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Cyclooxygenase inhibitors combined with deuterium-enriched water augment cytotoxicity in A549 lung cancer cell line via activation of apoptosis and MAPK pathways

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ABSTRACT

Objective(s): Combination chemotherapy is a rational strategy to increase patient response and tolerability and to decrease adverse effects and drug resistance. Recently, the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been reported to be associated with reduction in occurrence of a variety of cancers including lung cancer. On the other hand, growing evidences suggest that deuterium-enriched water (DEW, D2O) and deuterium-depleted water (DDW) play a role both in treatment and prevention of cancers. In the present study, we examined the effects of DEW and DDW in combination with two NSAIDs, celecoxib and indomethacin, on A549 human non-small lung cancer cell to identify novel treatment options. Materials and

Materials and Methods: The cytotoxicity of celecoxib or indomethacin, alone and in combination with DDW and DEW was determined. The COX-2, MAPK pathway proteins, the anti-apoptotic Bcl2 and pro-apoptotic Bax proteins and caspase-3 activity were studied for cytotoxic combinations.

Results: Co-administration of selective and non-selective COX-2 inhibitors with DEW led to a remarkable increase in cytotoxicity and apoptosis of A549 cells. These events were associated with activation of p38 and JNK MAPKs and decreasing pro-survival proteins Bcl-2, COX-2 and ERK1/2. Furthermore, the combination therapy activated caspase-3, and the apoptosis mediator, and disabled poly ADP-ribose polymerase (PARP), the key DNA repair enzyme, by cleaving it.

Conclusion: The combination of DEW with NSAIDs might be effective against lung cancer cells by influence on principal cell signalling pathways, and this has a potential to become a candidate for chemotherapy.

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Introduction

Lung cancer represents one of the most deadly diseases in the world (1). Therefore, intense efforts are being mounted to find new agents and combination therapies for treatment and prevention of human lung cancer (2, 3).

Recently, the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been reported to be associated with reduction in the occurrence of a variety of cancers including lung cancer (4-11). NSAIDs act as inhibitors of the COX enzymes that catalyze the conversion of arachidonic acid into prostanoids including prostaglandin E2, which is often associated with oncogenesis of lung tumors (12, 13). PGE2, the predominant prostaglandin, exerts its biological effects via some pathways including apoptosis and MAPKs (10, 14). Since anti-neoplastic effects of NSAIDs manifest only in high concentrations, so serious adverse effects and drug resistance do not let utilization of NSAIDs solely as a chemotherapeutic agent. Accordingly, several co-administrations of NSAIDs with different chemopreventive agents have previously been investigated in the lung cancers (15-18). However to the best of our knowledge, no study has been conducted to assess the combination effects of deuterium-enriched water (DEW) and or deuterium-depleted water (DDW) with NSAIDs on the cancer cells. Application of DEW and DDW is recently known as an opportunity in cancer therapy (19-30). Although growing *in vitro* and *in vivo* studies suggest that DEW and DDW might play a role both in treatment and prevention of cancers through inhibition of cancer cells proliferation (31), there is no study focusing on both combination therapy and the cellular events leading to these effects (32).

In the present study, we examined the cytotoxic effects of DEW and DDW, individually and in combination with celecoxib and indomethacin, on A549 cell line. Moreover, changes in the apoptosis and MAPKs pathways were examined to identify the possible molecular pathways.

Materials and Methods

Materials and reagents

Dulbecco's modified eagle medium (DMEM) (high glucose), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from PAA (Australia).

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Culture flask (25, 75 cm^2) was purchased from SPL company (Korea). Western blot detection kit and polyvinylidenedifluoride (PVDF) membrane were from Roche Applied Science (Germany). Anti-extracellular receptor kinase 1/2 (ERK1/2), phospho-ERK1/2, p38, phospho-p38, c-Jun N-terminal kinase (JNK), phospho-SAPK/JNK, Bax, Bcl-2, COX-2, Caspase-3 and β-actin antibodies were purchased from Cell Signaling Technology (USA). Poly ADP-ribose polymerase (PARP) and secondary antibodies were achieved from Roche (Germany). Bromophenol blue, Coomassie blue R-250 and G-250, MTT, and caspase-3 colorimetric assay kit were purchased from Sigma Chemical Company (UK). Indomethacin and celecoxib were kindly provided by a collaborative lab (as 98.8% purity) and dissolved in minimal amounts of dimethyl sulfoxide (DMSO), so that the final DMSO in tests did not exceed 1%. Centrifuge tube (15, 50 ml), micro centrifuge tube (1.5 ml), multiwall plates (6-well, 24-well, and 96-well) (microtitration) plates obtained from Nest company (China). Dithiothreitol (DTT) and all other chemicals were bought from Merck (Germany).

