Cytotoxicity of 2,3-Dichloro-5,8-dimethoxy-1,4-naphthoquinone in Androgen-dependent and -independent Prostate Cancer Cell Lines

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Abstract. *Background: Prostate cancer ranks third worldwide in cancer incidence and sixth in cancer mortality among men. A number of 1,4-naphthoquinone derivatives have been found to possess significant pharmacological effects associated with marked antimicrobial and antitumor activities. In the present study the in vitro effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) was evaluated on androgen-dependent (LNCaP, CWR-22) and androgen-independent (PC-3, DU-145) human prostate cancer cell lines, and/or a normal bone marrow cell line (HS-5). Moreover, the in vitro activity of this compound on cell cycle regulation and apoptosis was evaluated. Materials and Methods: Established methods of cell viability, cell cycle, Western blot and apoptosis were used. Results: The effect of DCDMNQ on LNCaP, CWR-22, PC-3, DU-145 and HS-5 cells revealed significant anti-tumor activities with IC_{50}s, of 1, 3, 1.5, 3 and 10 μM respectively. Cell cycle analysis showed that DCDMNQ inhibited progression through the cell cycle in PC-3 and DU-145 cell lines in a time-dependent manner. The result for the CWR-22 cell line showed that DCDMNQ arrested cells in the G_1-phase of the cell cycle with the greatest proportion of cells in the G_1-phase by day 5; however, the LNCaP cell line was inconsistent. The compound showed no effect on the cell cycle progression in the bone marrow HS-5 cell line. These findings were further validated using Western blot analysis. Furthermore, DCDMNQ induced apoptosis in the androgen-independent cells, preferentially over that of the androgen-dependent cell lines, in a time-dependent manner. Conclusion: Although the mechanism of action of this compound has not been completely elucidated, the effect on the cell cycle and the induction of apoptosis in different prostate cancer cell lines prompted us to carry out a more in-depth preclinical evaluation of it. This study suggests that DCDMNQ may have an impact on treatment of prostate cancer while protecting the bone marrow.*

While there have been major improvements in localized therapy for prostate cancer, the disease is uniformly fatal once it has spread outside of the prostate gland. Although androgen ablation is highly effective palliative therapy, most patients eventually relapse due in part to the presence of androgen-independent prostate cancer cells. Currently, there is no therapy that effectively eliminates these androgen-independent prostate cancer cells. In recent years, the progression of prostate cancer to hormone-refractory disease has often correlated with overexpression of growth factors and receptors capable of establishing autocrine and/or paracrine growth-stimulatory loops. Many of these growth factors engage the Ras/mitogen-activated protein kinase (MAPK) pathway as part of their signaling activities (1-3). The Ras-MAPK signaling cascade has been reported to be associated with about 30% of all human cancers (4, 5). It was recently suggested that inhibition of Ras function in conjunction with standard hormone ablation therapy might prove beneficial in treating advanced hormone-refractory prostate cancer (3)

Previous studies have demonstrated that metastatic androgen-independent prostate cancer cells have a remarkably low rate of cell proliferation (*i.e.* <5% cells proliferating/day) (6, 7). This low proliferative rate could explain the relative unresponsiveness of prostate cancer cells in humans to standard anti-proliferative chemotherapy,

Abbreviations: DCDMNQ: 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone; MAPK: mitogen-activated protein kinase; EGFR: epidermal growth factor receptor.

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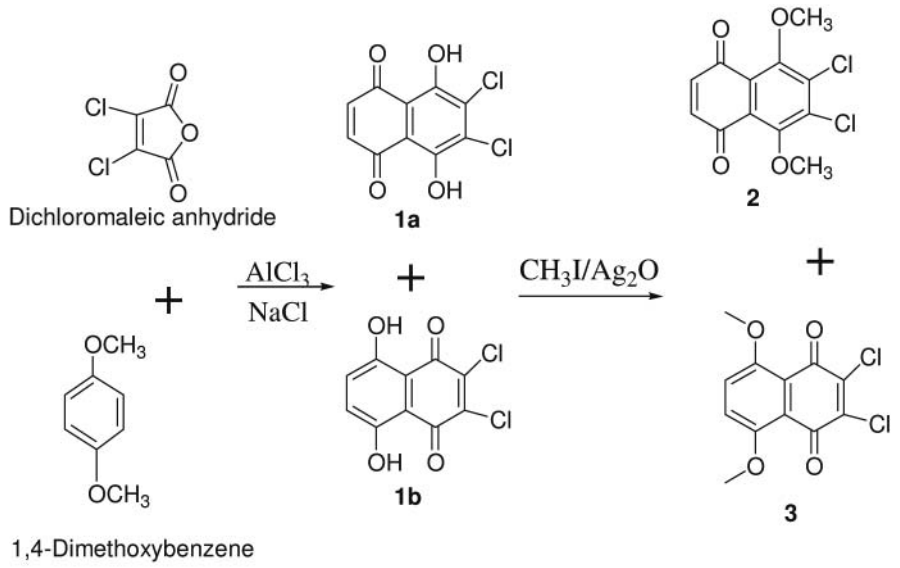

Figure 1. *Synthesis of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (3) and 6,7-dichloro-5,8-dimethoxy-1,4-naphthoquinone (2) via O-methylation of 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (**1b**) and 6,7-dichloro-5,8-dihydroxy-1,4-naphthoquinone (**1a**).*

while highly proliferative androgen-independent prostate cancer cell lines remain exquisitely sensitive to apoptosis induction in preclinical studies *in vitro* and *in vivo*. Therefore, new therapies for metastatic prostate cancer are urgently needed.

