

Cytotoxic Effects of *N*-(3-Chloro-1,4-dioxo 1,4-dihydro-naphthalen-2-yl)-benzamide on Androgen-dependent and -independent Prostate Cancer Cell Lines

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Abstract. *Background:* Worldwide among men, prostate cancer ranks third in cancer occurrence and sixth in cancer mortality. A number of 1, 4-naphthoquinone derivatives have been identified that possess significant pharmacological effects associated with antitumor activities. In this study, the *in vitro* effects of *N*-(3-chloro-1,4-dioxo 1,4-dihydro-naphthalen-2-yl)-benzamide (NCDDNB) were evaluated on androgen-dependent (CWR-22) and androgen-independent (PC-3, DU-145) human prostate cancer cell lines, and on a normal bone marrow cell line (HS-5). Specifically, 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 NCDDNB on CWR-22, PC-3, DU-145 and HS-5 cells revealed significant anti-tumor activities with IC_{50} s, of 2.5, 2.5, 6.5, and 25 μ M respectively. The results of cell cycle analysis showed that NCDDNB arrested PC-3, DU-145, and CWR-22 cells in the G_1 -phase of the cell cycle. The compound showed no effect on the cell cycle progression in the HS-5 bone marrow cell line. These findings were further validated using Western blot analysis. NCDDNB showed the greatest amount of apoptosis in the androgen-independent PC-3 cells in a time-dependent manner with the apoptotic apex at day 5 of treatment.

Abbreviations: *N*-(3-Chloro-1,4-dioxo 1,4-dihydro-naphthalen-2-yl)-benzamide (NCDDNB), phosphatidylserine (PS), retinoblastoma protein (Rb), poly(vinylidene fluoride) (PVDF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

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Furthermore, NCDDNB induced-apoptosis in DU-145 and CWR-22 cells peaked at day 3 of treatment. **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. This study suggests that NCDDNB may have an impact on treatment of prostate cancer while protecting the bone marrow.

Prostate cancer constitutes a major public health burden in Western countries. It is the most frequently diagnosed cancer in American men, and the second leading cause of cancer death in men, exceeded only by lung cancer (1). In 2008, an estimated 186,320 new cases of prostate cancer were diagnosed and 28,660 men died from the disease in the United States (2). 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. Early-stage prostate cancer is typically dependent on androgen for growth and therefore is responsive to the surgical or pharmacological ablation of circulating androgen (3). Although androgen ablation is highly effective palliative therapy, most men eventually relapse due to the presence of androgen independent prostate cancer cells within metastatic sites. Currently there is no therapy that effectively eliminates these androgen independent prostate cancer cells. Therefore, new therapies for metastatic prostate cancer are urgently needed.

1,4-Naphthoquinone derivatives possess a wide variety of biological responses that include antibacterial, antifungal, anti-inflammatory, antiviral, anticancer and apoptotic activity. The biological activity of 1,4-naphthoquinones is mainly due to the presence of two carbonyl groups that have the ability to accept one and/or two electrons to form the corresponding radical anion or di-anion species as well as their acid-base properties (4, 5). Although there are general

mechanisms of quinoid toxicity, such as redox cycling and conjugation reactions with bionucleophiles, the specific roles of the quinoid substructures in antitumor activity is as yet unknown (5).

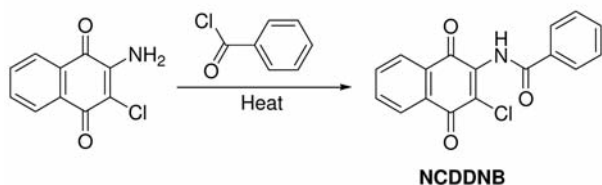
Bakare *et al.* (6) previously developed 2-chloro-3-(*N*-succinimidyl)-1,4-naphthoquinone as a specific MEK 1 inhibitor of the MAP kinase cascades. Whereas, Copeland *et al.* (7) showed in subsequent studies on prostate cancer cell lines the antitumor potency of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone. In continuation of our studies on the effects of the imido-substituted chloronaphthoquinones on prostate cancer cell lines, we found it necessary to screen some of the amidosubstituted derivatives for anticancer activity. This study was undertaken to show the cytotoxic and cell regulatory effects of *N*-(3-chloro-1,4-dioxo 1,4-dihydronaphthalen-2-yl)-benzamide [2-chloro-3-benzamido-1,4-naphthoquinone] on both androgen dependent and androgen independent human prostate cancer cell lines.