Preparation of media

Cell culture media with different concentrations of deuterium were prepared by dissolving DMEM powder, FBS powder and penestrep in water with different concentrations of deuterium.

Cell culture and growth inhibition assay

A549 human Non-Small Cell Lung cancer cell line was purchased from Cell Lines Service (Canada) and grown in monolayer cultures in DMEM containing phenol red supplemented with 10% FBS, 100 units/ml of penestrep and 5% CO_2 at 37 ˚C. For reduction of cell responses to stimulators and inhibitors, all the experiments were performed on the cells in the logarithmic phase (33). For cytotoxicity assay, 50 µl of the cell suspension containing 8×10^3 cells were seeded into each well of a flat-bottomed 96-well plate. Adhering to the surface of plates, A549 cells were treated with DEW (50000, 100000, 200000 and 300000 ppm of deuterium) and DDW (31, 69, 91, 109 and 127 ppm of deuterium), in addition to indomethacin (2- 800 µm) and celecoxibe (2- 400 µm) for 24, 48 and 72 hr. The cytotoxicity was determined using MTT assay by adding 25 µl of 3- (4,5- dimethylthiazol-2-yl)- 2, 5-diphenyl tetrazolium bromide solution (MTT) to the wells and then incubation for 4 hr at 37 °C in 5% CO_2 atmosphere. Absorbance of the formazan was measured at 570 nm using a microplate reader, and the viability was calculated from the equation, %viability = (1 - At/Ac) x 100), where At and Ac represent absorbencies of treated and control cultures, respectively. Solvent control trials were performed appropriately and exhibited no cytotoxic effects.

Combination therapy

After determination of IC_{50} (concentration causing 50% growth inhibition) for each of celecoxib and indomethacin solutions, four close concentrations to the calculated IC_{50} (for celecoxib: 10, 25, 75 and 100 µM and for indomethacin: 50, 100, 175 and 250 µM) were combined with minimum and maximum limit of DEW (50000, 300000 ppm) and DDW (31, 127 ppm) for combination therapy. In the control group, the cells were treated only with medium or DMSO. The viability of the cells was determined after 24, 48 and 72 hr of treatment.

Western blot analysis of protein expression using sodium dodecyl sulfate polyacrylamide

After treatment of the cells for 48 hr with drugs, the cells were harvested, washed with ice-cold PBS, and lysed in 100 µl lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT) at 4 °C for 15 min. Insoluble components were removed from lysates by centrifugation at 14,000×g for 5 min, and the supernatants were transferred to the fresh tubes. Protein concentrations were determined by the Bradford method. Thirty µg of protein was added to an equal volume of 2X SDS-sample buffer and then the mixture was electrophoretically separated through 10% SDS-polyacrylamide gel. Proteins were transferred to PVDF membranes (Roche), stained with 0.1% Ponceau S to ensure equal protein loading, and blocked with 25 µl blocking reagent 0.5% in TBS (50 mM Tris, 150 mM NaCl) for 1 hr at room temperature. After blocking, the membranes were probed with anti-human antibodies at appropriate dilutions against COX-2 (1 : 1500), caspase-3 (1 : 1500), Bcl-2 (1 : 1500), Bax (1 : 2500), P38 (1 : 1000), phospho-p38 (1 : 1000), ERK1/2 (1 : 2500), phospho-ERK (1 : 2500), SNAPK/JNK (1 : 1000), phospho-SNAPK/JNK (1 : 1500), β-actin (1 : 1500), and PARP (1 : 2000). Following washing the membranes for four times, 15 min each, by agitating with 200 ml TBS-T, the blots were incubated with a goat anti-mouse/rabbit-antibody-HRP conjugate (Roche) for 1 hr at room temperature. Then immunoreactive bands became visualized by adding luminal substrate to the blots and their exposure to the BioMax film (Kodak).

