromatography.

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
lary liquid chromatography-electrospray ionization mass spectrometry (capillary LC-ESI MS analysis), we further observed the products of NSC 663284 bound to the recombinant human Cdc25A catalytic domain.

EXPERIMENTAL PROCEDURES

Materials—Propidium iodide and RNase A (Cellular DNA Flow Cytometry Analysis Reagent Set) were from Roche Molecular Biochemicals. The anti-Cdk1(sc-54 and sc-54-AC), anti-Cdk2 (sc-163 and sc-163-AC), anti-Cdk4(sc-749 and sc-260-AC), anti-cyclin D1 (sc-486), anti-cyclin E (sc-481), anti-cyclin B1 (sc-245), anti-Cdc25B (sc-5619), anti-Cdc25C (sc-327), and transforming growth factor-β1/23 (sc-7892) antibodies were purchased from Santa Cruz Biotechnology, CA. Weel kinase and Cdk2/cyclin A complexes were from Upstate Biotechnology (Lake Placid, NY). Anti-c-Myc antibody (OP10) and histone 1 were purchased from Calbiochem (La Jolla, CA). (γ-32P)ATP (10 mM/m) was from Amer sham Biosciences. pET-21a (+) vector and bacteria strain BL21(DE3) were obtained from Novagen (Madison, WI). Plated Pdu DNA polymerase was obtained from Stratagene (La Jolla, CA). Restriction enzymes and calf intestinal alkaline phosphatase were from New England Biolabs (Beverly, MA). Acetic acid, HPLC grade water, and acetonitrile were purchased from Fisher. Sannapinic acid and apomyoglobin were from Sigma. Sequencing grade modified trypsin was purchased from Promega (Madison, WI).

Cell Cycle Analysis—tsfT210 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 1% γ-glutamate, and 1% penicillin/streptomycin (Life Technologies, Inc., Gaithersburg, MD) in a humidified atmosphere of 5% CO2 at 32 °C and plated at a density as described previously (21). The tsfT210 cells have two point mutations in the Cdk1 gene, which changes an isoleucine to a valine in the PSTAIR region, and a proline to a serine at the COOH terminus (22). Because of these mutations, Cdk1 becomes inactivated and degrades in tsfT210 cells at the restrictive temperature of 39.4 °C, and cell cycle progression arrests at the mid to late G2 phase. Therefore, tsfT210 cells are easily manipulated for cell cycle studies without the use of chemicals. Cell cycle progression was arrested at G2 phase by incubation at 37 °C for 17 h. For G2 arrest studies, the G2 phase synchronized cells were then treated with various concentrations of NSC 663284 (0.1–30 μM), nocodazole (1 μM), or C2d5 (20 μM) and reincubated at 32 °C for 6 h. For G2 arrest studies, the G2 phase synchronized cells were reincubated immediately at 32 °C. Cells were then treated with various concentrations of NSC 663284 (0.1–30 μM), Cpd 5 (15 μM), or roscovitine (50 μM), and incubated at 32 °C for another 6 h. The treated cells were harvested and fixed in 70% ethanol in phosphate-buffered saline at 5 °C for another 6 h. The treated cells were mixed with equal volume of 2× SDS-PAGE loading buffer. Proteins were resolved on 12% Tris-glycine SDS-PAGE and analyzed by autoradiography. Bar charts were plotted using Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

Whole cell lysates were mixed with equal volume of 2× SDS-PAGE loading buffer, fractionated on a 12.5% Tris-glycine SDS-PAGE gel (7 cm × 11 cm), and Western blotting for Cdk2, Cdk4, cyclin D1, cyclin B1, Cdc25A, Cdc25B, and Cdc25C was performed.

Cloning, Expression, and Purification of Recombinant Catalytic Domain of Human Cdc25A—The catalytic domain of human Cdc25A (residues 336–523) was cloned into pET-21a (+) vector at BamHI and XhoI sites with a His6 tag at the COOH terminus, and expressed in BL21(DE3). Protein was affinity-purified to homogeneity by fast protein liquid chromatography using nickel-nitrilotriacetic acid resin (Tiangen, Valencia, CA) followed by dialysis using 10-kDa cut-off dialysis tubing (Millipore, Bedford, MA).

In Vitro Phosphatase Assay—The phosphatase activity of recombinant human Cdc25A catalytic domain was assayed using O-methyl fluorescein monophosphate as a substrate (Molecular Probes Inc., Eugene, OR). Fluorescence emission from the product was measured over a 10–60-min reaction period at room temperature with a multichannel plate reader (PerSeptive Biosystems Cytofluor II; excitation filter, 458/20 nm; emission filter, 530/30 nm).

