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Naphthoquinone Analogs as Inactivators of cdc25 Phosphatase

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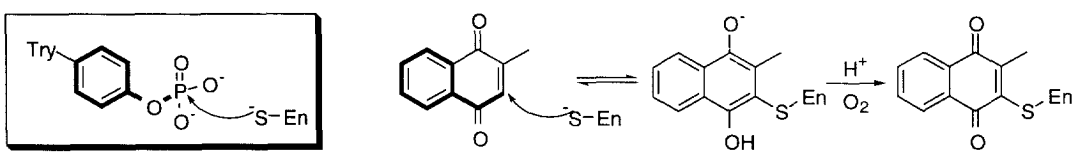
Abstract: cdc25A and cdc25B were significantly overexpressed in certain types of cancers, and they represent potential therapeutic targets for anticancer drug. In this study, naphthoquinone analogs as cdc25A phosphatase inactivators were investigated.

Keywords: Enzyme inhibitors; Quinones.

Cell cycle progression in eukaryotic cells is controlled by cyclin-dependent kinases, which are positively regulated by association with cyclins and negatively regulated by binding to inhibitory subunits [1]. Cyclin-dependent kinases are also inactivated by phosphorylation with protein kinase *wee1* [2], and activated by dephosphorylation with cdc25 phosphatases [3]. Mammalian or human cells contain three cdc25 genes named cdc25A, cdc25B and cdc25C [4]. Several lines of evidence suggest cdc25 phosphatases as potential human oncogenes [5]. Especially, cdc25A and cdc25B are known to play an important role in head, neck, and breast cancer development [5,6]. Therefore, inhibitors of these enzymes may become potential chemotherapeutic agents in the field of antitumor treatment.

Since menadione was found to induce alterations in the phosphorylation status and the activity of protein tyrosine phosphatase [7], and shares critical structural features with phosphotyrosine, we investigated the effects of menadione on the activity of cdc25A phosphatase [8]. We suggested that cdc25A phosphatase was inactivated by menadione and that the loss of enzyme activity was due to the modification of the active site. Recently, it has also been shown that a thioether analog of menadione inhibited cell growth and exerted its effect mainly through sulfhydryl arylation of cellular PTPases [9].



However, hydrophobicity of menadione and its thioether analog may have physicochemical properties of the cell membrane, leading to a cytotoxicity. For this reason, the potency of the naphthoquinones with the polar groups is compared to that of menadione to judge whether

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precursors to more active naphthoquinones could be generated in the design of possible inactivators by improving cellular transformation.

In search of new inactivators of cdc25A phosphatase, the naphthoquinone analogs with polar groups, which were either purchased or prepared according to described methods in the literature, were used. For example, 2-aminonaphthoquinone **3** [10] can be present in protonated form at physiological pH, and Michael addition of thiolate may therefore be activated toward nucleophilic attack, resulting in enzyme inactivation. However, when the assay for cdc25A phosphatase activity was performed, there was no detectable decrease in the enzyme activity. Similarly, compounds **4**, **5**, and **6** with the polar substituents such as 2-hydroxy or 2-thiol group as well as alkylamino group also displayed little or no loss in enzyme activity (*data not shown*). In contrast, the ethyl ether **7** or thioether **8** showed the similar inactivation to menadione.