A number of 1,4-naphthoquinone derivatives have been found to possess powerful pharmacological effects associated with marked antimicrobial and antitumor activities (8, 9). Bakare *et al.* (10) reported the MEK-1 specific inhibitory activity of 2-chloro-3-(*N*-succinimidyl)-1,4-naphthoquinones. These analogs have IC_{50}s against purified MEK-1 enzyme in the range of 0.4-10 μM, with IC_{50}s against other kinases (*e.g.* Erk, Raf, PKC) that were 10- to 200-fold higher and thus form a new class of MEK-1 inhibitors. Therefore, the present study was undertaken to determine the cytotoxic and cell cycle regulatory effects of such a compound on human prostate cancer cell lines.

Materials and Methods

Synthesis of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (3). This compound was synthesized as described by Kuo and coworkers (11) and outlined in Figure 1: A mixture of 6,7-dichloro-5,8-dihydroxy-1,4-naphthoquinone (**1a**) and 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone (**1b**) was obtained from the reaction of dichloromaleic anhydride and 1,4-dimethoxybenzene as shown in Figure 1. Subsequent *O*-methylation of the mixture of **1a** and **1b** with methyl iodide-silver oxide furnished the mixture of 6,7-dichloro-5,8-dimethoxy-1,4-naphthoquinone (**2**) and 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (**3**). Compounds **2** and **3** were easily separated by column chromatography on silica gel to obtain the target compound, 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ, **3**) in pure form. The structures of the compounds were established by melting point, infrared (IR) spectroscopy, proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) and electrospray mass spectrometry.

Reagents. The BCA Protein assay kit, PVDF membrane and Super Signal West Dura were purchased from Pierce, Rockford IL, U.S.A. Propidium iodide (PI), annexin V and primary (Rb) antibody were from BD Biosciences, CA, U.S.A. MTT, trypan blue dye and all other reagents were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. An early passage of human prostate cancer cells and human bone marrow (HS-5) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, U.S.A.

Cell culture. Stock cultures of the human androgen-dependent (LNCaP, CWR-22) and androgen-independent (PC-3, DU-145) cell lines and human bone marrow (HS-5) cells were grown in 75 cm^3 flasks and incubated in RPMI-1640 medium (Cellgro, Mediatech Inc., VA, USA) 10% fetal bovine serum incubated in 5% CO_2/95% air at 37˚C. Upon reaching confluence, the cells were trypsinized, staining with 0.2% trypan blue dye and counted in a hemacytometer.

Cell viability. Cell viability was quantitatively determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Cells ($1.5x10^4$ cells per well) were plated in a 96-well plate in RPMI-1640 media in the presence or absence of DCDMNQ. The cells were incubated for 24 h under 5% CO_2, 95% O_2 at 37˚C. The control plates received 0.001% DMSO-containing medium (concentration had no antiproliferative effect on any of the tested cell lines, data not shown) and treatment plates received a 3-log range concentration (10^{-1}-10^1 μM) of DCDMNQ-containing media to span the IC_{50} as determined by preliminary experiments. After 1, 3 and 5 days the medium was aspirated and

