NAD(P)H:quinone oxidoreductase-1 (NQO1)-dependent and –independent cytotoxicity of potent quinone Cdc25 phosphatase inhibitors[*]

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Running title: Quinone oxidoreductase-1 and Cdc25 phosphatase inhibitors

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Abbreviations

PBS, Phosphate-buffered saline;

IC_{50}, the drug concentration that inhibits cell growth by 50%;

SNP, single nucleotide polymorphism;

NQO1, NAD(P)H:quinone oxidoreductase-1;

NQO1*1, wild type NQO1; NQO1*2, 609C>T SNP; NQO1*3, 465C>T SNP;

FBS, fetal bovine serum;

CYP reductase, cytochrome P450 reductase

Cyto-b reductase, microsomal NADH:cytochrome b_5 reductase

Abstract

Cdc25 dual-specificity phosphatases coordinate cell cycle progression and cellular signaling.

Consequently, Cdc25 inhibitors represent potential anticancer agents. We evaluated >10,000

compounds for inhibition of human Cdc25 phosphatases and identified many potent and

selective inhibitors, which all contained a quinone. Bioreductive enzymes frequently detoxify or

activate quinones. Therefore, we evaluated the effect of NAD(P)H:quinone oxidoreductase-1

(NQO1) and reductase-rich microsomes on the activity of three quinone-containing Cdc25

inhibitors: compound 5 [Cpd 5, 2-(2-hydroxyethylsulfanyl)-3-methyl-1,4-naphthoquinone],

NSC95397 [2,3-bis-(2-hydroxyethylsulfanyl)-1,4-naphthoquinone] and NSC663284 [6-chloro-7-

(2-morpholin-4-yl-ethylamino)quinoline-5,8-dione]. Each inhibitor was reduced by human

NQO1 (K_m 0.3-0.5 µM) but none by microsomes. Compounds were evaluated with six cancer

cell lines containing different amounts of NQO1: HT-29 (1056 nmol/mg/min), HCT116 (660

nmol/mg/min), sub-lines HCT116-R30A (28 nmol/mg/min) and HCT-116R30A/NQ5 (934

nmol/mg/min), MDA-MB-231/Q2 (null NQO1) and sub-line MDA-MB-231/Q6 (124

nmol/mg/min), but containing similar amounts of microsomal cytochrome P450 reductase and

cytochrome-b_5 reductase. Growth inhibition and G2/M arrest by Cpd 5 was proportional to

NQO1 levels, requiring 4-5-fold more Cpd 5 to inhibit HCT-116 or HCT-116R30A/NQ5

compared with HCT-116R30A. In contrast, in all tested cell lines irrespective of NQO1 level,

growth inhibition and G2/M arrest by NSC95375 and NSC663284 were similar (average IC_{50} of

1.3 $\pm$ 0.3 µM and 2.6 $\pm$ 0.4 µM, respectively). NSC95375 and NSC663284 also caused similar

Cdk1 hyperphosphorylation, indicating similar Cdc25 inhibition. However, lower Cpd 5

concentrations were needed to produce Cdk1 hyperphosphorylation in sub-lines with minimal

NQO1. Thus, NQO1 detoxified Cpd 5, probably by reducing it to a less active hydroquinone,

while NSC95397- and NSC663284-generated cytotoxicity was unaffected by NQO1.

Mammalian cells depend on protein kinases and phosphatases for communication and growth. Among the protein phosphatases, the dual-specificity Cdc25 phosphatases regulate cell cycle progression (Nilsson and Hoffmann, 2000) and may participate in MAPK-mediated cell signaling (Vogt *et al*., 2001). The three human Cdc25 homologues have been assigned different roles in cell cycle progression (Sadhu *et al*., 1990; Galaktionov and Beach, 1991; Nagata *et al*., 1991). Cdc25C completes the G2/M transition by dephosphorylating and activating the mitotic kinase Cdk1/cyclin B, which is required for entry into mitosis (Millar *et al*., 1991). Cdc25A is important for the entry into S-phase (Jinno *et al*., 1994) and may participate in mitosis (Donzelli *et al*., 2002). Cdc25B is essential for pre-initiating G2/M transition and S-phase progression (Lammer *et al*., 1998).

Tumor cells frequently over express Cdc25 phosphatases (Wu *et al*., 1998; Cangi *et al*., 2000), making them attractive candidate targets for anticancer agents. Earlier studies (Tamura *et al*., 2000; Wang *et al*., 2001) showed that 2-(2-hydroxyethylsulfanyl)-3-methyl-1,4-naphthoquinone (Cpd 5, NSC 672121), a vitamin K analogue with arylating capability, inhibited Cdc25 and arrested cell cycle progression at G2/M and G1 phases. More recently, we evaluated >10,000 compounds in the NCI chemical repository for their ability to inhibit recombinant human Cdc25B phosphatase *in vitro*. A number of potent phosphatase inhibitors were identified with *in vitro* inhibitory concentrations < 1 μM (Lazo *et al*., 2001; Lazo *et al*., 2002). Many of these active compounds were shown to contain a quinone sub-structure. Besides Cpd 5, two other potent quinone-containing Cdc25 inhibitors, NSC 95397 [2,3-bis-(2-hydroxyethylsulfanyl)-1,4-naphthoquinone] and NSC 663284 [6-chloro-7-(2-morpholin-4-yl-ethylamino)quinoline-5,8-dione] (Figure 1) were also evaluated extensively (Lazo *et al*., 2001 and 2002).

