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G2/M accumulation in prostate cancer cell line PC-3 is induced by Cdc25 inhibitor 7-chloro-6-(2-morpholin-4-ylethylamino) quinoline-5, 8-dione (DA 3003-2)

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Abstract. Cdc25 phosphatases are dual-specific phosphatases that play a role in cell cycle progression. In many human cancers, Cdc25 phosphatases are overexpressed as compared with normal tissues. In addition, overexpression of Cdc25 phosphatases in prostate cancer is correlated with disease progression. The antiproliferative efficacy of Cdc25 phosphatase inhibitor 7-chloro-6-(2-morpholin-4-ylethylamino) quinoline-5, 8-dione (DA 3003-2) was investigated in the PC-3 asynchronous human prostate cancer cell line using a cell-based assay. The time course changes in cell cycle distribution and the modulation of cell cycle regulators after DA 3003-2 administration were analyzed using the MTT assay. We found that the relative IC_{50} of DA 3003-2 was 2-fold lower as compared with its congener (2-mercaptoethanol)-3-methyl-1, 4-naphthoquinone (NSC 672121). Asynchronous PC-3 cells accumulated in the G2/M phase at 24 h after treatment with 10 μM DA 3003-2 or 20 μM NSC 672121, which represent IC_{70} concentrations. Treatment of cells with DA 3003-2 caused hyperphosphorylation of Cdc2 tyr^{15} in cyclin B_1 and cyclin A complexes. DA 3003-2 did not downregulate the protein expression levels of Cdc25s, cyclins and cyclin-dependent kinases (Cdks). To conclude, after DA 3003-2 administration asynchronous PC-3 cells accumulated in the G2/M phase, with hyperphosphorylation of the G2/M cyclin-Cdk complex.

Introduction

Cell cycle progression and cell proliferation are controlled in part by a balance between cyclins, cyclin-dependent kinases (Cdks) and phosphatases. Various types of human cancers are characterized by uncontrolled cell growth which leads to poor patient prognosis. One of the mechanisms involved in uncontrolled cell growth is thought to be the change in basic cell cycle regulation caused by cancer-associated mutations, overexpression of cell cycle-regulated protein, as well as by the loss of Cdk inhibitor expression. Cdc25 is a dual-specificity protein tyrosine phosphatase which catalyzes the dephosphorylation and activation of cyclin-Cdk complexes through the removal of inhibitory phosphates. The Cdc25 phosphatase family comprises three related gene products, namely Cdc25A, Cdc25B and Cdc25C. Cdc25A and Cdc25B cooperate with activated Ras to induce oncogenic focus formation in rat embryonic fibroblasts. Moreover, overexpression of Cdc25 phosphatases in human cancers correlates with disease progression and is an indicator of poor patient prognosis (1,2). Taken together, these results suggest that Cdc25 phosphatases have potential as a potent small molecular target for cancer therapy.

In prostate cancer, Cdc25A, Cdc25B and Cdc25C are upregulated in cancerous lesions relative to non-cancerous lesions, and levels are increased in higher Gleason grade tumors (3-5). A number of novel Cdc25 inhibitors from the compounds library at the University of Pittsburgh have been screened with a focus on compound structures, such as quinine or naphthoquinone structures (6-8). In the present study, a more selective naphthoquinone Cdc25 inhibitor, 7-chloro-6-(2-morpholin-4-ylethylamino) quinoline-5, 8-dione (DA 3003-2), was generated from the drug library at the University of Pittsburgh, and its molecular mechanisms of action were investigated using the PC-3 human prostate cancer cell line.

