

# Redox Regulation of Cdc25B by Cell-Active Quinolinediones

Marni Brisson, Theresa Nguyen, Peter Wipf, Beomjun Joo, Billy W. Day, John S. Skoko, Emanuel M. Schreiber, Caleb Foster, Pallavi Bansal, and John S. Lazo

*Departments of Pharmacology (M.B., T.N., J.S.S., C.F., P.B., J.S.L.), Pharmaceutical Sciences (B.W.D., E.M.S.), Chemistry (P.W., B.J., B.W.D.), and the Center for Chemical Methodologies and Library Development (P.W., B.J.), University of Pittsburgh, Pittsburgh, Pennsylvania*

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## ABSTRACT

Intracellular reduction and oxidation pathways regulate protein functionality through both reversible and irreversible mechanisms. The Cdc25 phosphatases, which control cell cycle progression, are potential subjects of oxidative regulation. Many of the more potent Cdc25 phosphatase inhibitors reported to date are quinones, which are capable of redox cycling. Therefore, we used the previously characterized quinolinedione Cdc25 inhibitor DA3003-1 [NSC 663284 or 6-chloro-7-(2-morpholin-4-yl-ethylamino)-quinoline-5,8-dione] and a newly synthesized congener JUN1111 [7-(2-morpholin-4-yl-ethylamino)-quinoline-5,8-dione] to test the hypothesis that quinone inhibitors of Cdc25 regulate phosphatase activity through redox mechanisms. Like DA3003-1, JUN1111 selectively inhibited Cdc25 phosphatases in vitro in an irreversible, time-dependent man-

ner and arrested cells in the G<sub>1</sub> and G<sub>2</sub>/M phases of the cell cycle. It is noteworthy that both DA3003-1 and JUN1111 directly inhibited Cdc25B activity in cells. Depletion of glutathione increased cellular sensitivity to DA3003-1 and JUN1111, and in vitro Cdc25B inhibition by these compounds was sensitive to pH, catalase, and reductants (dithiothreitol and glutathione), consistent with oxidative inactivation. In addition, both DA3003-1 and JUN1111 rapidly generated intracellular reactive oxygen species. Analysis of Cdc25B by mass spectrometry revealed sulfonic acid formation on the catalytic cysteine of Cdc25B after in vitro treatment with DA3003-1. These results indicate that irreversible oxidation of the catalytic cysteine of Cdc25B is indeed a mechanism by which these quinolinediones inactivate this protein phosphatase.

The Cdc25 phosphatases are a subfamily of tyrosine phosphatases, which dephosphorylate inhibitory tyrosine and threonine residues on cyclin-dependent kinases (Cdk) (Lyon et al., 2002). All three human homologs of Cdc25 (Cdc25A, Cdc25B, and Cdc25C) participate intimately in checkpoint regulation of the cell cycle (Lyon et al., 2002). Cdc25A and Cdc25B have been implicated in the oncogenesis of many human tumor types (Galaktionov et al., 1995; Cangi et al., 2000; Lyon et al., 2002; Oguri et al., 2003), and their overexpression is thought to promote the loss of cell cycle checkpoint control, uncontrolled cell proliferation, and genomic instability (Lyon et al., 2002). Thus, there is considerable

interest in identifying potent and selective inhibitors of Cdc25 phosphatases.

The Cdc25 phosphatases contain a structural feature that distinguishes them from other protein tyrosine phosphatases (Buhrman et al., 2005). The crystal structures of the catalytic domains of Cdc25A and Cdc25B reveal a shallow and open active site pocket, making structure-specific inhibitor design difficult (Fauman et al., 1998; Reynolds et al., 1999). However, like other tyrosine-specific protein phosphatases, the Cdc25 phosphatases have the signature catalytic domain comprising -H-C-X<sub>5</sub>-R-, where X is any amino acid (Denu et al., 1996). The pK<sub>a</sub> of the active site cysteine residue of Cdc25B is unusually low (5.6–6.3) as a result of the unique polar environment created by a proximal  $\alpha$ -helix, the amides of the five X residues, and the conserved arginine (Chen et al., 2000; Sohn and Rudolph, 2003). This ensures that the active site cysteine exists as a thiolate anion to form a phosphocysteine intermediate, allowing for removal of the phos-

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**ABBREVIATIONS:** Cdk, cyclin-dependent kinase; ROS, reactive oxygen species; PTP1B, tyrosine phosphatase 1B; VHR, vaccinia H1-related phosphatase; DA3003-1, 6-chloro-7-(2-morpholin-4-yl-ethylamino)-quinoline-5,8-dione; JUN1111, 7-(2-morpholin-4-yl-ethylamino)-quinoline-5,8-dione; JUN1120-2, 6-(2-morpholin-4-yl-ethylamino)-quinoline-5,8-dione; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; OMFP, O-methyl fluorescein phosphate; PBS, phosphate-buffered saline; PI, propidium iodide; DCF, 2',7'-dichlorodihydrofluorescein; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; MKP, mitogen-activated protein kinase phosphatase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; VHR, vaccinia virus VH1-related human phosphatase.

phate from the substrate (Guan and Dixon, 1991; Cho et al., 1992; Rudolph, 2002). The low  $pK_a$  of the active site cysteine makes it a potential target for endogenous and exogenous oxidants (Salmeen et al., 2003; Wang et al., 2004). Reactive oxygen species (ROS), such as  $H_2O_2$ , have been shown to directly inhibit the activity of Cdc25B phosphatase in vitro with a second-order rate constant for oxidation that is  $\sim 400$  and  $\sim 15$  times faster than the oxidation of glutathione and tyrosine phosphatase 1B (PTP1B), respectively (Sohn and Rudolph, 2003).

Oxidative processes can profoundly influence both intracellular signaling and cell cycle progression (Tonks, 2005). For example, oxidative stress leads to mitotic cell arrest (Arrington et al., 2000; D'Agnillo and Alayash, 2001), which is reminiscent of the cell cycle arrest seen after addition of some Cdc25 phosphatase inhibitors (Lyon et al., 2002; Kristjansdottir and Rudolph, 2004). Addition of reducing agents (e.g., thioredoxin) or superoxide dismutase releases cells from mitotic arrest, coinciding with dephosphorylation of inhibitory tyrosine and threonine phosphates on Cdk1 (pCdk1), a known substrate for Cdc25 (Natsuyama et al., 1993). In addition, exposure of HeLa cells to  $H_2O_2$  results in oxidation of the active site cysteine in Cdc25C and protein degradation, leading to cell cycle arrest (Savitsky and Finkel, 2002).

Several benzoquinones and fused quinones have previously been identified as potent in vitro Cdc25 inhibitors by high-throughput in vitro screening (Lyon et al., 2002; Kristjansdottir and Rudolph, 2004). One of these compounds, DA3003-1 [NSC 663284 or 6-chloro-7-(2-morpholin-4-yl-ethylamino)quinoline-5,8-dione], is believed to inhibit the Cdc25A catalytic domain through covalent adduct formation between DA3003-1 and a serine residue adjacent to the catalytic cysteine in Cdc25A. This led to arylation of DA3003-1 and release of the halogen substituent (Kerns et al., 1995; Pu et al., 2002). However, based on our current studies, covalent adduct formation may not be the sole or even the major mechanism of Cdc25B inhibition by quinones.

Previous studies with other protein tyrosine phosphatases and quinoid agents suggest that Cdc25B inhibition could be caused by redox cycling of the quinones in the presence of oxygen (Wardman, 1990; Koster, 1991; O'Brien, 1991). Bova et al. (2004) measured direct production of  $H_2O_2$  by ortho-quinone inhibitors of protein tyrosine phosphatase  $\alpha$ . In addition, the active site cysteines of CD45 and protein tyrosine phosphatase 1B (PTP1B) can be irreversibly oxidized to sulfinic (Cys-SO<sub>2</sub><sup>-</sup>) and sulfonic acid (Cys-SO<sub>3</sub><sup>-</sup>) by quinones in the presence of oxygen, a course of action that is prevented by the addition of catalase or superoxide dismutase (Wang et al., 2004). Rudolph and colleagues (Sohn and Rudolph, 2003) have demonstrated the formation of a sulfenic acid (Cys-SO<sup>-</sup>) intermediate after a brief  $H_2O_2$  treatment of the catalytic domain of Cdc25B, and, more recently, the generation of sulfinic and sulfonic acid species after longer treatments with  $H_2O_2$  (Buhrman et al., 2005).

In the current study, we have examined the essential nature of the halogen substituent in DA3003-1 in Cdc25 inactivation and the possible role of oxidation induced by the quinolinediones. We provide the first direct evidence that quinolinediones can directly inactivate Cdc25B in cells by generating ROS, causing irreversible oxidization of the catalytic cysteine of Cdc25B.