Materials and Methods

Synthesis of *N*-(3-chloro-1,4-dioxo 1,4-dihydro-naphthalen-2-yl)-benzamide (scheme 1). A mixture of 2-amino-3-chloro-1,4-naphthoquinone (251 mg, 1.209 mmol) and benzoyl chloride (5 mL) was heated at 95°C for 1.5 hours. The reaction mixture was cooled to room temperature and 50% diethyl ether/hexane (60 ml) added. The resulting solution was placed in the freezer and the solid that precipitated was filtered under suction. The crude product was purified by column chromatography on silica gel using 20% ethyl acetate/hexane as the eluent to obtain a yellow solid (113 mg, 30%). Mp 192-194°C. IR (cm⁻¹) 3353, 3073, 2924, 1697, 1662, 1614, 1589, 1457, 1314, 1288, 1199, 1081, 922, 854, 786, 674. ¹H-NMR (CDCl₃) δ 7.50-7.58 (m, 2H), 7.61-7.68 (m, 1H), 7.74-7.86 (m, 2H), 7.95-8.02 (m, 2H), 8.11-8.17 (m, 1H), 8.20-8.26 (m, 1H), 8.36 (s, 1H). ¹³C-NMR (CDCl₃) δ 180.0, 177.6, 163.8, 139.6, 134.9, 134.1, 133.2, 132.8, 132.6, 131.7, 130.3, 129.0, 128.2, 127.6, 127.1.

The molecular weight of NCDDNB is 311.71. A 10 mM stock solution of NCDDNB was prepared by dissolving 3.1 mg of NCDDNB powder in 994.5 µL DMSO.

Scheme 1



Cell culture. The CWR22Rv1, DU-145, PC-3 and HS-5 cell lines (purchased from the American Type Culture Collection, Rockville, MD) were propagated in RPMI-1640 medium (Cellgro, Mediatech Inc. VA, USA) containing 10% heat-inactivated fetal bovine serum (Mediatech, VA), 100 IU/ml Penicillin and 100 µg/ml Streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂/95% air. For all the experiments,

cells were cultured in 75 cm² flasks until they reached 90% of confluence. Cells were then trypsinized with trypsin-EDTA solution (0.25% trypsin; Gibco), centrifuged at 110 × g for 5 min and resuspended in fresh complete medium. Cell viability was assessed by trypan blue exclusion and cell concentration was adjusted according to the requirements of each experiment.

Cytotoxicity assay. (a) *Drug concentration-range finding studies:* Following treatment of cells (12,000 cells/well) in 96 -well plates with a 3-log range concentration (0.5 µM, 5 µM and 50 µM) of NCDDNB for one, three, and five days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, MO, USA) was added to each well. Plates were incubated at 37°C for 4h, then the medium was aspirated, plates were dried for 1 h at 37°C. The formazan was solubilized with 100 µL of acidic isopropanol (0.04 M HCl in 70% v/v isopropanol). After 2 h incubation, absorbance in control untreated and treated wells were measured at a wavelength of 570 nm with background subtraction of 650 nm using the Wallac 1420 plate reader (Perkin Elmer, Finland).

(b) *Determination of IC₅₀ concentrations of NCDDNB:* cells (12,000 cells/well) in a 96 well plate treated with 1, 2, 4, 6, 8, 10, 12, 16, 20, and 30 µM of NCDDNB for five days. At each endpoint, the MTT assay was used as previously described to determine cell viability. All assays were done in triplicate. The IC₅₀ was determined from a plot of log (drug concentration) versus percentage of change from control using Prism Graphpad software (ver 4.0).

Analysis of apoptosis. The appearance of phosphatidylserine (PS) residues on the surface of the cell is an early indication of apoptosis. PS was analyzed using an annexin V-FITC/PI apoptosis detection kit (BD Biosciences, CA, USA) with a fluorescent conjugated annexin V anticoagulant which has a high affinity to PS.

The untreated and NCDDNB IC₈₀ treated cells (8×10⁵) cells were washed twice with cold 1× PBS and resuspended in 1X Binding Buffer (10 mM HEPES/NaOH [pH 7.4], 140 mM NaCl, 2.5 mM CaCl₂) and incubated with FITC Annexin V and 10 µl PI (50 µg/m) for 15 min at RT (25°C) in the dark. Four hundred µl of 1× Binding Buffer was added to each tube and samples were analyzed by flow cytometry within 1 h. The following controls were used to set up compensation and quadrants: a) unstained cells, b) cells stained with FITC Annexin V (no PI), and c) cells stained with PI (no FITC Annexin V). The resulting fluorescence was measured using BD FACScan flow cytometer.

Cell cycle analysis. Cell cycle perturbations induced by NCDDNB was analyzed by propidium iodide (PI) (BD Biosciences, CA, USA) DNA staining as previously described (6). The cells were grown in 25 cm² cell culture flasks at approximately (1×10⁵) cells per flask in the presence of IC₅₀ concentrations of NCDDNB for 3 or 5 days. At each end point, treated and untreated cells were collected and fixed in 70% (v/v) ethanol for 4 h at 4°C. Cells were washed twice with PBS and resuspended with 1 mL of staining solution [DPBS pH 7.4, 0.1% (v/v) Triton X-100, 0.05 mg/ml DNase-free RNase A, 50 µg/ml propidium iodide] and incubated at 37°C for 30 minutes. Cells were then analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data acquisition (10,000 events for each sample) was performed using CELLQuest Software (Becton Dickinson, Italy).

