Inhibition of Cdc25 Phosphatases by Indolyldihydroxyquinones

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Received February 19, 2003

Overexpression of the Cdc25A and Cdc25B dual-specificity phosphatases correlates with a wide variety of cancers, making the Cdc25s attractive drug targets for anticancer therapies. However, the search for good lead molecules has been hampered by the reactivity of the active site thiolate anion and the flat solvent-exposed active site region. We describe here the indolyldihydroxyquinones, a new class of inhibitors of Cdc25 that bind reversibly to the active site with submicromolar potency. Structure−activity relationships in the 50 derivatives of the lead molecule 2,5-dihydroxy-3-(1*H*-indol-3-yl)[1,4]benzoquinone show interesting and consistent trends identifying features required for inhibition of all three isoforms of Cdc25. The compounds do not show time-dependent inhibition, indicating that they form neither covalent adducts with nor oxidize the active site thiol. Our best compounds, 2,5-dihydroxy-3-(7-farnesyl-1*H*-indol-3-yl)[1,4]benzoquinone and 2,5-dihydroxy-3-(4,6-dichloro-7-farnesyl-1*H*-indol-3-yl)[1,4]-benzoquinone, are competitive with substrate for the active site and yield K_is of 640 and 470 nM, respectively. Binding of the indolylhydroxyquinones is diminished by three, but not by six other, specific mutations in the active site region. Additionally, the flexible C-terminal tail required for binding of protein substrate is also required for binding derivatives with hydrophobic modifications at the 7-position. The indolyldihydroxyquinones compete effectively with the protein substrate for Cdc25 in vitro and lead to rapid cell death in vivo. Thus, the indolyldihydroxyquinones will serve as useful lead molecules for drug discovery and further cell-based studies on the role of Cdc25s in cell cycle control.

Cancer at its most basic level is a rampant and undesired growth of cells that leads to a serious and life-threatening disruption of the normal function of multicellular organisms. For this reason, much effort has been devoted to understanding the processes of normal cell cycle regulation and how disruption of these processes contributes to uncontrolled cell growth. In the elucidation of these control mechanisms, the cyclin-dependent kinases (Cdks)[1] were found to be the central regulators of the eukaryotic cell cycle.[1] The kinase activity of the Cdks is directly responsible for the initiation and progression of successive phases of the cell cycle by modification of proteins responsible for the activation of numerous structural and regulatory genes. Given their central role in controlling cell cycle progression, it was not surprising to find that numerous other regulatory proteins modulate the activity of these protein kinases. Among these regulatory proteins, the Cdc25 phosphatases were found to be particularly important in that they are responsible for the final activation step of the Cdks by their dephosphorylation of two inhibitory phosphorylation sites on the Cdks.[2] There exist three phase-specific isoforms of Cdc25 in human cells. Cdc25A is responsible for the initiation of DNA synthesis in the S-phase of the cell cycle whereas Cdc25B and Cdc25C are regulators of the G2/M transition.

As important cell cycle regulators, Cdc25A and Cdc25B have been consistently linked to cancer in a variety of ways. The initial discovery of human Cdc25A and Cdc25B showed that they were proto-oncogenes and targets of c-myc.[3,4] Overproduction of both Cdc25A and Cdc25B has been found in a high percentage of breast cancer patients, where elevated expression levels correlate with reduced life expectancy.[3,5] Cdc25 overexpression has also been associated with gastric carcinomas,[6] colon cancer,[7,8] non small cell lung carcinoma,[9] and aggressive non-Hodgkin's lymphomas.[10] Additional support for the importance of Cdc25 phosphatase activity has come from forced overexpression of Cdc25B in mouse models[11,12] and studies correlating Cdc25 inhibition by small molecules with growth arrest or cell cycle blocks.[13−17]

Because of their important role in cell cycle regulation and their correlation with a wide variety of cancers, the Cdc25s have been attractive targets for drug development.[18,19] However, despite extensive efforts by both academic and corporate researchers, there has been a dearth of potent, specific, active site-directed inhibitors of Cdc25 for use in studies of Cdc25 function or in the development of pharmaceuticals. For many years, sodium orthovanadate, a broad-spectrum protein phosphatase inhibitor, was the only available Cdc25 phosphatase inhibitor, and it is still being used in many cell-based assays to achieve cell cycle blocks. Inhibitors derived from natural products such as dysidiolide [16,20,21], sulfiricin,[22] and vitamin K-derivatives [13,15,23] are either irreproducible, weak, or form irreversible adducts with Cdc25. Other inhibitors derived from combinatorial libraries based on Ser/Thr kinase inhibitors,[24] libraries of dipeptidyl phosphonates,[25] or steroidal derivatives[17,26] have been described that do not break the nanomolar

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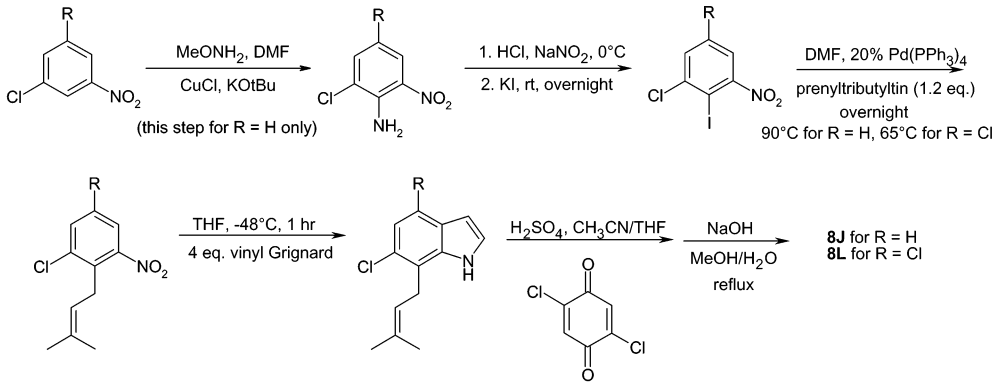
10.1021/jm0300835 CCC: $25.00
Published on Web 05/21/2003

Scheme 1. Synthetic Route to Compounds **8J** and **8L**



barrier, lack specificity toward Cdc25, and/or show noncompetitive inhibition. Even apparently more potent compounds, such as the quinolinediones[27] and the naphthoquinones,[28] derived from a screen of the National Cancer Institute Diversity Set, exhibit mixed inhibition kinetics and appear at least in part to be irreversible.[29]

The lack of real success in the discovery of reversible active-site directed inhibitors for Cdc25 can be attributed to two defining characteristics of this phosphatase. First, Cdc25 as a dual-specificity phosphatase containing the active site motif HCX_5R is highly susceptible to inactivation by covalent modification and oxidation. In this motif, H is a highly conserved histidine residue, C is the catalytic cysteine, the five X residues form a loop whose backbone amides hydrogen bond to the phosphate of the substrate, and R is a highly conserved arginine that is required for binding of the phosphate and transition state stabilization. The catalytic cysteine forms a covalent phosphocysteine intermediate in a two-step reaction mechanism and exists as a thiolate anion in the free enzyme.[30] This active-site thiolate is the source of Cdc25s susceptibility to covalent modification and oxidation.[31] Thus, assays of Cdc25 in the absence of reducing agents or containing reactive compounds are highly likely to demonstrate covalent and irreversible inhibition, a trait generally avoided in drug discovery and inhibitor development.