Kinase/Phosphatase/kinase Assay—Weel kinase was carried out in the kinase buffer of 50 mM Tris-HCl, 1 mM MgCl2, 1 mM EGTA, 2 mM dithiothreitol, pH 7.5, incubated at 30 °C for 30 min. Reactions were stopped by adding 2×SDS-PAGE loading buffer. Proteins were resolved on 12% Tris-glycine gel. Gels were dried and analyzed by autoradiography. The Cdk2/cyclin A kinase assay was carried out at 30 °C for 30 min in 25 mM Tris-HCl, 5 mM MgCl2, 0.5 mM EGTA, 1.65 mM dithiothreitol, 0.005% Brij 35, 25 mM HEPES, pH 7.5, containing 25 mM NaF, 2.5 mM β-glycerophosphate, 0.5 mM Na3VO4, 0.5 mM/ml BSA, 25 μM cold ATP, 20 units of Cdk2/cyclin A, 3 μg of histone 1, 60 μg/ml NSC 663284, and 10 μCi of (γ-32P)ATP. Proteins were resolved on 12% Tris-glycine gel. Gels were dried and exposed to film. The phosphorylated histone 1 bands were analyzed on a Molecular Dynamics personal SI densitometer and quantified using the ImageQuant software package (version 4.1; Molecular Dynamics, Sunnyvale, CA).

MALDI-TOF MS Analysis—NSC 663284 was dissolved in Me2SO and at various compound to protein molar ratios (0.1, 1, 1.1, 10, 10.1, 200:1) was incubated with 60 μM (final concentration) recombinant human Cdc25A catalytic domain for 1 h at room temperature. An equal volume of Me2SO without NSC 663284 was used as vehicle control. The reaction mixtures were desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedford, MA), which also removed non-covalently bound NSC 663284. The reaction mixtures were dried, and desalted with C18 Ziptips (Millipore, Bedfo
and stained with Coomassie Blue. The in-gel trypsin digestion was performed based on the published procedures (22) with modifications. Briefly, the spots of interest were excised from the two-dimensional gel, rehydrated with ammonium bicarbonate buffer, destained with 50% acetonitrile in ammonium bicarbonate, and subjected to overnight digestion at 37 °C in the presence of 25 ng/μl sequencing grade modified trypsin without reduction and alklylation. Peptides were extracted with bicarbonate buffer followed by three changes of 5% formic acid, 50% acetonitrile. Extract volumes were reduced using a SpeedVac.

Tryptic peptides were separated by microcapillary HPLC using a Rainin Gradient Bio-compatible HPLC system (Varian, Inc., Walnut Creek, CA) operating at 0.4 ml/min. The flow was split to a usable flow rate of 150 nl/min using an Accurate Microflow Processor (LC Packings, Inc., San Francisco, CA). Buffers for gradient elution consisted of 0.1% acetic acid in water (buffer A) and 100% acetonitrile containing 0.1% acetic acid (buffer B). A 35-min linear gradient was run. Peptides were pressure-loaded onto a microcapillary column (75 μm inner diameter × 11 cm) packed with C<sub>18</sub> Poros R2 10-μm diameter beads (PerSeptive Biosystems, Inc., Framingham, MA). A stainless steel zero dead volume union was fitted to the end of the column to cause electrospray ionization, and the capillary size was reduced to 20 μm inner diameter. Peptides were eluted directly into a Quattro II triple quadrupole mass spectrometer (Micromass, Inc., Beverly, MA). The capillary voltage was operated at 3.5 kV. Q1 was operated at unit resolution and Q3 slightly below unit resolution. The cone voltage was set to 25 V. The flow was paused as the peptides eluted. The collision energy (40 V) and CID gas (argon) were optimized during the runs. Scans in the MS mode were performed at 400 Da/s. Data were collected in the continuum mode and summed. Scans in the MS/MS mode were performed at 350 Da/s and stored as summed scans.

RESULTS

NSC 663284 Arrested Cell Cycle Progression at G<sub>2</sub>/M Phase—We first examined the effect of NSC 663284 on cell cycle progression of tsFT210 cells, which are a convenient cell model because they can be synchronized without the use of chemicals (22). The typical cell cycle distribution of G1 phase, 28% in S phase, and 23% in G2/M phase. Seventeen hours after incubation at the nonpermissive temperature of 39.4 °C, 68% of tsFT210 cells were arrested in G2/M phase. The medium was then replaced with medium containing Me2SO vehicle alone or various compound concentrations. The resulting population had a broad S phase peak, and the cell cycle distribution of G1 phase, 59%, S phase, 9%, and G2 phase (33%) (Fig. 2 and Table I) with 49% of cells at G2/M phase.

