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Biological evaluation of newly synthesized quinoline-5,8-quinones as Cdc25B inhibitors

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Abstract—Cdc25B protein phosphatase represents an attractive potential therapeutic target for small molecule intervention because of its central role in positively regulating cyclin dependent kinases and thus cell proliferation, as well as its elevated levels observed in many human tumors. Among the most potent previously identified Cdc25 inhibitors have been quinoline quinones, which have a rich legacy as therapeutic agents but have also been associated with nonspecific interactions. In this study, we have interrogated the structure–activity relationship of a focused series of C2-, C3-, or C4-modified quinoline-5,8-quinones on Cdc25B inhibition in vitro. Substitution at the C3-position in this small chemical series were slightly superior to substitutions at the C3-position. For all compounds, recombinant human Cdc25B was approximately 5-fold more sensitive compared to recombinant human PTP1B. Two compounds inhibited HeLa cell growth with IC_{50} values of approximately 2 μM. Consistent with other *para*-quinones, some members of this series generated intracellular reactive oxygen species and the in vitro enzyme inhibition was mitigated by addition of reductants or catalase. These results indicate that chemical modifications on the pyridine core are tolerated, providing additional sites for future structural modification of this biologically active pharmacophore.

1. Introduction

Mammalian cells commonly use the reversible phosphorylation of tyrosine residues to transmit extracellular signal information to intracellular targets. It is perhaps not surprising, therefore, that abnormalities in the mediators of these protein phosphorylation signaling pathways, namely kinases and phosphatases, are closely linked with many human diseases. In contrast to the recent success in identifying small molecules that inhibit disease-associated kinases, protein tyrosine phosphatases remain remarkably intractable to chemical inhibition.

Cdc25B protein phosphatase is an attractive potential therapeutic target for small molecule intervention due to its central role in controlling malignant cell proliferation by regulating cyclin dependent kinases and because it is highly expressed in many human tumors.[1] Using high-throughput screening methods or synthetic approaches, we previously identified the *para*-naphthoquinones, 7-aminoquinoline-5,8-quinones and Substituted isoquinoline-5,8-quinones, as promising core structures for potent Cdc25 inhibitors, exemplified by **DA3003-1**.[2–4] The *para*-quinone core structure is fundamental to the biological activity of at least 14 clinically used therapeutics and, thus, of interest in the further development of potential small molecule inhibitors of this class of disease-related enzymes. We have previously reported that some quinones inactivate Cdc25B either by Michael addition[5] or oxidation of the catalytic cysteine.[6] It may be possible to harness the chemically reactive quinone moiety with appropriate chemical substitutions to enhance specific interactions with Cdc25B. A necessary stage in this process will be to define the

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domains on the quinoline-5,8-quinone pharmacophore that can be chemically modified. Previously, we have focused our synthetic efforts exclusively on C6- and C7-modifications of this core structure. In the current study, we have examined a focused series of C2-, C3-, and C4-modified quinoline-5,8-quinones and analyzed their biochemical actions on Cdc25B and their ability to block proliferation of human tumor cells in culture.

2. Results and discussion

Nine newly synthesized quinoline-5,8-quinones were examined for their ability to inhibit the catalytic domain of human Cdc25B. The most potent inhibitors were the 2-methyl substituted **1**, the 4-methyl substituted **3**, and the 2-carbonitrile substituted **5** congeners with IC_{50} values of 4.6, 4.6, and 3.7 μM, respectively (Table 1). All of these compounds were comparable to the previously reported unsubstituted **DA276**, which had an IC_{50} value of 3.0 ± 1.4 μM against Cdc25B.[2] All congeners tested were 5-fold more potent in vitro against Cdc25B compared with human PTP1B (tyrosine phosphatase 1B), consistent with previous studies with other *para*-quinones.[2] When larger and polar moieties not conjugated with the pyridine were added to the C2-position as in **8** and **9**, the IC_{50} for Cdc25 was greater than 10 μM. The amide **7**, however, was noticed to be an exception in this series as this compound retained considerable potency in spite of the bulkiness of the C2-substituent. It is likely that the amide carbonyl is sterically incapable of direct conjugation with the pyridine ring in **7**, therefore changing the trajectory of this substituent versus other carbonyl derivatives. In this regard, compound **7** is comparable to the previously reported NSC 45384.[2] Aldehyde **4** and ester **6** substitutions at C2 retained activity within a factor of 3 or 2, respectively. Overall, the results in this series indicate that small modifications to the C2- as well as the C4-positions are well tolerated, whereas the C3-position appears to be more sensitive to steric bulk (Fig. 1).

