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Effects of a Naphthoquinone Analog on Tumor Growth and Apoptosis Induction

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Vitamin K-related analogs induce growth inhibition in various cancer cell lines. A naphthoquinone analog, termed 2,3-dichloro-5, 8-dihydroxy-1,4-naphthoquinone (DDN), induces apoptosis in human promyeloid leukemic HL-60 cells, and shows antitumor activity *in vivo*. Following treatment with DDN, evidence of apoptosis, including DNA fragmentation and cleavage of poly ADP ribose polymerase (PARP), was observed. DDN induced an upregulation of proapoptotic Bax protein, and Bid cleavage. Antiapoptotic Bcl-2 protein levels were not changed by DDN, but the expression of Bcl-xL was decreased. In addition, DDN reduced the mass of solid tumor in the Sarcoma 180 tumor-bearing mouse model. These results indicate that DDN exerts antitumor activity, which appears to be related to the induction of apoptosis by regulating Bcl-2 family proteins.

Key words: Naphthoquinone analog, Bcl-2, Bax, Bcl-xL, Bid, Apoptosis, Antitumor activity, HL-60 cells

INTRODUCTION

Menadione is a synthetic compound among the vitamin K family molecules. Menadione has increasingly been of interest because it has shown to exhibit a broad range of antitumor activity in human cells and has imposed lower levels of toxicity than other chemotherapeutic anticancer drugs of quinone structure, such as doxorubicin, daunorubicin, and mitomycin C (Okayasu *et al.*, 2001; Wu *et al.*, 1993) It has been reported that menadione induces cell growth arrest and apoptosis in various cancer cell lines. In human hepatoma cells, menadione has an inhibitory effect on the activity of cdc25 phosphatase, which is involved in cell cycle regulation through dephosphorylating cyclin-dependent kinases (Juan *et al.*, 1993; Nishikawa *et al.*, 1995).

Despite a broad-range effect of menadione on growth suppression of cancer cells, its hydrophobicity presents difficulties for use as an anticancer drug. Earlier experiments focusing on a search for new naphthoquinone analogs with polar groups led to the discovery of 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquione (DDN) (Ham *et al.*, 1998). Unlike Menadione that induces growth inhibition through producing reactive oxygen species (ROS), DDN-induced growth inhibition does not involve ROS in human hepatocarcinoma cells. Previous studies have shown that DDN induces an arrest in the G1 cell cycle phase through cdc25A phosphatase inhibition in hepatocarcinoma cells. DDN induces cell death in HeLa cells via an apoptotic pathway by inhibiting transcriptional activity of nuclear factor κB (Kang *et al.*, 2001).

Apoptosis is an important regulatory mechanism that eliminates unwanted cells during the development and maintenance of cell homeostasis. The failure of apoptotic cell death has been implicated in various cancers. The molecular mechanisms by which anticancer drugs induce apoptosis involve activation of proapoptotic signaling or inhibition of survival signaling (Gross *et al.*, 1999; Zhang *et al.*, 2000). The key regulatory proteins in apoptotic events are the Bcl-2 family proteins. Bax is a proapoptotic member of the Bcl-2 family protein that resides in the cytosol and translocates to the mitochondria upon induction of apoptosis (Green and Reed, 1998). In contrast, antiapoptotic Bcl-2 and Bcl-xL proteins reside on the outer membrane of the mitochondria and can inhibit apoptosis in the presence of apoptotic stimuli and promote cell

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survival (Finucane *et al.*, 1999). Since induction of apoptosis is an effective strategy for cancer chemotherapy, it is important to develop new drugs targeting apoptosis-related proteins and signal transduction pathways.

In this study, anticancer effects induced by a naphthoquinone analog, DDN, were examined *in vivo* and *in vitro*. DDN induced significant cell death in 8 cancer cell lines, including leukemia, breast, colon, lung and hepatoma (liver), ovary, and melanoma cell lines. DDN induces apoptotic cell death in HL-60 cells via regulating the expression of Bcl-2 family proteins. The effects of DDN on the growth inhibition of solid tumor were observed.

MATERIALS AND METHODS

Animals and cells

Six-week-old ICR mice weighing between 20 and 22 g were purchased from Hallim Experimental Animals Ltd. (Korea). The mice were acclimated for at least four days to the animal facilities, which were maintained at 23 °C and 12 h light/dark cycle. Food and water were freely accessible to the mice. The Sarcoma S-180 mouse, and the human cancer cell lines were obtained from Korea Cell Line Bank (Seoul, Korea).