Fig. 1. NSC 663284 arrested tsFT210 cell cycle progression at G2/M phase. tsFT210 cells were cultured at the permissive temperature of 32.0 °C, and then incubated for 17 h at the nonpermissive temperature of 39.4 °C. Cells were then switched to the medium containing Me2SO vehicle control, 1 μM nocodazole and 20 μM Cpd 5 (positive control), and 0.1–30 μM NSC 663284, respectively, and incubated at the permissive temperature of 32.0 °C for 6 h. The treated cells were stained with 50 μg/ml propidium iodide. Flow cytometry analysis was carried out using a BD Biosciences FACStar. Results are representative of three independent experiments. The x axis shows DNA content; the y axis shows the relative numbers.

15 μM Cpd 5 or 50 μM roscovitine did not enter S phase, with 68 and 61% cells arrested at G1 phase, respectively. NSC 663284 also arrested tsFT210 cells at G1 phase in a concentration-dependent manner. Cells treated with 0.1 μM NSC 663284 demonstrated a cell cycle distribution pattern similar to that seen with Me2SO treatment with 27% of cells at G1 phase. Cells exposed to 1 and 3 μM NSC 663284 demonstrated a small increase in the number of cells at G1 phase. Similar to the effects with Cpd 5 and roscovitine, treatment with NSC 663284 at either 10 or 30 μM arrested 67% of tsFT210 cells at G1 phase.

NSC 663284 Inhibited Dephosphorylation and Kinase Activity of Cdk2 in G1-arrested tsFT210 Cells—To delineate the molecular mechanisms of G1 phase-arrest induced by NSC 663284, we determined the kinase activity of Cdk2 in G1 phase-arrested tsFT210 cells. As illustrated in Fig. 3A, we observed a concentration-dependent inhibition of Cdk2 kinase activity in NSC 663284-treated tsFT210 cells (lanes 2–4) as compared with that in vehicle-treated tsFT210 cells (lane 1). The positive controls from Cpd 5-treated (lane 5) and roscovitine-treated (lane 6) cells demonstrated decreased kinase activity as expected. Exposure of cells to increasing concentrations of NSC 663284 caused a gradual appearance of a slower migrating Cdk2 band (Fig. 3B, lanes 2–4) when compared with that in vehicle-treated tsFT210 cells (lane 1), indicating the inhibition of Cdk2 dephosphorylation. The expression level of the other G1/S phase transition regulators such as Cde25A, c-Myc (16), transforming growth factor-β1/2/3, cyclin D1, and cyclin E did not change with NSC 663284 treatment (data not shown).

NSC 663284 Inhibited the Kinase Activity of Cdk4 in G1-arrested tsFT210 Cells—Because Cdk4 is also a G1/S phase transition regulator, we examined the kinase activity, the level, and phosphorylation status of Cdk4 in G1 phase-arrested tsFT210 cells. Fig. 4A showed a concentration-dependent inhi-
tsFT210 cells were cultured at the permissive temperature of 32.0 °C. Incubation at the nonpermissive temperature of 39.4 °C for 17 h resulted in a prominent G2/M arrest. Cells were then released by incubation at 32.0 °C in the presence of MeSO4, nocodazole, Cpd 5, or NSC 663284. Data are means from three independent experiments ± S.E.

| Time             | Treatment          | G0    | S     | G2/M  |
|------------------|--------------------|-------|-------|-------|
| 0 h              | 32.0 °C            | 49 ± 2| 28 ± 2| 23 ± 2|
| 17 h             | 39.4 °C            | 10 ± 2| 24 ± 4| 66 ± 5|
| 6 h after release| MeSO4              | 43 ± 1| 13 ± 1| 44 ± 1|
|                  | Nocodazole (1 μM)  | 7 ± 1 | 17 ± 1| 76 ± 2|
|                  | Cpd 5 (20 μM)      | 15 ± 2| 26 ± 5| 59 ± 4|
|                  | NSC 663284 (0.1 μM)| 41 ± 2| 14 ± 1| 46 ± 2|
|                  | NSC 663284 (1 μM)  | 41 ± 1| 13 ± 3| 45 ± 3|
|                  | NSC 663284 (3 μM)  | 26 ± 5| 21 ± 5| 53 ± 0|
|                  | NSC 663284 (10 μM) | 15 ± 1| 24 ± 6| 61 ± 6|
|                  | NSC 663284 (30 μM) | 19 ± 1| 25 ± 7| 62 ± 6|