Western blotting. The retinoblastoma protein (Rb), a cell cycle regulator, which when phosphorylated allows the progression of cells from the G₁- to the S-phase, was used as a marker to determine the effects of NCDDNB on cellular proliferation at the molecular level. Total cellular proteins from the IC₅₀ treated and non treated cells were extracted by using the M-PER Mammalian Protein Extraction Reagent (Pierce, IL, USA) lysis buffer containing Halt™ Protease Inhibitor Cocktail. Protein concentration was quantitated using BCA Protein Assay with bovine serum albumin as a standard (Pierce).

The protein extracts (30 µg/lane) were applied to electrophoresis on 12-14% Precast Ready Gel (BioRad, CA, USA), and then, a transferred poly(vinylidene fluoride) (PVDF) membrane (Millipore, Billerica, MA, USA) was blocked in 3% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (Sigma, St. Louis, MO, USA) overnight at room temperature followed by the hybridization to the primary mouse anti-human retinoblastoma protein Rb monoclonal antibody 1:1000 (BD Biosciences, CA, USA) at 4°C overnight. After being washed, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Goat anti-mouse IgG 1:10,000, BD Biosciences, CA, USA) and washed again. The specific binding signals were visualized by enhanced chemiluminescence's system (Super Signal, West Dura) according to manufacturer's instructions. The membrane was exposed to Hyperfilm MP (Amersham Biosciences, PA, USA) for five to ten minutes and developed using Kodak GBX developer and fixer. Densitometric analysis was conducted using Quantity One imaging software (BioRad, CA, USA).

Statistical analysis. 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 (Graphpad Software, Inc, San Diego, CA, USA).

Results

Determination of IC₅₀ concentrations of NCDDNB. To determine the IC₅₀ values of NCDDNB, cells were treated with several concentrations of drug for five days. At each endpoint, the MTT assay was used to determine cell viability. All assays were carried out in triplicate. The IC₅₀ was then determined for the compound from a plot of log (drug concentration) *versus* percentage of change from control (Figures 1). The IC₅₀ values for NCDDNB on CWR-22, PC-3, DU-145 and HS-5 were 2.5, 2.5, 6.5, and 25 µM respectively.

Effect of NCDDNB induced apoptosis. Apoptosis analysis of human prostate cancer and bone marrow cell lines treated with respective IC₈₀s of NCDDNB. After drug treatment, both floating and attached cells were combined and subjected to Annexin V/propidium iodide staining to distinguish between viable, early apoptotic, necrotic or late apoptotic cells. The NCDDNB induced apoptosis was measured using cell flow cytometry. The percentage of early plus late apoptotic cells only increased with increasing treatment time in PC-3 cell lines (Figures 2A and B). In PC-3 cells, both

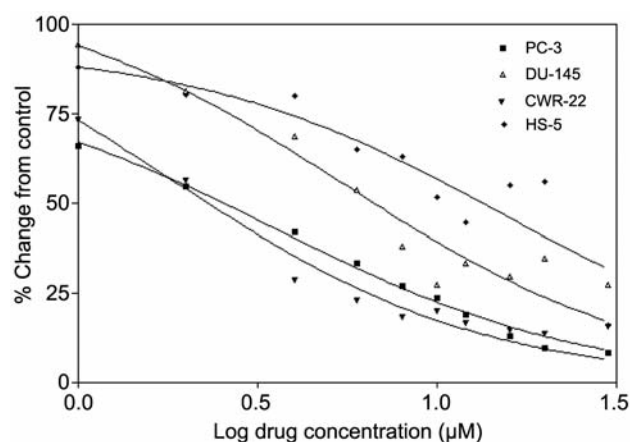


Figure 1. Determination of IC₅₀ concentrations of NCDDNB (day 5 treatment) on Human prostate cancer and bone marrow cell lines.

days three and five showed a significant increase ($p<0.001$) in apoptotic cells compared to control with $31\%\pm6\%$ and $43\%\pm7\%$ respectively (Figures 2B). In DU-145 and CWR-22 the highest percentage of early plus late apoptotic cells was reached after three days treatment with $18\%\pm7\%$ and $21\%\pm5\%$ respectively (Figure 2B). These cells then returned to control levels by day 5. Bone marrow cells showed no significant apoptosis ($p<0.05$) on day one – day five compared to the control, but overall they were not affected by NCDDNB treatment (Figures 2A and B).