Materials

2,3-dichloro-5,8-dihydroxy-1,4-naphtoquinone, the naphthoquinone analog (DDN) was synthesized at Chung-Ang University, College of Natural Sciences, Seoul, Korea. Fetal bovine serum was purchased from Gibco BRL, ECL kit from Amersham Pharmacia (Freiberg, Germany). Antibodies to Bax, Bcl-2, Bcl-xL, Bid, PARP and HRP-conjugated secondary antibody were from Santa Cruz Biotechnology, Inc (Heidelberg, Germany). All reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA), unless otherwise specified.

Cytotoxicity assay

The *in vitro* cytotoxicity tests against various cancer cell lines were evaluated by a tetrazolium-dye (MTT) assay. In brief, cells (1×10^4) were seeded in each well containing 100 µL of RPMI medium supplemented with 10% FBS in a 96-well micro titer plate and incubated overnight. Cells were then treated with DDN dissolved in DMSO in serial dilution. After incubation for 4 days at 37°C, 50 µL of 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (2.5 mg/mL) was added to each well. After 4 h of incubation at 37 °C, the medium was discarded. The formazan crystals formed were solubilized by adding 50 µL DMSO, and the optical density was measured at 540 nM.

Cell viability and internuclosomal DNA fragmentation

HL-60 cells were washed with serum-free RPMI. DDN or vehicle was diluted into serum-free RPMI at the indicated concentrations. Cell viability was determined by the trypan blue exclusion method at each time point. To assess DNA fragmentation, total genomic DNA was extracted using a lysis buffer (100 mM NaCl, 25 mM EDTA, 0.5% SDS and 0.1 mg/mL proteinase K) at 50 °C for 15 h. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation and then dissolved in a TE buffer (pH 8.0). After treatment with RNase A (0.1 mg/mL) for 1 h at 37 °C, the fragmentation of genomic DNA was evaluated by 1.5% agarose gel electrophoresis and ethidium bromide staining.

Western blot analysis

The cells were solubilized with an ice-cold lysis buffer containing 1% Triton-X-100, 50 mM NaCl, 25 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/mL leupeptin. Insoluble materials were removed by centrifugation at 10,000×g for 10 min. Extracted proteins (50 µg/well) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gels, and were electrophoretically transferred onto the immobilon-P membrane. Blocking was performed in Tris-buffered saline containing 5% skimmed-milk powder and 0.1% Tween-20. The membranes were probed with antibodies against PARP, Bcl-2, Bax, Bcl-xL and Bid proteins. Detection was performed with the ECL System. Protein content was determined by the Bradford method using bovine serum albumin as a standard.

RNA isolation and RT-PCR

Hl-60 cells were treated with DDN, harvested and washed with with PBS. Total RNA was extracted using Trizol reagent (Invitrogen). Semi-quantitative RT-PCR for Bcl-xL was performed using a Titan One Tube RT-PCR System according to the manufacturer's instruction. The sequences of primers used in this study were as follows: Bcl-xL sense primer, 5'-CAGCTGGAGTCAGTTTAG-3', and Bcl-xL antisense primer, 5'-CGCTTTCCACGCACAGTG-3'. A reverse transcription reaction was performed at 50°C for 30 min, and the cDNA were amplified for 30 cycles at 94 °C for 30s, 56 °C for 30s, and 72°C for 30s and then at 72 °C for 7 min for prolonged elongation. The amplified PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

Antitumor activity in ICR mice bearing sarcoma 180 cells

The following procedure was completed according to the protocol of the National Cancer Institute USA, 1972. The test samples dissolved in a predetermined amount of

50% PEG200 were stored at 4 °C. Sarcoma 180 cells (0.1 mL per mouse) suspended in saline (1×10^7 cells/mL) were injected subcutaneously into the male ICR mice. Twenty four hour after the transplantation, the mice were divided into groups of 8. The sample was administered into the intraperitoneal cavity of the mouse on the schedule. On the 12th day after the last treatment, all mice were sacrificed and their tumor masses were measured. The rate of growth inhibition (T/C, %) was calculated by the following equation (Park *et al.*, 1999: Hwang *et al.*, 1999).