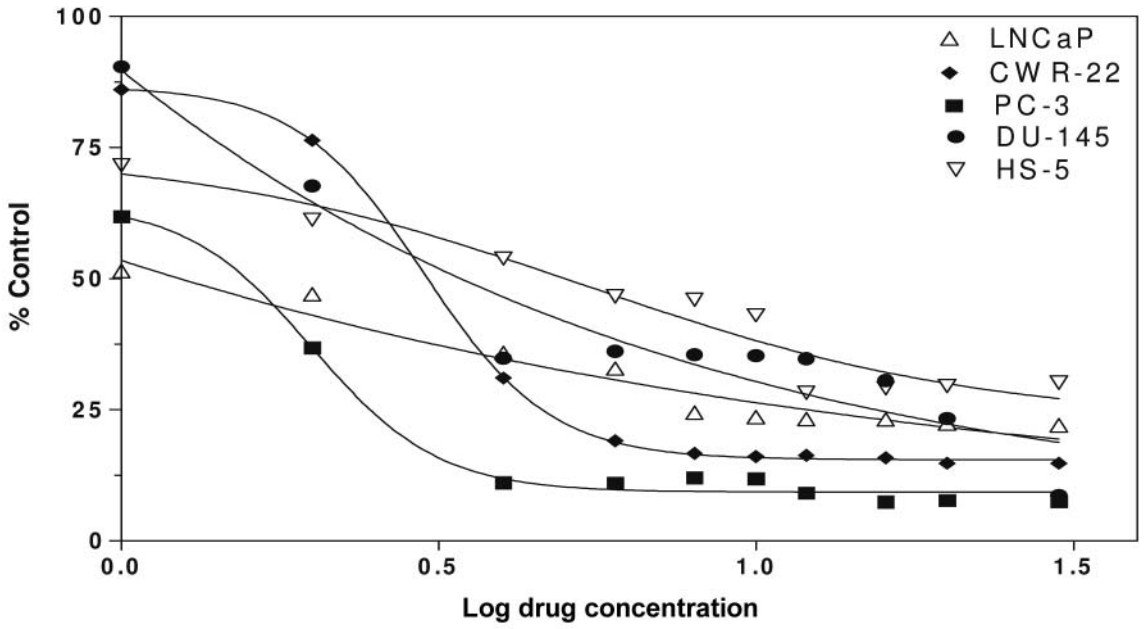

Figure 2. *The effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) on cell viability of human prostate cancer (androgen-dependent LNCaP, CWR-22, and androgen-independent PC-3, DU-145) and bone marrow (HS-5) cell lines.*

30 μl of MTT reagent (0.5 mg/mL) in PBS containing 10 mM HEPES was added to the plates. The plates were incubated for 3 h at 37˚C followed by aspiration. The plates were allowed to dry in the incubator for 1 h. The incorporated dye was solubilized in 100 μL of 0.04 N HCl in isopropanol. In order to determine cell number in each sample, the absorbance was measured spectrophotometrically in a plate reader at 570 nm with a background of 630 nm. Data points represent the means of eight wells. Cell viability was determined by subtracting the test results from the background as a percentage of the control.

Cell cycle analysis. Cell cycle perturbations were assessed using flow cytometry to measure the proportion of cells in the G_1-, S- and G_2/M- phases. Cell cycle perturbations induced by DCDMNQ were analyzed using propidium iodide (PI) DNA staining as described elsewhere (12). Approximately $1x10^5$ cells per well were plated in 6-well plates and allowed to attach overnight and then cells were exposed to IC_{50}s (calculated from cell viability experiment, the concentration that kills 50% of cells) of DCDMNQ. After 1, 3 or 5 days of exposure, the cells were collected, plated and fixed in ice-cold 70% ethanol for 4 h and stored at 4˚C until PI staining. Ethanol-suspended cells were then centrifuged at 200 xg for 5 min and washed twice in PBS to remove residual ethanol. Pellets were suspended in 1 mL of propidium iodide/RNase A reagent and incubated at 37˚C for 30 min. Cell cycle profiles were obtained using a BD FACScan Cell flow Cytometer (Becton Dickinson Italy). Data were analyzed by ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Determination of apoptotic cells. To quantify drug-induced apoptosis, annexin V/propidium iodide staining was performed using flow cytometry. Briefly, after drug treatments, both floating and attached cells were combined and subjected to annexin V/propidium iodide staining with an annexin V-FITC apoptosis detection kit according to the protocol provided by the manufacturer. Untreated control cells were maintained in parallel to the drug treatment group. The cells were exposed to IC_{80}s (calculated from cell viability experiment, the concentration that kills 80% of cells) of DCDMNQ for 3 or 5 days. Double staining was used to distinguish between viable, early apoptotic and necrotic or late apoptotic cells. The resulting fluorescence was measured using flow cytometry using a FACScan flow cytometer.

Western blot analysis. Approximately $1x10^6$ cells were plated in 60-mm Petri-dishes and allowed to attach overnight and then cells were exposed to IC_{50} of DCDMNQ for 3 or 5 days of exposure (control was without any treatment) cells were collected by scraping and homogenizing using lysis buffer containing protease inhibitor (cocktail tablet complete, from Roche). The cell lysates were centrifuged at 5000 xg for 10 min at 4˚C. The supernatant was collected and protein concentration was determined using the BCA Protein Assay with bovine serum albumin as a standard. Equal amounts of protein (20 μg) were loaded onto a 4-12% SDS-PAGE then transferred to a polyvinylidenedifluoride (PVDF) membrane. The membrane was probed with primary antibody (mouse anti-human retinoblastoma protein Rb monoclonal antibody 1:1000), followed by horseradish peroxidase-conjugated secondary antibody (Goat anti-mouse IgG, 1:10,000). Antibody detection was performed using enhanced Chemoluminesence Reagents Super Signal West Dura. The membrane was exposed to Hyperfilm MP (Amersham Biosciences, USA) and developed using Kodak GBX developer and fixer. Densitometric analysis was conducted using the AIS densitometric computer based Imaging System (Imaging Research Inc. Canada) program to quantify the intensity of bands from five independent Western blots.