**Fig. 2.** NSC 663284 arrested tsFT210 cell cycle progression at G1 phase. tsFT210 cells were synchronized at G2/M phase by incubating at the nonpermissive temperature 39.4 °C for 17 h. Cells were then released from G2/M phase arrest by shifting back to the permissive temperature of 32.0 °C. Subsequently, the cells were switched to the medium containing MeSO4 vehicle (negative control), 15 μM Cpd 5 and 50 μM roscovitine (positive control), and 0.1–30 μM NSC 663284, respectively, and incubated for another 6 h. The treated cells were stained with 50 μg/ml propidium iodide. Flow cytometry analysis was carried out using a BD Biosciences FACStar. Results are representative of three independent experiments. The x axis shows DNA content; the y axis shows the relative cell numbers.

That in vehicle-treated tsFT210 cells (Fig. 5B, lane 1), suggesting the hyperphosphorylation of Cdk1. The expression level and phosphorylation status of the other G2/M phase transition regulators such as Cdc25B, Cdc25C, and cyclin B1 did not change with NSC 663284 treatment (data not shown). The expression level and phosphorylation status of Cdk2 was also unchanged (Fig. 5C), which is consistent with the role of Cdk2 in G1/S, and S/G2 phase transitions, but not in G2/M phase transition.

**NSC 663284 Inhibited the Phosphatase Activity of Recombinant Human Cdc25A Catalytic Domain**—Because Cdc25A is involved in G1/S transition, it is conceivable that NSC 663284 arrested cell cycle progression at G1 phase by inhibiting Cdc25A phosphatase activity and then blocking the dephosphorylation of the inhibitory Thr14 and Tyr15 of Cdk2. Cdc25A consists of a catalytic domain located in the carboxyl terminus and a regulatory domain in the amino terminus. To test our hypothesis that NSC 663284 interacted with the catalytic domain of Cdc25A, we cloned and purified to homogeneity the recombinant catalytic domain of human Cdc25A (residues 336–523). We then examined whether NSC 663284 inhibited the phosphatase activity of the recombinant human Cdc25A catalytic domain in vitro with an artificial phosphatase substrate, O-methyl fluorescein monophosphate. As expected, NSC 663284 inhibited the phosphatase activity of the recombinant human Cdc25A catalytic domain in a concentration-dependent manner with an IC50 = 0.45 μM (Fig. 6).

**NSC 663284 Blocked the Reactivation of Cdk2/Cyclin A Kinase Activity by Recombinant Human Cdc25A Catalytic Domain**—As we described earlier, Cdk2/cyclin A is a biological substrate of Cdc25A. Wee1 is the kinase that phosphorylates Tyr15 of Cdk2 and thus inhibits its activity (6, 7). To address whether NSC 663284 arrested G1/S phase cell cycle progression through the inhibition of Cdc25A, we developed an in vitro kinase/phosphatase/kinase reaction assay, which used the appropriate in vivo biological substrate. After phosphorylation of Cdk2/cyclin A at Tyr15 with Wee1 kinase, we examined the reactivation of the protein complex by the recombinant human Cdc25A catalytic domain with and without NSC 663284 or vanadate.

As shown in Fig. 7 (A and B), Wee1 kinase decreased the kinase activity of Cdk2/cyclin A to ~39% (lane 2) of the initial activity (lane 1). In contrast, recombinant human Cdc25A catalytic domain restored the kinase activity of Cdk2/cyclin A to ~66% (lane 3) of the initial activity (lane 1). MeSO4 vehicle did not affect the restoration of the Cdk2/cyclin A kinase activity by recombinant human Cdc25A catalytic domain (lane 4). NSC 663284 at 0.1:1 molar ratio of compound to recombinant human Cdc25A catalytic domain also had no effect (data not shown).
cell cycle analysis of G1 arrest by NSC 663284

| Time          | Treatment | Total cell population | G1 (%) | S (%) | G2/M (%) |
|---------------|-----------|-----------------------|--------|-------|----------|
| 0 h           | 32.0 °C   | 49 ± 2                | 28 ± 2 | 23 ± 2|
| 17 h          | 39.4 °C   | 10 ± 2                | 24 ± 4 | 66 ± 5|
| 6 h after release | 32 °C | 56 ± 6                | 9 ± 1  | 35 ± 5|
| Another 6 h after release | MeSO | 29 ± 0                | 43 ± 1 | 28 ± 1|
| Cpd 5 (15 μM) |           | 68 ± 0                | 5 ± 1  | 27 ± 1|
| Roscovitine (50 μM) |       | 82 ± 6                | 18 ± 2 | 20 ± 1|
| NSC 663284 (0.1 μM) |      | 27 ± 1                | 43 ± 1 | 30 ± 0|
| NSC 663284 (1 μM) |         | 33 ± 2                | 42 ± 1 | 25 ± 3|
| NSC 663284 (3 μM) |         | 43 ± 5                | 40 ± 3 | 17 ± 2|
| NSC 663284 (10 μM) |       | 67 ± 1                | 8 ± 2  | 25 ± 2|
| NSC 663284 (30 μM) |       | 67 ± 1                | 6 ± 1  | 27 ± 1|