Tumor growth inhibition ratio (%)= $(C_w - T_w)/C_w \times 100$

C_w: tumor weight of control group
T_w: tumor weight of experimental group

Tumor volume (V_{tm}) was estimated using a slide caliper according to the following equation (Giuliani *et al.*, 1981): V_{tm} (mm^3) = 0.5 × length × width^2

DDN was administered when the implanted tumors were growing progressively and their volumes were between 80 and 120 mm^3. The changes in body weights of the mice were observed daily for five weeks.

Statistical analysis

The statistical significance of differences among the values was evaluated by ANOVA and Duncans multiple range test. P value <0.05 was defined as significant.

RESULTS

Inhibition of tumor cell proliferation by DDN

DDN was evaluated for its cytotoxicity against 8 human cancer cell lines following a continuous 4-day exposure. Marked growth inhibition was observed after treatment with DDN. DDN induced the inhibition of cell growth of a wide range of cultured cancer cells including leukemia, breast, colon, lung and hepatoma cells. Of these, the HL-60 cells showed high sensitivity in response to DDN with IC_{50} of 0.7 μM (Table I).

Table I. IC_{50} valuse for the inhibition of cancer cell lines by DDN

Cell line	IC_{50} (μM)	
	DDN	Menadione
HL-60	0.71	15.97
U937	0.91	36.0
MCF-7	4.81	>100
HepG2	9.60	38.29
SK-OV-3	2.69	>100
HT-29	4.33	61.01
A549	10.94	31.17
SK-MEL-5	3.80	>100

DDN Induces Apoptotic Cell Death in HL-60 cells

The growth inhibitory effects of DDN were assessed in the human promyelocytic leukemia HL-60 cells. Exposure of the cells to 1 μM DDN for 24 h resulted in a time-dependent decrease in cell viability (Fig. 1A). In order to determine whether cells are killed via apoptotic processes, we examined an internucleosomal DNA fragmentation. Cell death induced by DDN was associated with marked DNA fragmentation (Fig. 1 B). DNA laddering of nucleosomal size (180-200 bp) was revealed in the cells treated with 1 μM DDN, suggesting that a population of the cells underwent apoptotic cell death (Fig. 1B). Proteolytic cleavage of PARP by caspase-3 activation is a predominant biochemical hallmark of apoptosis (Kaufmann *et al.*, 1993). The cleavage of PARP into a 89 kDa fragment was observed after DDN treatment, suggesting that DDN induces caspase-3 activation in HL-60 cells (Fig. 1C).

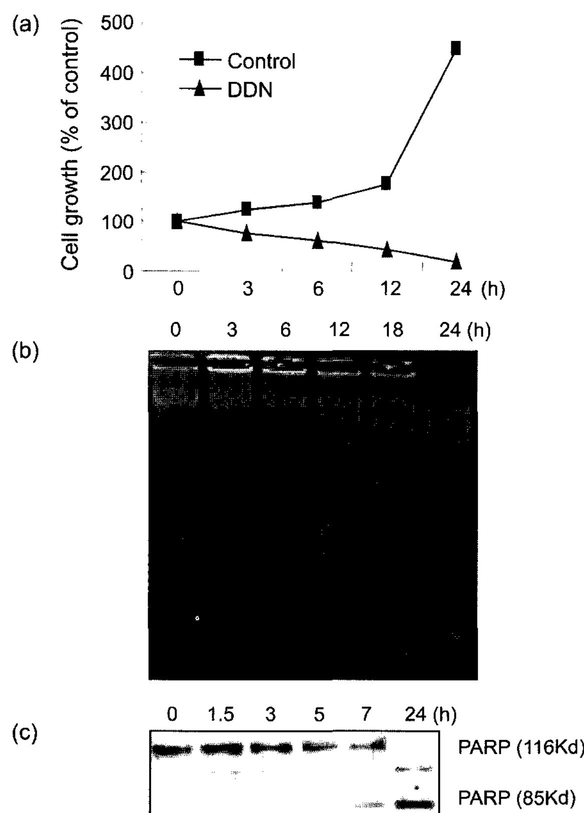

Fig. 1. DDN induces apoptosis in HL-60 cells. A: HL-60 cells were treated with 1 μM DDN, and cell viability was determined by trypan blue exclusion method at the indicated time points. B: Internucelosomal DNA fragmentation was investigated in a time-dependent manner. Cells were treated with 1 μM DDN for the indicated time periods. Total genomic DNA was prepared, separated on a 1.5% agarose gel and visualized by ethidium bromide staining. C: Cleavage of PARP after treatment with DDN was analyzed by western blot using anti-PARP antibody.