Materials and methods

Cell culture, chemicals and antibodies. PC-3 human prostate cancer cells were obtained from the American Type Culture Collection (Bethesda, MD). The cells were cultured at 37˚C in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2. DA 3003-2 and (2-mercaptoethanol)-3-methyl-1, 4-naphthoquinone (NSC 672121) were generated as described previously (7). These compounds were solubilized so that the final concentration of dimethyl sulfoxide (DMSO) was <0.1% when added to the cells. The following antibodies were used: anti-Cdc25A (Ab3) purchased from NeoMarkers, Inc. (Fremont, CA);

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anti-Cdc25B from Transduction Laboratories (Lexington, KY); and anti-Cdc25C (C-2), anti-Cdk2 (D-12), anti-Cdc2 (17), anti-phospho-Cdc2/Cdk2 (Tyr^{15}), anti-cyclin A (H-432), anti-cyclin B_1 (GNS1) and anti-actin (C-2) from Santa Cruz Biotechnology (Santa Cruz, CA).

MTT assay. The sensitivity of the cells to NSC 672121 and DA 3003-2 was determined by a microtiter assay. Cells ($4x10^3$) were plated in 96-well microtiter plates, cultured for 24 h and exposed continuously to 0.3-30 μM of NSC 672121 or DA 3003-2 for 48 h. The viability of the cells was assayed by determining the color development resulting from the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) spectrophotometrically at 540 nm, as described previously (7).

Flow cytometry. PC-3 cells were plated at $5x10^5$ cells/dish and maintained for 24 h. Cells were treated with DMSO, NSC 672121 or DA 3003-2. After 24 h, cells were trypsinized and washed with phosphate-buffered saline (PBS). The harvested cells were stained with a solution containing 50 μg/ml propidium iodide (PI) and 250 μg/ml RNase A. Flow cytometric analysis was conducted using EPICS XL™ and XL-MCL™ systems (Beckman Coulter, Inc.).

Western blotting and immunoprecipitation. Vehicle- or compound-treated cells were harvested by scraping and were resuspended in lysis buffer (30 mM HEPES, 1% Triton X-100, 10 mM NaCl, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA and 0.2 mM Na_3VO_4, pH 7.6) with protease inhibitors [10 μg/ml leupeptin, 10 μg/ml aprotinin and 100 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride]. The samples were briefly vortexed and centrifuged at 13,000 x g for 30 min. The total protein concentration was determined using the Bradford protein assay (BioRad, CA). To immunoprecipitate cyclin B_1, cyclin A, Cdc2 and Cdk2 proteins, 2 μg of anti-cyclin B_1, anti-cyclin A, anti-Cdc2 or anti-Cdk2 antibodies and Protein G Sepharose 4B were incubated with 1 mg of lysates for 16 h. Beads were washed three times by vortexing with lysis buffer at each step. Equal amounts of protein or supernatant were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. A chemiluminescence detection system (Western Lightning™; Perkin Elmer Life Sciences, Boston, MA) was used for immunocomplex detection.

Results

Cdc25 inhibitor induced G2/M accumulation in asynchronous PC-3 cells. To determine the cytotoxicity of DA 3003-2 in PC-3 cells as compared with the cytotoxic effects of the non-specific Cdc25 inhibitor NSC 672121 [2-(2-mercaptoethanol)-3-methyl-1, 4-naphthoquinone], the MTT assay was performed. The IC_{50} of DA 3003-2 in PC-3 cells was 2-fold higher than that of NSC 672121, and the IC_{50} values of NSC 672121 and DA 3003-2 were ~10 and 5 μM, respectively (Fig. 1). Tamura *et al* reported that NSC 672121 induced dual G1 and G2/M arrest in synchronized tsFT210 cells (9). Next, the changes in cell cycle distribution induced in asynchronous PC-3 cells by NSC 672121 and DA 3003-2 were analyzed. After 24 h, PC-3 cells had accumulated in the

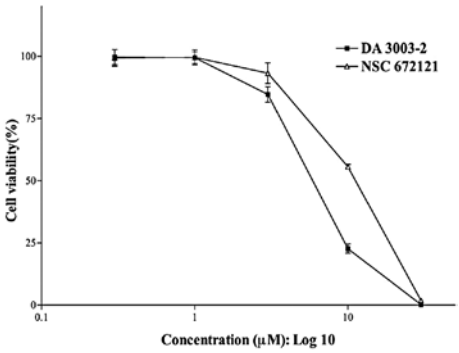

Figure 1. MTT assay of PC-3 cell viability at 48 h after DA 3003-2 and NSC 672121 administration.

