Abstract. Background: Breast cancer is the most frequent cancer and the second leading cause of cancer deaths in women today. 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 estrogen-positive MCF-7 and estrogen-negative MDA-MB-436 and Hs-578T human breast cancer cell lines. Moreover, the in vitro activity of this compound on cell cycle regulation and apoptosis were evaluated. Materials and Methods: Established methods of cell viability, cell cycle, Western blot and apoptosis were used. Results: The effect of DCDMNQ on MCF-7, MDA-MB-436 and Hs-578T cells revealed significant antitumor activities with IC50s, of 0.6±0.02, 1.4±0.25 and 3.1±0.4 μM respectively. Cell cycle analysis showed that DCDMNQ inhibited progression through the cell cycle in MCF-7 and MDA-MB-436 cell lines in a time-dependent manner. DCDMNQ arrested cells in the S-phase of the cell cycle with the greatest proportion of cells in the S-phase by day 5. This cell-cycle arrest was corroborated by inhibition of topoisomerase I induced by DCDMNQ. These findings were further validated using Western blot analysis of retinoblastoma protein time-dependent phosphorylation. Furthermore, DCDMNQ induced apoptosis in both estrogen-positive and -negative cell lines in a time-dependent manner. However, the highest percentages of apoptotic cells were observed in the MDA-MB-436 cell line. Conclusion: Although the mechanism of action of DCDMNQ has not been completely elucidated, it appears that this compound can inhibit topoisomerase I in a concentration-dependent manner. These promising results to explore novel naphthoquinone analogues as potential breast cancer agents. This study suggests that DCDMNQ may have an impact on treatment of estrogen-positive and -negative breast cancer while protecting the bone marrow.

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

Correspondence to: Dr. Robert L. Copeland Jr, Department of Pharmacology, College of Medicine, Howard University, Adams Bldg., 520 W. Street NW, Washington, D.C. 20059, U.S.A. Tel: +1 2028066311, Fax: +1 2028064453, e-mail: rlcopeland@howard.edu

Key Words: 2,3-Dichloro-5,8-dimethoxy-1,4-naphthoquinone, estrogen positive/negative breast cancer cell lines, cytotoxicity, cell cycle, apoptosis.

Breast cancer is a leading cause of morbidity and mortality in women, not only in developed countries but increasingly also in developing countries (1). It is estimated that in 2008 there would be approximately 182,460 women diagnosed with the disease and 40,480 deaths from breast cancer (2). In the new millennium, the expected number of annual new cases of breast cancer worldwide could exceed 1.5 million (3). These statistics emphasize the urgent need for improvements in detection, diagnosis and treatment of breast cancer. Several studies have shown that the mortality rate with breast cancer is about three times higher in African-American women than in other populations (4). In addition, the available data also indicate that the tumors are very aggressive and poorly differentiated, with a very low frequency of hormone receptors, higher S-phase fraction and tumor necrosis (5).

Breast cancer is highly curable if diagnosed at early stage. It is now well established that adjuvant systemic therapy improves survival in patients with early-stage breast cancer (5). Current treatment methods of breast cancer, depending on the stage of cancer upon diagnosis, include surgery, radiation therapy, biological therapy, hormone therapy (e.g. tamoxifen, aromatase inhibitor) and chemotheraphy (e.g. anthracyclines, taxanes). Recent progress in diagnosis and therapy has increased the survival of women with estrogen-dependent breast cancer. However, the treatment options available for estrogen-independent tumors are far from satisfactory and consequently carry a poorer prognosis.

The intricate details of breast cell growth control have not been completely elucidated. Estrogens are well recognized
as playing the predominant role in breast cancer development and growth and much effort has been devoted to block estrogen formation and action (6). The most widely used therapy for breast cancer is the use of antiestrogen such as tamoxifen. However, the present breast cancer therapies achieve meaningful clinical results in only 30–40% of patients (7) because drug resistance is linked to the presence of estrogen-independent pathways for breast cancer cell growth (8). Therefore, more potent anti-breast cancer agents that combine the desired, tissue-selective effects with novel structures or new mechanism(s) of action must be developed.