A number of clinically used antitumor drugs, such as mitomycin C and doxorubicin, are also quinones. They are known to be activated or inactivated by a 2e$^-$ reduction catalyzed by NAD(P)H:quinone oxidoreductase-1 (NQO1) or by a 1e$^-$ reduction catalyzed by NADPH:cytochrome P450 reductase (CYP reductase) (Bachur *et al*., 1979; Pan *et al*., 1984; Siegel *et al*., 1990; Walton *et al*., 1991). In addition, cytochrome b5 reductase (Powis and Appel, 1980; Hodnick and Sartorelli, 1993) and carbonyl reductase (Wermuth *el al*., 1986) also are capable of reducing quinones. NQO1 is frequently elevated in human tumors when compared to the surrounding normal tissue (Schlager and Powis, 1990; Cresteil and Jaiswal, 1991; Malkinson *et al*., 1992; Jarrett *et al*., 1998; Mikami *et al*., 1998). Furthermore, the level of NQO1 in human tumors can be 5- to 25-fold greater than other reductive enzymes, including CYP reductase and cytochrome b5 reductase (Smitskamp-Wilms *et al*., 1995). Therefore, sensitivity of quinones to NQO1 reduction could markedly alter, limiting or enhancing, the potential utility of any new quinone anticancer agents. Because several newly found Cdc25 inhibitors have a quinone sub-structure, we examined the role NQO1 might have in determining their cellular action in tumor cells. Using cell lines containing different levels of NQO1 activity, we evaluated three potent quinone-containing Cdc25 inhibitors, NSC 672121 (Cpd 5), NSC 95397, and NSC 663284 (Figure 1), for their inhibition of cell growth, arrest of cell cycle progression and inhibition of Cdk1 dephosphorylation.

Materials and Methods

Reagents.

Cpd 5 (NSC 672121) was synthesized as described earlier (Tamura *et al*., 2000) (Figure 1). NSC 95397 and NSC 663284 (Figure 1) were provided by Dan Zaharevitz and Jill Johnson from the Development Therapeutics Program of the National Cancer Institute (Bethesda, MD). Mouse monoclonal anti-Cdk1 antibody (SC 54) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho-Cdk1 (#9111) was purchased from New England BioLab (Boston, MA). Sheep anti-mouse IgG and donkey anti-rabbit IgG, both conjugated to horseradish peroxidase (HRP), recombinant human microsomal cytochrome b_5 and other biochemicals were purchased from Sigma (St. Louis, MO). Renaissance Kits were purchase from New Life Science (Boston, MA). Hybond-ECL nitrocellulose membranes were purchase from Amersham (Patacasway, NJ).

Cell lines.

Six human tumor cell lines were selected to study the effect of Cdc25 inhibitors on cellular growth. These cell lines provide a broad spectrum of different NQO1 genotypes and levels of activity (Table 1). Thus, the three known human NQO1 genotypes are represented: 1) wild type (NQO1*1), 2) 609C>T single nucleotide polymorphism (NSP) (NQO1*2), and 3) 465C>T NSP (NQO1*3) (Ross *et al*., 2000; Pan *et al*., 2002). HCT-116R30A, a mitomycin C-resistant subline with minimum NQO1 activity, was derived from human colon cancer cell line HCT-116 (Pan *et al*., 1992). HCT-116R30A/NQ5 cells were generated from HCT-116R30A cells by a transfection of human NQO1*1 cDNA and selected for stable expression (Pan *et al*., 2002). HCT-116 and all of the sub-lines contain one or two alleles of NQO1*3. MDA-MB-231/NQ2 and MDA-MB-231/NQ6, sublines of human breast cancer cell line MDA-MB-231 with homozygous NQO1*2,

contain stably transfected vector or a human NQO1*1 cDNA, respectively (Gifts from Dr. David A. Boothman, Department of Radiation Oncology, Case Western Reserve University, Cleveland, OH). The human colon cancer cell line HT-29 contains two NQO1*1 alleles with high amounts of NQO1 activity. All colorectal cancer cell lines were maintained and sub-cultured in modified McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 100 units/ml penicillin and 100 µg/ml streptomycin. The MDA-MB-231 sublines were maintained in RPMI 1640 medium containing the same supplements as McCoy's 5A. All cell lines were incubated at 37°C at 5% CO_2 and 95% humidity.

Cytotoxicity of Cdc25 inhibitors.

The cytotoxicity of Cdc25 inhibitors was assessed by a modified colony formation assay described elsewhere (Pan *et al*., 1992). Briefly, cells were plated in six-well plates at 500 to 1,000 cells per well in 3 ml medium. After overnight incubation, designated concentrations of compounds were added to the wells, and an equal volume of vehicle (ethanol) was added to the control well. After further incubation for 5 days to allow colony formation, medium was removed. Wells were washed with phosphate-buffered saline (PBS) and stained with 0.25% crystal violet in 10% formalin and 90% methanol. Excess stain was removed and the wells were washed with water and dried. Colonies (>50 cells) were counted. The drug concentration producing 50% inhibition of cell growth for each cell line was calculated with a Sigmoid-E_{max} model implemented in the ADAPT II computer program (D'Argenio and Schumitzky, 1979).

Isolation and assays of NQO1.

Recombinant human NQO1 was prepared and purified as described elsewhere (Pan *et al*., 1995) and used to determine the kinetics of reduction of Cdc25 inhibitors. Purified NQO1 had a specific activity of 598 µmol/mg/min in reducing menadione-cytochrome c at pH 7.5 at room

temperature. Due to interfering absorbance peaks of Cdc25 inhibitors and to allow for comparison with menadione, we adapted a method described to measure reduction of menadione by NQO1 (Ernster, 1967). Cdc25 inhibitors were used as intermediate electron acceptors, and cytochrome c was the terminal electron acceptor. The reaction mixture of 1 ml contained 50 mM Tris-HCl buffer at pH 7.5, 200 μM NADH, 80 μM cytochrome c, 5 µM FAD, 0.07% BSA, various concentrations of Cdc25 inhibitors and 100 ng NQO1. The rate of reduction of cytochrome c was measured at room temperature by monitoring the increase of absorbance at 550 nm for 2 min. Absorbance was measured with a Beckman DU-640 spectrophotometer and each assay was performed in triplicate. Each assay was also repeated in the presence of 10 µM dicoumarol, a NQO1 inhibitor. Enzymatic activity attributed to NQO1 was that inhibited by dicoumarol. We also assayed the reduction of menadione, the commonly used substrate of NQO1 containing the core naphthoquinone structure. The best curve fit for Lineweaver-Burk plots, and values of K_m and V_{max} were determined using Sigma Plot 8.0 (SPSS, Inc., Chicago, IL). NQO1 activity in the cytosol was determined by the same assay while 20 µl cytosol was used in place of purified NQO1. Cytosol of each cell line was prepared as described elsewhere (Pan *et al*., 1992).