Although we are unaware of any previously published information on the effects of this class of compounds on mammalian cell proliferation, the US National Cancer Institute (NCI) has found that a quinoline-5,

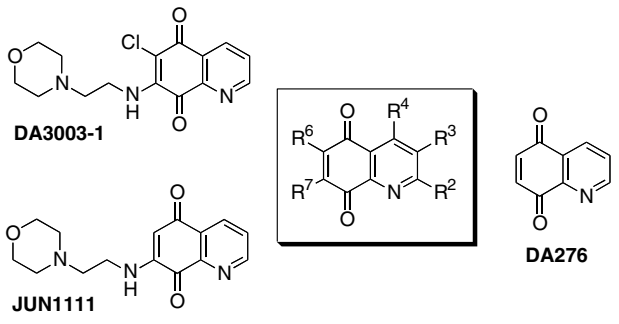


Figure 1. Structures of **DA276**, **DA3003-1**, and **JUN1111** and general substitution pattern of quinoline-5,8-quinones. Previous structure–activity relationship studies did not investigate the influence of substituents at the C2-, C3-, and C4-positions of the pyridine core in any detail.

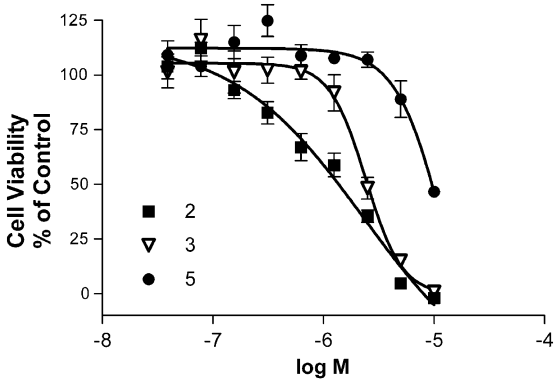


Figure 2. Antiproliferative effects of compounds on HeLa cells. HeLa cells were treated for 72 h with compounds using 3-fold dilutions ranging from 40 nM to 10 μM. The viable cell number was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide. All results were normalized to vehicle treated control values and are expressed as mean values ± SEM ($N = 4$). Compound **2**, ■; compound **3**, ∇; compound **5**, ●.

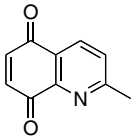
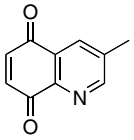
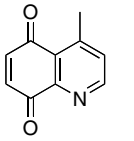
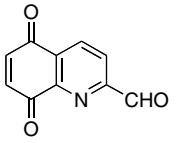
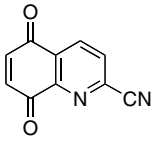
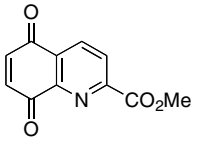
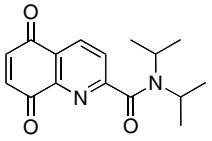
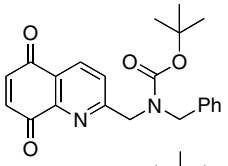
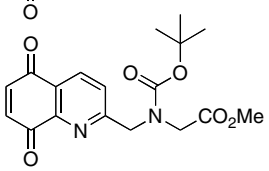
6-quinone (NSC 400944) and 7-amino-6-methoxy-2-methyl-quinoline-5,8-quinone (NSC 132493) inhibit the growth of human cancer cells (see http://pubchem.ncbi.nlm.nih.gov/). Thus, we examined the ability of the congeners to inhibit the growth of HeLa cells. Of the newly synthesized compounds, **2** and **3** were the most potent inhibitors of cell proliferation with IC_{50} values of 2.3 and 2.4 μM, respectively (Table 1 and Fig. 2).