## Materials and Methods

**Chemical Synthesis.** The preparation of DA3003-1 has been reported previously (Lazo et al., 2001). To synthesize JUN1111 and JUN1120-2, we dissolved quinoline-5,8-dione (Barret and Daudon, 1990) (0.33 g; 2.1 mmol) in ethanol (20 ml) and added 4-(2-aminoethyl)-morpholine (0.27 ml; 2.1 mmol) at room temperature. The reaction mixture was stirred for 16 h and concentrated under reduced pressure. The crude residue was purified by chromatography on SiO<sub>2</sub> (methanol/CH<sub>2</sub>Cl<sub>2</sub>; 1:30) to give an  $\sim 2:3$  mixture of JUN1111 and JUN1120-2 (0.36 g; 60%). Pure JUN1111 and JUN1120-2 were obtained as red solids by further chromatographic separation on SiO<sub>2</sub> (MeOH/CH<sub>2</sub>Cl<sub>2</sub>; 1:100). JUN1111 had a melting point of 180°C (dec.); IR (neat) 3329, 2966, 2837, 1696, 1618, 1598 cm<sup>-1</sup>; <sup>1</sup>H NMR  $\delta$  8.88 (dd, 1 H,  $J = 4.5, 1.6$  Hz), 8.39 (dd, 1 H,  $J = 7.9, 1.6$  Hz), 7.63 (dd, 1 H,  $J = 7.9, 4.5$  Hz), 6.62 (bs, 1 H), 5.75 (s, 1 H), 3.80–3.60 (m, 4 H), 3.27–3.21 (m, 2 H), 2.69 (t, 2 H,  $J = 6.1$  Hz), 2.55–2.35 (m, 4 H); <sup>13</sup>C NMR  $\delta$  181.5, 180.1, 153.0, 148.3, 146.8, 134.3, 130.8, 128.3, 100.6, 66.9 (2C), 55.5, 53.3 (2C), 38.8; MS (EI)  $m/z$  (relative intensity) 289 ([M + 2H]<sup>++</sup>, 1), 189 (4), 160 (2), 100 (100); HRMS (EI)  $m/z$  calculated for C<sub>15</sub>H<sub>19</sub>N<sub>3</sub>O<sub>3</sub> ([M + 2H]<sup>++</sup>) 289.1426, found 289.1427. JUN1120-2 had a melting point of 182°C (dec.); IR (neat) 3293, 2945, 2837, 1685, 1588, 1562, 1490 cm<sup>-1</sup>; <sup>1</sup>H NMR  $\delta$  8.97 (dd, 1 H,  $J = 4.8, 1.6$  Hz), 8.33 (dd, 1 H,  $J = 8.0, 1.6$  Hz), 7.55 (dd, 1 H,  $J = 8.0, 4.8$  Hz), 6.53 (bs, 1 H), 5.87 (s, 1 H), 3.80–3.60 (m, 4 H), 3.27–3.18 (m, 2 H), 2.69 (t, 2 H,  $J = 6.0$  Hz), 2.55–2.35 (m, 4 H); <sup>13</sup>C NMR  $\delta$  181.5, 181.2, 155.1, 149.3, 147.6, 134.2, 127.4, 126.3, 102.1, 66.9 (2C), 55.5, 53.2 (2C), 38.4; MS (EI)  $m/z$  (relative intensity) 289 ([M + 2H]<sup>++</sup>, 2), 261 (2), 100 (75), 91 (100); HRMS (EI)  $m/z$  calculated for C<sub>15</sub>H<sub>19</sub>N<sub>3</sub>O<sub>3</sub> ([M + 2H]<sup>++</sup>) 289.1426, found 289.1429.

**In Vitro Enzyme Assays.** Epitope-tagged His<sub>6</sub>Cdc25A<sub>1</sub>, His<sub>6</sub>Cdc25B<sub>2</sub>, and GST-Cdc25C<sub>1</sub> were expressed in *Escherichia coli* and purified by nickel-nitrilotriacetic acid (His<sub>6</sub>) or glutathione-Sepharose resin (glutathione S-transferase) as described previously (Lazo et al., 2001). Human recombinant VHR and PTP1B were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). A serine-to-alanine point mutation in the Cdc25B<sub>2</sub> catalytic domain cDNA at Ser<sup>450</sup> (GenBank accession no. NM\_021872) was accomplished using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and complementary custom oligonucleotide primers from Invitrogen (Carlsbad, CA). The resulting DNA was sequenced by the University of Pittsburgh Core DNA Sequencing Facility to ensure proper integration of primers. Enzyme activities in the absence and presence of inhibitors were measured using the artificial substrate 3-O-methylfluorescein phosphate (Sigma-Aldrich, St. Louis, MO) at concentrations equal to the  $K_m$  of each enzyme and at the optimal pH for individual enzyme activity in a 96-well microtiter plate assay based on methods described previously (Lazo et al., 2001). Fluorescence emission from the product was measured after a 20-min (VHR and PTP1B) or 60 min (Cdc25) incubation period at ambient temperature with a multiwell plate reader (Cytofluor II; Applied Biosystems, Foster City, CA; excitation filter, 485 nm/20-nm bandwidth; emission filter, 530 nm/30-nm bandwidth). IC<sub>50</sub> concentrations were determined using Prism 3.0 (GraphPad Software Inc., San Diego, CA). For dithiothreitol (DTT) studies, enzyme activities for full-length Cdc25B<sub>2</sub> in the absence or presence of quinones was assessed in the presence of 2 to 100 mM DTT in the assay buffer. The pH of the assay buffer was adjusted from pH 7.0 to 8.3 to determine the effect of pH on compound inhibition. The effect of glutathione concentration on in vitro inhibition was measured by the addition of 0, 5, or 10 mM glutathione to the assay buffer. For reversibility studies with inhibitors, we used a protocol similar to a dilution method described previously (Sohn et al., 2003). Cdc25B<sub>2</sub> full-length enzyme (60 mM Tris, 2 mM EDTA, and 150 mM NaCl, pH 8.0) was preincubated with  $\sim 3$  times the IC<sub>50</sub> concentration for each inhibitor: DA3003-1 (3  $\mu$ M) or 6  $\mu$ M JUN1111 for 0, 5, or 20 min at room temperature. The enzyme was also incubated separately with the DMSO vehicle. After preincubation,

the enzyme from each treatment was diluted >10-fold, and remaining enzyme activity was determined by the above-mentioned phosphatase assay using 3-*O*-methyl fluorescein phosphate (OMFP).

**Flow Cytometric Analysis.** tsFT210 cell synchronization and flow cytometry assays were performed as described previously (Osada et al., 1997) on a FACSCalibur flow cytometer (BD Biosciences Pharmingen, San Diego, CA), and data were analyzed using ModFit LT cell-cycle analysis software (Verity Software House, Topsham, ME).

**Western Blotting and Immunoprecipitation of pCdk1.** tsFT210 cells were arrested at G<sub>2</sub>/M by incubation at 39.4°C for 17 h. DA3003-1 (1 or 10  $\mu$ M) or JUN1111 (10 or 30  $\mu$ M) or DMSO vehicle was added to cells for 1 h at 32°C. Cells were vortexed every 10 min in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, containing 250 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100) supplemented with various protease and phosphatase inhibitors. Total protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA), and lysates were incubated with 50  $\mu$ l of an anti-Cdc2 p34 IgG<sub>2A</sub> mouse monoclonal antibody-agarose conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C on an orbital rocker. Immunocomplexes were washed three times in ice-cold PBS supplemented with protease and phosphatase inhibitors. Immunocomplexes were boiled in SDS electrophoresis loading buffer and supernatants were resolved on a 12% Tris-glycine gel. Proteins were transferred to a nitrocellulose membrane and blotted with anti-phospho-Cdc2 (Tyr15) rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA) for detection of hyperphosphorylated Cdk1. Membranes were stripped and reprobed with an anti-Cdk1 mouse monoclonal antibody (Santa Cruz Biotechnology) for detection of total levels of Cdk1 (loading control).