The second reason Cdc25 has proven to be a difficult target in the development of inhibitors is the open and somewhat featureless architecture of its active site. The crystal structures of the catalytic domains of Cdc25A[32] and Cdc25B[33] show an exposed active site region not suitable for structure-based design of compounds with complementary binding surfaces. There are no apparent phospho-threonine or phospho-tyrosine binding pockets, let alone two adjacent pockets as suggested by the preference of Cdc25 for bis-phosphorylated protein substrate.[34] The open architecture of the active site is reflected in the low and nonspecific activity of Cdc25 toward phospho-peptide substrates and suggests that peptide mimetics are not a viable route to Cdc25 inhibition.[34]

In this manuscript we describe the discovery and characterization of a novel class of compounds, the indolyldihydroxyquinones, that inhibit the Cdc25 phosphatases with surprising potency. Extensive and consistent structure−activity relationships with 50+ derivatives of the parent molecule (2,5-dihydroxy-3-(1*H*-indol-3-yl)[1,4]benzoquinone), reversible active-site directed inhibition, submicromolar binding constants, and effective inhibition of the natural protein substrate make this family of compounds an attractive lead for the development of specific Cdc25 inhibitors.

Experimental Section

Compounds. Synthesis, purification, and characterization of all the indolyldihydroxyquinones derivatives except **8J** and **8L** have been described previously.[35] Syntheses of **8J** and **8L** were performed using materials and methods as described previously,[35] as shown in Scheme 1, and as further elucidated below. **NSC668394** (Figure 1) was obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute.

2-Chloro-6-nitrophenylamine.[36] 1-Chloro-3-nitrobenzene (3.1546 g, 20 mmol) and methoxyamine hydrochloride (2.02 g, 24 mmol) were dissolved in DMF (15 mL). The solution was added dropwise in 15 min to a suspension of cuprous chloride (209.5 mg, 2.1 mmol) and potassium *tert*-butoxide (11.07 g, 98.6 mmol) in DMF (35 mL) cooled in a 15 °C bath. The cold bath was removed, and the mixture was stirred for 1 h. The reaction was quenched with aqueous saturated ammonium chloride and extracted with ethyl acetate. After being washed with brine, the organic layer was separated and dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography, the desired product was obtained as a light yellow solid (1.601 g, 46%). mp 72−73 °C. ^{1}H NMR ($CDCl_3$): 8.06 (1H, dd, J = 8.7, 1.5 Hz), 7.50 (1H, dd, J = 7.8, 1.5 Hz), 6.64 (1H, dd, J = 8.7, 7.8 Hz), 6.4−6.5 (2H, br s).

1-Chloro-2-iodo-3-nitrobenzene.[37] 2-Chloro-6-nitrophenylamine (1.87 g, 10.8 mmol) was dissolved in HCl (37%, 20 mL) and cooled in an ice bath. Then a solution of $NaNO_2$ (1.27 g, 18.4 mmol) in water (8 mL) was added very slowly with stirring. After 15 min the mixture was filtered through glass wool into a solution of KI (7.8 g, 47 mmol) in water (20 mL). The resulting orange mixture was stirred at room temperature overnight, then extracted with ethyl acetate and subsequently washed twice with 10% NaOH and once with brine. The organic layer was dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography, the desired product was obtained as a yellow solid (2.29 g, 75%). mp 101−102 °C. ^{1}H NMR ($CDCl_3$): 7.66 (1H, dd, J = 7.8, 1.8 Hz), 7.52 (1H, dd, J = 7.8, 1.8 Hz), 7.42 (1H, t, J = 7.8 Hz).

1-Chloro-2-(3-methylbut-2-enyl)-3-nitrobenzene. 1-Chloro-2-iodo-3-nitrobenzene (711.8 mg, 2.5 mmol) and $Pd(PPh_3)_4$ (580.3 mg, 0.5 mmol) were dissolved in DMF (10 mL). Argon was bubbled through the solution for 10 min to remove oxygen. Tributyl(3-methyl-but-2-enyl)stannane (1.06 g, 2.95 mmol) was added dropwise. The solution was then heated to 90 °C. After incubation overnight, the mixture was diluted with ethyl acetate and washed with saturated aqueous KF solution twice.

The organic layer was dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography, the desired product was obtained as a light yellow oil (496.5 mg, 88%). ^{1}H NMR ($CDCl_3$): 7.63 (1H, dd, J = 8.1, 1.2 Hz), 7.58 (1H, dd, J = 8.1, 1.2 Hz), 7.27 (1H, t, 8.1 Hz), 5.05−5.14 (1H, m), 3.66 (2H, d, J = 6.6 Hz), 1.74 (3H, m), 1.70 (3H, m). ^{13}C NMR ($CDCl_3$): 151.4, 136.4, 134.4, 133.8, 133.5, 127.3, 122.6, 119.1, 28.7, 25.6, 17.5.

6-Chloro-7-(3-methylbut-2-enyl)-1*H*-indole. 1-Chloro-2-(3-methylbut-2-enyl)-3-nitrobenzene (184.7 mg, 0.82 mmol) was dissolved in THF (8 mL) and cooled to −48 °C. Under a N_2 stream ,vinylmagnesium bromide (0.8 M, 4.5 mL, 3.6 mmol) was added in one portion. After being stirred for 30 min at the same temperature, the reaction was quenched with saturated aqueous NH_4Cl before allowing the mixture to warm to room temperature and extracting it with ethyl acetate. The organic layer was washed with saturated aqueous NH_4Cl and brine and dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography, the desired product was isolated as a light yellow oil (68 mg, 38%). ^{1}H NMR ($CDCl_3$): 8.21 (1H, br s), 7.43 (1H, d, J = 8.7 Hz), 7.19 (1H, dd, J = 3.0, 2.4 Hz), 7.14 (1H, d, J = 8.4 Hz), 6.54 (1H, dd, J = 3.0, 2.1 Hz), 5.27−5.38 (1H, m), 3.75 (2H, d, J = 6.9 Hz), 1.90 (3H, m), 1.78 (3H, m). ^{13}C NMR ($CDCl_3$): 136.1, 133.8, 127.2, 126.8, 124.8, 121.8, 121.7, 121.5, 119.6, 103.3, 28.4, 25.9, 18.4.