Effect of NCDDNB on the cell cycle progression. Cell flow cytometry was used to determine the effect of NCDDNB on the cell cycle progression. The cell cycle profile in Figure 3A is representative of three independent experiments inclusive of the three treatment groups in all the cell lines. The highest percentage of cells from control, days three and five treatments were found in the G₁-phase (Figure 3B). Figure 3B also shows that there were significant decreases in the number of cells in the G₁-phase after day one treatment in PC-3 cells ($p<0.001$), CWR-22 cells ($p<0.001$), and HS-5 cells ($p<0.05$). Figure 3C 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 occurred at the day one treatment for all cell lines with the highest number occurring in PC-3 cells ($40\%\pm2\%$) followed by DU-145 cells ($38\%\pm0.3\%$). In addition, there was a significant increase ($p<0.001$) in PC-3, CWR-22, and HS-5 cells in the S-phase on day one (Figure 3C).

Western blot analysis. The Western blot of NCDDNB treated cells (Figure 4A) is representative of three independent experiments of the three treatment groups (days 1, 3, 5). The relative optical density (ROD) of Rb protein in NCDDNB

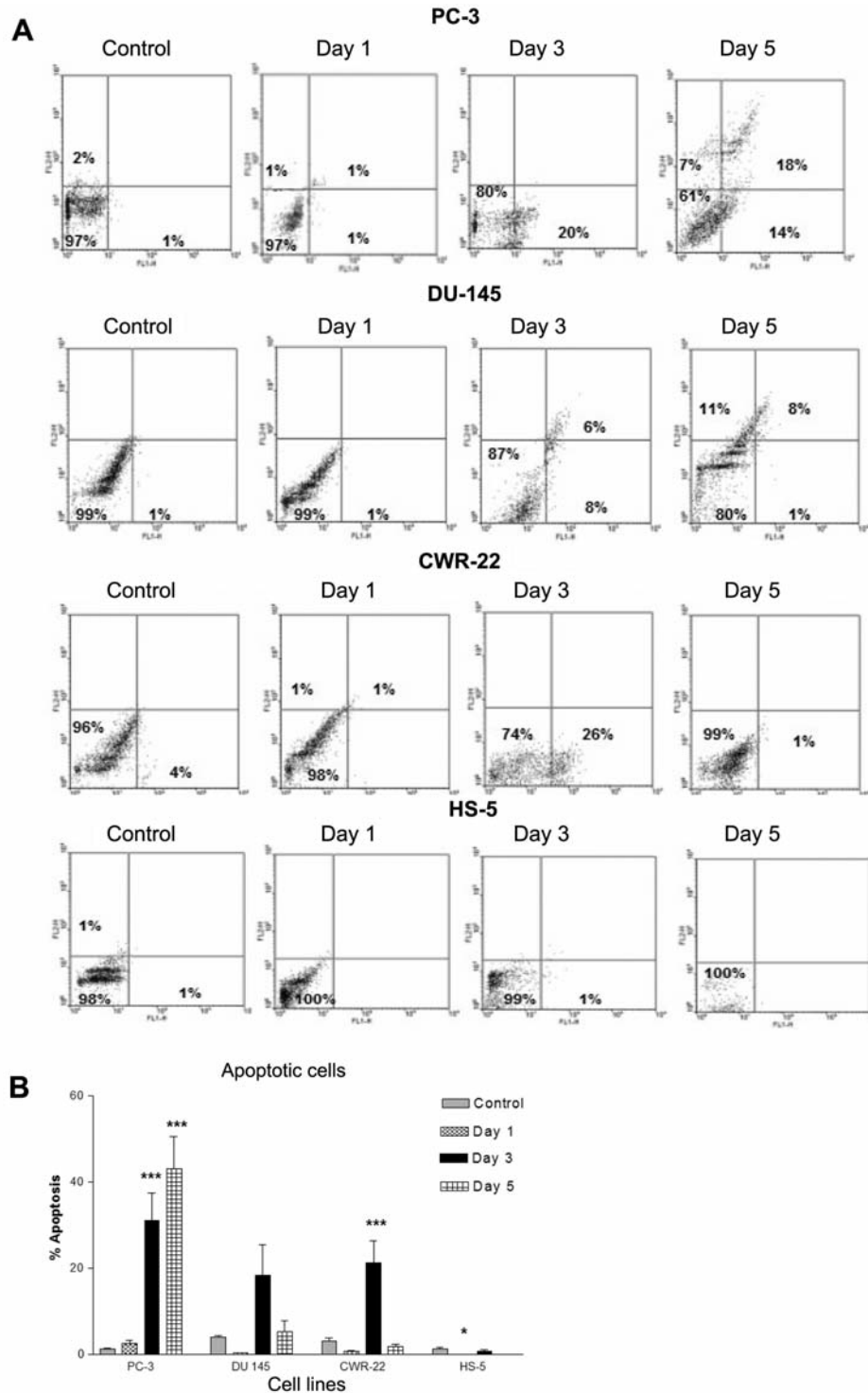


Figure 2. A: NCDDNB induced apoptosis on human prostate cancer and bone marrow cell lines treated with respective IC_{50} s. The apoptosis profile is representative of three independent experiments. Double staining was used to distinguish between viable, early apoptotic, necrotic or late apoptotic cells. 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. B: NCDDNB induced apoptosis on human prostate cancer and bone marrow cell lines. After drug treatments both floating and attached cells were combined and subjected to annexin V/propidium iodide staining. Control cells shown were measured at day 1. Double staining was used to distinguish between viable, early and late apoptotic cells or necrotic cells. The resulting fluorescence was measured by flow cytometry using a FACScan flow cytometer. Results represent mean \pm SEM of three independent experiments. Analysis of variance indicated a significant increase compared with control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

treated cells is shown in Figure 4B. The highest density was observed after one day of treatment in HS-5 cells, followed by DU-145, PC-3, and then CWR-22, indicating higher phosphorylation. Significant phosphorylation ($p < 0.001$) was observed in both day one and day three treatment groups of PC-3 and DU-145 cells when compared to the control results. Similar results were observed with the S-phase cell cycle flow cytometry analysis of NCDDNB treated prostate cancer and human bone marrow cells.

Discussion

This study represents the first to report, the effects of NCDDNB on prostate cancer cell proliferation, cell cycle and apoptosis. NCDDNB is a 1,4 naphthoquinone derivative. Naphthoquinones are organic compounds that form the central chemical structure of many natural compounds, most notably K vitamins. They have been reported to possess interesting biological activities including antibacterial (10), antiviral (11), antifungal (12), antimalarial (13), antihypoxic (14), antiplatelet, anti-inflammatory and antiallergic activities (15). Naphthoquinones have also been reported to induce topoisomerase-I- and II-mediated DNA cleavage (16).