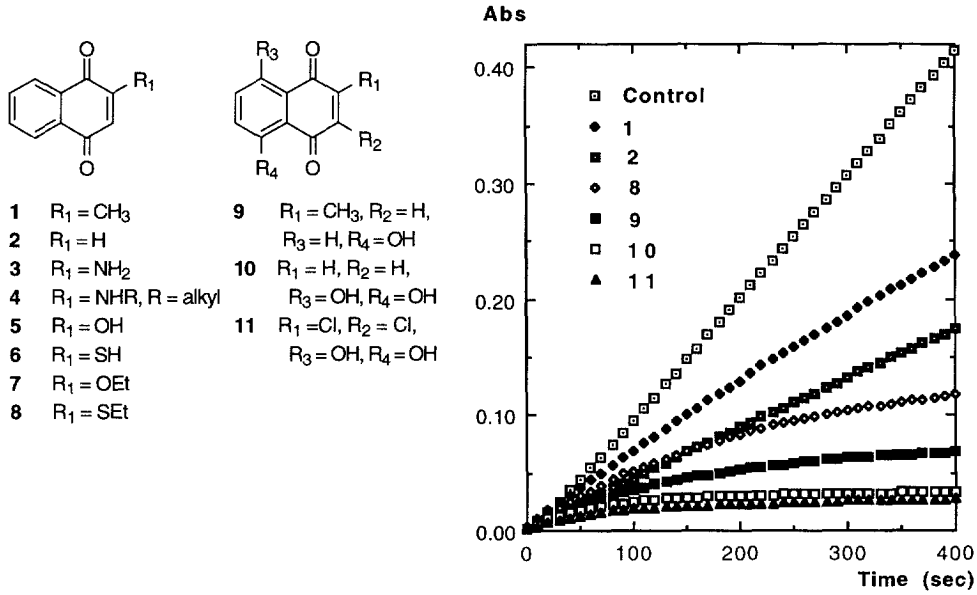
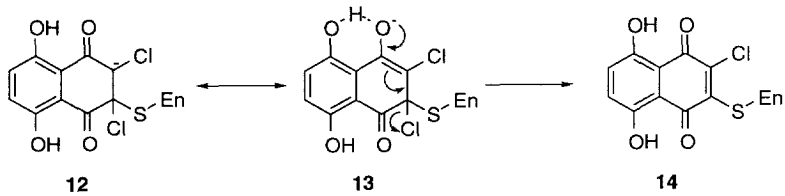


Figure 1. Purified GST-cdc25A phosphatase (0.2 mg/mL) was incubated with the chromogenic substrate [11], 40 μM *p*-nitrophenyl phosphate in 20 mM Tris (pH 8.0), 1 mM EDTA, and 0.2 mM DTT. To determine the efficiency of enzyme inactivation by 5 μM naphthoquinone derivatives, we continuously monitored at 410 nm and 37.0 ± 0.1°C by a JASCO UV/Vis spectrophotometer equipped with a JASCO peltier type thermostatic cell holder.

We next turned our attention to the naphthoquinone derivatives with the hydroxyl group at C-5 or C-8 of benzene ring to increase the water-solubility. In addition, the resulting enolic anion **12** might be stabilized by hydrogen bonding with the hydroxy group, thereby increasing the ability of Michael addition. Moreover, in the case of compound **11**, a sequence of modification might easily proceed by addition of thiolate to more electrophilic carbon atom



from the chloro group. It may also eject a chloride ion to promote the irreversible inactivation. Indeed, this appears to be the case. As shown in Figure 1, compounds **9**, **10**, and **11** offered the opportunity to manipulate physical properties without deleterious influence on inhibition of the enzymatic activity. In particular, compound **11** was the most active in this series.

On the basis of the above results, compound **11** was selected to further investigate the role in cell-cycle progression. Since cdc25A is a crucial factor that is required for cells to enter or to progress in the S phase [12], inactivation of cdc25A phosphatase block cell to start the cell cycle and the cells are expected to remain in G_1. Thus, asynchronized human hepatoma SK-hep-1 cells were therefore treated with 4 μM compound **11** for 1 h and then allowed to recover for various times. After 0, 6, 12, and 24 h, DNA content was measured by flow cytometry using a Becton-Dickinson fluorescence activated cell analyzer to determine cell cycle distribution [13]. Flow cytometric analysis shows that a cell cycle delay at the G_1/S phase occurred in compound **11**-treated cells as compared with control cells (Table1), and supports that decreases in cdc25A phosphatase activity caused by compound **11** correlate with cell cycle progression delay in **11**-treated human SK-hep-1 cells.