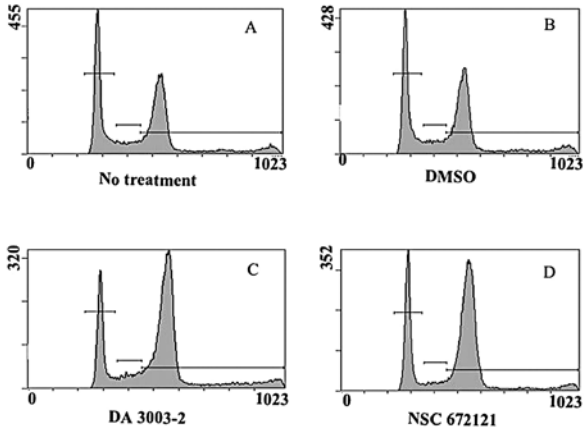

Figure 2. Changes in cell cycle distribution without treatment (A) and 24 h after treatment with (B) DMSO (C) DA 3003-2 or (D) NSC 672121.

Table I. Cell cycle distribution 24 h after treatment with and without Cdc25 inhibitors.

	G1	S	G2/M
No treatment	35.8±2.4	8.2±0.4	52.5±2.8
DMSO	35.7±1.5	8.0±1.1	53.2±1.0
DA 3003-2			
5 μM	33.9±2.3	12.3±1.5	46.8±1.6
10 μM	19.6±1.8	7.3±0.6	70.3±1.4
NSC 672121			
10 μM	36.3±1.0	7.9±0.6	54.9±1.8
20 μM	25.7±2.0	5.5±0.7	67.2±1.3

G2/M phase in a concentration-dependent manner as a result of the administration of both compounds (Fig. 2, Table I). DA 3003-2 induced G2/M accumulation at a concentration that was lower than that for NSC 672121 supporting the results of the MTT assay.

Hyperphosphorylation of Cdc2 Tyr^{15} in the cyclin-Cdk complex by DA 3003-2. Dephosphorylation of Cdk tyrosine by Cdc25s is necessary for the full activation of the cyclin-Cdk complex. NSC 672121 and 6-chloro-7-(2-morpholin-4-

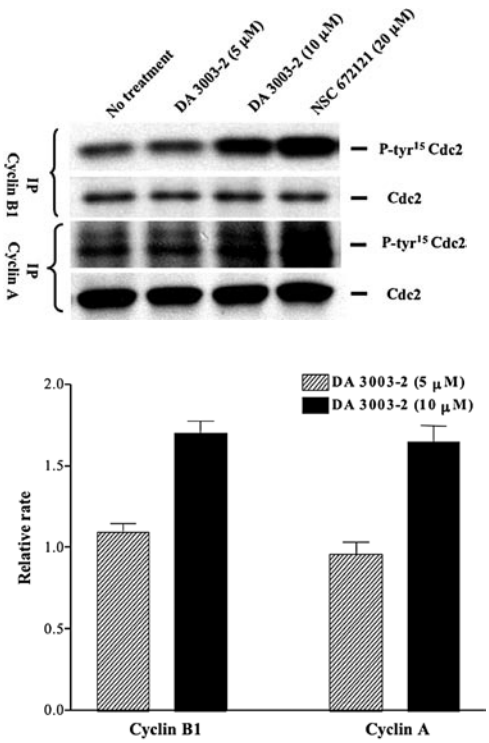

Figure 3. Hyperphosphorylation of Cdc2 Tyr[15] in the cyclin-Cdk complex by DA3003-2 after 1 h.