Isolation of microsomes and assay for cytochrome P450 reductase (CYP reductase) and cytochrome b reductase (cyto-b reductase).

Microsomes, rich in CYP reductase and cyto-b reductase, were isolated from each cell line according to a method described elsewhere (Pan *et al*., 1992) Freshly prepared microsomes were used to assay the activity of CYP reductase and cytochrome b reductase. All reactions were carried out at room temperature. Each enzymatic activity was determined with microsomes from three independent preparations and each determination was carried out in triplicate.

CYP reductase activity was determined by cytochrome c reduction using NADPH as the electron donor. The reaction mixture of 1 ml contained 100 mM phosphate buffer at pH 7.5, 400 µM NADPH, 80 µM cytochrome c, 20 µM KCN (to inhibit cytochrome c oxidase) and 100 µg microsome. Reduction of cytochrome c was monitored at 550 nm for 2 min. To analyze the reduction of Cdc25 inhibitors by microsomes, the reaction mixture was identical except that NADPH or NADH concentration was decreased to 150 µM, and cytochrome c was replaced with various concentrations of Cdc25 inhibitors. The rate of reducing Cdc25 inhibitors was monitored by the oxidation of NADPH or NADH as reflected by the decrease of absorbance at 340 nm for 10 min.

The activity of cyto-b reductase was determined in a 1ml reaction mixture containing 10 mM phosphate buffer at pH 6.6, 1 mM NADH, 500 µM ferricyanide and 100 µg microsome as described (Hodnick and Sartorelli, 1993). Reduction of ferricyanide was monitored at 420 nm. For confirmation, cyto-b reductase activity was also determined in a 100 µl reaction mixture containing 10 mM phosphate buffer at pH 6.6, 100 µM NADH, 2 µM cytochrome b_5 and 10 µg microsome. Reduction of cytochrome b5 was monitored at 427 nm for 10 min as described (Yubisui and Takeshita, 1982; Holmans *et al*., 1994). Purified rat CYP reductase was analysed for its ability to reduce ferricyanide in the same fashion with NADPH or NADH as an electron donor.

Flow cytometric analysis.

The analysis of cell cycle by flow cytometry was conducted with asynchronous cells. All cell lines were plated at 4 x 10^6 cells in 75 cm^2 flask containing 10 ml of medium. After cells were allowed to adhere to the monolayer by overnight incubation at 37° C, either the compounds or vehicle was added to cells, which were incubated for an additional 12 h. Treated cells were

harvested by trypsin digestion, washed twice with cold PBS, suspended in cold 70% ethanol and incubated at -20° C for 30 min. Cells were washed again with PBS containing 1% bovine serum albumin before being stained at 5×10^5 cells/ml with a PBS solution containing 50 μg/ml propidium iodide and 250 μg/ml RNase A. After incubating overnight at 4° C, cells were analyzed with a Beckman-Coulter Epics XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Each Cdc25 inhibitor was analyzed at least three independent times. Cells treated with the vehicle, 0.5% DMSO, served as a negative control.

Preparations of cell lysates for the analysis of Cdk1.

HCT-116, HCT116R30A, MDA-MB-231/Q6 and MDA-MB-231/Q2 cells were plated at 4×10^6 cells in 75 cm^2 flask (10 ml of medium) and incubated overnight at 37° C to allow cells to adhere. Compounds at 10 and 20 μM or vehicle (ethanol) were added to cells, which were incubated for an additional 6 h, medium removed, and cells washed twice with cold PBS. Drug-treated cells were lysed with 1.5 ml lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NT40, 0.1% sodium deoxycholate, and protease inhibitors including 100 μg phenylmethylsulfonyl fluoride, 100 μg benzamide hydrochloride and 2 μg/ml each of aprotinin, leupeptin, antipain, and pepstatin). Protein concentrations were determined by the Bradford method (Bradford, 1976).

Western blotting.

 Proteins (40 μg) were separated by SDS-polyacrylamide gel and transfer from the gel to Hybond-ECL nitrocellulose membranes as described previously (Pan *et al*., 1995). Total Cdk1 was detected by anti-Cdk1 at 1:100 dilution, and phosphorylated Cdk1 was detected by anti-phospho-Cdk1 at 1:1,000 dilution. Anti-mouse IgG and anti-rabbit IgG conjugated to HRP at 1:2,000 dilution were used as secondary antibodies, respectively. All incubations were performed

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at room temperature. Membranes were exposed to primary antibodies for 2 h, blocked with a

PBS solution containing 5% nonfat milk and 1% Tween 20 for 1 h, and washed with PBS until

free of milk. Then membranes were exposed to secondary antibody for 1 h and rinsed 2 times

with PBS. Protein bands in membranes were detected by chemiluminescence produced by the

oxidation of luminol, using a Renaissance Kit, and captured by exposing blots to Kodak X-

OMAT Blue film. Loading equivalence was monitored by the detection of β-actin bands on the

same membrane.

Results

Activities of reductases in cell fractions of different cell lines.