Statistical analysis

Statistical analysis was performed using SPSS software. Data were expressed as Mean±SD. One-way analysis of variance (ANOVA) was used to assess significant differences between treatment groups. The differences were considered as significant when $P<0.05$. The IC_{50} was calculated using master plex software (MiraiBio Group of Hitachi Solutions America, version: 2.0.0.73).

Results

Cytotoxicity effects of celecoxib and indomethacin on A549 cell line

The cytotoxicity of celecoxib and indomethacin at different times has been shown in Figure 1. The calculated IC_{50}s for celecoxib after 24, 48 and 72 hr treatment were 102.47, 58.96 and 27.62 µM, respectively. These values for indomethacin were 236.7, 149.98 and 140.11 µM. Subsequently, four concentrations of celecoxib and indomethacin close to their IC_{50}s combined with DEW and DDW. As it is depicted in Figure 2, combination of celecoxib and indomethacin with DEW, but not DDW, could significantly ($P<0.05$) increase the cytotoxicity of different concentrations of celecoxib and indomethacin in a dose dependent manner. Since the 24, 48 and 72 hr treatments had significant cytotoxicity, the assays were performed 48 hr after treatment. Furthermore,

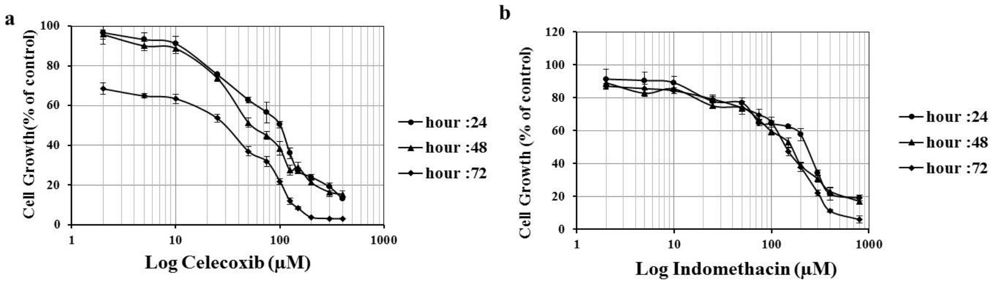

Figure 1. The logarithmic cytotoxic effect of celecoxib (a) and indomethacin (b) on the A549 cell line after 24, 48 and 72 hour treatment. Cell growth, assessed by MTT method, is expressed as the percent of control (DMSO-treated) cells against logarithm of drug concentration. Each data set is the mean±SD value from eight identical wells