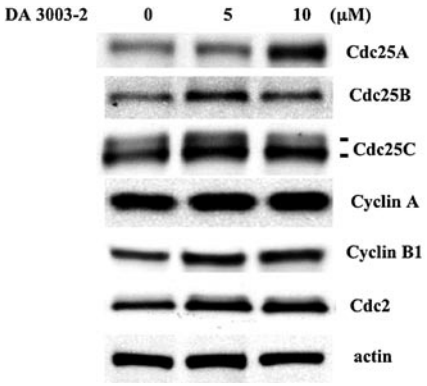

Figure 4. Modulation of the expression levels of cell cycle-regulating proteins by DA3003-2 after 24 h.

ylethylamino)quinoline-5, 8-dione (NSC 663284), which is a regioisomer of DA 3003-2, were previously found to increase total Cdc2 Tyr[15] levels in parallel with the inhibition of Cdc2 kinase activity. In the present study, whether DA 3003-2 and NSC 672121 affect the Tyr[15] phosphorylation status of Cdc2 in cyclin-Cdc2 complexes was investigated using a cell-based assay. Cyclin B_1 or cyclin A were immunoprecipitated from whole cell lysates harvested 1 h after exposure to 5 or 10 μM DA 3003-2 or 20 μM NSC 672121. The lysate proteins were separated by SDS-PAGE and immunoblotted with antibodies to both Cdc2 phospho-Tyr[15] and Cdc2. As shown in Fig. 3, 10 μM DA 3003-2 and 20 μM NSC 672121 hyperphosphorylated Cdc2 Tyr[15] in both cyclin-Cdc2 complexes within 1 h. Cdc25 is an unstable protein and is easily degraded by the proteasome pathway. Therefore, the modulation of cell cycle regulator expression levels was investigated using Western blotting. As shown in Fig. 4, Cdc25, G2/M cyclins and Cdc2 levels were not downregulated by DA 3003-2 administration.

Discussion

DA 3003-2 obtained from the compounds library at the University of Pittsburgh was screened using an *in vitro* phosphatase assay and was previously found to be more selective against Cdc25B2 relative to dual phosphatase VHR or protein tyrosine phosphatase PTP1B (6). It has already been revealed that NSC 663284, which is a regioisomer of DA 3003-2, inhibited Cdc25 activity by binding directly to the Cdc25 catalytic domain (10). On the other hand, Brisson *et al* suggested that the detection of differences in the cell cycle profile of asynchronous cells using Cdc25 inhibitors was difficult (11). In the present study, it was revealed that G2/M accumulation was induced in asynchronous cells using the Cdc25 inhibitor DA 3003-2 due to the hyperphosphorylation of the G2/M cyclin-Cdk complex. A Cdc25 overexpression study will be required to fully clarify the relationship between the inhibition of Cdc25 activity and G2/M accumulation using a cell-based assay. Moreover, as many different pathways feed into cell cycle regulation, non-Cdc25-specific cellular insults are also expected to cause G2/M arrest. However, the results of the present study confirm the potency of the Cdc25 inhibitor.

In both early and advanced stage prostate cancer etiology, the androgen receptor plays an important role (12,13). Androgen ablation remains the primary course of treatment for all patients with metastatic disease. These therapies are initially effective. However, recurrent tumors arise within a median time of 2-3 years. The balance of androgen receptor co-regulators is associated with the androgen refractory mechanism. Cdc25B directly acts as the co-activator of the androgen receptor and, in contrast, Cdc25A acts as the co-repressor (3,4). Unfortunately, it is difficult to use previously screened Cdc25 inhibitors as molecular targeting agents for androgen co-regulators in androgen refractory prostate cancer. This is due to the fact that the actions of Cdc25s related to androgen receptors have nothing to do with Cdc25 phosphatase activity. On the other hand, several investigators have shown increased expression of cyclin B_1 in human prostate cancer (14-16). In addition, Maddison *et al* demonstrated increased cyclin B_1 in poorly differentiated and androgen refractory cancers in the TRAMP mouse model of prostate cancer (16). The levels of Cdc25C, which is an activator of the Cdc2/cyclin B complex, increased in prostate cancer and decreased after anti-androgen therapy. Taken together these results suggest that G2/M transition activators, including Cdc25, are one of the important small molecular targets for androgen refractory prostate cancer treatment. The clinical application of the Cdc25 inhibitor as an anti-cancer drug is expected in the near future.

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