**Direct Inhibition of Cdc25B in HeLa Cells.** The pCMV-myc-Cdc25B<sub>2</sub> vector was constructed by subcloning Cdc25B<sub>2</sub> cDNA from the pQE30-H25B plasmid and subsequent ligation into the BamHI/HindIII sites of the pCMV-Tag 2-5 vector from Stratagene (La Jolla, CA). The pCMV-myc-Cdc25B<sub>2</sub> plasmid was expressed in HeLa cells for 24 h followed by the addition of the vehicle control (DMSO), 10  $\mu$ M DA3003-1 or JUN1111 for 2 h, or 1 mM H<sub>2</sub>O<sub>2</sub> for 15 min. A pCMV-myc-vector was expressed in HeLa cells and treated with DMSO as a negative control. Cells were lysed as stated above and ectopically expressed myc-Cdc25B<sub>2</sub> was immunoprecipitated with an agarose-conjugated mouse monoclonal anti-myc (9E10) antibody (Santa Cruz Biotechnology). The beads were washed three times in ice-cold phosphate-buffered saline (PBS) supplemented with protease inhibitors. Remaining enzyme activity was determined with an *in vitro* OMFP assay similar to methods described previously (Lazo et al., 2001). Percentage of inhibition was calculated using the following formula for enzyme activity in the immunoprecipitates:  $[100 - (\text{Cdc25B-transfected cells after treatment with DA3003-1 or JUN1111} - \text{vector-transfected cells}) / (\text{Cdc25B-transfected cells after treatment with DMSO} - \text{vector-transfected cells})] \times 100\%$ .

**Glutathione Reduction in tsFT210 Cells.** Cellular glutathione was reduced 50% in  $1 \times 10^6$  tsFT210 cells after a 24-h incubation at 32°C with 50  $\mu$ M L-buthionine sulfoximine. Glutathione levels were tested using the ApoAlert glutathione detection kit from BD Biosciences Clontech (Palo Alto, CA). Cells were then treated with DMSO, DA3003-1 (0.1–10  $\mu$ M), or JUN1111 (1–30  $\mu$ M) for 48 h followed by counting of live and dead cells by trypan blue exclusion. Percentage of inhibition of cell growth at each inhibitor concentration was calculated by  $100 - [\text{number of live cells treated with inhibitor} / \text{number of live cells treated with DMSO}] \times 100\%$ . IC<sub>50</sub> concentrations were determined using Prism 3.0 (GraphPad Software Inc.).

**Measurement of Cellular ROS Generation and Calculation of Reduction Potential.** tsFT210 cells ( $1 \times 10^6$ ) were suspended in PBS and preloaded with chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester dye (Invitrogen). Cells were washed in PBS and resuspended in PBS buffer containing 3  $\mu$ M PI. Cells were then treated for 10 min with DMSO, 1 mM H<sub>2</sub>O<sub>2</sub>, or 10  $\mu$ M DA3003-1

or JUN1111. DCF and PI fluorescence was measured by flow cytometry using the FACSCalibur flow cytometer (BD Biosciences Pharmingen). The one-electron reduction potential was calculated using the quantum chemical modeling program Spartan from Wavefunction, Inc. (Irvine, CA), based on Austin-model 1/lowest unoccupied molecular orbital energies.

**Mass Spectrometry.** Recombinant Cdc25B<sub>2</sub> catalytic domain was purified as described previously in Guan and Dixon (1991), with one exception: the size exclusion buffer included 50 mM Tris-HCl, pH 7.5, containing 300 mM NaCl, 1 mM EDTA, and 1 mM DTT. Recombinant Cdc25B<sub>2</sub> catalytic domain protein in size exclusion buffer was treated with the DMSO vehicle or a 30-fold molar excess of DA3003-1 for 1 h at 25°C. An aliquot from each reaction was removed, diluted 3-fold in 1:1 H<sub>2</sub>O-CH<sub>3</sub>CN containing 0.1% CF<sub>3</sub>CO<sub>2</sub>H, mixed with an equal volume of a sinapinic acid-saturated solution of the same solvents, spotted on a stainless steel target, and analyzed by MALDI-TOF-MS in the linear mode on a Voyager DE Pro spectrometer (Applied Biosystems). Another aliquot of each solution was treated with iodoacetamide, precipitated in cold (−20°C) acidic acetone, resuspended in loading buffer and segregated by SDS-polyacrylamide gel electrophoresis on a 12% Tris-glycine gel with Coomassie Blue staining. Bands containing the protein as well as a band from a lane in which no protein was loaded, were excised, destained with dilute methanolic aqueous acetic acid, dried, and treated with sequencing grade trypsin (Promega, Madison, WI) in aqueous NH<sub>4</sub>HCO<sub>3</sub> for 12 h at 37°C. The gel pieces were subjected to centrifugation, and the supernatant was removed and lyophilized. The digest was resuspended in 1% aqueous HCO<sub>2</sub>H. One portion was diluted 3-fold in 1:1 H<sub>2</sub>O-CH<sub>3</sub>CN containing 0.1% CF<sub>3</sub>CO<sub>2</sub>H, mixed with an equal volume of a  $\alpha$ -cyano-4-hydroxycinnamic acid-saturated solution of the same solvents, spotted on a stainless steel target, and analyzed by MALDI-TOF-MS and MALDI-TOF/TOF-MS/MS in reflector mode on a spectrometer (model 4700; Applied Biosystems). The remainder of each sample was analyzed by nanoflow LC-electrospray ionization trap MS<sup>n</sup> (where  $n = 1$  or 2, for single or tandem MS) on either an LCQ DECA XP Plus or an LTQ system with a Surveyor LC (all from Thermo Electron Corporation, Waltham, MA).

## Results

Previous investigations with DA3003-1 proposed the potential importance of Ser<sup>435</sup> (accession no. NM\_001789) in adduct formation and halogen release from DA3003-1, resulting in Cdc25A inhibition (Pu et al., 2002). To investigate this mechanism further, we generated an S450A mutant Cdc25B construct (equivalent to Ser<sup>435</sup> in Cdc25A), expressed the recombinant protein, and evaluated inhibition with DA3003-1. The Ser<sup>450</sup> mutation produced no significant difference in the Cdc25B catalytic activity and, remarkably, did not alter the ability of DA3003-1 to inhibit the enzyme activity *in vitro* (data not shown). In addition, we synthesized the dehalogenated congener of DA3003-1, namely, JUN1111, and its regioisomer JUN1120-2 as small molecule tools (Table 1). A comparison of the IC<sub>50</sub> values for all three Cdc25 isoforms for DA3003-1 and JUN1111 revealed that the chloride substituent was not essential for inhibition (Table 1). JUN1111 was slightly more selective than DA3003-1, with higher *in vitro* IC<sub>50</sub> values against VHR and PTP1B. We have observed a substantial reduction in inhibition with the regioisomer of DA3003-1 and proposed that this reflected different steric or electrostatic effects (Lazo et al., 2001). This effect also extended to the regioisomer of JUN1111, namely, JUN1120-2, which was markedly less active against the Cdc25, VHR, and PTP1B phosphatases (Table 1). Although it has been suggested by others (Ham et al., 2004) that reduc-



tion potentials can be a factor in quinone-based inhibition of Cdc25 phosphatases, the potency for in vitro inhibition of Cdc25 did not correlate with the calculated one electron reduction potentials ( $E_{1/7}$ ) for these compounds (Table 1). Thus, the difference in  $E_{1/7}$  values between JUN1111 and JUN1120-2 ( $-193 \text{ mV} - (-146 \text{ mV}) = -47 \text{ mV}$ ) was much smaller than that between JUN1111 and DA3003-1 ( $-193 \text{ mV} - (-78 \text{ mV}) = -115 \text{ mV}$ ), although the difference in  $IC_{50}$

values for Cdc25 phosphatases was much greater between the two regioisomers.