Cytosolic NQO1 activity in six cell lines showed a broad spectrum of level of NQO1 (Table 1). The difference between cell lines with high and low activity was as much as 60-fold. However, the activities of microsomal CYP reductase and cyto-b reductase activity among the cell lines did not vary significantly (Table 1). To address the possibility that other NADH-utilizing reductases might contribute to the reduction of ferricyanide which was utilized for measuring the activity of cyto-b reductase we analyzed purified CYP reductase for its ability to reduce ferricyanide. We found that purified CYP reductase with activity equivalent to microsomes of HCT-116 reduced only 0.05 μM ferricyanide using NADPH or NADH as electron donor. Therefore, CYP reductase in microsomes appeared to contribute only minimally to the reduction of ferricyanide. The reduction of cytochrome b_5 by microsomes also showed that cyto-b reductase activity among the tested cell lines did not vary significantly. Thus, the ferricyanide-reductase activity shown by microsomes (Table 1), which represents total one–electron reduction by microsomes, appears to be mainly a reflection of cyto-b reductase activity.

Reduction of Cdc25 inhibitors by recombinant human NQO1 and microsomes.

All Cdc25 inhibitors shared similar K_m values for reduction by purified recombinant human NQO1 (Table 2). Menadione, which is a substrate commonly used to measure the activity of NQO1 had a significantly higher K_m. Cpd 5 exhibited a 2-fold and 6-fold higher maximum rate of reduction than did NSC 95397 and NSC 663284, respectively. The V_{max} values of Cpd 5 and menadione for human NQO1 were similar. Oxidation of NADPH as well as NADH could not be detected after incubating microsomes for 10 min with Cdc25 inhibitors. Therefore, reduction of

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the three Cdc25 inhibitors by microsomes was minimal under conditions employed in this study.

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Relationship between NQO1 activity and cytotoxicity of Cdc25 inhibitors.

The three Cdc25 inhibitors caused a concentration dependent cytotoxicity in all six cell lines tested with a colony formation assay (Figure 2). Comparing the isogenic cell groups, we found HCT-116 and MDA-MB-231/NQ6, which had higher NQO1 activity, were more resistant to Cpd 5 than were HCT-116R30A and MDA-MB-231/NQ2 cells (Figure 2A). HCT-116 cells were approximately 5-fold more resistant compared to HCT-116R30A cells and MDA-MB-231/NQ6 cells were 2-fold more resistant compared to MDA-MB-231/Q2 cells (Table 3). Restoration of NQO1 activity in HCT-116R30A/NQ5 cells regenerated resistance to Cpd 5 as reflected by an IC_{50} value similar to that seen in the parental HCT-116 cells. Among the six cell lines, HT-29 had the highest NQO1 activity and was the most resistant toward Cpd 5. In contrast, all cell lines irrespective of NQO1 content were equally sensitive to NSC 95397 and also equally sensitive to NSC 663284 (Figure 2 C and D). The IC_{50} values of NSC 95397 and NSC 663284 for all six cell-lines did not show significant differences, exhibiting average values of 1-1.8 μM and 2-3 μM, respectively.

Effect of dicoumarol on IC_{50} of Cpd 5.

Treatment with 10 μM dicoumarol, a potent inhibitor of NQO1, enhanced the sensitivity of HT-29 and HCT-116 cells to Cpd 5 (Figure 2B), decreasing their IC_{50} values from 6.7 $\pm$ 0.3 and 2.3 $\pm$ 0.3 to 0.5 $\pm$ 0.1 and less that 0.5, respectively. Treatment with dicoumarol did not alter the IC_{50} values of Cpd 5 for HCT-116R30A and MDA-MB-231/Q2 cells (data not shown) consistent with the hypothesis that NQO1 inactivates Cpd 5 in cells. In addition, dicoumarol treatment did

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not alter the IC_{50} values of NSC 95397 and NSC 663284 (data not shown) consistent with the hypothesis that these two compounds were not affected by NQO1.

Cell cycle progression at G2/M

Because Cdc25 has an essential role at the G_2/M interface, mitotic arrest is a valuable beacon of Cdc25 inhibition within cells. HCT-116 and HCT-116R30A cells were used to analyze the effect of NQO1 activity on the ability of Cdc25 inhibitors to cause cell cycle arrest. All three Cdc25 inhibitors blocked cell cycle progression of the two cell lines at the G2/M interface (Figure 3). As anticipated by the cytotoxicity data, HCT-116R30A cells were more sensitive to Cpd 5 than HCT-116 cells. Cpd 5 at 2.5 µM clearly blocked HCT-116R30A cells at G2/M while only slightly affecting HCT-116 cells (Figure 3A and B). Cpd 5 at 5.0 µM caused 92% of HCT-116R30A cells to arrest in G2/M phase, but only 32% of HCT-116 cells. Too few HCT-116R30A cells survived exposure to 7.5 µM Cpd 5 to permit an accurate analysis while 34% HCT-116 cells accumulated in G2/M phase without prominent cell loss. HCT-116 and HCT-116R30A cells showed similar sensitivity to G2/M block by NSC 95397 and NSC 663284. (Figure 3, C-F). More than 40% of the total cells were arrested at G2/M with either 7.5 µM NSC 95397 or NSC 663284.

Inhibition of Cdk1 dephosphorylation

Dephosphorylation of the mitotic inhibitor Cdk1 by Cdc25 B and C is obligatory for entry into mitosis (Nilsson and Hoffmann, 2000; Sakhu *et al.*, 1990). Thus, inhibition of Cdc25 phosphatases should prevent the dephosphorylation of Cdk1. Consequently, we compared the effect of the three quinoid Cdc25 inhibitors on the phosphorylation status of Cdk1 in the isogenic HCT-116 pair. Exposure of HCT-116R30A cells to Cpd 5 resulted in a pronounced increase of phosphorylated Cdk1 but only a modest change in Cdk1 phosphorylation status in

HCT-116 cells (Figure 4A). In contrast, Cdk1 phosphorylation was markedly increased in both

HCT-116 and HCT-116R30A cells after either NSC 95397 or NSC 663284 exposure (Figure 4B

and C). A similar inhibition of dephosphorylation of Cdk1 was seen in MDA-MB-231/Q2 and

MDA-MB-231/Q6 cells after treatment by the three inhibitors (data not shown).