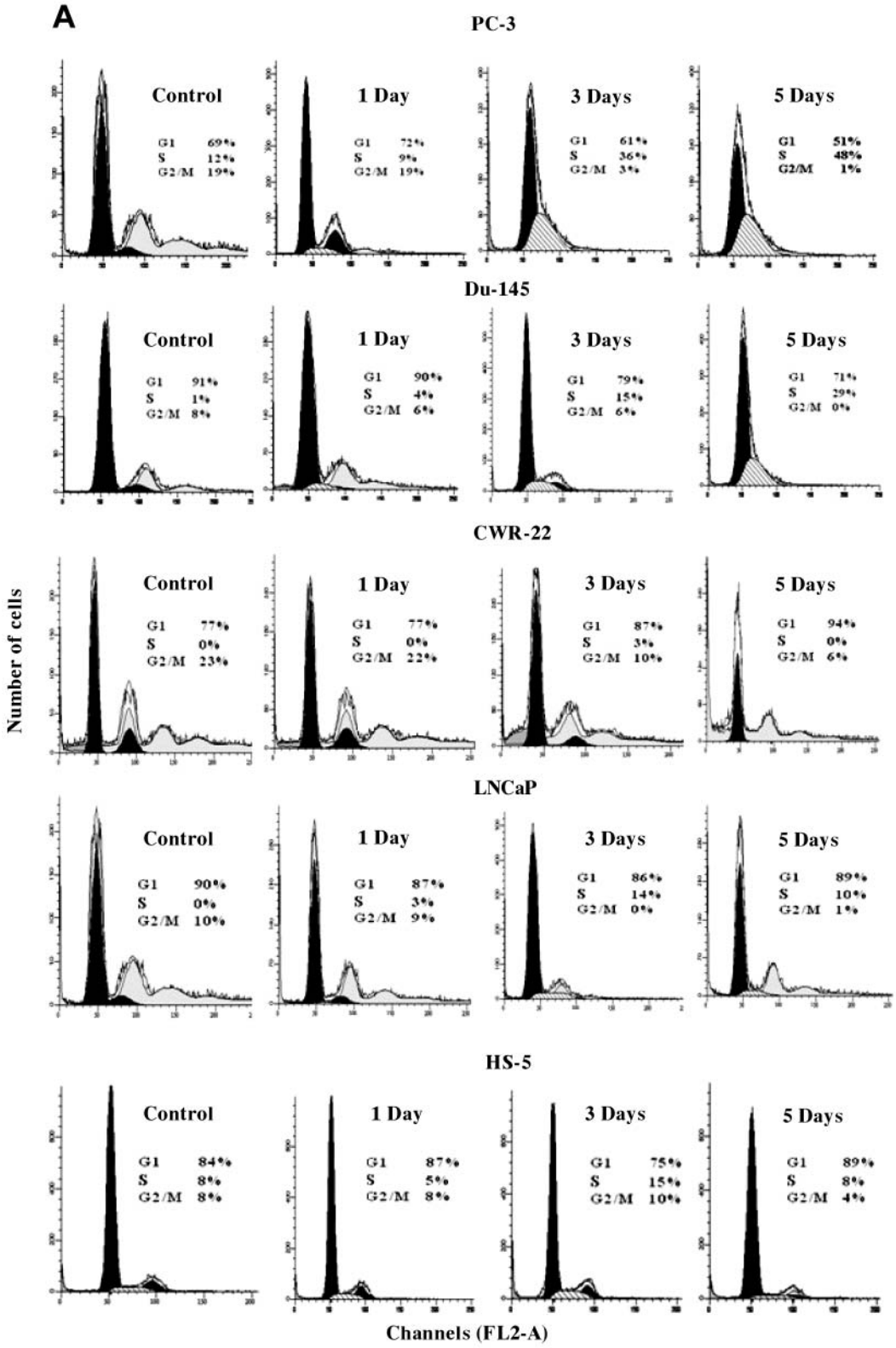

Figure 3. *continued*

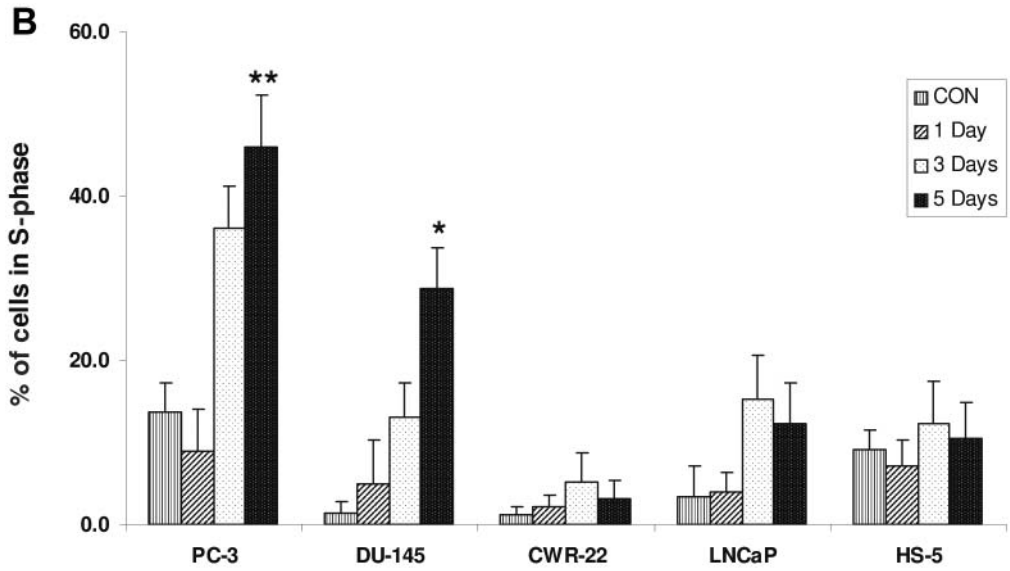

Figure 3. *Effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) on the cell cycle progression of human prostate cancer and human bone marrow cell lines. Cells were exposed for 1, 3 and 5 days in the presence or absence of the drug at respective IC_{50}s. Control cells shown were measured at 1 day and as expected no significant changes were observed in the control cells at 3 and 5 days. (A) The cell cycle profile is representative of three independent experiments. (B) Percentage of cells in S-phase. Results represent mean±SEM of three independent experiments. Analysis of variance indicated a significant increase compared with the control (*p<0.05, **p<0.01).*

Statistical analyses. Data were expressed as mean±standard error. One-way ANOVA followed by *post hoc* Dunnett's comparison test determined statistical differences between control and treatment groups. $P<0.05$ was considered statistically significant. Data were analyzed for both control and treatment groups using Graphpad InStat (Graphpad Software, Inc, San Diego, CA, USA).

Results

Effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) on the cell viability, cell cycle progression and apoptosis of human prostate cancer and bone marrow cell lines.

Cell viability. 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone inhibited the growth of the androgen-dependent (LNCaP, CWR-22) and androgen-independent (PC-3, DU-145) human prostate cancer, and normal bone marrow (HS-5) cell lines in a dose-dependent manner following treatment for 5 days. These *in vitro* studies of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone on human prostate cancer cell lines revealed significant anti-tumor activities with IC_{50}s for LNCaP, CWR-22, PC-3, DU-145 and HS-5 of 1, 3, 1.5, 3 and 10 mM respectively (Figure 2).