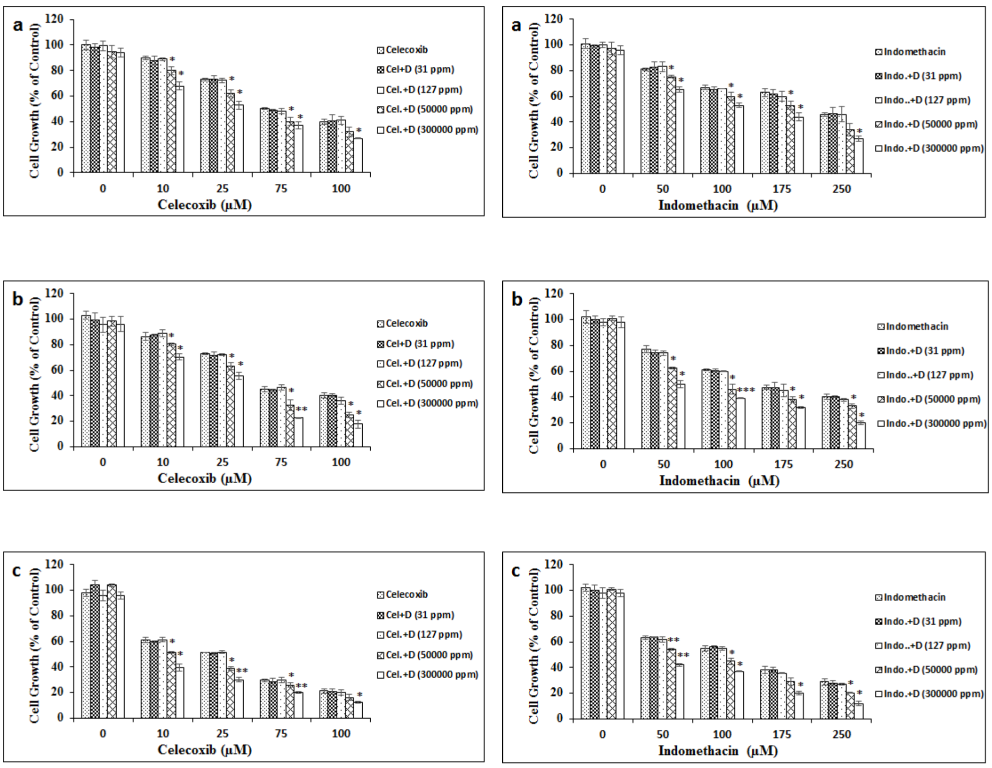

Figure 2. The cytotoxic effect of celecoxib or indomethacinin in combination with deuterium-depleted water (DDW: 31 and 127 ppm of deuterium) and deuterium-enriched water (DEW: 50000 and 300000 ppm) after 24 (a), 48 (b) and 72 (c) hours on A549 cell line. Data are shown as Mean± SD of triplicate wells of two independent experiments. The statistical significance of the differences was determined by one way ANOVA. * P<0.05, ** P<0.01. The cells treated with only celecoxib or indomethacin were considered as reference

we selected concentrations of less than IC_{50} (45 μM for celecoxib and 110 μM for indomethacin) for combination therapy.

Expression of COX-2 protein in the cells treated with DDW and DEW

Considering the role of COX-2 inhibition in lung cancer, we initially conducted preliminary experiments to determine the role of DDW and DEW in expression of COX -2 protein. As shown in Figure 3, both DDW and DEW could decrease the COX-2 protein expression in contrast to the control medium containing normal concentrations of deuterium (Figure 3).

Expression of COX-2 protein in the cells treated with celecoxib, celecoxib/DDW and celecoxib/DEW

After 48 hr treatment with 45 μM celecoxib alone, the expression of COX-2 protein increased. The combination of celecoxib-DEW decreased the expression of COX-2 protein more than each drug treatment alone (Figure 3).

Expression of Cox-2 protein in the cells treated with indomethacin, indomethacin/DDW and indomethacin/ DEW

The treatment with 110 μM indomethacin decreased the expression of COX-2 protein. Neither DDW nor DEW in combination with indomethacin could increase the effect of indomethacin itself, albeit the effect was clear as referred to the control (Figure 3).

Expression of Bcl2 and Bax proteins in the cells treated with DEW and DDW

The pro- and anti-apoptotic proteins of Bcl-2 family constitute a critical control point for apoptosis. To address the role of proteins involved in the apoptosis, the expression of anti-apoptotic Bcl2 and pro-apoptotic Bax proteins was determined. The level of the pro-apoptotic molecule Bax was significantly increased

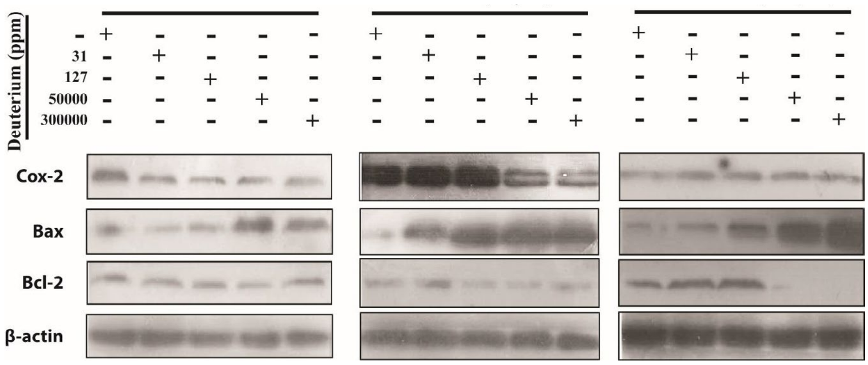

Figure 3. Western blot analysis of COX-2, Bax and Bcl-2 proteins in the A549 cells treated with celecoxib and indomethacin alone or in combination with deuterium-depleted water (DDW) and deuterium-enriched water (DEW)

in response to DEW, but it appeared that DDW had no impact on the Bax expression. Moreover, none of DEW and DDW affected the anti-apoptotic Bcl2 protein expression (Figure 3).