Discussion

Utilizing relatively small, publicly available compound libraries, we have previously identified the para-naphthoquinone and quinolinedione scaffolds as promising lead structures for the design of inhibitors of dual-specificity Cdc25 phosphatases (Lazo *et al*., 2001 and 2002). Both electrostatic and steric issues are important for potent and selective inhibition of Cdc25. However, the quinone sub-structure can be reduced by various enzymes and such metabolism might enhance or reduce the activity of a compound.

In this report, we have evaluated the ability of microsomes and an important reductive enzyme, NQO1, to affect the cytotoxicity and growth arrest of three potent, quinone-containing Cdc25 inhibitors. Microsomes are rich in both CYP reductase and cyto-b reductase that catalyze one-electron reduction of quinones to semi-quinones. The failure to detect consumption of NADPH or NADH when microsomes were incubated with Cdc25 inhibitors strongly suggests that reduction of Cpd 5, NSC 95397 and NSC 663284 by microsomes isolated from human tumor cell lines was minimal. The amount of these microsomal reductases in the tested cell lines did not vary significantly although the cells exhibited different sensitivity to the cytotoxic effects of the compounds. Thus, the cytotoxicity of the three inhibitors can not be easily explained by CYP reductase or cyto-b reductase. In contrast, recombinant human NQO1 was capable of reducing all three Cdc25 inhibitors, although with some differences in the rate of their reduction. The fact that the K_m values of these inhibitors are in the range of 0.3 to 0.5 μM indicates that the affinity of these compounds toward the recombinant NQO1 is similar, but much higher than that of the commonly used substrate and vitamin K analogue, menadione. Earlier publications (Tamura *et al*., 2000; Lazo *et al*., 2001 and 2002) showed that *in vitro* K_i values for Cdc25A, Cdc25B and Cdc25C were 15, 1.7 and 1.3 μM for Cpd 5, 32, 96 and 40 nM for NSC 95397, and

29, 95 and 89 nM for NSC 663284, respectively. The K_m values for NQO1 are about 100- and 50-fold higher than the K_i values for Cdc25 of NSC 95397 and NSC 663284, respectively. This difference may influence the effect of these compounds on cells.

Indeed, our studies of Cdc25 inhibitors in selected human tumor cells showed that the effect of NQO1 on the activity of Cpd 5 differed from its effects on the activity of NSC 95397 and NSC 663284. The presence of NQO1 in each cell line had a minimal effect on the inhibition of cell growth by NSC 95397 and NSC 663284. On the other hand, cell lines with more NQO1 activity, such as HT-29 and HCT-116, were more resistant to Cpd 5 than cell lines with minimal NQO1 activity. The involvement of NQO1 in the cytotoxicity of Cpd 5 was confirmed by two additional experimental approaches. First, restoration of NQO1 activity increased the resistance for the HCT-116R30A/NQ5 cells to Cpd 5. Second, inhibition of NQO1 by dicoumarol rendered HT-29 and HCT-116 cells more susceptible to Cpd 5. Two other analyses were conducted to study the effect of cellular NQO1 on the function of Cdc25 inhibitors. Arrest of cell cycle progression at G2/M and inhibition of Cdk1 dephosphorylation, indicative of inhibition of Cdc25B and Cdc25C (Nilsson and Hoffmann, 2000;Lammer *et al*., 1998; Tamura *et al*., 2000), were observed for cells treated with Cpd 5, NSC 95397 and NSC 663284. Again, the activities of NSC 95397 and NSC 663284 were unaffected by the presence of NQO1 in cells while the activity of Cpd 5 was decreased by the presence of NQO1. Reduction of Cpd 5 by cellular NQO1 decreases its activity against Cdc25 phosphatase and its cytotoxicity, indicating a detoxification process of Cpd 5.

The loss of Cpd 5 function may be due to its reduction to the hydroquinone. Cpd 5 is an analogue of vitamin K with arylating capability. Among several vitamin K analogues, only those with arylating capability inhibited the action of Cdc25 (Wang *et al*., 2001), and as a consequence

of the reduction by NQO1, Cpd 5 hydroquinone has lost its electrophilic properties. The chemical structure of NSC 95397 differs from that of Cpd 5 by one additional thioethanol residue, while NSC 663284 differs from Cpd 5 by having a quinolinedione rather than a naphthoquinone sub-structure. Both NSC 95397 and NSC 663284 were reduced by NQO1 at a slower rate than Cpd 5. However, the Vmax and Km values of both compounds observed are not different enough from Cpd 5 to account for the difference in NQO1 independence for the two compounds in tumor cells. Apparently, the reduction of these two compounds in cells, if any, does not effect their activity in inhibiting Cdc25 phosphatases. One possible factor attributed to the difference between Cpd 5 and the other two compounds may be the K_i values mentioned above. The much lower K_i values of NSC 95397 and NSC 663284, compared with Cpd 5, for Cdc25 phosphotases probably make these two compounds more effective inhibitors in cells which is independent of NQO1. Another factor may be redox-cycling. After reduction by NQO1 or other reductases, these three compounds may be capable of undergoing redox-cycling at a different rate in the presence of oxygen to cause cytotoxicity. Cell lines with less NQO1 are more susceptible to Cpd 5, which may be an indication that reduced Cpd 5 is not capable being recyclec and becomes less toxic. All tested cell lines being equally sensitive to NSC 95397 and NSC 663284 could mean that the inhibitory function of these two compounds are not affected by the rate of redox-cycling. In addition to NQO1 and microsomes, mitochondrial reductases also could have a role in the toxicity of these Cdc25 inhibitors.