Cytotoxicity of NCDDNB. NCDDNB was cytotoxic to prostate cancer cells *in vitro*. The cell number of cell lines treated was found to decrease when treated with a 3-log range concentration (0.5 μ M, 5 μ M and 50 μ M) of each drug for one, three and five days. More detailed analysis showed that NCDDNB had IC_{50} s below 10 μ M.

Quinone moieties are present in several chemotherapeutic drugs such as anthracyclines, daunorubicin, doxorubicin, mitomycin, mitoxantrones and saintopin, which are used clinically in the therapy of solid tumors. The cytotoxic effects of these quinones are primarily due to: (a) inhibition of DNA topoisomerase-II and, (b) formation of semiquinone hydroxyl radicals that can transfer an electron to oxygen to produce super oxide; which is catalyzed by flavoenzymes such as NADPH-cytochrome-P-450 reductase. Both semiquinone and super oxide of quinones can generate the hydroxyl radical, which can cause DNA strand breaks. 1,4-Naphthoquinone contains two quinone groups that have the ability to accept one or two electrons to form the corresponding radical anion or di-anion species. It is most likely dependent on the quinone redox cycling that yields reactive oxygen species (ROS) as well as arylation reactions.

Effect of NCDDNB on cell cycle progression. NCDDNB treatment arrested the androgen-independent cells, PC-3 and DU-145 and the androgen-dependent cells, CWR-22 cells in the G_1 -phase of the cell cycle. Notably, the different cell lines used in the present study displayed different cell cycle perturbations following treatment. Similar observations were

also reported with different drugs and were attributed to different cell cycle checkpoint status and susceptibility to apoptosis (17-19).

Apoptotic potential of NCDDNB. 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 (20, 21). Based on annexin V/propidium iodide staining, NCDDNB caused the greatest amount of apoptosis in PC-3 cells; whereas, in DU-145 and CWR-22 the maximum amount occurred in day 3 and apoptotic levels decreased by day 5. This would suggest that the later cell types may have developed some form of early resistance to the compound. PC3 was most affected *via* 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 . While in DU-145 and CWR-22 the reverse may be true especially by day 5. This would also make NCDDNB a potential candidate for combination chemotherapeutic study. The amount of apoptosis shown in HS-5 cells was minimal. Interestingly, it was recently reported that plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone induces apoptosis with concomitant inactivation of Bcl-2 and the DNA binding activity of NF-kappaB (22). Moreover, Liou *et al.* demonstrated that labedipinedilol-A (selective $\alpha(1)$ -adrenoceptor antagonist)-induced inhibition of growth on prostate cancer cells. This was associated with G_0/G_1 arrest, and G_2/M arrest depending upon concentration. In addition, cell cycle blockade was associated with reduced amounts of cyclin D1/2, cyclin E, Cdk2, Cdk4, and Cdk6 and increased levels of the Cdk inhibitory proteins (Cip1/p21 and Kip1/p27) (23). Retinoblastoma (RB) normally remains in the hyperphosphorylated (inactivated) state until mitosis ends, then the RB tumor suppression is reverted by phosphatase activity. It may be speculated that NCDDNB's anti-proliferative effect could result in RB dephosphorylation (activation), thereby inducing RB-dependent termination of cell cycle progression. Thus the cell cycle alterations indicate that cell cycle arrest may be one of the primary mechanisms responsible for the anti-proliferative action of NCDDNB in prostate cancer cells *in vitro*.