Cell flow cytometry analysis. Cell flow cytometry was used to determine the effect of the DCDMNQ compound on the progression of the cell cycle. The cell cycle profile in Figure 3A is representative of three independent experiments inclusive of the three treatment groups in all the cell lines. Figure 3B shows the percentage of cells in the S-phase at different time points under each treatment. Analysis revealed that the highest number of cells progressing to the S-phase of the cell cycle was found in PC-3 cells (46%±6.2%) followed by DU-145 cells (28.7%±4.9%). They were statistically significantly higher than the corresponding control values. In contrast, there was a decreased proportion of CWR-22 cells in the S-phase. The cells with lower DNA content, as shown by PI staining less than G1, were defined as apoptotic (sub G0/G1 population). The greatest sub-G_0/G_1 population was observed in cells of DU-145 (47%±7.1%) followed by PC-3 (29%±5.2%) then CWR-22 (25%±4.5%), LNCaP (20%±3.4%) and the lowest number of apoptotic cells was observed in HS-5 (Figure 4). The percentages of apoptotic cells increased with increasing treatment time and were significant when compared with their respective controls.

Western Blot analysis. The retinoblastoma protein (Rb), a cell cycle regulator which when phosphorylated allows the progression of cells from the G_1- to the S-phase, was used as a marker to determine the effects of DCDMNQ on cellular progression at the molecular level. The Western blot (Figure 5A) is the representative of five independent experiments of the two treatment groups.

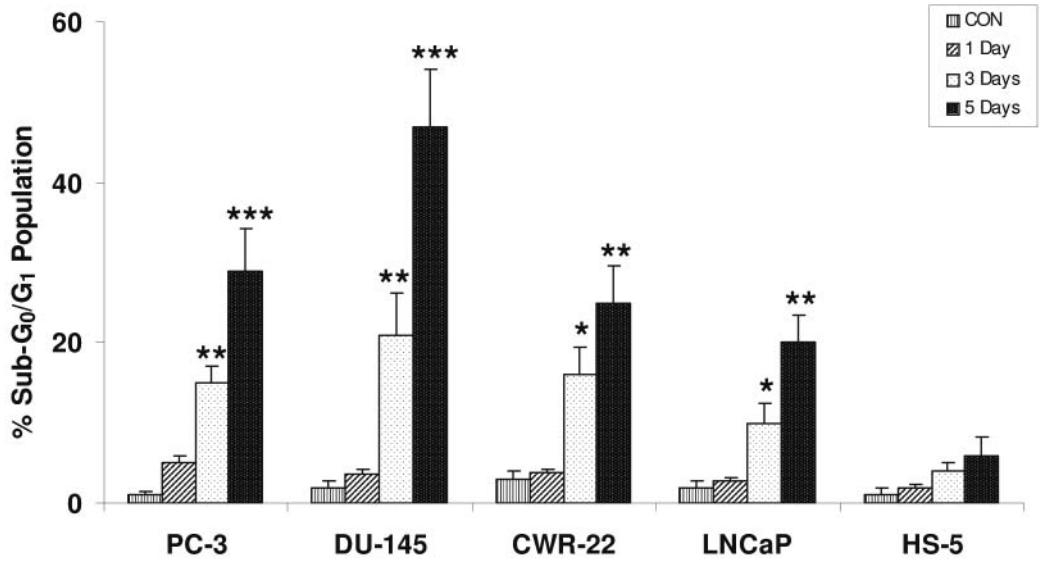

Figure 4. *Percentage of apoptosis calculated by measuring the sub-G0/G1 population using cell flow cytometry in all the cell lines mentioned. The cells with a lower DNA content showing less PI staining than G_1 were defined as apoptotic. The graph represents the number of apoptotic cells as a proportion of the total (%); *p<0.05, **p<0.01, ***p<0.001 compared with the control.*