3-[6-Chloro-7-(3-methylbut-2-enyl)-1*H*-indol-3-yl]-2,5-dihydroxy-[1,4]benzoquinone (8J). 6-Chloro-7-(3-methylbut-2-enyl)-1*H*-indole (33 mg, 0.15 mmol) was dissolved in acetonitrile (0.2 mL) and THF (1 mL) at room temperature. H_2SO_4 (8 μL, 0.15 mmol) was added, followed by 2,5-dichloro-[1,4]benzoquinone (53.1 mg, 1.0 mmol) under a N_2 stream. The solution was stirred for 24 h followed by addition of silver carbonate (∼50 wt % on Celite, 165 mg, 0.3 mmol). After 2 h, the mixture was diluted with ethyl acetate and filtered. The filtrate was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, and the solvent was removed under vacuum. After flash chromatography, the desired product was isolated and dissolved in methanol (3 mL). The solution was brought to reflux and 10% NaOH (1.5 mL) was added dropwise. After 30 min, the solution was poured into cold water (20 mL), acidified with 10% H_2SO_4, and extracted with ethyl acetate. The organic layer was washed with brine and dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography (oxalic acid-coated silica gel), the desired product was isolated as a red solid (10 mg, two-step yield 19%). ^{1}H NMR (d_6-acetone): 10.55 (1H, br s), 9.0−10.2 (2H, br s), 7.39 (1H, d, J = 3.0 Hz), 7.35 (1H, d, J = 8.4 Hz), 7.04 (1H, d, J = 8.4 Hz), 6.01 (1H, s), 5.19−5.27 (1H, m), 3.76 (1H, d, J = 6.9 Hz), 1.83 (3H, d, J = 0.9 Hz), 1.67 (3H, d, J = 0.9 Hz).

1,5-Dichloro-2-iodo-3-nitrobenzene.[38] 2,4-Dichloro-6-nitrophenylamine (2.14 g, 10.3 mmol) was dissolved in HCl (37%, 20 mL) and cooled in an ice bath. A solution of $NaNO_2$ (1.27 g, 18.4 mmol) in water (8 mL) was added very slowly with stirring. After 15 min the mixture was filtered through glass wool into a solution of KI (7.5 g, 45.2 mmol) in water (20 mL). The resulting orange mixture was stirred at room-temperature overnight and extracted with ethyl acetate. The organic layer was washed twice with 10% NaOH and once with brine. It was then dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography, the desired product was obtained as a yellow solid (2.42 g, 76%). Mp 56−57 °C. ^{1}H NMR ($CDCl_3$): δ 7.63 (1H, d, J = 2.1 Hz), 7.50 (1H, d, J = 2.4 Hz). MS (EI): calcd for $C_6H_2Cl_2INO_2$: 316.85, found 317.

1,5-Dichloro-2-(3-methylbut-2-enyl)-3-nitrobenzene. 1,5-Dichloro-2-iodo-3-nitrobenzene (830.5 mg, 2.6 mmol) and Pd(PPh_3)$_4$ (604.5 mg, 0.5 mmol) were dissolved in DMF (10 mL). Argon was bubbled through the solution for 10 min to remove oxygen. Tributyl(3-methyl-but-2-enyl)stannane (1.28 g, 3.6 mmol) was added dropwise. The solution was then heated to 65 °C. After incubation overnight, the mixture was diluted with ethyl acetate and washed with saturated aqueous KF solution twice. The organic layer was dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography, the desired product was obtained as a light yellow oil (565.9 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 7.62 (1H, d, J = 2.4 Hz), 7.57 (1H, d, J = 2.1 Hz), 4.8−5.6 (1H, m), 3.60 (2H, d, J = 6.6 Hz), 1.71 (3H, m), 1.67 (3H, m). ^{13}C NMR ($CDCl_3$): δ 151.6, 137.1, 134.9, 134.5, 133.2, 132.6, 122.9, 118.6, 28.4, 25.6, 18.1.

4,6-Dichloro-7-(3-methylbut-2-enyl)-1*H*-indole. 1,5-Dichloro-2-(3-methylbut-2-enyl)-3-nitrobenzene (582.4 mg, 2.2 mmol) was dissolved in THF (16 mL) and cooled to −48 °C under N_2. Vinylmagnesium bromide (0.8 M, 8.4 mL, 6.7 mmol) was added in one portion. After being stirred for 30 min, the reaction was quenched with saturated aqueous NH_4Cl before allowing the mixture to warm to room temperature. After extraction with ethyl acetate, the organic layer was washed with saturated aqueous NH_4Cl and brine and dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography, the desired product was isolated as a light yellow oil (220 mg, 39%). ^{1}H NMR ($CDCl_3$): δ 8.29 (1H, br s), 7.19 (1H, dd, J = 2.4, 3.3 Hz), 7.15 (1H, s), 6.59 (1H, dd, J = 2.1, 3.3 Hz), 5.2−5.3 (1H, m), 3.67 (2H, d, J = 6.9 Hz), 1.84 (3H, m), 1.74 (3H, m). ^{13}C NMR ($CDCl_3$): δ 136.0, 134.0, 126.7, 125.5, 125.0, 124.0, 120.8, 120.7, 120.5, 101.8, 27.8, 25.6, 18.1.