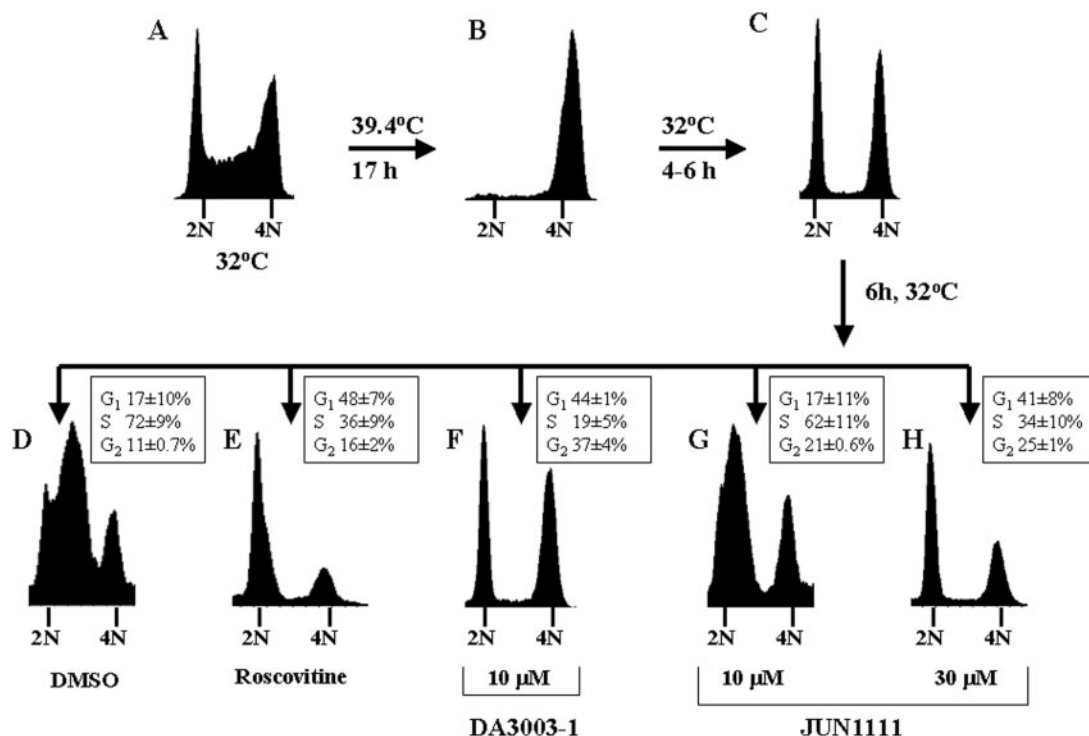
The halogen was also dispensable for cell cycle arrest. We demonstrated previously that DA3003-1 caused both a  $G_1$  and  $G_2/M$  arrest in tsFT210 cells, consistent with its ability to inhibit the Cdc25 family of phosphatases (Pu et al., 2002). JUN1111 also caused both  $G_1$  (Fig. 1) and  $G_2/M$  (Fig. 2) phase arrest. The  $G_2/M$  arrest induced by DA3003-1 and JUN1111

TABLE 1

Chemical structures and  $IC_{50}$  values for the quinolinediones assayed against Cdc25A, Cdc25B, Cdc25C, VHR, and PTP1B

Results are presented as mean  $\pm$  S.E.M. ( $n = 4$ ). The one-electron reduction potential ( $E_{1/7}$ ) was calculated using a quantum chemical modeling program as described under *Materials and Methods*.

Name	Structure	$IC_{50}$ ( $\mu\text{M}$ )					$E_{1/7}$ (mV)
		Cdc25A	Cdc25B	Cdc25C	VHR	PTP1B	
DA3003-1		$0.50 \pm 0.01$	$0.91 \pm 0.36$	$0.47 \pm 0.09$	$10 \pm 0.2$	$15 \pm 0.3$	-78
JUN1111		$0.38 \pm 0.06$	$1.8 \pm 0.7$	$0.66 \pm 0.27$	$28 \pm 1.7$	$37 \pm 0.2$	-193
JUN1120-2		$3.0 \pm 0.05$	$20 \pm 8.1$	$16 \pm 0.48$	$320 \pm 9.8$	$370 \pm 23$	-146



**Fig. 1.**  $G_1$  phase arrest with DA3003-1 and JUN1111. tsFT210 cells were cultured at the permissive temperature of 32°C and then incubated at the nonpermissive temperature (39.4°C) for 17 h to synchronize cells at  $G_2/M$ . Cells were then released for 4 to 6 h at 32°C to arrest cells at the  $G_1$  checkpoint. DMSO or inhibitor was added for an additional 6 h at 32°C. A, asynchronous control. B,  $G_2/M$ -arrested cells after temperature shift for 17 h at 39.4°C. C,  $G_1$ -arrested cells after temperature shift back to 32°C. D, DMSO-treated cells. E, cells treated with 50  $\mu\text{M}$  roscovitine (positive control). F, cells treated with 10  $\mu\text{M}$  DA3003-1. G and H, cells treated with 10 or 30  $\mu\text{M}$  JUN1111. Data are representative of three independent experiments.

coincided with Cdk1 hyperphosphorylation, further supporting cellular inhibition of Cdc25 phosphatase activity (Fig. 3). Overall, JUN1111 was slightly less potent in the tsFT210 mouse cell line compared with DA3003-1. Nonetheless, JUN1111 was more potent than DA3003-1 in inhibiting human MDA-MB-435 breast (JUN1111,  $IC_{50} = 0.1 \mu M$ ; DA3003-1,  $IC_{50} = 0.5 \mu M$ ) and PC-3 prostate (JUN1111,  $IC_{50} = 0.8 \mu M$ ; DA3003-1,  $IC_{50} = 1 \mu M$ ) cell growth.

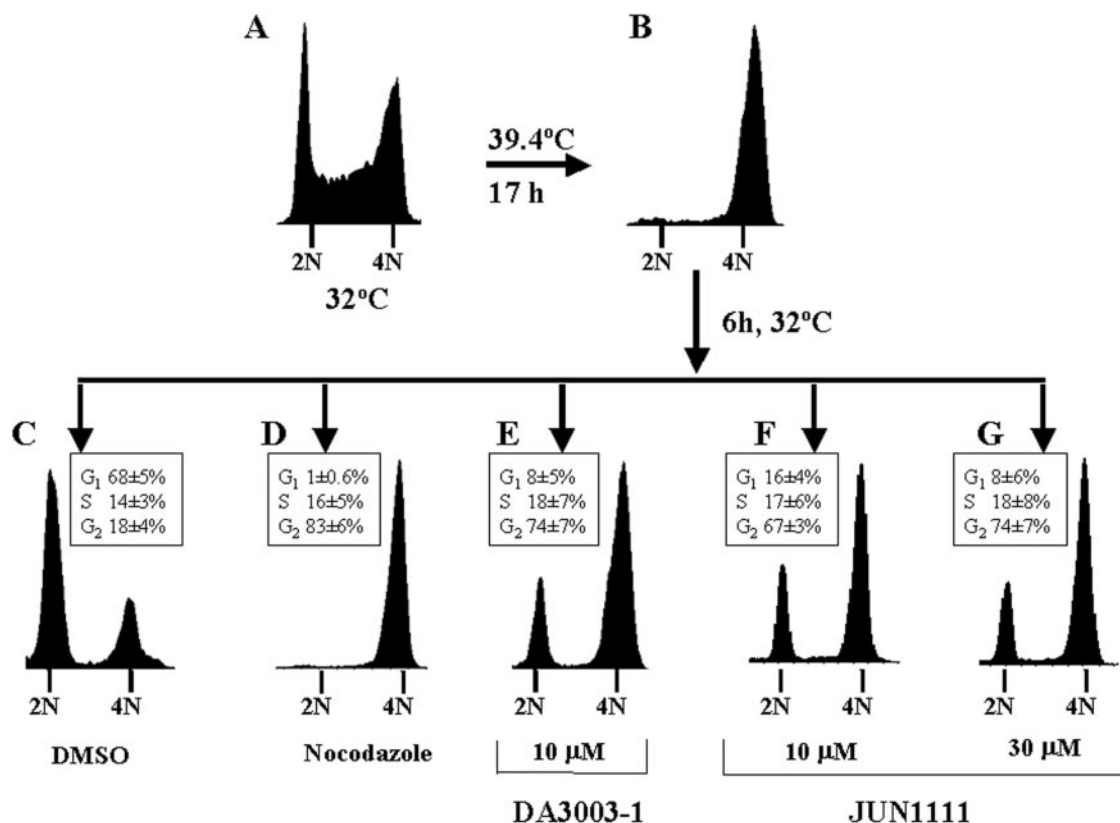
To confirm direct cellular inhibition of Cdc25B by DA3003-1 and JUN1111, we transfected HeLa cells with a vector containing or lacking myc-Cdc25B, before treatment of cells for 2 h with DMSO vehicle, DA3003-1, or JUN1111 or a 15-min treatment with  $H_2O_2$ . Ectopically expressed Cdc25B activity was measured by immunoprecipitation of myc-Cdc25 followed by in vitro phosphatase assays using OMFP as a substrate. DA3003-1 and JUN1111 inhibited the ectopically expressed Cdc25B in HeLa cells by 77 and 56%, respectively, which was comparable with the 73% inhibition seen with 1 mM  $H_2O_2$  (Fig. 4). The loss of cellular enzyme activity in the immunoprecipitates of Cdc25B from cells treated with DA3003-1 and JUN1111 is consistent with either prolonged or irreversible enzyme inhibition.

Because quinones DA3003-1 and JUN1111 clearly demonstrated both in vitro and cellular inhibition of Cdc25B, we investigated possible mechanisms of action for these compounds. Cdc25B was preincubated with DA3003-1 and JUN1111 with greater than 3 times their  $IC_{50}$  concentrations for 0, 5, and 20 min before diluting the enzyme/inhibitor mixture 10-fold and assaying for remaining enzyme activity.