Conclusion

From these studies, NCDDNB may be a candidate for a drug combination study, considering its potential as a stand alone chemotherapeutic agent is nominal. It may be speculated that, with the exception of PC-3, the other cell lines were able to recover from NCDDNB probably due to a change in Bax/Bcl-2 ratios toward antiapoptosis. It is well known that late-stage prostate cancer metastasizes to bone, and in this study HS-5 bone marrow cells were much less sensitive to this compound than were the three prostatic cancer cell lines. Therefore, considering the cytotoxicity profile in different prostate cancer

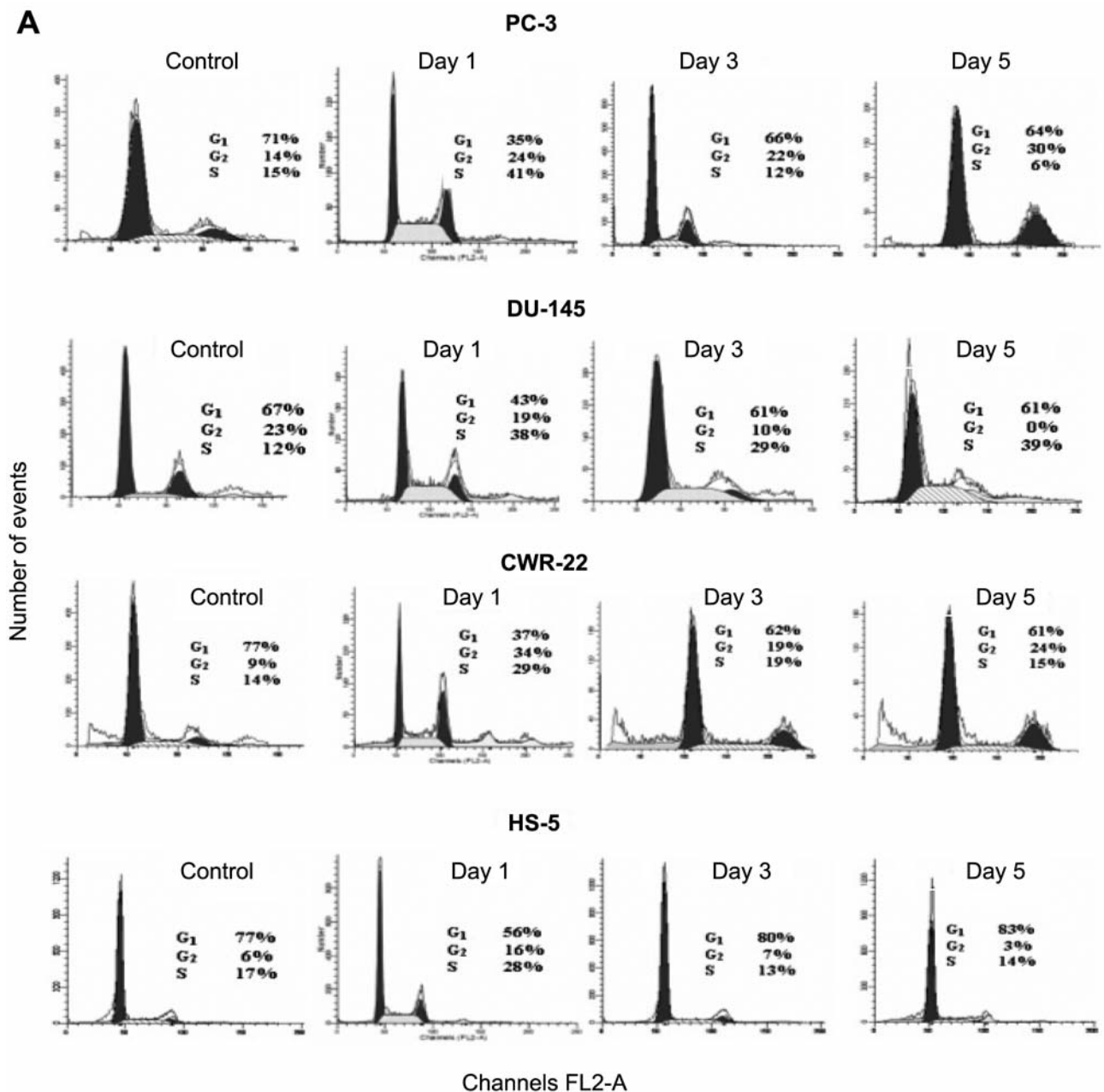


Figure 3. continued

and bone marrow cell lines, the ability to arrest cell cycle progression and induce apoptosis, NCDDNB may represent a class of specific compounds useful in the treatment of metastatic prostate cancer. Future *in vivo* studies may ascertain whether this cell growth inhibitory effect of NCDDNB might contribute to its overall chemotherapy effect in the fight against prostate cancer and its possible future therapeutic applications.