Fig. 3. NSC 663284 induced hyperphosphorylation and decreased the kinase activity of Cdk2 in G1-arrested tsFT210 cells. G1/M synchronous tsFT210 cells were shifted back to the permissive temperature of 32.0 °C for 6 h, and subsequently treated with vehicle or various compounds as described in Fig. 2 and incubated at the permissive temperature of 32.0 °C for another 6 h. Cdk2 was immunoprecipitated using anti-Cdk2 agarose conjugate, and the immunoprecipitates were used for kinase assay. Whole cell lysates were used for Cdk2 Western blot as described under “Experimental Procedures.” A, representative Cdk2 kinase activity using histone H1 as a substrate. B, representative Cdk2 Western blot.

Contrast, NSC 663284 at 1:1, 10:1, 30:1, and 100:1 molar ratios of compound to recombinant human Cdc25A catalytic domain blocked the recovery of the Cdk2/cyclin A kinase activity by recombinant human Cdc25A catalytic domain (lanes 5–8). Similarly, vanadate at 20:1 and 100:1 molar ratios of compound to recombinant human Cdc25A catalytic domain blocked the recovery of the Cdk2/cyclin A kinase activity by recombinant human Cdc25A catalytic domain (lanes 9 and 10). The decrease in Cdk2/cyclin A activity seen with NSC 663284 was not the result of a direct effect on the kinase, as no inhibition in kinase activity was seen with 60 μM NSC 663284, which corresponds to a 1:1 molar ratio of compound to target protein (Fig. 8).

NSC 663284 Bound to Cdc25A Catalytic Domain—We next examined whether NSC 663284 (Mz = 321 Da) bound to recombinant human Cdc25A catalytic domain using mass spectrometry techniques. We obtained a MALDI-TOF signal of m/z 24,927 ± 40 for recombinant human Cdc25A catalytic domain after incubation with MeSO (Fig. 9A). When NSC 663284 was incubated with catalytic domain of Cdc25A in 30-fold molar excess, the MALDI-TOF mass spectrum exhibited an increase in peak width and a new base peak mass assignment of 24,960 ± 50 Da. In addition an unresolved shoulder mass peak at 25,153 Da of the [M + (NSC 663284n + H)]+ adduct ion (Fig. 9B) was also observed. As illustrated in Fig. 9C, the MALDI-TOF mass spectrum from the reaction products of the recombinant human Cdc25A catalytic domain with NSC 663284 at a
molar ratio of 200:1 had an increase in the width of the assigned peak of ~50% and the appearance of a new base peak at 25,186 Da. The front shoulder of the main peak was assigned as free recombinant human Cdc25A catalytic domain (24,950 Da).

To further identify the accurate molecular mass of the reaction adducts of recombinant human Cdc25A catalytic domain with NSC 663284 and to provide additional evidence for binding, we collected the capillary LC-ESI mass spectra of the adduct. Fig. 10A shows a capillary LC-ESI mass spectrum obtained from the recombinant human Cdc25A catalytic domain incubated with Me2SO vehicle. The multiply charged mass spectrum was deconvoluted to 24,906 Da (Fig. 10A, inset). Compared with the calculated mass of 25,036.1 Da of the recombinant catalytic domain of human Cdc25A, there was a mass difference of 130.1 Da, suggesting that the initiator fMet residue (131.21 Da) in the recombinant catalytic domain of human Cdc25A was removed. Fig. 10B showed a capillary LC-ESI mass spectrum obtained from the free NSC 663284, which eluted early in the capillary LC-ESI run of the reaction products. We observed the base peak at m/z 322.1 with a chlorine isotope pattern corresponding to the [NSC 663284(Cl) + H]⁺ ion. The 37Cl isotope signal was evident at the predicted 32% abundance at m/z 324.1. Some minor signals were observed at m/z 286.2 and 363.1. The mass differences of ~36 Da was assigned to the loss of HCl from NSC 663284. The ion at m/z 363.1 was an unassigned doubly charged ion. These minor components could be NSC 663284 fragments or contaminants produced during transmission of ions through the tube lens skimmer region of the mass spectrometer.