Currently, our study has demonstrated that reduction of NSC 95397 and NSC 663284 by NQO1 had little effect on their inhibitory functions. NSC 95397 and NSC 663284 are not efficiently reduced by microsomes. However, the full extent of cellular metabolism of these compounds requires additional studies. It is unclear whether these compounds can be substrates

for other quinone reductases or dehydrogenase, such as carbonyl reductase and xanthine dehydrogenase. Hypoxia may also play a role in the metabolism of these compounds. In addition, the effect of phase II metabolism, such as glucuronidation of reduced products of quinones, could occur *in vivo*. Studies are continuing to address these issues.

Acknowledgments

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Footnote

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Legends for figures:

Figure 1.   Chemical structures of Cdc25 inhibitors.

Figure 2.   Cytotoxicity of Cdc25 inhibitors for tumor cell lines containing different amounts of
NQO1 activity. Cells were treated for 5 days with A) Cpd 5, B) Cpd 5 in the
presence of 10 μM dicoumarol, C) NSC 95397, and D) NSC 663284. Cell survival
was determined by colony formation assay. Each data point represents the mean $\pm$
SD of three independent experiments with duplicate wells.

Figure 3.   Blockage by Cdc25 inhibitors of HCT-116 and HCT-116R30A cell cycle
progression. Cells were treated with each agent at 0 (vehicle control), 2.5, 5.0 or 7.5
μM for 12 h and then stained for flow cytometric analysis. The cell cycle
distribution, shown in the tables below each figure, was measured as the percentage
of cells containing DNA of G1, S and G2/M phases. Panels A, C and E) HCT-116
cells treated with Cpd 5, NSC 95397 and NSC 663284, respectively. Panels B, D
and F) HCT-116R30A cells treated with Cpd 5, NSC 95397 and NSC 663284,
respectively.

Figure 4.   Inhibition by Cdc25 inhibitors of dephosphorylation of Cdk1 in HCT-116 and HCT-
116R30A cells. Cells were exposed to Cdc25 inhibitors for 6 h, after which protein
lysates were prepared for Western blotting. Cdc25 inhibitors used for: Panel A, Cpd
5; Panel B, NSC 95397; and Panel C, NSC 663284. Lanes 1, 2 and 3, HCT-116 cells
treated with vehicle (ethanol) control, 10 and 20 μM Cdc25 inhibitor, respectively.
Lanes 4, 5 and 6, HCT-116R30A cells treated with vehicle control, 10 and 20 μM
Cdc25 inhibitor, respectively. Each lane represents 40 μg protein separated by SDS-
polyacrylamide gel.

Table 1.   Differences in NQO1 in different human tumor cell lines. NQO1 activity was determined by the reduction of menadione-cytochrome c using NADH as the electron donor. Final enzymatic activity attributed to NQO1 was calculated by the rate of cytochrome c reduction that was inhibited by 10 μM dicoumarol. CYP reductase was determined by the reduction of cytochrome c using NADPH as the electron donor. Cyto-b reductase was determined by the reduction of ferricyanide using NADH as the electron donor.

Cell lines	NQO1 Geno- type /Insert	NQO1 Activity (nmol/mg/min)	CYP 450 Reductase (nmol/mg/ml)	Cyto-b_5 Reductase (μmol/mg/min)
HCT-116	NQO1*1/NQO1*3/none	660 $\pm$ 82	11.4 $\pm$ 0.6	612 $\pm$ 23
HCT-116R30A	NQO1*3/ NQO1*3/none	28 $\pm$ 2	9.5 $\pm$ 0.7	681 $\pm$ 32
HCT-116R30A/NQ5	NQO1*3/ NQO1*3/ NQO1*1	934 $\pm$ 166	10.2 $\pm$ 0.7	716 $\pm$ 35
MDA-MB-231/Q2	NQO1*2/ NQO1*2/vector	17 $\pm$ 2	8.9 + 1.7	658 $\pm$ 36
MDA-MB-231/Q6	NQO1*2/ NQO1*2/ NQO1*1	124 $\pm$ 14	9.2 $\pm$ 0.9	609 $\pm$ 47
HT-29	NQO1* 1/ NQO1* 1/none	1056 $\pm$ 146	7.5 $\pm$ 0.5	701 $\pm$ 37

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Table 2. Specific activity in reducing Cdc25 inhibitors by recombinant human NQO1. Details

of the assay are described in the text. Sigma Plot 8.0 was used to calculate values of

K_m and V_{max} (mean $\pm$ SD) based on data from three separate experiments, each

performed in duplicate.
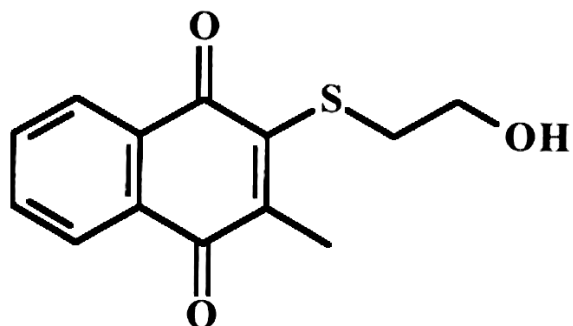
Compounds	K_m (μM)	V_{max} (μmol/mg/min)
Cpd 5	0.5 ± 0.04	242 ± 28
NSC 95397	0.3 ± 0.05	98 ± 12
NSC 663284	0.3 ± 0.03	40 ± 5
Menadione	27.9 ± 3.2	305 ± 24