3-[4,6-Dichloro-7-(3-methylbut-2-enyl)-1*H*-indol-3-yl]-2,5-dihydroxy-[1,4]benzoquinone (8L). 4,6-Dichloro-7-(3-methylbut-2-enyl)-1*H*-indole (130.4 mg, 0.5 mmol) was dissolved in acetonitrile (2 mL) and THF (0.5 mL) at room temperature under N_2. H_2SO_4 (26 μL, 0.5 mmol) was added, followed by 2,5-dichloro-[1,4]benzoquinone (176.7 mg, 1.0 mmol). The solution was stirred for 5 d, and then silver carbonate (∼50 wt. % on Celite, 550 mg, 1 mmol) was added. After 2 h, the mixture was diluted with ethyl acetate and filtered. The filtrate was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, and the solvent was removed under vacuum. After flash chromatography, the desired product was isolated and dissolved in methanol (3 mL). The solution was brought to reflux and 10% NaOH (1.5 mL) was added dropwise. After 30 min, the solution was poured into cold water (20 mL), acidified with 10% H_2SO_4, and extracted with ethyl acetate. The organic layer was washed with brine, and dried over Na_2SO_4, and the solvent was removed under vacuum. After flash column chromatography (oxalic acid-coated silica gel), the desired product was isolated as a red solid (27 mg, two-step yield 15%). ^{1}H NMR ($CDCl_3$): δ 10.74 (1H, br s), 8.4−10.0 (2H, br s), 7.39 (1H, d, J = 3.0 Hz), 7.07 (1H, s), 6.04 (1H, s), 5.16−5.25 (1H, m), 3.74 (1H, d, J = 6.9 Hz), 1.82 (3H, d, J = 0.9 Hz), 1.68 (3H, d, J = 1.2 Hz).

Enzymes. Cdc25A, Cdc25B, and Cdc25C were prepared as previously described.[34,39,40] All three isoforms are prepared as native proteins consisting of the catalytic domains starting from residue 323 for Cdc25A, 377 for Cdc25B, and residue 280 for Cdc25C and terminating at the natural C-termini. The truncated catalytic domain of Cdc25B has been described previously.[39] Site-directed mutations of Cdc25B were generated using the appropriate primers and site-directed mutagenesis as described previously.[39] Expression, purification, and detailed characterization of F475A, R482L, R492L, Y528F, M531A, N532A, and R544L will be described elsewhere (manuscript in preparation).

Inhibition Assays. Cdc25 was assayed using 3-*O*-methylfluorescein phosphate (mFP) as a substrate in time-dependent assays monitoring product formation at 477 nm following the reaction under initial velocity conditions (2−5% turnover). IC_{50} determinations were performed using eight different concentrations of inhibitor at an mFP concentration of 50 μM (∼5 × K_m) and the data were fitted to eq 1. K_i determinations were performed using mFP concentrations from 2 to 150 μM and inhibitor concentrations as indicated in the figure legends. The data were fitted to models for competitive, uncompetitive, and noncompetitive inhibition using Cleland's equations in the Kinet-A-Syst package.[41] Assays using the protein substrate

Table 1. Percent Inhibition of Cdc25A, Cdc25B, and Cdc25C at 50 μM Indolyldihydroxyquinones[a]

compd	modification	Cdc25A	Cdc25B	Cdc25C	compd	modification	Cdc25A	Cdc25B	Cdc25C
2A	none	33	75	69	**5C**	6-benzyloxy	82	97	92
2B	1-methyl	18	42	19	**5D**	6-methyl	66	16	28
2C	2-methyl	24	9	5	**5E**	7-fluoro	100	67	68
2D	2-ethyl	15	36	14	**5F**	7-chloro	0	23	54
2E	2-cyclopropyl	22	32	0	**5G**	7-bromo	11	45	11
2F	2-isopropyl	7	13	0	**5H**	7-methyl	0	0	1
2G	2-(1-methylcyclopropyl)	0	17	0	**6A**	7-propyl	72	23	79
2H	2-*tert*-butyl	30	11	0	**6B**	7-prenyl	100	99	84
3A	2-(1-methylcyclohexyl)	44	11	22	**6C**	7-geranyl	86	95	96
3B	2-phenyl	40	38	18	**6D**	7-farnesyl	96	89	85
3C	2-(1,1, dimethyl-allyl)	12	0	21	**6E**	7-benzyl	100	97	98
3D	4-fluoro	20	5	0	**6F**	7-(2-methyl-benzyl)	100	97	94
3E	4-chloro	22	21	0	**6G**	7-*tert*-butyl	93	33	37
3F	4-bromo	51	6	54	**6H**	7-phenyl	99	92	91
3G	4-methoxy	40	21	19	**7A**	7-methoxy	0	15	13
3H	4-benzyloxy	48	0	2	**7B**	7-benzyloxy	98	97	85
4A	4-methyl	39	9	28	**7C**	2,5-dimethyl	18	20	25
4B	5-fluoro	39	32	23	**7D**	2-methyl-5-methoxy	0	0	31
4C	5-chloro	59	32	45	**7E**	2-methyl-5-chloro	0	42	30
4D	5-bromo	83	55	26	**7F**	2,6-dimethyl	0	16	0
4F	5-methoxy	0	0	0	**7G**	2,7-dimethyl	0	0	0
4G	5-benzyloxy	100	50	26	**7H**	5,6-methylenedioxy	99	88	63
4H	5-methyl	100	61	55	**8A**	5,6-dimethoxy	54	19	20
5A	6-fluoro	97	90	48	**8B**	6,7-dimethyl	9	32	20
5B	6-chloro	100	92	81	**8C**	benzo[*g*]	99	79	75

[a] Each compound was tested in a time-dependent assay and compared with a simultaneous control reaction containing no inhibitor. By testing selected compounds in duplicate the average error is estimated at ±7%.

Cdk2-pTpY/CycA were performed as IC_{50}'s using eight different inhibitor concentrations in time-dependent assays with 300 nM Cdk2-pTpY/CycA in a modification of the previously described procedure.[34] To avoid assaying compounds in the presence of 1 mg/mL bovine serum albumin, this carrier protein was added after the TCA quench. Assays using protein tyrosine phosphatase 1B, a generous gift from Zhong-Yin Zhang (Albert Einstein College of Medicine), were performed in the same buffer as for Cdc25, and product formation was monitored at 410 nm using 200 μM *p*-nitrophenyl phosphate as a substrate.

Cell-Based Assays. HEK293 cells were cultured on poly-L-lysine coated plates in MEM media supplemented with 10% fetal bovine serum and 0.1 mM nonessential amino acids. Cell synchronization in G1/S was achieved using a double thymidine block.[42] Cell lysis was performed in RIPA buffer supplemented with protease and phosphatase inhibitors. The phospho-Cdk-specific antibody was obtained from Cell Signaling Laboratories. Cell staining for apoptosis using TUNEL was performed according to the manufacturer's instructions (Boehringer Mannheim). Caspase activation was measured using the Apo-ONE homogeneous caspase-3/7 assay and 50000 HEK293 cells/well according to the manufacturer's instructions (Promega).