Fig. 10C shows the LC-ESI mass spectrum from the reac-
tion products formed by NSC 663284 with recombinant human Cdc25A catalytic domain at a reagent molar ratio of 30:1. When compared with the mass spectrum from a MeSO control (Fig. 10A), the LC-ESI mass spectrum from adducts (Fig. 10C) exhibited a lower signal height because of heterogeneous adducts formation as shown in Fig. 10D. Interestingly, we obtained several deconvoluted masses of 24,963 ± 40, 25,085 ± 40, 25,233 ± 20, and 25,342 ± 20 Da (Fig. 10D). These masses suggested that NSC 663284 bound to recombinant human Cdc25A catalytic domain forming several heterogeneous products.

Identification of Specific Binding Sites and Chemical Structure—To further identify the specific binding sites and the binding chemical structure, we performed in-gel trypsin digestion of recombinant human Cdc25A catalytic domain incubated with NSC 663284 and examined the tryptic digests using microcapillary LC-ESI MS and MS/MS analyses. Both MeSO control and NSC 663284-treated samples exhibited the following mass ions that corresponded to Cdc25A residues: m/z 425.8, 658.5, 705.4, and 761.9 (Table III). In addition, the NSC 663284-treated sample contained a mass ion of 663.8 (Table III). The mass ion at 663.8 is a triply charged ion corresponding to Gly431 and Ser133 residues (corresponding to Gly431 and Ser133 of full-length Cdc25A). This ion occurred at the NH2 terminus. Missed cleavage sites typically occur during in-gel digestions.

MS/MS analysis of the 663.8 mass ion indicated characteristic low mass ions for Val and Arg residues (m/z 72 and 129, respectively; Table IV). As shown in Fig. 11 and Table IV, fragment ions of m/z 374, 478, 621, and 763 were also detected. These correspond to y type ions of the following sequences: SER-NH3, SSER, (VFHCEFSSER)2+, and SSER + NSC 663284 – HCl (478 + 321 – 36). The latter mass ion indicated that the modification occurred at one of the two Ser residues, suggesting formation of a peptide-O-NSC 663284 ether linkage. In addition, internal fragment ions occurring at m/z values of 563, 881, 931, and 1126 were present which suffered losses of one or two H2O molecules (Table IV). Neutral losses of water typically occur at serine, threonine, and glutamic acid residues upon collision-induced dissociation of peptides. Given the fact that two water molecules were lost from the internal fragment VIVVFHCEF (m/z 1126), which could only occur from the Gly111 and Ser113 residues (corresponding to Gly331 and Ser333 residues of full-length Cdc25A), it indicates that there is a high probability that the modification did not occur at the Ser113 residue, but at the Ser114 residue (corresponding to Ser134 in full-length Cdc25A).

**DISCUSSION**

NSC 663284 is a potent in vitro Cdc25 inhibitor that was identified using a high throughput screen of the Compound
Library of the National Cancer Institute (20). Because of the proposed important role Cdc25A has in cell cycle progression through G1 phase, we examined the actions of NSC 663284 in greater detail.

Based on the chemical structure of NSC 663284, we have hypothesized three possible interactions between NSC 663284 and Cdc25A. First, because NSC 663284 is a potential electrophile, it might form a covalent bond with amino acid residues, e.g. sulfhydryl arylation of a cysteine or ether linkage of a serine in the catalytic domain of Cdc25A. Second, NSC 663284 might have noncovalent interactions with Cdc25A, such as H-bonding and salt bridging. Finally, NSC 663284 might inactive Cdc25A by inducing disulfide bond linkage through quinone redox reaction and, as a consequence, change the conformation of Cdc25A. To test the first potential mechanism, we developed a novel in vitro Wee1/Cdc25A/Cdk2 reaction assay and found that NSC 663284 blocked the recovery of Wee1-phosphorylated Cdk2/cyclin A kinase activity by recombinant human Cdc25A catalytic domain (Fig. 7). This is the first evidence showing that Cdc25A is a direct molecular target of the

TABLE III
Microcapillary LC-ESI MS analysis of in-gel trypsin digests

| m/z       | z  | Cdc25A residues                                      |
|-----------|----|----------------------------------------------------|
| 425.8     | 3  | 137–146 (LHYPELYVKK)                               |
| 658.5     | 3  | 77–93 (GAVNLMEEVEFDRLLK)                           |
| 663.8*    | 3  | 103–116 (RVVVFHCEFSSER) + NSC 663284               |
| 705.4     | 1  | 52–57 (FANLIK)                                    |
| 761.9     | 2  | 38–51 (YISPEIMASVLNGK)                             |
| 987.1     | 2  | 77–93 (GAVNLMEEVEFDRLLK)                           |