As illustrated in Fig. 5, both DA3003-1 and JUN1111 inhibited Cdc25B enzyme activity in an irreversible, time-dependent manner.

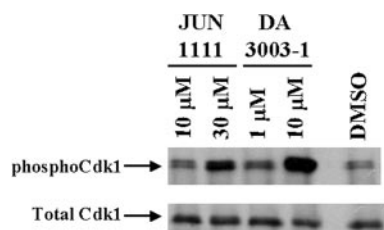
Recent studies have suggested that Cdc25 phosphatases are potential targets for both reversible as well as irreversible oxidation (Sohn and Rudolph, 2003; Buhrman et al., 2005). In addition, quinones are known to generate ROS (Bova et al., 2004). To investigate a possible role for redox cycling in Cdc25B inhibition, we evaluated the ability of reductant concentration and pH to alter in vitro Cdc25 inhibition by DA3003-1 and JUN1111. Increasing the concentration of DTT from the standard concentration used in our in vitro assay (1–2 mM) to 100 mM did not affect Cdc25B enzyme activity (data not shown). However, the  $IC_{50}$  values for both DA3003-1 and JUN1111 increased with increasing DTT concentrations (Fig. 6A). Previous studies have also shown that pH has a direct effect on increased oxygen consumption and enzyme oxidation by quinones (Molina Portela and Stoppani, 1996; Wang et al., 2004). Thus, we also tested pH dependence on inhibition of Cdc25 phosphatase by our quinones by varying the pH of the assay buffer from 7.0 to 8.3. The difference in pH had no effect on Cdc25B enzyme activity itself (data not shown); however, the  $IC_{50}$  values for both inhibitors continuously decreased with increasing pH, resulting in the compounds being more potent at higher pH (Fig. 6B).

The reducing agent glutathione also affected Cdc25B inhibition by DA3003-1 and JUN111 both in vitro and in cells. Concentrations of DA3003-1 (1.6  $\mu M$ ) and JUN1111

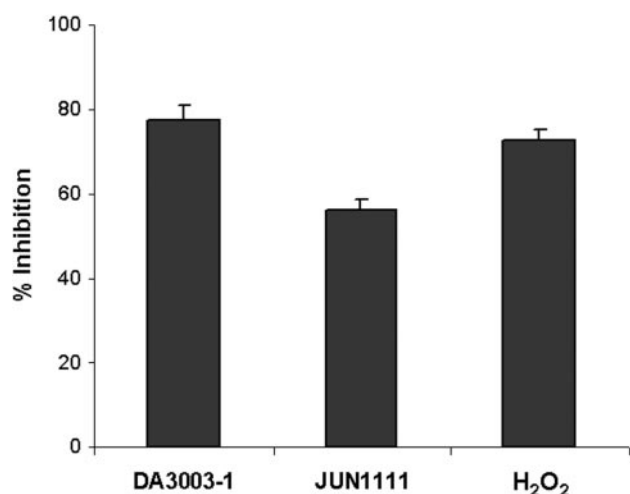


**Fig. 2.** G<sub>2</sub>/M arrest with DA3003-1 and JUN1111. tsFT210 cells were cultured at the permissive temperature of 32°C and then incubated for 17 h at the nonpermissive temperature (39.4°C). DMSO or drug was added to cells for an additional 6 h at 32°C. A, asynchronous control. B, G<sub>2</sub>/M-arrested cells after temperature shift for 17 h at 39.4°C. C, DMSO-treated cells. D, cells treated with 1  $\mu M$  nocodazole (positive control). E, cells treated with 10  $\mu M$  DA3003-1. F and G, cells treated with 10 or 30  $\mu M$  JUN1111. Data are representative of three independent experiments.

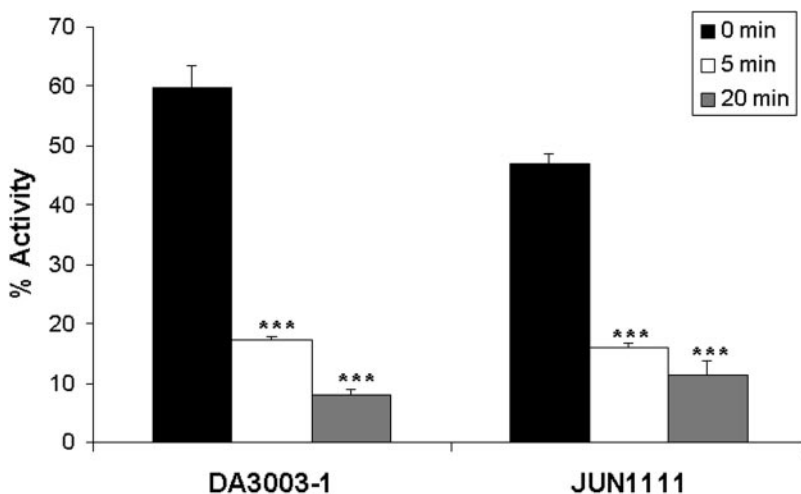
(3.1  $\mu$ M) that would inhibit Cdc25B by  $\sim$ 80% were assayed in the presence of 0, 5, or 10 mM glutathione using the in vitro phosphatase assay. Inhibition with both compounds decreased with increasing concentrations of glutathione (Fig. 7). Glutathione concentrations of  $\leq$ 10 mM did not



**Fig. 3.** Cdk1 is hyperphosphorylated in the presence of DA3003-1 and JUN1111.  $G_2/M$ -arrested tsFT210 cells were released for 1 h in the presence of 10 and 30  $\mu$ M JUN1111 or 1 and 10  $\mu$ M DA3003-1 or DMSO vehicle followed by immunoprecipitation with anti-Cdk1 mouse monoclonal antibody. Western blots were probed with phospho-Cdk1 (Tyr15) antibody. Blots were stripped and reprobed with total Cdk1 antibody as a loading control.



**Fig. 4.** DA3003-1 and JUN1111 directly inhibit transfected Cdc25B in HeLa cells. Transfected cells were treated with DA3003-1 (10  $\mu$ M), JUN1111 (10  $\mu$ M), or H<sub>2</sub>O<sub>2</sub> (1 mM) followed by immunoprecipitation of myc-Cdc25B. The remaining enzyme activity was tested in vitro using OMFP as a substrate. Percentage of inhibition was calculated by comparing to DMSO-treated myc-Cdc25B transfected cells (positive control) and DMSO-treated myc-vector transfected cells (negative control). Results are mean  $\pm$  S.E.M. ( $n = 4$ ).



**Fig. 5.** DA3003-1 and JUN1111 are irreversible inhibitors of Cdc25B. Recombinant Cdc25B was preincubated with  $\sim$ 3 times the  $IC_{50}$  of DA3003-1 (2.5  $\mu$ M) or JUN1111 (6  $\mu$ M) for 0, 5, or 20 min in the absence of substrate and then diluted 10-fold and assayed for enzyme activity. Results are mean  $\pm$  S.E.M. ( $n = 3$ ). Statistical significance was assessed by one-way analysis of variance. \*\*\*,  $p < 0.001$  compared with DMSO control.

affect basal Cdc25B enzyme activity in the absence of inhibitor (data not shown). When glutathione levels were decreased by 50% in tsFT210 cells using buthionine sulfoximine, the  $IC_{50}$  values were decreased 5-fold with DA3003-1 and 4-fold with JUN1111. These data provide evidence that the presence of a cellular reducing agent such as glutathione decreased the potency of DA3003-1 and JUN1111 inhibitors against Cdc25B phosphatase activity.

The above-mentioned data are consistent with the hypothesis that the Cdc25 inhibition by DA3003-1 and JUN1111 could be due to oxidation via production of ROS. To determine whether H<sub>2</sub>O<sub>2</sub> was involved in quinone inhibition, we added catalase (80 U/ml) to the in vitro phosphatase assay. Catalase eliminated the inhibitory activity of DA3003-1 and JUN1111 against Cdc25B, indicating that H<sub>2</sub>O<sub>2</sub> was at least one potential ROS involved in Cdc25B inhibition. To assess direct ROS production by the quinone inhibitors, we pre-loaded tsFT210 cells with dichlorodihydrofluorescein diacetate (chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) dye, a cell-permeant indicator of ROS. A 10-min treatment with 10  $\mu$ M DA3003-1 or JUN1111 produced a 3-fold increase in DCF fluorescence, reflecting a rapid and marked oxidative burst (Fig. 8). Simultaneous staining with PI confirmed that no significant cell death was caused by compound exposure (data not shown). Therefore, both DA3003-1 and JUN1111 are capable of rapidly generating ROS in cells.