The relative optical density (ROD) is shown in Figure 5B. The highest density was observed in PC-3 cells, followed by CWR-22, HS-5, and then DU-145, indicating higher phosphorylation. The lowest density was observed in LNCaP cells indicating fewer phosphorylated Rb proteins were present. Significant phosphorylation was observed in PC-3 and DU-145 cells when compared to the control results. Similar results were observed with the cell flow cytometry analysis.

Apoptosis. The drug-induced apoptosis was measured using cell flow cytometry. With this method, as shown in Figure 6, the percentage of early plus late apoptotic cells reached its highest in DU-145 cells after 3 days treatment (55%) followed by PC-3 cells (30%). The 5-day treatment groups showed more than half of the cells formed debris in PC-3 and DU-145 cell lines. In the case of CWR-22 and LNCaP, the percentage of apoptotic cells increased with increasing treatment time, whereas bone marrow cells did not show any significant change compared to the control.

Discussion

In the present study, we investigated the effects of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) on cell proliferation, cell cycle and apoptosis. Consistent with the effect of this compound on cell growth inhibition, the cell number was found to decrease with increasing concentrations ranging from 10^{-1}-10^{1} μM after 1, 3 and 5 days of treatment. The mechanism by which DCDMNQ causes growth inhibition remains unclear, while our studies suggest that it is an effective inhibitor of both androgen-dependent and -independent prostate cancer cells. Our data also showed the ability of this compound to arrest cell cycle progression.

Notably, the different cell lines used in the present study displayed different cell cycle perturbations following treatment. Treatment arrested the androgen-independent, PC-3 and DU-145 cells in the S-phase and the androgen-dependent, CWR-22 in the G_1- phase of the cell cycle. Similar observations were also reported with different drugs and were attributed to different cell cycle checkpoint status and susceptibility to apoptosis (13-15).

The results of cell flow cytometry and Western blot showed significantly fewer cells entering the S-phase of the cell cycle in bone marrow cells. Hence, DCDMNQ may interfere with the transition between the G_1- and S-phases and the release of an E2F transcription factor thereby decreasing the activity of the compound in the bone marrow, which arrests cells in the S-phase.

This property of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone opens the possibility of investigating innovative combinations with other agents acting at different stages of the cell cycle. ZD1839 (Iressa) is an orally active low molecular weight tyrosine kinase inhibitor highly selective for EGFR tyrosine kinase. It prevents EGFR autophosphorylation in a variety of EGFR- expressing human cancer cell lines and competitively interferes with signaling by