Table 3. IC$_{50}$ of Cdc25 inhibitors for different human tumor cell lines. Cytotoxicity was

      determined by colony formation assay as described in the text. IC$_{50}$ values, representing

      mean $\pm$ SD of three separate experiments, were calculated using a Sigmoid-E$_{max}$ model
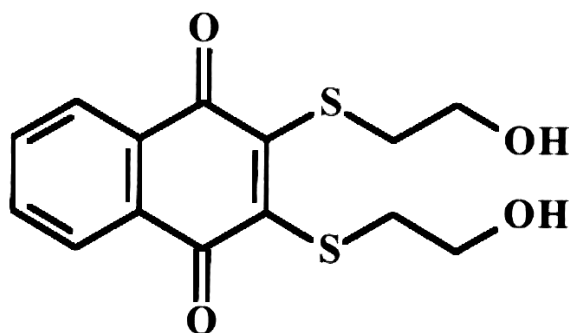
      as implemented by the ADAPT II computer program.

Cell Lines	IC$_{50}$ (μM)		
	Cpd 5	NSC 95397	NSC 663284
HCT-116	2.3 ± 0.3	1.3 ± 0.1	2.3 ± 0.2
HCT-116R30A	0.4 ± 0.1	1.1 ± 0.1	2.4 ± 0.2
HCT-116R30A/NQ5	1.7 ± 0.2	1.3 ± 0.1	2.1 ± 0.3
MDA-MB-231/Q2	0.3 ± 0.1	0.9 ± 0.1	3.2 ± 0.4
MDA-MB-231/Q6	0.7 ± 0.1	1.1 ± 0.1	2.9 ± 0.5
HT-29	6.7 ± 0.3	1.8 ± 0.3	2.7 ± 0.3

Figure 1

Figure 2

Figure 3

	HCT-116				HCT-116R30A			
	Control	2.5 µM	5.0 µM	7.5 µM	Control	2.5 µM	5.0 µM	7.5 µM
A		**Cpd 5**			**B**		**Cpd 5**	
G0/G1	64	58	56	52	61	48	8	0[a]
S	14	16	14	14	17	1	0	0
G2/M	22	26	30	34	22	51	92	0
C		**NSC 95397**			**D**		**NSC 95397**	
G0/G1	63	52	40	39	63	47	39	38
S	19	26	27	21	14	24	28	24
G2/M	18	22	33	40	23	29	33	38
E		**NSC 663284**			**F**		**NSC 663284**	
G0/G1	65	56	41	40	64	45	42	44
S	17	19	27	19	17	29	22	13
G2/M	18	25	32	41	19	26	36	43