TABLE IV
Microcapillary LC-ESI MS/MS analysis of adduct ion m/z 663.8

| m/z       | Fragment assignment                                      |
|-----------|---------------------------------------------------------|
| 72        | V (characteristic low mass ion)                         |
| 129       | R (characteristic low mass ion)                         |
| 374       | SEER-NH$_2$                                            |
| 478       | SSER                                                  |
| 563       | (VIVVFHCEFSSER-H$_2$O)$^{2+}$                         |
| 621       | (VFHCEFSSER)$^{+}$                                    |
| 664       | Parent ion                                            |
| 763       | SSER + NSC 663284 − HCl                               |
| 881       | VIVVFHCE-H$_2$O−CO                                     |
| 931       | VIVVFHCEFSSER-H$_2$O                                   |
| 1126      | VIVVFHCEFSSER-H$_2$O                                   |

FIG. 11. Microcapillary LC-ESI MS/MS analysis of the adduct ion of m/z 663.8. NSC 663284 was incubated with 60 µM (final concentration) recombinant human Cdc25A catalytic domain for 1 h at room temperature at a compound to protein molar ratio of 30:1. An equal volume of Me$_2$SO without NSC 663284 was used as vehicle control. The reaction mixtures were fractionated by two-dimensional electrophoresis. The spots of interest were excised and subjected to overnight digestion at 37 °C in the presence of 25 ng/µl trypsin without reduction and alkylation. A parent adduct ion of m/z 663.8 was detected from microcapillary LC-ESI MS analyses (Table III) was further analyzed by microcapillary LC-ESI MS/MS as described under “Experimental Procedures.”
antiproliferative agent NSC 663284 using a natural protein substrate, namely Wee1-phosphorylated Cdk2/cyclin A. We also demonstrated that NSC 663284 did not directly inhibit Cdk2/cyclin A kinase activity (Fig. 8). These in vitro results are consistent with the in vivo observation of hyperphosphorylation and decreased kinase activity of Cdk2. Moreover, they support the hypothesis that the G1 phase arrest induced by NSC 663284 was, at least in part, caused by its interaction with Cdc25A catalytic domain and subsequent inhibition of the phosphatase activity of Cdc25A. Whether the inhibition of Cdc25B or Cdc25C is involved in G1/M arrest needs to be further investigated, but NSC 663284 clearly can block these isoforms in vitro when using an artificial substrate (20).

Both MALDI-TOF MS and capillary LC-ESI MS analysis suggested that NSC 663284 bound to recombinant human Cdc25A catalytic domain in a 1:1 binding stoichiometry. In-gel trypsin digestion followed by capillary LC-ESI MS and MS/MS analysis further revealed that NSC 663284 formed ether linkage at one of the two serine residues in the signature motif (HCEFSSER) of the Cdc25A catalytic domain with the concomitant loss of one molecule of HCl (Fig. 12). Given the fact that losses of water occurred at Glu\textsuperscript{111} and Ser\textsuperscript{113} (Table IV), it is likely that the NSC 663284 modification occurred at Ser\textsuperscript{114}. This finding soundly supported our first hypothesis that NSC 663284 bound covalently to the Cdc25A catalytic domain rather than the third possibility that redox induced disulfide bond formation. The formation of a covalent adduct near the catalytic cysteine and in the active site could explain the mixed inhibition kinetics seen previously with NSC 663284 (20). We cannot, however, eliminate the possibility that other noncovalent binding or modification at other hydroxyl- or thiol-containing residues occurred.

In conclusion, the present study, we demonstrated that NSC 663284 arrested tsFT210 cell cycle progression at both G\textsubscript{1} and G\textsubscript{2}/M phase, and inhibited dephosphorylation and kinase activity of Cdk2 and Cdk1. Using a natural biological substrate, namely Wee1-phosphorylated Cdk2/cyclin A, we also showed that Cdc25A rather than Cdk2/cyclin A was a molecular target of NSC 663284. NSC 663284 appeared to bind covalently to the recombinant human Cdc25A catalytic domain, as analyzed by capillary LC-ESI MS and MS/MS of the tryptic in-gel digesta. We proposed herein that the antiproliferative activity of NSC 663284 is, at least in part, the result of its ability to bind covalently to Cdc25A catalytic domain and inhibit its phosphatase activity, thereby blocking the dephosphorylation of inhibitory Tyr(P)\textsuperscript{15} on Cdk2, thus arresting the cell cycle progression. The present study also illustrated how NSC 663284 might be an important tool to probe the biological roles of the Cdc25A pathway. Finally, the structure of NSC 663284 could serve as a valuable lead for future searches for more selective and potent Cdc25 inhibitors and possible anticancer agents.