Table 1. [a] Flow cytometry analysis of asynchronized SK-hep-1 cells that were untreated or treated with compound **11**

Time in culture (h)	Cell Cycle Fraction (%)					
	untreated cell			compound **11** treated cell		
	G_1	S	G_2/M	G1	S	G_2/M
0	48	21	31	48	21	31
6	47	25	28	54	20	26
12	45	25	30	70	14	16
24	46	23	31	71	13	16

[a]Cell culture and cell cycle analysis: Human SK-hep-1 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum , 100 units/mL of penicillin and 100 μg/mL of streptomycin at 37°C in 5% CO_2 humidified incubator. The cultured cells were plated at a density of 3 x 10^5/mL on a 100 mm culture dish. When the cells were approximately 60-70% confluent, they were washed once in phosphate buffered saline (pH 7.4), and then replaced with fresh medium containing 2% fetal bovine serum. After addition of 4 μM compound **11**, the cells were harvested at the indicated time points, fixed in 70% ethanol, incubated with RNase A (0.1 mg/mL) for 30 min, and stained with propidium iodide (50 μg/mL) in PBS. The stained cells were analyzed for DNA content on a Becton Dickinson fluorescence-activated cell .

Other possible mechanism of the growth inhibitory actions was also examined. In general, quinones including menadione are thought to undergo redox cycling and to generate reactive oxygen species [14]. This possibility was then tested by co-incubation of compound **11** and catalase, deferoxamine mesylate, and superoxide dismutase (SOD), which are the scavengers of hydrogen peroxide, hydroxyl radical, and superoxide anion, respectively. However, these antioxidants did not antagonize the growth inhibitory effects of **11** (*data not shown*). The other low molecular weight antioxidants (butylated hydroxyanisole, nordihydroguaiaretic acid, and vitamin E) also had no effect on compound **11**.

It has been shown that cdc25A phosphatase regulates cdk2 kinase activity by dephosphorylation. In this respect, an obvious question concerned whether cdk2 was also inhibited via hyperphosphorylation. Thus, the extracts of compound **11**-treated cells were first immunoprecipitated with anti-cdk2 antibody. The activity of cdk2 kinase was then measured by this immunoprecipitate's capacity to phosphorylate the histone H1 substrate. As shown in

Figure 2, the compound **11**- treated cell extracts following various recovery times resulted in decreasing the activity of cdk2 kinase. To detect the phosphorylation level of tyrosine, cdk2 protein was also immunoprecipitated with anti-cdk2 antibody from the cell extracts treated with compound **11** at the indicated times, and immunoblotted with anti-phosphotyrosine antibody. Figure 2 shows that prolonged incubation with compound **11** increased in the degree of phosphorylation of cdk2 kinase, suggesting that compound **11** inhibits the growth of SK-hep-1 cells via a cell cycle delay due to decreased activity of cdk2 kinase caused by cdc25A phosphatase inactivation.

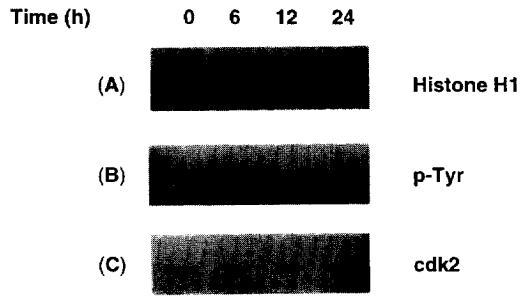


Figure 2. Effects of compound **11** on cdk2 kinase activity and phosphorylation level in SK-hep-1 cells. Cells were treated with compound **11** (15 μM) for the indicated times. (A) Ten lysates were immunoprecipitated with 2 μg of anti-cdk2 antibody followed by kinase assay with histone H1 as a substrate. (B) The lysates were immunoprecipitated with 2 μg of anti-cdk2 antibody followed by Western blotting with phosphotyrosine antibody. (C) The immunoprecipitates were subjected to Western blotting for cdk2.

In summary, this study has shown that modification of menadione could produce compounds with enhanced inactivation for cdc25A phosphatase, resulting in cell growth inhibition. We are in process of investigating compound **11** with a variety of different protein tyrosine phosphatases in addition to designing improved versions of these inactivators.

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