Results

The core molecule in the library is 2,5-dihydroxy-3-(1*H*-indol-3-yl)[1,4]benzoquinone (**2A**) (Figure 1). The library of 50 derivatives with a variety of single and double modifications of differing positions on the indole ring are listed in Table 1. We first screened the library of 50 compounds against Cdc25A, Cdc25B, and Cdc25C by measuring the amount of inhibition seen against the artificial substrate mFP (50 μM). Although formation of methylfluorescein can be readily measured by fluorescence spectroscopy, we have chosen to perform our assays using UV−vis spectroscopy to avoid potential artifacts induced by fluorescence quenching of methylfluorescein by the inhibitors. All measured activities in the presence of inhibitors were linear over the time course of the assay indicating that these compounds were not causing time-dependent inactivation of Cdc25 (Figure S1, Supporting Information). The percent inhibition at 50 μM ranged from 0 to 100%, indicating a broad range of inhibitory activities for this class of compounds (Table 1). Using protein tyrosine phosphatase 1b (PTP1b) and compounds **2A** and **6B**, we saw no significant inhibition at 50 μM, indicating that this family of compounds is not a general nonspecific inhibitor of cysteine phosphatases (data not shown).

We next tested selected compounds using eight different inhibitor concentrations ranging from 0.1 to 50 μM to address in more detail the interactions of these compounds with Cdc25. Again, all activities were linear in the presence of the compounds. These studies provide reproducible IC_{50}s along with Hill slopes and display some differentiation among the three Cdc25s (Figure S2, Supporting Information, and Table 2). To compare our compounds against other reported inhibitors of Cdc25, we tested **NSC668394** (Figure 1) under our assay conditions and obtained IC_{50} ranging from 15 to 35 μM (Table 2). The origin of the 50-fold discrepancy with the published report is unknown.[27]

On the basis of the significantly improved potency for modifications in the 7-position and the favorable Hill slope characteristics of chloro-modifications to the indole ring, we generated two second generation compounds, namely **8J** and **8L**. Compounds **8J** and **8L** were subjected to the same eight-point IC_{50} tests as the other compounds and displayed improved potency toward Cdc25B and Cdc25C but not Cdc25A (Table 2).

To address specifically in more depth the question of reversibility, we further tested compounds **2A**, **6B**, and **8L**. Following preincubation of 320 nM Cdc25B with 4−50 μM inhibitor for varying lengths of time, the enzyme was diluted 100-fold into a reaction mixture containing 50 μM mFP. In comparison to control experiments lacking inhibitor, there was no significant loss in activity, in particular for **6B** and **8L**, indicating that these inhibitors are reversible (Figure 2). Additionally,

Table 2. Eight-Point IC_{50}'s (μM) of Selected Indolyldihydroxyquinones against Cdc25A, Cdc25B, and Cdc25C[a]

compd	modification	Cdc25A	Cdc25B	Cdc25C
2A	none	25	18	30
2E	2-cyclopropyl	35	>50	>50
3E	4-chloro	28	>50	>50
4D	5-bromo	5.8	n.d.	n.d.
4G	5-benzyloxy	5.5	n.d.	n.d.
4H	5-methyl	8.1	n.d.	n.d.
5B	6-chloro	4.2	14	40
6A	7-propyl	4.2	59	>50
6B	7-prenyl	4.1	5.6	15
6C	7-geranyl	1.0	n.d.	10
6D	7-farnesyl	2.4	2.4	3.5
6E	7-benzyl	2.3	2.3	15
6F	7-(2-methyl-benzyl)	1.0	1.0	3.5
6G	7-*tert*-butyl	7.2	7.2	>50
6H	7-phenyl	2.5	2.5	27
7B	7-benzyloxy	4.2	4.2	6.9
8C	benzo[*g*]	2.5	24	15
8J	6-chloro-7-prenyl	4.0	7.2	11
8L	4,6-dichloro-7-prenyl	5.0	2.3	8.8
NSC668394		15	35	35

[a] The values shown are the mean of three separate determinations with errors averaging 20%.

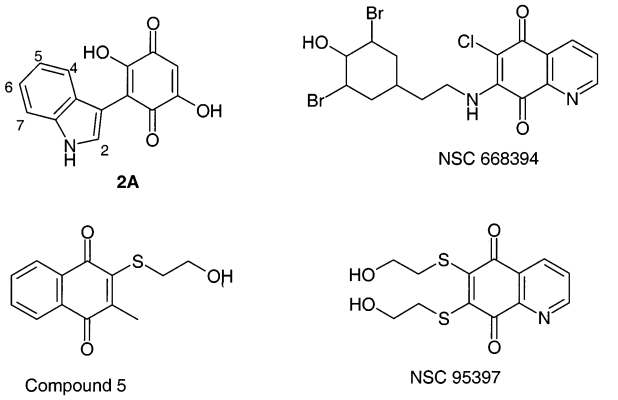


Figure 1. Structures of Cdc25 inhibitors. The unmodified indolyldihydroxyquinone that forms the core structure for the compounds described in this report is **2A**. The carbons on the indole ring are numbered in accord with the substitutions indicated in Table 1. Compound 5, **NSC668394**, and **NSC95397** have been described previously.[15,27,28]

incubation of 1 μM of **8J** in the presence of 10 μM Cdc25B did not change the UV−vis absorbance spectra of **8J**, suggesting that a reversible covalent adduct is not formed (data not shown). Control experiments using **NSC668394** (25 μM) or hydrogen peroxide (200 μM) that modify the active site cysteine by alkylation or oxidation, respectively, showed time-dependent loss of activity (Figure 2).

To elucidate the kinetic mechanism of inhibition, we tested compounds **2A**, **6B**, and **8L** by varying the concentrations of both the substrate mFP and the inhibitors. All the compounds exhibited competitive inhibition (Figure S3, Supporting Information). No adequate fits could be obtained when the primary data were fitted to equations for noncompetitive or uncompetitive inhibition as judged by variance, relative errors in fitted values (>100%), and/or prediction of negative inhibition constants. As shown in Table 3, the inhibition constants (K_i), equal to the dissociation constants (K_d) for competitive inhibitors, are approximately 5−10-fold lower than the IC_{50} values, consistent with the assay

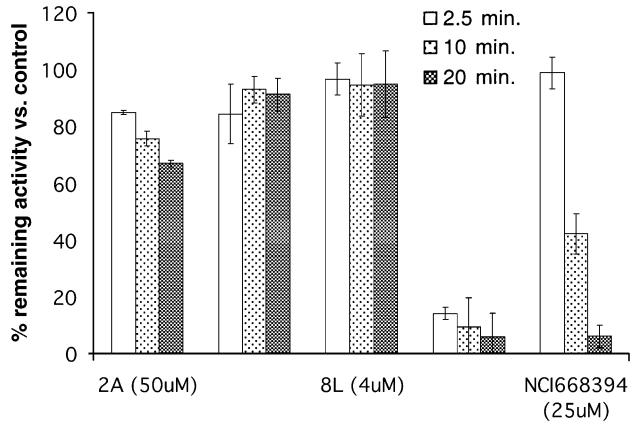


Figure 2. Reversibility of Cdc25 Inhibition. Cdc25B was incubated with an excess of inhibitor at the concentration indicated for varying lengths of time, as indicated. The remaining activity was measured by dilution of the enzyme 100-fold into a reaction mixture and measuring the activity with mFP as a substrate. The data represent the average of 2−5 separate determinations, depending on the compound tested.