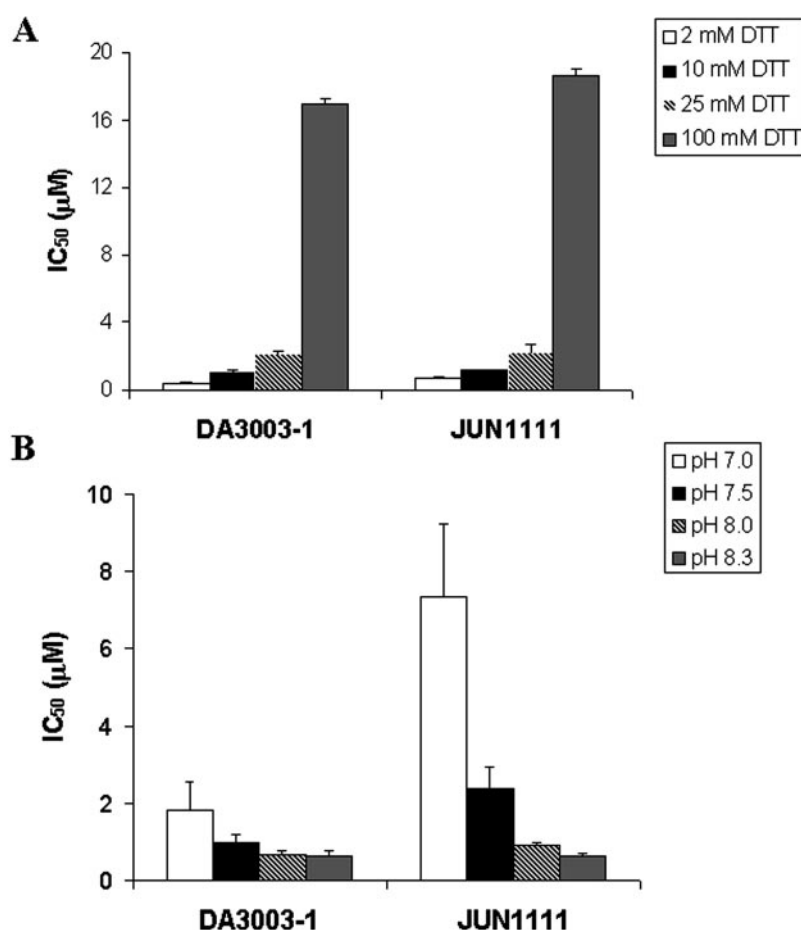
To directly test for Cdc25B oxidation by DA3003-1, we incubated the catalytic domain of Cdc25B<sub>2</sub> (residues 350–539; accession no. NM\_021872) in the presence or absence of a 30-fold excess of the DA3003-1 quinone at 25°C for 1 h followed by protein isolation and mass spectrometric analyses. Direct analysis of vehicle-treated intact protein samples by MALDI-TOF-MS in linear, positive ion detection mode showed sharp  $m/z$  signals for ions of 22407 and 22490 (expected  $[M + H]^+$   $m/z = 22407$ ). The spectrum of the DA3003-1-treated protein, in contrast, had a broad, unresolved ion signal from  $m/z$  22000 to 23600. These results suggested multiple modifications of the protein had occurred as a result of the treatment with DA3003-1. To investigate these modifications further, the protein samples were treated with iodoacetamide to block free sulfhydryls, followed by separation using SDS-polyacrylamide

gel electrophoresis, and excision of the protein band migrating at approximately 23 kDa. The protein band was digested with trypsin, and the tryptic fragments were analyzed by MALDI-TOF-MS and nanoflow LC-electrospray ionization-MS on an ion trap spectrometer. Compared with the vehicle-treated sample, there was a general increase in the presence of methionine sulfoximine-containing peptides in the DA3003-1-treated protein samples. The control protein sample gave abundant ion signals from two tryptic peptides containing the iodoacetamide-blocked active site cysteine (VILIFHC<sup>446</sup>\*EFSSER and RVILIFHC<sup>446</sup>\*EFSSER, \* is carbamidomethylated). Results from MALDI-TOF/TOF-MS/MS analysis of VILIFHC<sup>446</sup>\*EFSSER are shown in Table 2. These ion signals as well as any others corresponding to a peptide containing carbamidomethylated Cys<sup>446</sup> were absent in the MALDI-TOF-MS and LC-MS profiles of the DA3003-1-treated sample. Instead, ions corresponding to three new peptides occurred with *m/z* values and corresponding MS/MS spectra entirely consistent with the conversion of Cys<sup>446</sup> to its fully oxidized, sulfonic acid form (RVILIFHC<sup>446</sup>‡EFSSER, VILIFHC<sup>446</sup>‡EFSSERGPR and VILIFHC<sup>446</sup>‡EFSSERGPRMCR, ‡ is Cys-SO<sub>3</sub><sup>-</sup>). Results from MALDI-TOF/TOF-MS/MS analysis of RVILIFHC<sup>446</sup>‡EFSSER are also given in Table 2. No evidence of the addition product of DA3003-1 (e.g., DA3003-1 + protein → protein adduct + HCl), neither with nor without aromatization of the product (Pu et al., 2002) was found in these spectra.

## Discussion

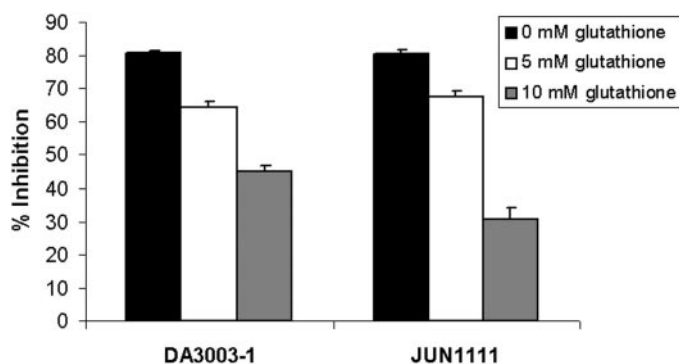
The physiological role of redox regulation of protein phosphatases including PTP1B, low molecular weight phosphatases PTEN and SHP-2 has gained considerable support (Lee et al., 1998; Mahadev et al., 2001; Meng et al., 2002; Cho et al., 2004). Mild oxidation of protein tyrosine phosphatases at low concentrations of ROS leads to reversible oxidation of the catalytic cysteine residue to Cys-SO<sup>-</sup>, which is readily reversible by cellular reductants. Further oxidation of the catalytic cysteine to Cys-SO<sub>2</sub><sup>-</sup> or Cys-SO<sub>3</sub><sup>-</sup> leads to irreversible inactivation of the enzyme. This mechanism of oxidation has been demonstrated with Cdc25B upon exposure to H<sub>2</sub>O<sub>2</sub> (Sohn and Rudolph, 2003; Buhrman et al., 2005). Cdc25B can be protected from irreversible oxidation through formation of the reversible sulfenic acid species followed by rapid formation of a disulfide bridge between the active-site cysteine and a neighboring, backdoor cysteine residue. The oxidized sulfenic acid and the disulfide bridge forms of the enzyme can be reduced by DTT and cellular reductants, such as thioredoxin/thioredoxin reductase, with concomitant enzyme reactivation. With increased exposure or higher concentrations of ROS (H<sub>2</sub>O<sub>2</sub>), further oxidation of Cdc25B occurs, resulting in irreversible deactivation.

Quinoid arenes are highly electrophilic compounds that participate in redox cycling, producing ROS that can inactivate phosphatases at their active site cysteine. The most potent and selective Cdc25 inhibitors reported to date are

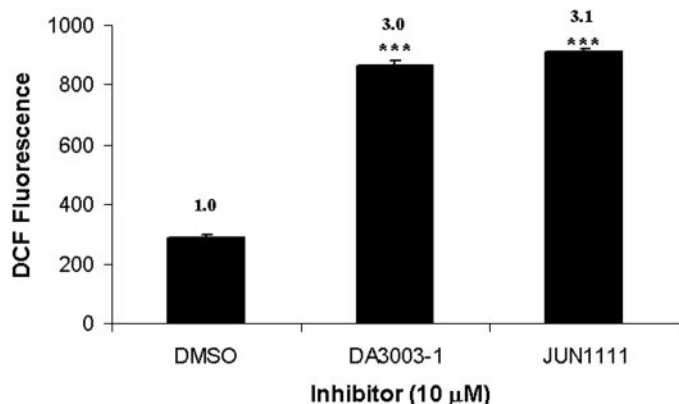


**Fig. 6.** Effect of DTT concentration and pH on phosphatase inhibition. A, Cdc25B phosphatase inhibition by DA3003-1 and JUN111 was tested using our *in vitro* enzyme assay as described under *Materials and Methods* at DTT concentrations of 2, 10, 25, and 100 mM. Results are mean  $\pm$  S.E.M. ( $n \geq 3$ ). B, Cdc25B phosphatase inhibition by DA3003-1 and JUN111 was tested using our *in vitro* enzyme assay as described under *Materials and Methods* under various pH conditions (pH 7–8.3). Results are mean  $\pm$  S.E.M. ( $n = 3$ ). IC<sub>50</sub> values were calculated using GraphPad Prism software. A positive correlation of +1 (effect of DTT on IC<sub>50</sub>) and a negative correlation of -1 (effect of pH on IC<sub>50</sub>) were determined by Spearman's rank correlation.