Expression of Bcl2 and Bax proteins in the cells treated with celecoxib, celecoxib/DDW and celecoxib/DEW

Celecoxib solely decreased the expression of the Bax, but did not affect the level of Bcl2 protein expression. As a combination, both celecoxib/DDW and celecoxib/DEW co-treated cells obviously enhanced the Bax expression. The Bcl2 protein was not influenced by the celecoxib/DDW or celecoxib/DEW treatment (Figure 3).

Expression of Bcl2 and Bax proteins in the cells treated with indomethacin, indomethacin/DDW and indomethacin/DEW

As a single agent, indomethacin had no significant effect on Bcl2 and Bax proteins levels. In combination with DEW, indomethacin resulted in a marked increase in the level of Bax protein and a decrease in Bcl2 expression. Indomethacin/DDW did not alter the Bax and Bcl2 protein levels (Figure 3).

Expression of ERK, JNK and p38 MAPKs proteins in the cells treated with DEW and DDW

To determine the role of MAPK pathway, we examined the expression of ERK, JNK and p38 proteins. Since the changes in the expression of total ERK, JNK and p38 proteins were not remarkable, we also investigated the phosphorylation level of these proteins. As depicted in the Figure 4, none of the proteins involved in MAPK pathway (ERK, JNK and p38) were affected by DDW; however, DEW, dose dependently, decreased the ERK phosphorylation and increased the JNK phosphorylation (Figure 4).

Expression of ERK, JNK and p38/MAPK proteins in the cells treated with celecoxib, celecoxib/DDW and celecoxib/DEW

Treatment with 45 µM celecoxib and celecoxib/DDW combination for 48 hr had no distinctive effect on total ERK, JNK and p38 and their phosphorylation. Celecoxib,

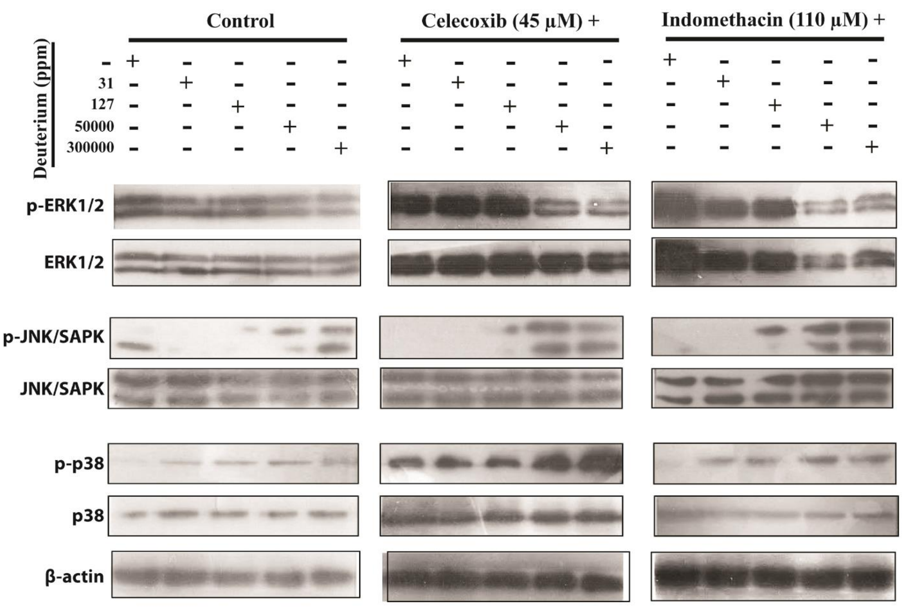

Figure 4. Western blot analysis of ERK1/2 and p- ERK1/2 proteins, JNK and p- JNK proteins, and p38 and p- p38 MAPK proteins in the A549 cells treated with celecoxib and indomethacin, alone or in combination with deuterium-depleted water (DDW) and deuterium-enriched water (DEW)