Table 3. K_i Values Obtained from Fits to the Equation Describing Competitive Inhibition for Selected Indolyldihydroxyquinones Using MFP as a Substrate and IC_{50} Determinations Using Cdk2-pTpY/CycA as a Substrate against Cdc25B

copmd	modification	K_i vs mFP	IC_{50} vs Cdk2-pTpY/CycA
2A	none	13.0 ± 1.9 μM	>50 μM
6B	6-prenyl	0.64 ± 0.13 μM	28.2 ± 0.6 μM
8L	4,6-dichloro-7-prenyl	0.43 ± 0.07 μM	8.6 ± 3.1 μM

conditions and the equation that applies to reversible competitive inhibitors.[43]

To provide further confirmation that the inhibitors were active site directed and in order to map the binding site for these inhibitors, we tested the activity of compound **6B** in eight-point IC_{50} assays using nine different site-directed mutants of Cdc25B. All but Arg492 are located in close proximity to the active site thiol as seen in Figure 3. Additionally, we tested the activity toward a truncated form of Cdc25B lacking the last 17 residues. The mutants E474Q, F475A, and R482L and the truncated mutant showed significantly altered activity toward **6B** without demonstrating significantly altered activity toward the small molecule substrate mFP (Table 4). When we further tested the three site-directed mutants with altered potencies using the parent molecule **2A**, we observed similar losses in potency (Table 4). In contrast, the truncated mutant showed no significant difference in its inhibition by **2A** or **6B** (Table 4).

Given the much higher reactivity of Cdc25 toward its protein substrate Cdk2-pTpY/CycA in comparison to the artificial substrate mFP,[34] we tested the activity of compounds **2A**, **6B**, and **8L** using the native protein substrate. The IC_{50} were found to be >50 μM, 28.2 ± 0.6 and 8.6 ± 3.1, respectively (Table 3).

Finally, to evaluate the in vivo effect of these compounds, we tested these compounds on the human cell line HEK293, embryonic kidney cells. Incubation of 50 but not 25 μM of **6B** in the absence of serum led to cell death within 4−6 h as indicated by rounding up of cells

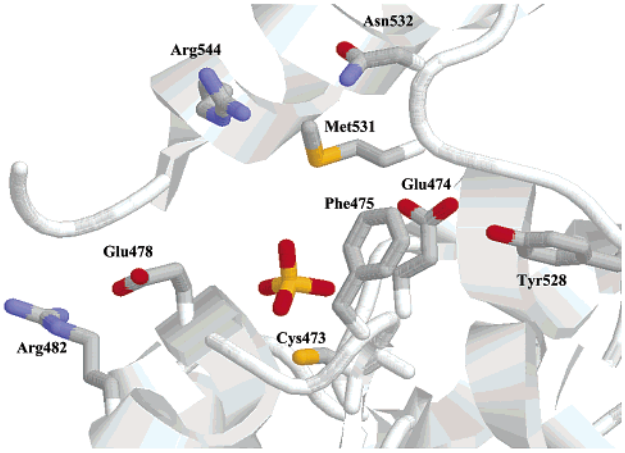


Figure 3. Active site region of Cdc25B. Mutated residues near the active site are labeled and shown as sticks, with the remainder of the protein shown as a cartoon. (Arg492 is not seen in this view.) A sulfate ion as seen in the crystal structure of the catalytic domain of Cdc25B is shown bound in the active site pocket. The coordinates of Cdc25B were taken from 1qb0 and the figure prepared using RasMol.[33]

Table 4. Eight-Point IC_{50}'s (μM) vs Various Mutants of Cdc25B for Compounds **2A** and **6B**[a]

mutant	IC_{50} vs **6B**	IC_{50} vs **2A**
WT	6.4	18
E474Q	>50	200
F475A	>50	76
E478Q	6.5	n.d.
R482L	40	73
R492L	11	n.d.
Y528F	15	n.d.
M531A	6.5	n.d.
N532A	3.9	n.d.
R544L	12	n.d.
truncated	33	34

[a] The values shown are the mean of two separate determinations with errors averaging 28%.

and their loss of adhesion to the plate. HEK293 cells assayed for apoptosis by monitoring caspase activation showed a significant increase in caspase activity following 4 h of treatment with 50 μM, but not 25 μM, of **6B** (Figure 4). Similarly, staining of HEK293 cells with TUNEL following 4 h of treatment with 6B showed 10% of the cells in late apoptosis compared to 0% in the control (data not shown). Attempts to correlate these cellular effects with phosphorylation levels on the Cdks showed no significant differences with control samples using either commercial or in-house phospho-Cdk-specific antibodies using unsynchronized or synchronized cultures.

Discussion

We have discovered a novel class of compounds that appear to be excellent lead molecules for the design of specific and potent inhibitors of Cdc25 phosphatases. These indolyldihydroxyquinones share some similarity yet critical differences with other recently described quinone inhibitors of Cdc25 (Figure 1).[15,27−29] Interestingly, all of these compounds share a substituted quinone and an extended aromatic ring structure. This suggests a paradigm for inhibition of Cdc25 based on either one or both of these shared features. We believe that the quinone ring is the only important common

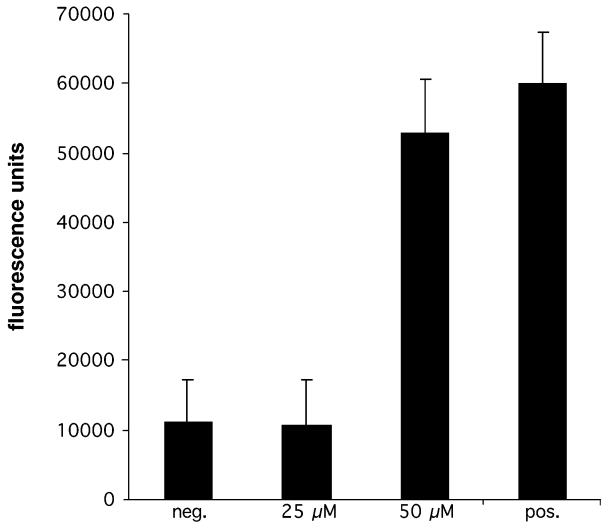


Figure 4. Rapid apoptosis as measured by caspase 3/7 activation is caused by treatment with **6B** for 4 h at 50 μM, but not 25 μM following. The negative control contains only DMSO and the positive control consists of overnight incubation with 5 μM staurosporine.