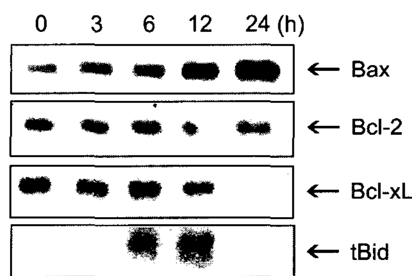

Fig. 2. Effects of DDN on Bcl-2 family protein levels in HL-60 cells. Cells were treated with 1 μM DDN for the indicated time points. Protein levels were examined by western blot using anti-Bax, anti-Bcl-2, anti-Bcl-xL and anti-Bid antibodies.

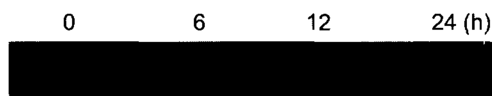

Fig. 3. Effect of DDN on Bcl-xL mRNA level. HL-60 cells were treated with 1 μM DDN and harvested at the times indicated. Total RNA was isolated using Trizol reagent and I μg of RNA was transcribed into single-strand DNA. PCR was performed for 30 cycles using specific primers as described under 'Materials and Methods'.

DDN regulates the expression of Bcl-2 family proteins

To determine whether Bcl-2 family proteins are involved in DDN-induced apoptosis, the expression of antiapoptotic and proapoptotic Bcl-2 proteins in DDN-treated HL-60 cells was investigated. As shown in Fig. 2, the expression of the Bcl-2 protein was not changed by DDN. However, the expression of Bcl-xL protein was substantially reduced whereas the level of Bax protein was increased. A decrease in Bcl-xL level was detected at 12 h and then a significant decrease was detected at 24 h after treatment with 1 μM DDN. The cleavage of caspase-8 substrate, Bid, was also observed within 6 h of DDN treatment. To identify if downregulation of Bcl-xL results from a decreased mRNA level, a semi-quantitative RT-PCR was performed. As shown in Fig. 3, the mRNA level of Bcl-xL was unchanged by DDN treatment, suggesting a posttranscriptional effect on the Bcl-xL protein.

Effects of DDN on the growth of solid tumors in mice

Since the potential of DDN as a treatment for solid tumors had not been examined previously, in this study the effectiveness of this agent against the Sarcoma 180 was evaluated. As shown in Table II, DDN significantly inhibited the growth of Sarcoma-180 in mice of the 2d×4 (10 mg/kg) group by 66% compared to control group. The every-second-day schedule was found to be more effective than any other schedule of administration and produced tumor growth delays of 10 days compared to that of the untreated control mice (Data not shown). A significant decrease in tumor volume was detected in mice given DDN at 10 mg/kg four times every 2 days (Table II). The body weight of the mice was also significantly reduced compared to that of control mice at the end of the experiment (Fig. 4). These results indicated an anticancer effect of DDN, evidenced by a decrease in the tumor size as well as its mass.

Table II. Growth-inhibitory effect of DDN in sarcoma-180 bearing ICR mice

Dosing Schedule	Tumor volume (mm^3)	Inhibition rate (%)	Tumor weight (mg)	Inhibition rate (%)
Control	2101±434	–	54±5.2	–
2D×4 5 mg/kg	1624±421*	22	31±4.0*	42
2D×4 10 mg/kg	695±92*	66	18±8.0*	66
4D×4 5 mg/kg	1685±495	19	48±4.3	11
4D×4 10 mg/kg	1128±417*	46	24±3.4*	55

values are means±SEM (n=6) *P<0.05 vs control.
Mice were treated with DDN four times every second days (2D×4) or four times every fourth days (4D×4)