Acknowledgements

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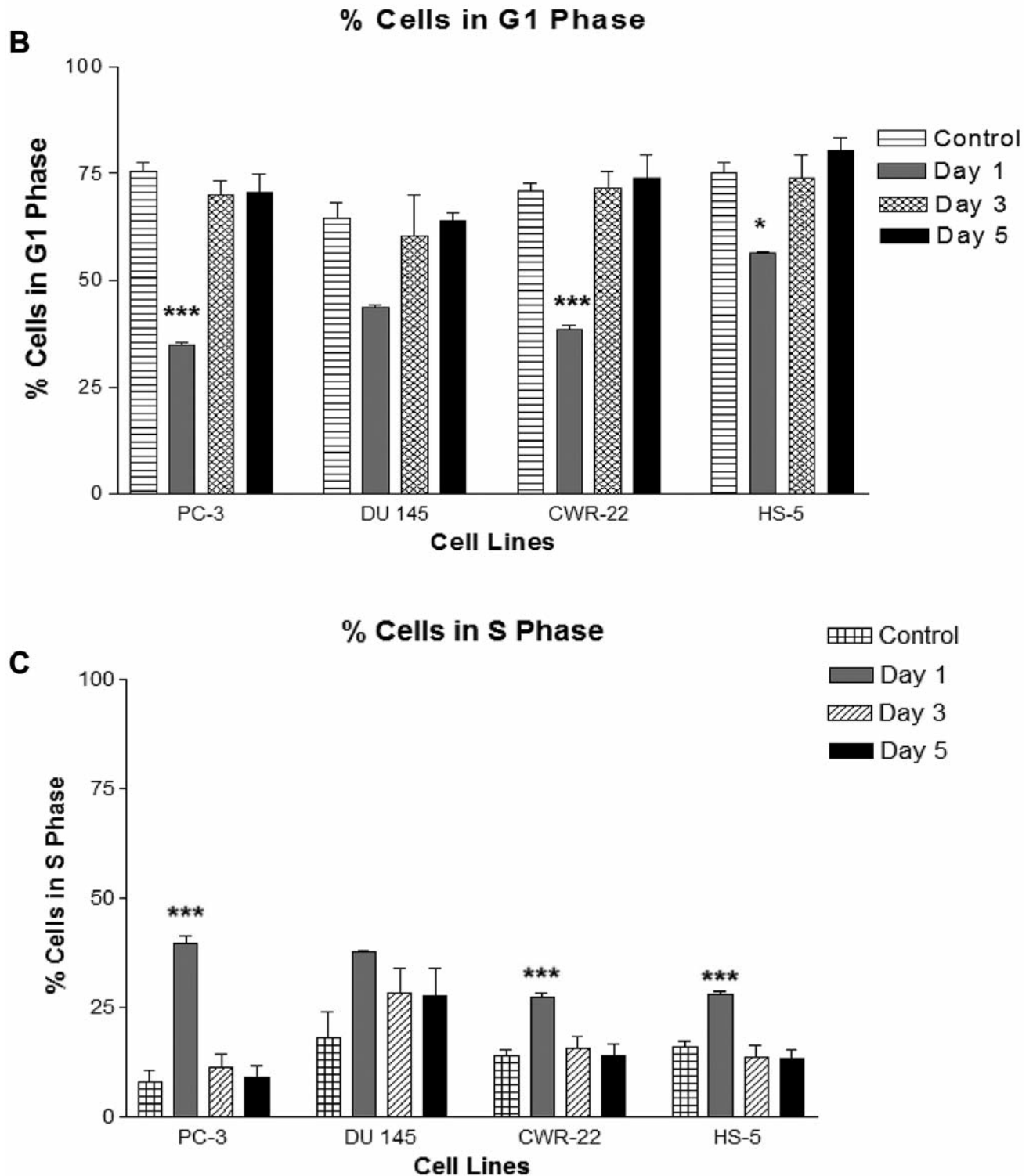


Figure 3. A: Effect of NCDDNB on the cell cycle progression of human prostate cancer and bone marrow cell lines. B & C, Effect of NCDDNB on the cell cycle progression of human prostate cancer and bone marrow cell lines. Cells were exposed for 1, 3 and 5 days in the presence or absence of the drug at respective IC_{50} s. (A) The cell cycle profile is representative of three independent experiments. B: Percentage of cells in G1-phase. C: Percentage of cells in S-phase. Results represent mean \pm SEM of three independent experiments. Analysis of variance indicated a significant increase compared with control (* p <0.05, *** p <0.001).