Naphthoquinones are widely distributed in nature and play important physiological roles in animals and plants. Quinone derivatives may be toxic to cells by a number of mechanisms including redox cycling, arylation, intercalation, induction of DNA strands breaks, generation of free radicals and alkylation via quinone methide formation (9). As a consequence, the molecular framework of a great number of pharmaceuticals and biologically important compounds contain a quinone moiety. Representative examples of this class of compounds are the well-known anticancer drugs of the anthracycline series, doxorubicin and mitoxanthrone, the action of which is believed to occur via topoisomerase II inhibition (10, 11). In addition, a number of naphthoquinone analogs such as plumbagin, shikonin and naphthazarin, as well as β-lapachone, have also been found to inhibit topoisomerase (12–14). DNA topoisomerases are a class of enzymes that alter DNA conformation through a concerted breaking and rejoining of DNA molecule, thereby controlling the topological state of DNA. There are two major categories of topoisomerases, topoisomerase I (Topo I) and topoisomerase II (Topo II). They are reported to be involved in many important processes of DNA metabolism including replication, transcription, recombination and chromosome segregation (15). They have been identified as important antitumor targets because of their essential physiological functions.

A number of 1,4-naphthoquinone derivatives have been found to possess powerful pharmacological effects associated with marked antimicrobial and antitumor activities (16, 17). Bakare et al. (18) reported the MEK-1 specific inhibitory activity of 2-chloro-3-(N-succinimidyl)-1,4-naphthoquinones. In our earlier paper, we reported the synthesis of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) which showed significant cytotoxicity against prostate cancer cell lines and at the same time protected bone marrow cells (19). Therefore, the present study was undertaken to determine the cytotoxic and cell cycle regulatory effects of DCDMNQ on human breast cancer cell lines.

Materials and Methods

Synthesis of DCDMNQ. This compound was synthesized as described elsewhere (19).

Cell viability. Cell viability was quantitatively determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described elsewhere (19). Briefly, estrogen-positive MCF-7 and estrogen-negative MDA-MB-436 (1.5×10^4 cells) were grown in 96-multi-well plates in RPMI-1640 media in the presence or absence of DCDMNQ in a 3-log concentration range (20 nM to 20 μM). The formazan dye produced by viable cells was quantified by measuring the absorbance of the dye solution at 440 nm using a microtiter plate reader.

Cell cycle analysis. Cell cycle perturbations induced by DCDMNQ were analyzed by propidium iodide (PI) DNA staining as described elsewhere (19). The cells were grown in 6-well plates (1×10^5 cells per well) in the presence or absence of the DCDMNQ. After 48 h, of exposure cells were collected and evaluated after propidium iodide staining and cell cycle profiles were obtained using a BD FACScan flow cytometer (Becton Dickinson San Jose, CA, USA). Data were analyzed by ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Western blot analysis. The retinoblastoma protein (Rb) is an important regulator of cell cycle when phosphorylated cells progress from the G1- to the S-phase. To quantify the total Rb protein in breast cancer cells, Western blot analysis was performed. Approximately 1×10^6 cells were plated in 60-mm Petri-dishes and allowed to attach overnight and then cells were exposed to IC_{50} (the concentration which kills 50% of cells calculated from cell viability experiment) of DCDMNQ for 3 or 5 days of exposure; the control was without any treatment. Cells were collected by scraping and homogenized using lysis buffer containing protease inhibitor (Roche, IN, USA). The cell lysates were centrifuged at 5000g for 10 min at 4°C. The supernatant was collected and protein concentration was measured using BCA Protein assay with bovine serum albumin as standard. Equal amounts of protein (20 μg) were loaded onto a 4–12% SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane and cut according to the size of total Rb protein and actin. The membranes were probed with primary antibody (mouse anti-human retinoblastoma protein Rh monoclonal antibody, 1:1,000) and β-actin (mouse monoclonal antibody, 1:5,000) to correct for unequal loading and followed by horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG, 1:10,000). Antibody detection and densitometric analysis were performed as described elsewhere (19).
Determination of apoptotic cells. To quantify drug-induced apoptosis, annexin V/propidium iodide staining was performed using flow cytometry. Briefly, after DCDMNQ treatment, both floating and attached cells were combined and subjected to annexin V/FITC apoptosis detection kit according to the protocol provided by the manufacturer. Untreated control cells were maintained in parallel to the DCDMNQ treatment group. The cells with lower DNA content, as shown by PI staining less than G1, were defined as apoptotic (sub G0/G1 population). The maximum sub-G0/G1 population was observed in the two treatment groups in all the cell lines. Figure 2A is representative of three independent experiments inclusive of the three treatment groups in all the cell lines.

Cell viability. 2,3-Dichloro-5,8-dimethoxy-1,4-naphthoquinone inhibited the growth of the estrogen-positive MCF-7 and estrogen-negative MDA-MB-436, Hs-578T cell lines.

Results

Effect of DCDMNQ on the cell viability, cell cycle progression, apoptosis and topoisomerase assay of human breast cancer cell lines.