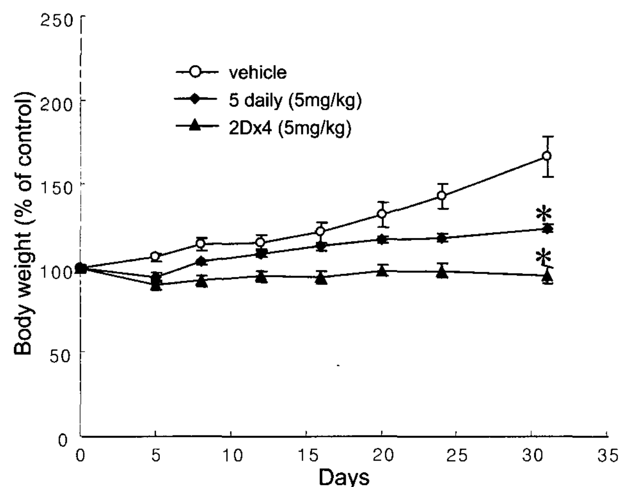

Fig. 4. Responsiveness on body weight change of DDN in sarcoma-180-bearing ICR mice. Mice were injected subcutaneously with Sarcoma 180 cells. After 24 h, DDN or vehicle was administered intraperitoneally into the mice on dose schedule. Control: body weight at day 1. Values are means±SEM (n= 6). P<0.05 vs untreated group.

DISCUSSION

K vitamins, especially vitamin K3 (menadione), have been reported to inhibit the growth of various tumor cell lines (Okayasu *et al.*, 2001; Wu *et al.*, 1993). Menadione has known to inhibit growth of many cell types through the inhibitory effect on the activity of cdc25 phosphatase, which is involved in cell cycle regulation (Ham *et al.*, 1997). A thioether analog of menadione that inhibits cell growth exerts its effects via sulfhydryl arylation of the cellular pro-

tein tyrosine phosphatase. In addition, menadione induces a G1 arrest by generating superoxides in several cell lines. Recently, several vitamin K analogs were synthesized, and the modification of menadione could produce compounds with enhanced activities for the inhibition of cdc25A phosphatase and cell growth (Ham *et al.*, 1998; Nishikawa *et al.*, 1995). Of these, DDN induced growth inhibition more potently than the natural vitamin K3 and induced a G1 arrest through cdc25A phosphatase inhibition in hepatocarcinoma cells (Ham *et al.*, 1998). We found that DDN showed a more potent effect than menadione in inducing cell death of several cancer cell lines. Moreover, the *in vivo* experiments showed that DDN treatment could cause a decrease in the solid tumor's size as well as its mass. These results clearly indicated an anticancer effect of DDN.

The possible mechanisms for the growth inhibition by DDN were also investigated. DDN inhibited cell growth through the induction of apoptosis in HL-60 cells. DDN treatment caused time-dependent DNA fragmentation and cleavage of PARP, a caspase-3 substrate. Furthermore, DDN has shown changes in protein levels of the Bcl-2 family which include Bax and Bcl-xL proteins. This indicates that proapoptotic- and antiapoptotic Bcl-2 family proteins may be involved in regulating on cell death by DDN in HL-60 cells. Bcl-2 family proteins regulate apoptosis and function as the molecular determinant of cell survival (Green *et al.*, 1998; Gross *et al.*, 1999).

Bcl-2 family proteins consist of three subfamilies; proapoptotic proteins, antiapoptotic proteins, and the BH-3 subfamily proteins. (Gross *et al.*, 1999). The proapoptotic subfamily includes Bax and Bak proteins, the antiapoptotic subfamily includes Bcl-2 and Bcl-xL proteins, and the BH-3 subfamily includes Bid proteins. Overexpression of Bcl-2 is associated with many types of cancers including leukemia (Kim *et al.*, 2002). Recent study has shown that alterations in the ratio between proapoptotic and antiapoptotic members of the Bcl-2 family proteins, rather than the absolute expression level of any single Bcl-2 member protein, can determine apoptotic sensitivity (Zhang *et al.*, 2000). Our results have shown that although the Bcl-2 levels remain unchanged during a 24 h treatment with DDN, the Bax level was increased, resulting in change in the Bcl-2/Bax ratio. In addition, the proapoptotic Bcl-xL protein level was decreased, and the proapoptotic carboxy-terminal fragment tBid was observed. Bid is reported to be cleaved by caspase-8 and efficient for triggering translocation of Bax into the mitochondrial membrane (Li *et al.*, 1998). These results indicate that DDN-induced apoptosis is regulated by caspase activation, and by multiple signaling pathways that involve decreasing survival signals in addition to increasing death signals. DDN has potent cell death effects on several cancer cell lines, with enhanced activity on apoptosis-controlling pathways, as compared to menadione. DDN may warrant further research as a promising candidate for cancer chemotherapy.

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