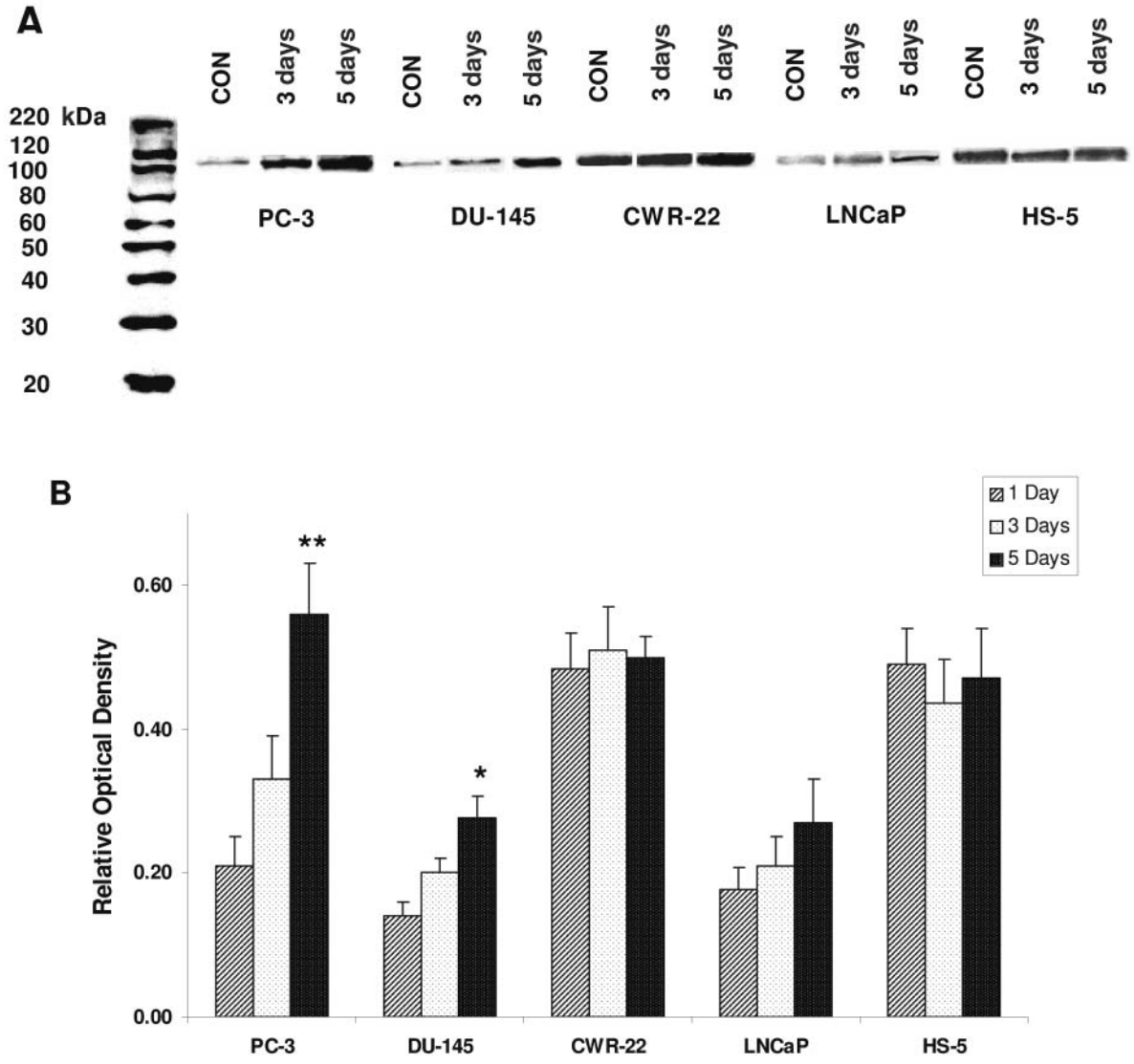

Figure 5. *Effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) on the level of retinoblastoma protein (Rb) as phosphorylated protein marker in human prostate cancer and bone marrow cells. Cells were exposed for 3 or 5 days. (A) The gel image is representative of five independent experiments. (B) Relative optical density and *p<0.05, **p<0.01 compared with the control.*

EGFR ligands (16). The effects of ZD1839 on androgen-dependant (LNCaP) and androgen-independent (PC-3 and DU-145) cell lines investigated by Sgambato *et al.* (17) caused a dose- and time-dependent growth inhibition with an IC_{50} of 15.6 μM. The results suggested that ZD1839 inhibited EGFR phosphorylation in androgen-sensitive and -insensitive prostate cancer cells and exerted a growth inhibitory effect by blocking the cells in the G_0/G_1 phase of the cell cycle.

It is known that apoptosis-signaling pathways and cellular events controlling them have a profound effect both on cancer progression and in response to chemotherapy (18, 19). Based on annexin V/propidium iodide staining it is clear that the highest proportion of apoptotic cell death was observed in DU-145 followed by PC-3 treated cells. DCDMNQ significantly inhibited the growth of prostate cancer cells *in vitro* possibly by either up-regulation of apoptotic Bax and down-regulation of anti-apoptotic Bcl-X_L, or by increasing the $p21^{waf1/cip1}$ protein which is involved in cell cycle arrest in G_1 or G_2 (20). The cell cycle alterations indicate that cell cycle arrest is one of the primary mechanisms responsible for the anti-proliferative action of DCDMNQ in prostate tumor cells *in vitro*.