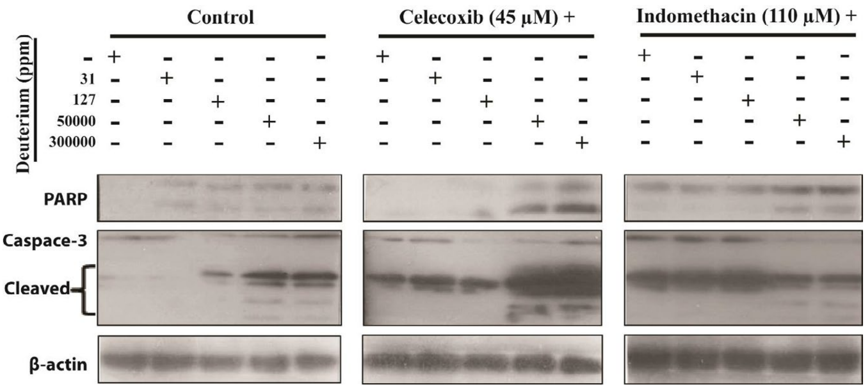

Figure 5. Western blot analysis of poly ADP-ribose polymerase (PARP) and Caspase-3 proteins in the A549 cells treated with celecoxib, indomethacin alone or in combination with different concentration of deuterium

only in combination with DEW, could substantially reduce the ERK phosphorylation and enhance JNK and p38 phosphorylation (Figure 4).

Expression of ERK, JNK and p38/MAPK proteins in the cells treated with indomethacin, indomethacin/DDW and indomethacin/DEW

Compared with the cells in control medium, only indomethacin/DEW could decrease the total ERK level and its phosphorylation. Indomethacin solely or in combination with DDW exhibited no obvious impact on the proteins involved in MAPK pathway (Figure 4).

Activation of caspase-3 and degradation of PARP proteins in the cells treated with DEW and DDW

To find out whether apoptosis has occurred during treatment of lung cancer cells with DEW and DDW, the activation of caspase-3 and PARP degradation was investigated. As shown in Figure 5, there was an increment in the activation of caspase-3 in DEW-treated cells, which is confirmed by an increase in PARP cleavage and degradation (Figure 5).

Activation of caspase-3 and degradation of PARP proteins in the cells treated with celecoxib, celecoxib/DDW and celecoxib/DEW

Although both celecoxib and celecoxib/DDW slightly enhanced the caspase-3 activity, celecoxib/DEW predominantly increased caspase-3 activation, PARP cleavage and degradation as well (Figure 5).

Activation of caspase-3 and degradation of PARP proteins in the cells treated with indomethacin, indomethacin/DDW and indomethacin/DEW

Indomethacin, indomethacin/DDW and indomethacin /DEW could notably agitate the activity of caspase-3. In addition, combination of indomethacin/DEW increased the PARP cleavage and degradation (Figure 5).