Acknowledgments—We sincerely appreciate the helpful discussions with Drs. Peter Wipf (Department of Chemistry, University of Pittsburgh, Pittsburgh, PA) and Billy W. Day (Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA). We acknowledge Daniel Zaharevitz and Jill Johnson (Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, Bethesda, MD) for generously providing NSC 663284.

REFERENCES

1. Pines, J. (1999) Nat. Cell Biol. 1, E73–E79
2. Vidal, A., and Koff, A. (2000) Gene (Amst.) 247, 1–15
3. Endicott, J. A., Noble, M. E. M., and Tucker, J. A. (1999) Curr. Opin. Struct. Biol. 9, 738–744
4. Gu, Y., Roenthall, J., and Morgan, D. (1992) EMBO J. 11, 3995–4005
5. Hagopian, J. C., Kirtley, M. P., Stevenson, L. M., Gergis, R. M., Russo, A. A., Perletich, N. P., Parsons, S. M., and Lew, J. (2001) J. Biol. Chem. 276, 275–280
6. Watanabe, N., Broome, M., and Hunter, T. (1995) EMBO J. 14, 1878–1891
7. Booser, R. N., Holman, P. S., and Fattaey, A. (1997) J. Biol. Chem. 272, 22300–22306
8. Nurse, P., Masui, Y., and Hartwell, L. (1998) Nat. Med. 4, 1103–1106
9. Russel, P. (1998) Trends Biochem. Sci. 23, 399–402
10. Hoffmann, I., Draetta, G., and Karsenti, E. (1994) EMBO J. 13, 4302–4310
11. Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H., and Okayama, H. (1994) EMBO J. 13, 1549–1556
12. Lammer, C., Wagerer, S., Saffrich, R., Mertens, D., Ansorge, W., and Hoffmann, I. (1998) J. Cell Biol. 111, 2445–2453
13. Vigo, E., Muller, H., Prosperi, E., Haeboer, G., Cartwright, P., Moroni, M. C., and Holin, K. (1999) Mol. Cell. Biol. 19, 6379–6395
14. Galaktionov, K., and Beach, D. (1995) Cell 67, 1181–1194
15. Blomberg, I., and Hoffmann, I. (1999) Mol. Cell. Biol. 19, 6183–6194
16. Galaktionov, K., Chen, X., and Beach, D. (1996) Nature 382, 511–517
17. Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M., and Beach, D. (1995) Science 269, 1575–1577
18. Dixon, D., Moyana, T., and King, M. J. (1998) Exp. Cell Res. 240, 236–243
19. Gasparotto, D., Maestro, R., Piccinin, S., Vukoaviljevic, T., Barran, L., Sulfaro, S., and Boiocchi, M. (1997) Cancer Res. 57, 2366–2368
20. Lazlo, J. S., Aslan, D. C., Southwick, E. C., Cooley, K. A., Ducruet, A. P., Joo, B., Vogt, A., and Wipf, P. (2001) J. Med. Chem. 44, 4042–4049
21. Tamura, K., Southwick, E. C., Kerns, J., Rost, K., Carr, B. I., Wilcox, C., and Lazlo, J. S. (2000) Cancer Res. 60, 1317–1325
22. Thng, J. P. H., Wright, P. S., Hamaeguchi, J., Lee, M. G., Norbury, C. J., Nurse, P., and Bradbury, E. M. (1996) Cell 63, 313–324
23. Wilm, M., Shevchenko, A., Houthaetve, T., Breit, S., Schweigerer, L., Fotais, T., and Mann, M. (1996) Nature 379, 466–469
Dual G₁ and G₂ Phase Inhibition by a Novel, Selective Cdc25 Inhibitor
7-Chloro-6-(2-morpholin-4-ylethylamino)-quinoline-5,8-dione
Lixia Pu, Andrew A. Amoscato, Mark E. Bier and John S. Lazo

J. Biol. Chem. 2002, 277:46877-46885.
doi: 10.1074/jbc.M207902200 originally published online September 27, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207902200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 23 references, 8 of which can be accessed free at
http://www.jbc.org/content/277/49/46877.full.html#ref-list-1