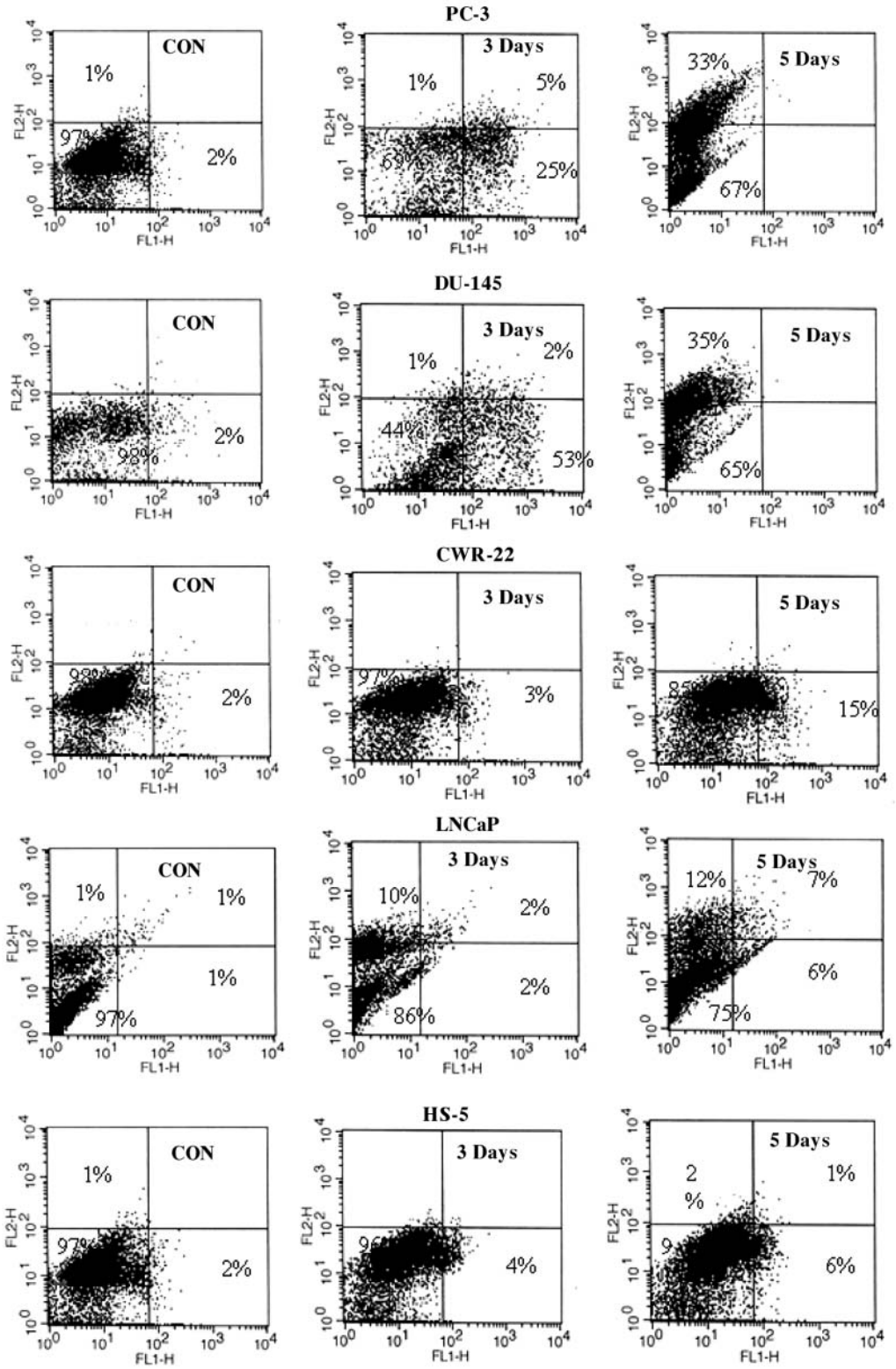

Figure 6. *Apoptosis analysis of human prostate cancer and bone marrow cell lines treated with respective IC_{80}s. Cells were exposed for 3 or 5 days. Control cells shown were measured at 3 days and as expected no significant changes were observed in the control cells at 5 days. Double staining was used to distinguish between viable, early apoptotic, necrotic or late apoptotic cells. The lower left quadrant shows the viable cells, the upper left quadrant shows cell debris, the lower right quadrant shows the early apoptotic cells and the upper right quadrant shows the late apoptotic and necrotic cells.*

We recently reported 2-chloro-3-(*N*-succinimidyl)-1,4-naphthoquinone and some of its analogs as MEK1-specific inhibitors of the Ras-MAPK pathway (10). An open chain analog was also reported to exhibit cytotoxicity against some renal cancer cell lines (10). Some compounds with the chloro-1,4-naphthoquinone skeleton have been reported to possess interesting biological activities including antiviral, (21) antifungal (22), antineoplastic (21-23), antihypoxic (24), antiischemic (24), antiplatelet, antiinflammatory and antiallergic activities (11, 25); others have been shown to inhibit the human cytomegalovirus (HCMV) protease (26).

Quinones are widely distributed in nature and many clinical important antitumor drugs containing the quinine nucleus, such as anthracyclines, mitoxanthenes and saintopin, show excellent anticancer activity. These anticancer agents are effective inhibitors of DNA topoisomerase and it is generally known that the cytotoxity of quinine analogs results from the inhibition of DNA topoisomerase-II (27). The quinine analogs can also induce the formation of the semiquinone radical which can transfer an electron to oxygen to produce superoxide. This process is catalyzed by flavonenzymes such as NADPH-cytochrome-P-450 reductase. Both superoxide and semiquinone radical anions of naphthoquinone analogs can generate the hydroxyl radical which is known to cause DNA strand breaks (28).

Conclusion

2,3-Dichloro-5,8-dimethoxy-1,4-naphthoquinone was more cytotoxic to and showed more specificity in androgen-independent than androgen-dependent prostate cancer cell lines as confirmed in cell-cycle and apoptosis experiments. Moreover, HS-5 bone marrow cells were less sensitive to this compound by at least one-log order of concentration magnitude than were all four prostate cancer cell lines. It is well known that late-stage prostate cancer metastasizes to bone.

Therefore, considering the cytotoxicity profile in different prostate cancer and bone marrow cell lines and the ability to arrest cell cycle progression and induce apoptosis, this compound represents the first in a class of specific compounds useful in the treatment of metastatic prostate cancer.

Acknowledgements

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