**Fig. 7.** Glutathione decreases the potency of DA3003-1 and JUN1111. In vitro inhibition of Cdc25B phosphatase by DA3003-1 (1.56  $\mu$ M) and JUN1111 (3.13  $\mu$ M) was tested in the presence of increasing concentrations of glutathione (0, 5, and 10 mM). Results are mean  $\pm$  S.E.M. ( $n = 6$ ). A negative correlation of  $-1$  (effect of glutathione on percentage of inhibition of enzyme) was determined by Spearman's rank correlation.



**Fig. 8.** DA3003-1 and JUN1111 produce ROS in cells. tsFT210 cells were preloaded with DCF dye (to measure ROS) and propidium iodide (to measure cell death) before treatment with DMSO, DA3003-1 (10  $\mu$ M), or JUN1111 (10  $\mu$ M) for 10 min. Resulting DCF and PI fluorescence was measured by flow cytometry. Results are mean  $\pm$  S.E.M. ( $n = 3$ ). Statistical significance was assessed by one-way analysis of variance. \*\*\*,  $p < 0.001$  compared with DMSO control.

TABLE 2

Collision-induced dissociation fragment ions from 1-kV MALDI-TOF/TOF-MS/MS experiments on the  $[M + H]^+$  ions containing the active site cysteine seen in the MALDI-TOF-MS spectra of tryptic digests from Cdc25B<sub>2</sub> incubated with DMSO or with a 30-fold molar excess of DA3003-1

RVILFHC(SO <sub>3</sub> )EFSSER ( <i>m/z</i> found 1783.8821, expected 1783.8801)			Carbamidomethylated-VILFHC(EFSSER) ( <i>m/z</i> found 1636.8134, expected 1636.8157)		
Ion Signal Observed ( <i>m/z</i> ) <sup>a</sup>	Ion Type	Expected <i>m/z</i>	Ion Signal Observed ( <i>m/z</i> ) <sup>a</sup>	Ion Type	Expected <i>m/z</i>
70.081	R immonium	70.07	70.084	R immonium	70.07
72.088	V immonium	72.08	72.079	V immonium	72.08
157.071	SS-H <sub>2</sub> O	157.06	158.079	y <sub>1</sub> -NH <sub>3</sub>	158.09
175.065	SS internal (or y <sub>1</sub> )	175.07 (or 175.12)	175.066	SS internal (or y <sub>1</sub> )	175.07 (or 175.12)
304.145	y <sub>2</sub>	304.16	304.149	y <sub>2</sub>	304.16
369.287	b <sub>3</sub>	369.26	326.313	b <sub>3</sub>	326.24
584.163	C(SO <sub>3</sub> )EFSS-H <sub>2</sub> O	584.17	391.214	y <sub>3</sub>	391.19
595.464	b <sub>5</sub>	595.43	439.312	b <sub>4</sub>	439.33
625.306	y <sub>5</sub>	625.29	625.298	y <sub>5</sub>	625.29
742.513	b <sub>6</sub>	742.50	754.513	y <sub>6</sub>	754.34
879.568	b <sub>7</sub>	879.56	914.488	y <sub>7</sub>	914.37
905.326	y <sub>7</sub>	905.33	1051.511	y <sub>8</sub>	1051.43
1030.590	b <sub>8</sub>	1030.55	1198.631	y <sub>9</sub>	1198.50
1189.501	y <sub>9</sub>	1189.46	1311.578	y <sub>10</sub>	1311.58
1302.524	y <sub>10</sub>	1302.54	1425.002	y <sub>11</sub>	1424.66
1415.707	y <sub>11</sub>	1415.63	1537.699	y <sub>12</sub>	1537.75
1510.719	y <sub>12</sub> -H <sub>2</sub> O	1510.70			
1528.814	y <sub>12</sub>	1528.71			
1627.821	y <sub>13</sub>	1627.78			

<sup>a</sup> Averaged from 3000 MS/MS spectra.

quinones. Most seem to be irreversible inhibitors of Cdc25 phosphatases (Lyon et al., 2002), with the possible exception of the naphthofurandione 5169131 (Brisson et al., 2004) and some indolylhydroxyquinones (Sohn et al., 2003). It is noteworthy that not all quinones are potent in vitro Cdc25 inhibitors, indicating that there is some specificity in the chemical structure of the quinones found to inhibit this enzyme. Previous studies with DA3003-1 (Pu et al., 2002) indicated that dehalogenation of the inhibitor occurred, with formation of a covalent ether adduct with a serine residue corresponding to Ser<sup>435</sup> of Cdc25A (equivalent to Ser<sup>450</sup> in Cdc25B). Therefore, it is interesting that in the current study, the S450A mutation in Cdc25B did not affect DA3003-1 inhibition (data not shown) and that the chlorine moiety was not obligatory for inhibition of Cdc25A, Cdc25B, or Cdc25C (JUN1111; Table 1). We also saw no evidence of a covalent adduct after incubation of Cdc25B with DA3003-1 (Table 2). This difference may be caused by possible alternate conformations of the active sites between the two Cdc25 isoforms as revealed in the X-ray crystal structures (Reynolds et al., 1999). It is also possible that redox regulation and adduct formation occur under different reaction conditions. A low concentration of reducing agent is required to reduce the quinone inhibitor and initiate redox cycling and ROS production (Bova et al., 2004). In the previous investigations of adduct formation, a reductant was not included in the buffer conditions when incubating DA3003-1 with the enzyme; therefore, in the absence of redox cycling, it is possible that arylation of DA3003-1 and adduct formation was favored. Because DTT was added in the current experiment, oxidation probably became the dominant mechanism of inhibition.

We have provided considerable experimental evidence suggesting that quinolinediones participate in redox cycling, leading to Cdc25B inactivation. First, increasing concentrations of DTT (Fig. 6A) and glutathione (Fig. 7) caused a subsequent decrease in Cdc25B inhibition by DA3003-1 and JUN1111. Second, when DA3003-1 and JUN1111 were incubated with Cdc25B phosphatase in the absence of reducing