Because previous studies suggested that quinoline-5,8-quinones can inactivate the Cdc25 family of phosphatases by irreversible oxidation of the catalytic cysteine,[6] we determined the sensitivity of in vitro Cdc25B inhibition by selected congeners in the presence of catalase or the reductant DTT. As indicated in Table 2, compound **2** was particularly affected by both catalase and DTT, consistent with the generation of reactive oxygen species (ROS) in vitro. Both compounds **3** and **5** were also affected by DTT concentrations but were minimally affected by catalase as compared with **2**, suggesting that compounds **3** and **5** produced ROS other than H_2O_2. The cell-permeable ROS indicator, H_2DCF, is often used to detect intracellular oxidation.[6] Incubation of HeLa cells with H_2O_2 leads to profound oxidation of DCF (Fig. 3). We observed significant formation of ROS with **5** (2.7-fold over DMSO) that was comparable or exceeded that seen with the previously reported **JUN1111**.[6] In contrast, **2** produced less intracellular ROS (1.4-fold over DMSO), while **3** did not generate significant intracellular ROS. It should be noted, however, that only a single time point was measured and it is possible that the kinetics of oxidation for these two congeners could be more or less rapid than **5**. Thus, we cannot exclude the possibility that **2** and **3** cause intracellular oxidation. Nonetheless, it appears that each of the three congeners has a distinct redox profile.

3. Conclusion

Quinoid substructures continue to dominate the field of small molecule inhibitors of dual-specificity phospha-

Table 1. In vitro inhibition of Cdc25B and PTP1B phosphatases and growth inhibition of HeLa cells

Compound	Structure	IC_{50} (μM)		
		Cdc25B	PTP1B	HeLa
DA3003-1	See Figure 1	0.91 ± 0.36[c]	>10[c]	ND[d]
DA276	See Figure 1	3.0 ± 1.4[b]	>10[b]	ND
JUN1111	See Figure 1	1.8 ± 0.7[c]	>10[c]	ND
1		4.6 ± 0. 5[a]	23.3 ± 2.1[a]	>10
2		10.1 ± 0. 6	55.9 ± 1.8	2.3 ± 0.3[a]
3		4.6 ± 0. 3	22.5 ± 0. 9	2.4 ± 0.1
4		11.3 ± 0. 8	62.7 ± 6. 5	>10
5		3.7 ± 0. 4	14.2 ± 1.4	>10
6		8.5 ± 0. 4	31.6 ± 3.1	>10
7		5.2 ± 0. 5	26.0 ± 2.3	>10
8		>10	ND	>10
9		>10	ND	>10

[a] Mean ± SEM ($N = 4$).
[b] Data from Ref. 2.
[c] Data from Ref. 6.
[d] ND, not determined.

tases.[8–21] In light of the potential of toxic or nonspecific side effects of the quinone pharmacophore,[22] the structure–activity relationship of any lead compound must be carefully evaluated, and ultimately the therapeutic potential will greatly depend on compound tolerance in animal models followed by side effects in human trials. Encouragingly, a significant number of clinically used and well-tolerated pharmaceuticals with

Table 2. Effect of catalase and DTT on in vitro inhibition of Cdc25B

Compound	IC_{50} (μM)		
	Catalase[a] (80 U/mL)	DTT[b] (12.5 mM)	DTT[b] (25 mM)
2	60.6 ± 7.7	43.8 ± 6.0	74.7 ± 3.6
3	6.0 ± 0.1	24.6 ± 5.5	49.7 ± 2.2
5	4.7 ± 3.2	11.6 ± 0.4	36.7 ± 2.3

[a] Means ± range ($N = 2$).
[b] Means ± SEM ($N = 3$).