Cell viability. 2,3-Dichloro-5,8-dimethoxy-1,4-naphthoquinone inhibited the growth of the estrogen-positive MCF-7 and estrogen-negative MDA-MB-436 and Hs-578T human breast cancer cell lines in a dose-dependent manner following treatment for 5 days. These in vitro studies of DCDMNQ on human breast cancer cell lines revealed significant antitumor activities with IC_{50} for MCF-7, MDA-MB-436 and Hs-578T cells of 0.6±0.02, 1.4±0.25 and 3.1±0.4 μM respectively (Figure 1).

Cell flow cytometric 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 2A is representative of three independent experiments inclusive of the three treatment groups in all the cell lines. Figure 2B 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 MCF-7 cells (55%±7.2%) followed by MDA-MB-436 cells (44%±5.6%) and Hs-578T (38%±5.4%) cells. The proportions of S-phase cells in treated groups were significantly higher than the corresponding control values.

Western blot analysis. 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 DCDMNQ on cellular progression at the molecular level. The Western blot shown in Figure 3A is representative of five independent experiments of the two treatment groups. The relative optical density (ROD) is shown in Figure 3B. The highest density was observed in MCF-7 cells, followed by MDA-Mb-436 and Hs-578T, indicating higher phosphorylation. Significant phosphorylation was observed in all the cell lines when compared to the control results. Similar results were observed with the cell flow cytometric analysis.

Aptoptosis. The cells with lower DNA content, as shown by PI staining less than G1, were defined as apoptotic (sub G0/G1 population). The maximum sub-G0/G1 population was observed in cells of MDA-MB-436 (70%±8.3%) followed by MCF-7 (42%±5.2%) and the lowest number of apoptotic cells were observed in Hs-578T (17%±3.4%) as shown in Figure 4. The percentage of apoptotic cells increased in a time-dependent manner when compared with their respective controls.

To quantify drug-induced apoptosis, annexin V/propidium iodide staining was performed using flow cytometry. The
Figure 2. Effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) on the cell cycle progression of human breast cancer cell lines. Cells were exposed for 3 and 5 days in presence and absence of drug at their respective IC_{50}. Control cells shown were measured at 3 days and as expected no significant changes were observed in the control cells at 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 in DCDMNQ-treated cells compared with the control (**p<0.01, ***p<0.001).
Figure 3. Effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) on the level of retinoblastoma protein (Rb) and β-actin in human breast cancer cells. Cells were exposed for 3 and 5 days. A. The gel image is representative of five independent experiments. B. Relative optical density of Rb protein. Analysis of variance indicated a significant increase in DCDMNQ-treated cells compared with the control (*p<0.05, **p<0.01, ***p<0.001).
Figure 4. Percentage of apoptosis calculated by measuring the sub-G2/G1 population using cell flow cytometry in all the cell lines mentioned. The cells with lower DNA content showing less PI staining than G1 were defined as apoptotic. A, The cell cycle profile with M1 gate is representative of three independent experiments. B, Percentage of apoptotic cells in the M1 (sub-G2/G1 population). Analysis of variance indicated a significant increase in DCDMNQ-treated cells compared with the control (*p<0.05, **p<0.01, ***p<0.001).
combination of early and late apoptotic cells was significantly higher in MDA-MB-436 cells following DCDMNQ treatment for 5 days as shown in Figure 5. The lowest number of apoptotic cells was observed in Hs-578T cells.

Topoisomerase I assay. To determine DCDMNQ as an inhibitor of topoisomerase I, we tested its effect on the catalytic activity of topoisomerase I. Figure 6A is representative of three independent experiments and Figure 6B shows the relative

Figure 5. Apoptosis analysis of human breast cancer cell lines treated at their respective IC_{50}s. Cells were exposed for 3 and 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 and 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.

Figure 6. Effect of 2,3-dichloro-5,8-dimethoxy-1,4-naphthoquinone (DCDMNQ) on the topoisomerase I activity. A, The gel image is representative of three independent experiments. B, Relative optical density of supercoiled DNA and linear DNA. Analysis of variance indicated a significant decrease in the supercoiled DNA (*)p<0.05) and an increase (p<0.05) in the linear DNA of DCDMNQ-treated cells as compared with control.
optical density of supercoiled DNA and linear DNA. Results indicated that DCDMNQ inhibits topoisomerase I in a concentration-dependent manner. The significant increase in linear DNA and reduction of supercoiled DNA was observed with 50 and 100 μM of DCDMNQ.

Discussion

The cytotoxicity results revealed that DCDMNQ is a potent inhibitor of the growth of both estrogen-positive and -negative human breast cancer cell lines. The results of cell flow cytometry and Western blot showed significantly higher numbers of cells entering the S-phase of the cell cycle in MDA-MB-436 cells followed by MCF-7 then Hs-578T, increasing in a time-dependent manner. Therefore, DCDMNQ arrested cells in the S-phase of the cell cycle in both estrogen-positive and -negative human breast cancer cells.