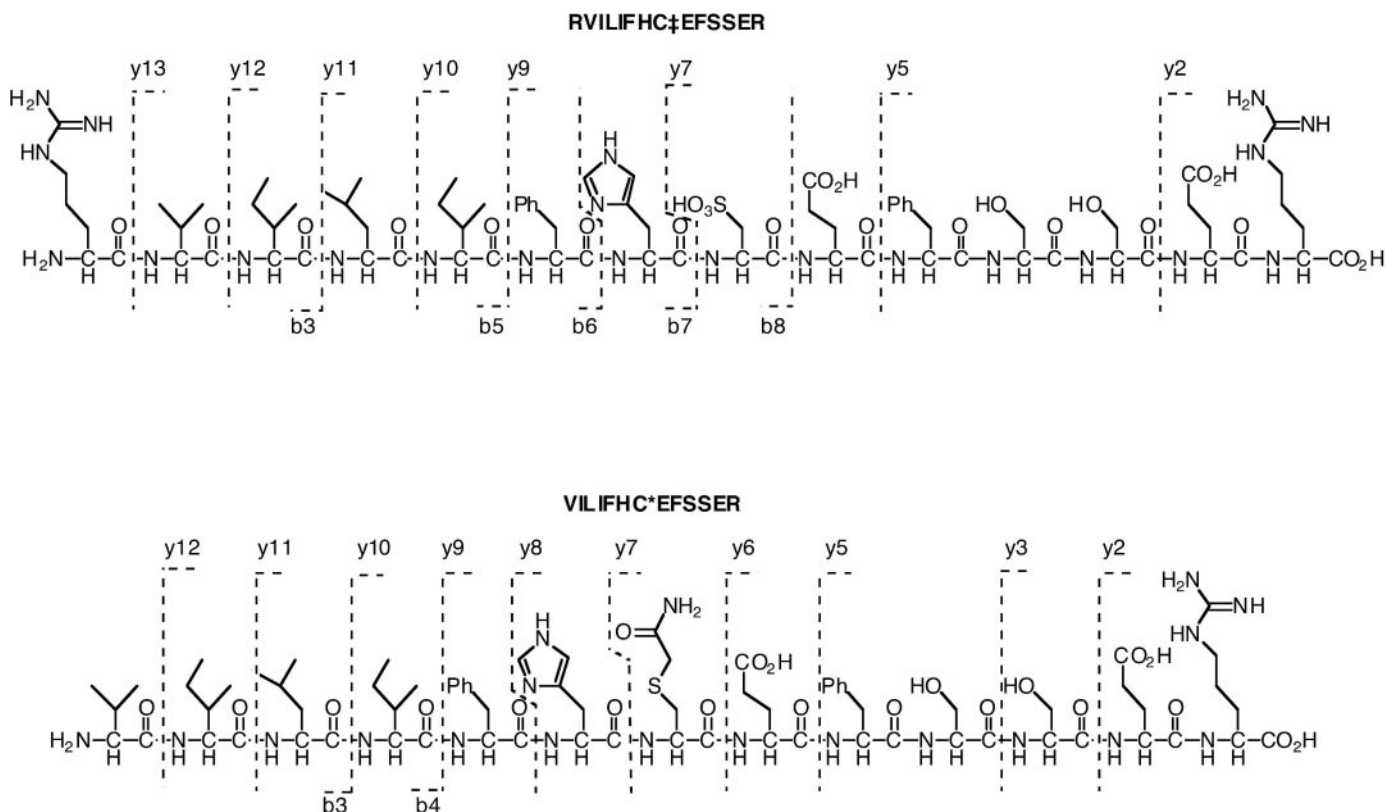


agents, the quinones were not capable of inhibiting Cdc25B, presumably because redox cycling was not initiated. Therefore, the presence of high concentrations of reducing agents interfered with Cdc25B oxidation and inactivation by quinolinediones, whereas low reductant levels were required to induce inhibition by initiating quinone redox cycling and subsequent production of ROS. Third, the presence of catalase abolished inhibition by DA3003-1 and JUN1111, indicating that  $H_2O_2$  production was important in inhibiting Cdc25B phosphatase activity. Fourth, the pH of the reaction also directly affected enzyme oxidation. Raising the reaction pH from pH 6.0 up to pH 8.0 enhances oxygen consumption by quinones, thereby increasing the rate of oxidation of enzymes (Molina Portela and Stoppani, 1996; Wang et al., 2004). Furthermore, increasing the pH above 7.0 increases hydroquinone oxidation by superoxide, resulting in increased production of  $H_2O_2$  (Ordóñez and Cadenas, 1992; Bova et al., 2004). Thus, the  $IC_{50}$  concentrations of DA3003-1 and JUN1111 decreased with increasing pH from pH 7.0 to 8.3 (Fig. 6B). Finally, we observed rapid ROS formation in cells treated with both DA3003-1 and JUN1111 (Fig. 8). Although DA3003-1 seemed to participate in redox cycling in cells, cell lines differing in levels of NAD(P)H:quinone oxidoreductase-1 are equally sensitive to DA3003-1 cytotoxic effects (Han et al., 2004). Therefore, these compounds may be substrates for other quinone reductases or dehydrogenases, such as carbonyl reductase and xanthine dehydrogenase.

The oxidation caused by quinolinediones may exploit the extreme reactivity of the Cdc25 phosphatases to oxidants and their susceptibility to form higher, irreversibly oxidized

sulfinic and sulfonic acid states, compared with other protein tyrosine phosphatases (PTP1B and VHR). This may be due, in part, to the open, exposed active site pocket of Cdc25 (Denu and Tanner, 1998; Reynolds et al., 1999; Rudolph, 2004). For example, the deeper active site pocket in PTP1B causes the rate of inactivation of the PTP1B to be 15-fold less than that of Cdc25B (Sohn and Rudolph, 2003). This differential reactivity provides a possible explanation for the selectivity of our quinolinediones for inhibition of Cdc25 phosphatases over VHR and PTP1B (Table 1). ROS have also been shown to oxidize and inactivate mitogen-activated protein kinase phosphatases or MKPs resulting in c-Jun NH<sub>2</sub>-terminal kinase (JNK) activation (Kamata et al., 2005). Therefore, we examined the effects of DA3003-1 and JUN1111 on MKP-1 both in vitro and in cells. Both compounds had no effect on in vitro MKP-1 activity up to 100  $\mu$ M. Furthermore, addition of DA3003-1 (10  $\mu$ M) and JUN1111 (30  $\mu$ M) to HeLa cells for 1 h did not induce activation of JNK when lysates were probed with an anti-phospho-stress-activated protein kinase/JNK (Thr183/Tyr185) antibody (data not shown). Although these studies indicate some selectivity of the compounds for Cdc25 phosphatases, we recognize that more of the almost 100 human protein tyrosine phosphatases would need to be evaluated to securely state a high degree of selectivity both in vitro and in vivo.

The ability of a particular quinone to redox cycle and produce ROS is dependent on the redox potential of the compound (Bova et al., 2004). Both DA3003-1 and JUN1111 were equally competent in generating cellular ROS as measured by the indicator dye DCF. Both rapidly caused a 3-fold



**Fig. 9.** Fragmentation patterns for peptides generated from tryptic digests of Cdc25B<sub>2</sub> incubated with DMSO vehicle or with a 30-fold molar excess of DA3003-1 for MALDI-TOF-MS spectra analysis. RVILIFHC<sup>46</sup>\*EFSSER (\* is Cys-sulfonic acid) treated with DA3003-1. VILIFHC<sup>46</sup>\*EFSSER (\* is carbamidomethylated) containing the iodoacetamide-blocked active site cysteine.

increase in ROS compared with DMSO-treated cells (Fig. 8), which is somewhat surprising because DA3003-1 had a much higher calculated E1/7 (−78 mV) compared with JUN1111 (−193 mV). Although other chemical factors such as cellular uptake or steric properties enabling docking with the targeted phosphatase (Lazo et al., 2002) could be important, both compounds are sufficiently close to the one-electron reduction potential of oxygen (−155 mV) to generate significant amounts of superoxide.

We have provided direct evidence of irreversible inactivation and oxidation of Cdc25B after exposure to DA3003-1. DA3003-1 and JUN1111 irreversibly inactivated Cdc25B enzyme activity in a time-dependent manner, similar to inactivation seen with H<sub>2</sub>O<sub>2</sub> (Figs. 4 and 5). Mass spectrometry analysis of the Cdc25B catalytic domain after a 1-h in vitro incubation with DA3003-1 revealed that the catalytic cysteine was irreversibly oxidized to sulfonic acid (Fig. 9; Table 2). Figure 9 shows the fragmentation patterns of the tryptic peptide containing the iodoacetamide-blocked active site cysteine (\* is carbamidomethylated) as well as the DA3003-1-treated peptide that revealed the oxidized catalytic cysteine (‡ is Cys-sulfonic acid). A total of seven fragments as well as an internal ion fragment—H<sub>2</sub>O of mass 584 (Table 2)—confirmed an increase in mass coinciding with conversion of the catalytic cysteine to its sulfonic acid form. These data are in agreement with irreversible oxidation of the catalytic cysteines of other phosphatases by *ortho*- and *para*-quinone inhibitors producing ROS (Bova et al., 2004; Wang et al., 2004). We recognize that additional post-translational modifications, such as S-glutathionylation, may regulate Cdc25 phosphatase activity as seen with regulation of PTP1B (Barrett et al., 1999). This possibility should be addressed in future studies.

In conclusion, in vitro inhibition of Cdc25B by *para*-quinolinediones DA3003-1 and JUN1111 seems to be due, at least in part, to ROS production and irreversible oxidation of the catalytic cysteine thiol to sulfonic acid. Differences in Cdc25 phosphatase inhibition are not directly correlated to the redox potentials of quinones, allowing for the possibility that interactions controlled by steric and electronic factors may confer some specificity for inhibition. Future synthesis of analogs of the quinolinediones to promote selective inhibition of Cdc25 could entail the addition of moieties that would better direct the generated ROS to the active site of Cdc25.

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**Address correspondence to:** Dr. John S. Lazo; Department of Pharmacology, University of Pittsburgh, Biomedical Science Tower 3-Suite 1032, 3501 Fifth Ave, Pittsburgh, PA 15260. E-mail: lazo@pitt.edu

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