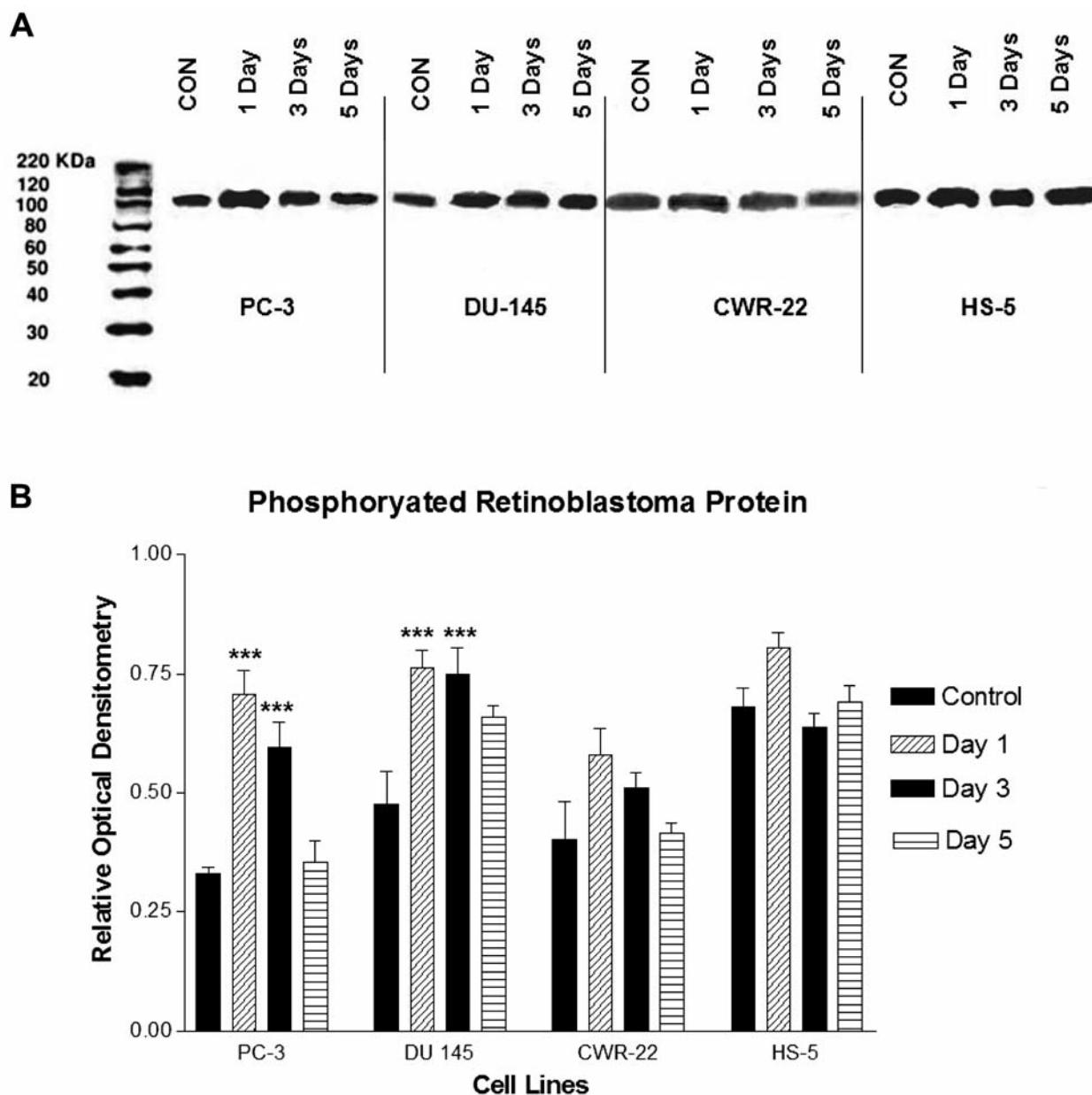


Figure 4. A, B: Effect of NCDDNB on the levels of phosphorylated retinoblastoma protein (Rb) in human prostate cancer and bone marrow cells. Cells were exposed for 1, 3 or 5 days. A: The phosphorylated Rb in this gel image has a 116 kDa molecular weight. The gel image is representative of three independent experiments. B: Relative optical density results represent mean \pm SEM of three independent experiments. Analysis of variance indicated a significant increase compared with control (*** p <0.001).

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