It is known that apoptosis-signaling pathways and cellular events controlling them have a profound effect both on cancer progression and on response to chemotherapy (20, 21). Based on annexin V/propidium iodide staining, it is clear that the highest proportion of apoptotic cell death was observed in MDA-MB-436 cells followed by MCF-7 then Hs-578T, which is involved in cell cycle arrest in G1 or G2 (22). It has also been reported that bis-type aziridinynaphthoquinone induced cell death in human breast adenocarcinoma BC-M1 cell line by mediating the apoptotic pathway. Yang et al. (23) reported that apoptosis induced by aziridinynaphthoquinone was initially triggered by the activation of p53 protein followed by up-regulation of the p21waf1/cip1 protein, which is involved in cell cycle arrest in G1 or G2 (22). It has also been reported that bis-type aziridinynaphthoquinone induced cell death in human breast adenocarcinoma BC-M1 cell line by mediating the apoptotic pathway. Yang et al. (23) reported that apoptosis induced by aziridinynaphthoquinone was initially triggered by the activation of p53 protein followed by up-regulation of the p21 protein to inhibit cyclin kinase cdk2 expression to arrest the BC-M1 cell cycle, leading finally to progression into the apoptotic process. Hou et al. (24) reported that the cytotoxic effect of shikokin (naphthoquinone) on MCF-7 cells occurred by an apoptotic process. The cell cycle alterations indicate that cell cycle arrest is one of the primary mechanisms responsible for the antiproliferative action of DCDMNQ in breast tumor cells in vitro.

Topoisomerase I catalyzes changes in the linkage of DNA strands via the formation of covalent enzyme-DNA intermediates. A phosphotyrosyl linkage of Topo I to the 3’-end of the cleaved DNA strand allows the free 5’ DNA end to rotate. The covalent Topo I-DNA intermediate is the cellular target of Topo I cytotoxins which reversibly stabilize the covalent Topo I-DNA intermediate by inhibiting DNA religation. During S-phase, these ternary Topo I-DNA-drug intermediates are transformed into potentially lethal lesions, which induce cell cycle arrest and cell death (25). The cell-cycle arrest induced by DCDMNQ in the S-phase is corroborated by the concentration-dependent inhibition of Topo I. It thus appears that DCDMNQ inhibits Topo I activity by stabilizing the cleavable complex.

However, Topo II inhibitors do not exhibit the same S-phase specificity as Topo I inhibitors (25). Other antineoplastic drugs dramatically increase levels of Topo II cleaved-DNA complexes, resulting in permanent DNA damage followed by G2/M-phase cell cycle arrest and apoptotic cell death (26). Earlier reports have suggested that 1,2-naphthoquinone thiosemicarbazone and its metal derivatives inhibit DNA Topo II by stabilizing the intermediate forms of enzyme-DNA complexes (cleavable complexes) in MCF-7 human breast cancer cells (27, 28). The stabilizing effect is mainly due to the alkylation of thiol residues on the Topo II-DNA complex (28). Protein denaturation of this cleavable complex results in single-stranded and double-stranded DNA breaks that lead to apoptosis. It is through this cellular process that Topo II inhibitors exert their antitumor activity (29). DCDMNQ appeared to have only weak Topo II inhibitor activity (data not shown).

Conclusion

2,3-Dichloro-5,8-dimethoxy-1,4-naphthoquinone showed potent cytotoxicity against both estrogen-positive and -negative human breast cancer cell lines as confirmed in cell cycle and apoptosis experiments. Moreover, HS-5 bone marrow cells were reported to be less sensitive to DCDMNQ (19). It is well known that late-stage breast cancer metastasizes to bone.

Therefore, considering the cytotoxicity profile in these different breast cancer cell lines and the ability to arrest cell cycle progression and induce apoptosis, this compound represents the first in a class of specific compounds which may be useful in the treatment of estrogen-positive and -negative breast cancer.

Acknowledgements

This work was supported in part by grant number 5-U54-CA914-31 (Howard University/Johns Hopkins Cancer Center Partnership) and the RCMI/NIH grant G12RR003048. We are grateful to the Department of Pharmacology, College of Medicine, Howard University for allowing us to use their core facility to conduct the research work.

References


Kanaan et al: Cytotoxic Effect of DCDMNQ on Human Breast Cancer Cell Lines

Received March 18, 2008
Revised August 21, 2008
Accepted October 10, 2008