feature based on the following observations regarding the extended aromatic ring structure. First, although Cdc25 has good activity toward the aromatic substrate mFP, burst kinetics have shown that the affinity (K_d) for mFP is very weak (>1 mM).[39,44] Thus, the high reactivity toward mFP probably relies mostly on the low pK_a of the leaving group (pK_a = 4.5) and the unusual pH-dependence of this enzyme, not on any intrinsic affinity for large aromatic ring structures.[30,39] Second, in overlaying the quinone rings of our indolyldihydroxyquinones with those of **NSC668394** or **NSC95397**, for example, the extended aromatic structures, the indole ring in the former and the naphthoquinone ring in the latter, do not overlap. Similarly, overlaying the aromatic parts of these two classes of molecules yields highly divergent structures in three-dimensional space.

Although the quinone ring appears to be the common feature between the previously described Cdc25 inhibitors and our indolyldihydroxyquinones, we believe that the detailed mode of inhibition is fundamentally different by being reversible and noncovalent. The broadly known electrophilic properties of quinones suggest the possibility of a reaction with nucleophilic groups such as the essential thiolate in the active site of Cdc25. Alternatively, the ability of quinones to be readily reduced might be related to their activity toward Cdc25, as large classes of anticancer agents (such as anthroquinones) are known to undergo reductive activation, and the reactions of quinones with thiols have been investigated.[45,46] However, the reactivity of the indolyldihydroxyquinones and the naphthoquinones are divergent in both these respects. First, the former are quite electron rich, with two electron-donating hydroxy groups and an electron-donating indole substitutent making them much less likely to accept nucleophiles.[47] Second, the redox properties of the indolyldihydroxyquinones have been investigated. It has been reported that the indolyldihydroxyquinones do not sustain redox cycling (cyclic voltammetry) in aqueous DMF.[48] For example, we have observed that the chemically similar molecule demethylasterriquinone B1 is oxidized at

+0.18 V and +0.394 V vs Ag^+ at a glassy carbon electrode in MeOH with lithium perchlorate as the supporting electrolyte. We see it reduced at a Pt electrode at −0.58 V vs Ag^+. None of these potentials are close to those of biologically relevant redox cofactors. It has also been observed that demethylasterriquinone B1 is reduced by hydrogen sulfide but readily converted back to the dihydroxybenzoquinone oxidation state in air.[48] Last, the indolyldihydroxyquinones have strong intramolecular hydrogen bonds further significantly reducing their reactivity.[49] It is for these reasons that we believe our compounds are significantly different than the previously described quinones and thus do not show time-dependent inhibition of Cdc25 (see below). That is, although the initial affinity toward the active site may be governed by recognition of the quinone ring, the final mode of binding is significantly different as the indolyldihydroxyquinones do not covalently modify any active site residues and are therefore classical reversible inhibitors.

From the initial screen of the library one can derive interesting structure−activity relationships, both in terms of the overall effects of modifications on the indole ring as well as specific differences between the three different isoforms of Cdc25. We discuss first the effects that appear to be applicable to Cdc25 inhibition by the indolyldihydroxyquinones in general (Table 1, Table 2). Modification of the nitrogen of the indole by a methyl group appears to be only slightly detrimental and further explorations at this position need to be undertaken. Modification of the 2- or 4-position by anything ranging from a methyl to a *tert*-butyl to a halide to the aromatic phenyl group leads to a significant and consistent loss of potency. Given that these modifications, in particular substituents at the 2-position, greatly increase the barrier to rotation around the biaryl bond and force the dihedral angle to 90°, these results suggest that some angle other than 90° may be required for optimal positioning onto Cdc25.[35] These self-consistent results among monosubstitutions at the 2-position are further confirmed by the loss of potency seen in the compounds with a second modification in addition to a methyl at the 2-position (e.g., **7C**−**G**). Modifications at the 5-position appear to have little effect, except for Cdc25A (see below), as if this position is pointed away from the active site and out into the solvent. Modifications at the 6-position show a significant increase in potency for the halides and benzyloxy, whereas a methyl group is detrimental. At the 7-position, all substituents of size greater than propyl appear to increase potency significantly. As exemplified by **6B** (Table 4), this increase in potency for modification at the 7-position requires an intact C-terminus on Cdc25B. This suggests that the C-terminal tail, not seen in the crystal structures of either Cdc25A or Cdc25B, forms an important part of the binding site for these inhibitors. This is particularly intriguing given that we have previously shown that the C-terminal tail is not important for activity with small molecule substrates, but is required for interaction with the protein substrate.[40] It is of interest to understand how the indolyldihydroxyquinones compounds take advantage of this C-terminal tail in their binding, as they may mimic the not yet understood interaction between Cdc25 and its protein substrate. Attempts to cocrystallize selected indolyldihydroxyquinones with the catalytic domain are underway and should yield insight into the details of the protein−protein interaction involved in recognition of the Cdk2-pTpY/CycA substrate.

Given the high sequence identity among the three Cdc25 isoforms, especially in the active site region, it was not surprising to find that the structure−activity relationships we observed applied to all three Cdc25s. However, we did detect a difference among the three isoforms for modifications at the 5-position. Three compounds with substitutions of bromo- (**4D**), benzyloxy- (**4G**), and methyl- (**4H**) are significantly improved in their inhibition of Cdc25A compared to Cdc25B and Cdc25C. Interestingly, this increased potency requires an intact C-terminal tail, as assays with a truncated form of Cdc25A do not show a similar increase in potency with these modifications at the 5-position (12%, 40%, and 17% remaining activity at 50 μM compound for **4D**, **4G**, and **4H**, respectively). This provides further support that the 5-position is aimed outward toward solvent, and in this case makes isoform-specific contacts with the relatively divergent C-terminal tail of Cdc25A.