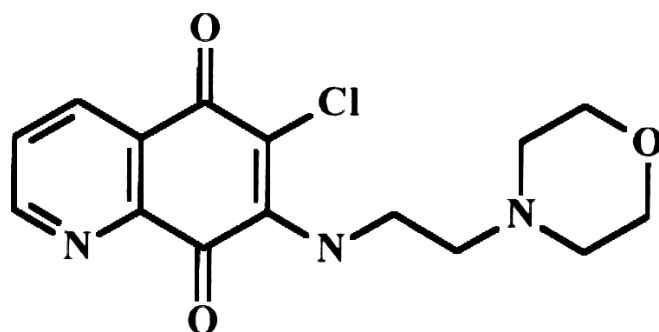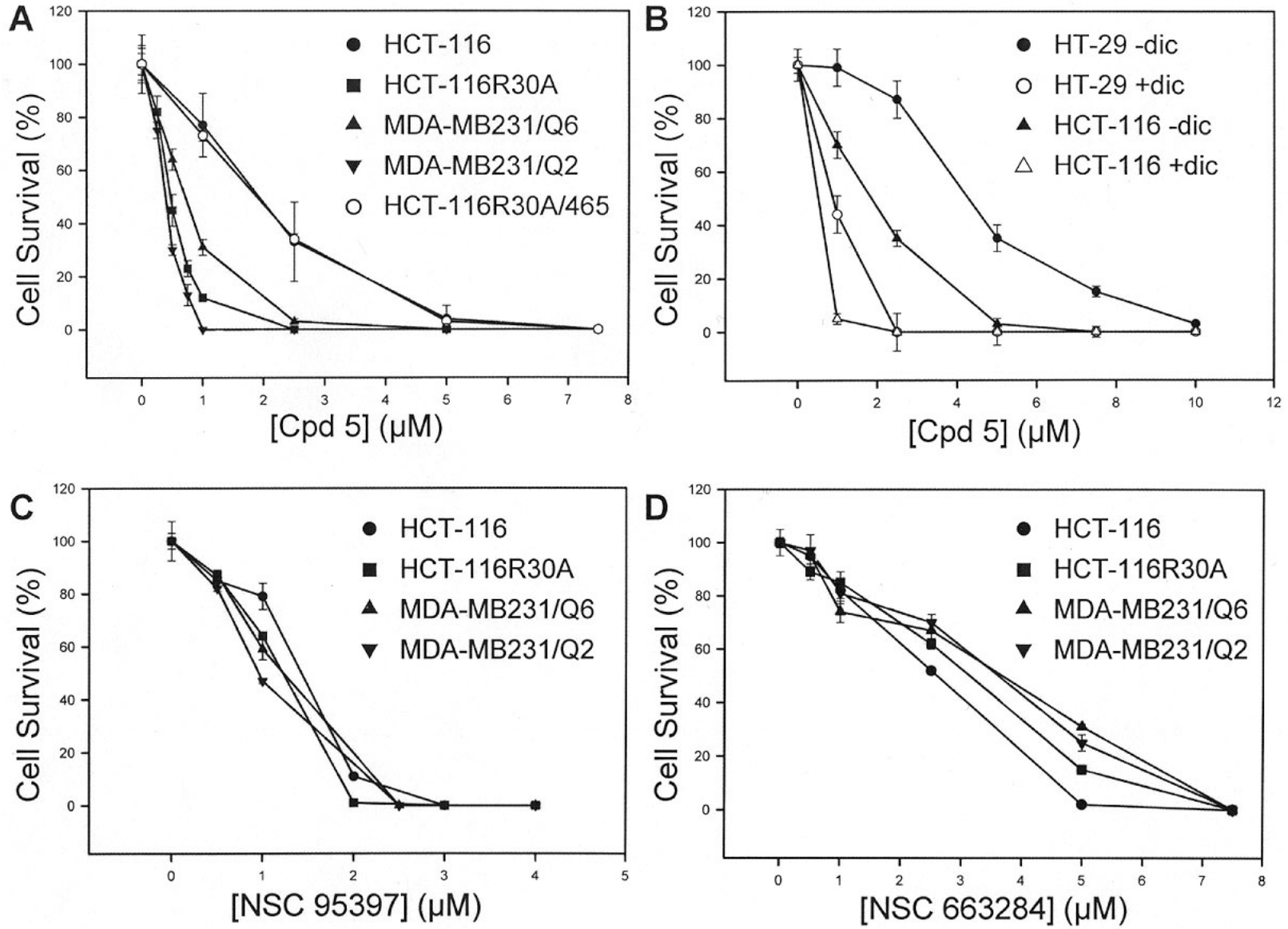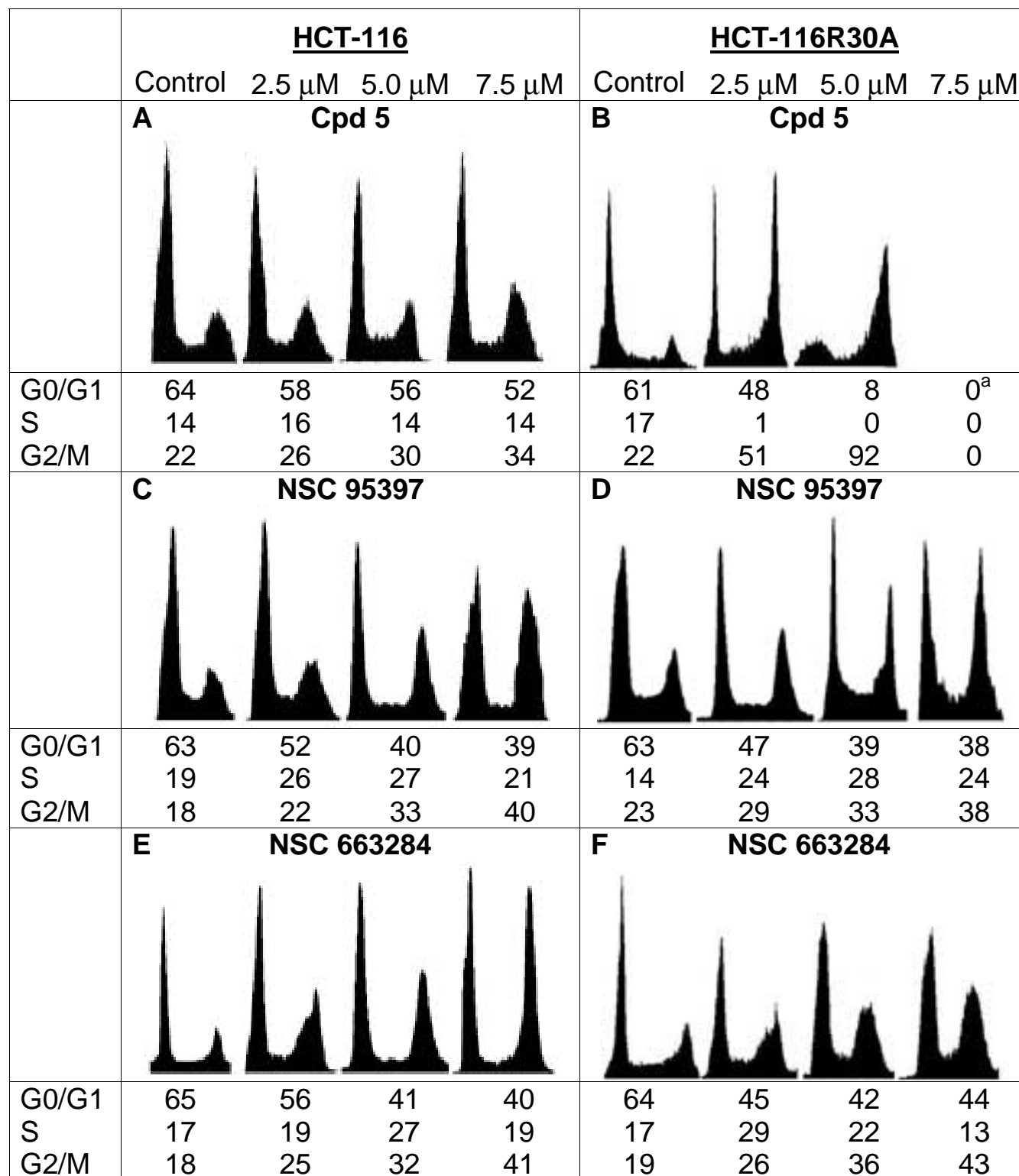[a] Cpd 5 at 7.5 µM killed most HCT-116R30A cells after 12 h exposure.

Figure 4

	HCT-116			HCT-116R30A		
Concentration [µM]	0	10	20	0	10	20
A. Cpd 5	1	2	3	4	5	6

P-Cdk1

total Cdk1

actin

B. NSC 95397

P-Cdk1

total Cdk1

actin

C. NSC 663284

P-Cdk1

total Cdk1

actin