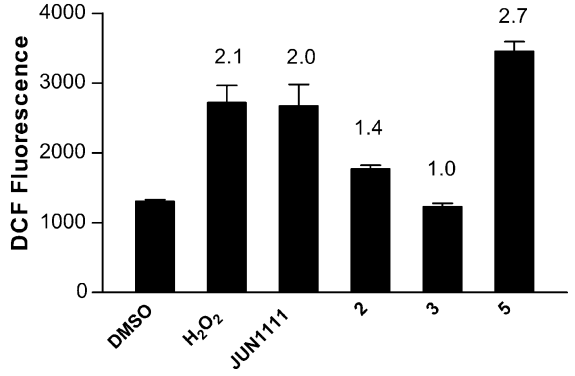


Figure 3. Generation of ROS by compounds in HeLa cells. HeLa cells were preloaded with H_2DCF dye before exposure to compounds for 10–15 min. DCF fluorescence was measured by flow cytometry and ROS generation was determined as the fold increase in DCF fluorescence over the DMSO control. Results are expressed as mean arbitrary unit values ± SEM ($N = 4$) from two individual experiments. The concentration of H_2O_2 was 1 mM and 10 μM for all other compounds. The fold increase over the DMSO vehicle control is indicated above the corresponding compound bar.

quinone substructures are on the market, therefore demonstrating the feasibility of this chemical fine tuning approach.

4. Materials and methods

4.1. Chemical synthesis

The preparation of **DA276**, **DA3003-1**, and **JUN1111** has been reported previously.[2,6] All other quinoline-5,8-quinones were synthesized by photooxygenation from 8-hydroxyquinolines.[7]

4.2. In vitro enzyme assays

Epitope-tagged (6× histidine) Cdc25B catalytic domain was expressed in *Escherichia coli* and purified by Ni–NTA as previously described.[2] Human recombinant PTP1B was purchased from BIOMOL (Plymouth Meeting, PA). Inhibition of enzyme activity was determined with a minimum of five concentrations of each compound using the artificial substrate *O*-methyl fluorescein phosphate (Sigma, St. Louis, MO) in a 96-well microtiter plate assay based on previously described methods.[2] Fluorescence emission from the product was measured after a 20-min (PTP1B) or 60-min (Cdc25B) 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_{50} concentrations were determined using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). In some studies, catalase (80 U/mL) or dithiothreitol (DTT) (12.5 or 25.0 mM) was increased above the normal 1–2 mM as previously described.[2]

4.3. Antiproliferative assays

HeLa cells (human cervical cancer) from the American Type Culture Collection (Manassas, VA) were used to determine the effect of compounds on cell viability and proliferation with a previously described colorimetric assay.[2] Briefly, cells (2000 per well) were plated in 96 microtiter plate wells in Dulbecco's minimal essential medium containing 10% fetal bovine serum with increasing concentrations of compound (3-fold serial dilutions from 40 nM to 10 μM). Cells were incubated at 37 °C in 5% CO and 95% air for 72 h (approximately three population doubling times). Viable cell numbers were determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide. All results were normalized to vehicle treated control values and are expressed as mean values ± standard error of the mean ($N = 4$).

4.4. Measurement of cellular ROS generation

HeLa cells (1×10^6) were detached from monolayer cultures using EDTA/trypsin, re-suspended in phosphate-buffered saline (PBS) and preloaded for 10–15 min with chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, (H_2DCF) dye (Molecular Probes, Eugene, OR) as previously described.[6] Cells were then washed in PBS and re-suspended in PBS buffer containing 3 μM propidium iodide (for the detection of dead cells). Cells were then treated for 10 min with DMSO (vehicle control) or with 1 mM H_2O_2, **JUN1111**, **2**, **3**, or **5**. DCF and propidium iodide (PI) fluorescence was measured by flow cytometry using the FACSCalibur flow cytometer (BD Pharmingen) as previously described.[6]

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