IC_{50} determinations using eight different substrate concentrations were used to further evaluate selected compounds from the library as well as the second generation compounds **8J** and **8L**. The IC_{50}s we found under identical assay conditions are among the most potent reported for Cdc25 in the literature at this time (Table 2). The IC_{50} determinations also yield Hill slope data that can point to potential artifacts such as multiple inhibitors binding to each protein and/or protein or inhibitor aggregation.[50−52] We noted reasonable Hill slopes (0.7−1.5) with all of the compounds, except those with very large hydrophobic substituents at the 7-position. In particular, substitution at this position with farnesyl (**6D**), phenyl (**6H**), and benzyloxy (**7B**), although giving a nice increase in potency over the parent molecule **2A**, yielded Hill slopes greater than 3. Given that such compounds are difficult to work with and that the origin of the Hill slope can have diverse causes, we avoided further experimentation with these compounds. Fortunately, addition of the smaller prenyl group (**6B**) still generated much of the increase in potency over the parent molecule **2A** while retaining a reasonable Hill slope (1.2−2.0). The compounds **8J** and **8L** were designed to test the effects of making multiple beneficial substitutions. Although simultaneous incorporation of a 6-chloro and a 7-prenyl in compound **8J** did not show a significant increase in activity against any of the Cdc25s, the addition of a 4-chloro to the 6-chloro-7-prenyl in **8L** did show increased potency toward Cdc25B and Cdc25C, but not Cdc25A. We did note an increased potency for **8J** over **6B** (IC_{50} = 11 μM vs 33 μM) with the truncated form of Cdc25B, confirming the benefit of the 6-chloro substitution seen for **5B**. These experiments demonstrate that not all multiple substitutions of the indolyldihydroxyquinones follow simple additivity rules. Thus we are pursuing larger and more diverse libraries so as to rapidly screen further combinations.

Time-dependent inhibition is a significant issue when working with protein tyrosine phosphatases and dual-specificity phosphatases containing an active site thi-

olate anion. Although we observed linear rates of reaction in the presence of inhibitors, we specifically tested for time-dependent inhibition in a separate series of experiments so as to ensure that our determination of K_i's in subsequent experiments would not become titrations of enzyme concentrations. Incubation of compounds **2A**, **6B**, or **8L** in excess over enzyme at concentrations greater than the K_i with Cdc25B followed by dilution into assay buffer led to an almost complete recovery of activity (Figure 2). Thus, the indolyldihydroxyquinones appear to behave significantly different than the naphthoquinones that showed significant loss of activity in related experiments.[28]

Given the open architecture of the active site region of Cdc25, it has been difficult to envision tight binding of a small molecule near the catalytic cysteine. Therefore, two other types of experiments were completed to determine if the binding of the indolyldihydroxyquinones was occurring at the active site of Cdc25. First, classical K_i determinations performed by simultaneous variation of substrate and inhibitor were found to fit only to equations for competitive inhibition (Table 3). This is in stark contrast to other published inhibitors of Cdc25 that appear to bind via a mixed inhibition mechanism that include a time-dependent component.[27,28] Therefore **6B** and **8L** present the first true submicromolar small molecule binders of Cdc25. Second, using a series of site-directed mutants located near the active site and in the water pocket adjacent to the catalytic site of Cdc25B (Figure 3), we found that mutation of Glu474, Phe475, and Arg482, but not Glu478, Arg492, Tyr528, Met531, or Asn532, reduced the amount of inhibition seen for the indolyldihydroxyquinone class of inhibitors (Table 4). Glu474 and Phe475 are a part of the HCX_5R loop that cradles the phosphate of the substrate. We have recently provided evidence that the former plays a critical role as a catalytic base in the reaction mechanism.[30] Interestingly, Arg482 is one of the two residues suggested previously by molecular modeling to be required for the binding of the large anionic quinone part of the naphthoquinone inhibitors.[28] These data thus bolster this particular docking model, provide strong support for the conclusion that these compounds are active-site directed, and provide further evidence for the concept of the quinone moiety serving as a paradigm for Cdc25 inhibition.

Given our promising results with the indolyldihydroxyquinones as potent and reversible inhibitors of Cdc25, we sought to extend the experiments to more physiologically relevant assays. We monitored the effect of selected compounds on Cdc25s ability to dephosphorylate its native substrate, Cdk2-pTpY/CycA, in vitro. This in vitro assay allowed us to determine the potency of our compounds without concern for membrane permeability or cellular stability. The parent molecule (**2A**), that apparently does not interact with the C-terminus of Cdc25, was not effective in competing with the natural substrate, where we have shown that the interaction with the C-terminal tail is important.[40] Although somewhat less potent than toward mFP, **6B** and in particular **8L** showed good inhibition of Cdc25 with IC_{50}'s of 20 μM and 8 μM, respectively (Table 3). Thus, these compounds can compete effectively with the physiological substrate that is 2 orders of magnitude better than the artificial mFP substrate as determined by k_{cat}/K_m measurements.[34] In associated control experiments we found that our class of compounds exhibit significant nonspecific binding to bovine serum albumin, a carrier protein normally used in the assay with the Cdk2-pTpY/CycA substrate. Thus, if this class of compounds is to be pursued further for drug development, it may be necessary to incorporate a modification that reduces nonspecific protein binding.[53] Incorporation of such simple modifications may be possible on the 5-position of the indole ring, which was seen to tolerate a wide variety of modifications (Table 1). Given that demethylasterriquinones with symmetrical indole rings attached to the remaining unmodified position of the quinone ring are also potent Cdc25 inhibitors (data not shown), modifications that reduce nonspecific protein binding could also be added on the quinone ring.

Our in vivo test of the indolyldihydroxyquinones showed effective killing of cells at 50 μM, but not 25 μM, of **6B** that may be mediated by apoptotic mechanisms that are under further investigation. This cell killing was not accompanied by an increase in phosphorylation of the Cdks, pointing to a mechanism of cell death mediated by some as yet unknown mechanism.

In summary, we have discovered the indolyldihydroxyquinones as a new class of submicromolar inhibitors of the Cdc25phosphatases that bind at the active site and are reversible. Additionally, they show activity in competition with the natural protein substrate in vitro and rapidly lead to cell death. Thus, these compounds serve as attrractive lead molecules for drug development efforts against the Cdc25 phosphatase, an important anticancer target.

Acknowledgment. This work was supported by NIH GM61822-01 grant to Johannes Rudolph and NIH AI48521 grant to Michael Pirrung.

Abbreviations

PTPase, protein tyrosine phosphatase; DSP, dual-specificity phosphatase; Cdk, cyclin-dependent kinase; Cyc, cyclin; Cdk2-pTpY/CycA, Cdk2/CycA complex phosphorylated on Thr14 and Tyr15; mFP, 3-*O*-methylfluorescein phosphate.

Supporting Information Available: Figures S1, S2, and S3, showing representative kinetic inhibition data, are available free of charge via the Internet at http://pubs.acs.org.

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JM0300835