Discussion

Combination chemotherapy is a rationale strategy to increase response and tolerability and to decrease adverse effects and drug resistance. *In vitro* studies have shown increased cytotoxicity of combination therapy in comparison with monotherapy in different cell lines (34-37). As a single agent, both COX inhibitors and deuterated-depleted/enriched water (DEW and DDW) have shown cytotoxicity and apoptosis induction; however, they have limited efficacy when used as a single therapeutic agent (12, 16, 32, 38-40). Many studies have demonstrated the inhibitory effects of COX-2 selective NSAIDs in tumor development and progression (41-43), whereas few others have pointed out the role of non-selective NSAIDs (15, 44, 45). Here, we first showed and compared the cytotoxicity of two selective and non-selective COX-2 inhibitors, celecoxib and indomethacin, on A549 lung cancer cell line. The results showed that celecoxib could produce more potent cytotoxicity compared to indomethacin (IC_{50}s of 58.96 and 149.98 µM, respectively, after 48 hr treatment). As expected, both celecoxib and indomethacin intensely inhibited the expression of COX-2 protein. The prognostic and predictive role of COX-2 expression in NSCLC in preclinical and clinical studies has been suggested (46-49). The increased expression of COX-2 leads to an increase in the production of PGE2, which has been demonstrated in colorectal, pancreatic, and lung cancers (47, 50, 51). PGE2 stimulates angiogenesis, cell invasion, and the formation of metastasis and cell survival (50, 52). Therefore, the use of NSAIDs would be regarded as an effective approach for cancer chemoprevention, as demonstrated by a bulk of clinical and experimental evidence. However, the clinical use of these drugs as chemopreventive agents encounters with issues regarding to optimal drug dose, adverse effects and the knowledge about the mechanism(s) upon which these drugs act (8). Considering that NSAIDs mediate their activity via both COX-dependent and -independent pathways, many attentions have been paid to COX-independent mechanism. Therefore, we also evaluated some important involved mechanisms in apoptosis and survival. Our finding revealed that both celecoxib and indomethacin could mediate their effects through caspase-3 over-activation. Moreover, celecoxib activated the p38 by its phosphorylation. Similar to these results, Yoshinaka *et al.* reported that the COX-2 inhibitor celecoxib suppresses tumor growth and lung metastasis of a murine mammary cancer with significantly elevated activities of caspase-3 (53). In addition, potentiating the anti-tumor effects of both selective cyclooxygenase-1 and cyclooxygenase-2 inhibitors in human hepatic cancer cells is attributed to activation of caspase3, concurrent cleavage of PARP, a known caspase3 substrate and a biochemical marker of apoptosis, and decreased Bcl2 protein expression (54). Besides, consistent to our findings, it is denoted that NSAIDs might mediate their effects through alterations of the ERK, and p38 MAPK activities (55). An attempt to define the relationship between the ERK1/2 MAPK cascade and NSAIDmediated anti-tumor effects encounters complication by conflicting reports pointing that exposure to non-selective NSAIDs or selective COX2 inhibitors can induce either an increase or a decrease in ERK1/2 activity, depending on the cell type (56-60). Our findings additionally revealed that NSAIDs did not affect the expression of ERK1/2 MAPK in NSCLC A549 cells.

Moreover, in the present study, we showed the ability of DEW as a single agent to induce apoptosis mediated through COX inhibition, ERK deactivation, and induction of JNK, Bax and caspase. Besides, DDW exerted its effects through COX inhibition, caspase activation and subsequent PARP degradation. Our findings were in accordance with previous studies which demonstrated the anti-proliferative and anti-neoplastic effects of DEW and DDW. Bahk and his coworkers affirmed that DEW has anti-proliferative, anti-adhesive and anti-invasive effects and therefore it can be considered as a potential chemotherapeutic agent with low systemic toxicity for a postoperative intravesical instillation in a superficial bladder cancer (40). Hartmann *et al.* showed that DEW is a useful agent against human pancreatic carcinoma cells, a fact that makes it a potential candidate for the treatment of pancreatic tumors (39). Bader showed that DEW in combination with gemcitabin yields highly synergistic effects in human pancreatic adenocarcinoma cells *in vitro* (32). Uemura *et al.* demonstrated that DEW exerts its cytotoxicity in RSVM cells by induction of apoptosis via the caspase activation (61). Furthermore, in agreement with our results, Cong proved that DDW inhibits human lung carcinoma cell growth by apoptosis (31). It has been reported that DDW mediates its cytotoxic effect by induction of apoptosis via expression of Kras and Bcl2 in mouse lung (38).

Lastly, because of limited efficacy of both NSAIDs and water with various D contents, when used as a single therapeutic agent, in addition to to obtain more efficacy, limited doses, and less adverse effects, we attempted to develop an effective combination regimen with COX inhibitors and DEW or DDW. Clinical protocols for cancer chemotherapy usually combine two or more agents to achieve therapeutic effects greater than those provided by a single drug. As a result, combination of celecoxib and indomethacin with DEW, but not DDW, could significantly increase the cytotoxicity of different concentrations of celecoxib and indomethacin in a concentration dependent mode. Based on western blot data, either celecoxib or indomethacin when co-administrated with DEW, led to a remarkable activation in apoptosis pathways of A549 human non-small cell lung cancer cell in comparison with their co-treatment with DDW. These events were associated with activation of p38, JNK and Bax as pro-apoptotic proteins and decreasing in pro-survival proteins COX-2 and ERK1/2.

Furthermore, these combinations activated caspase-3, the apoptosis mediator, and disabled PARP, the key DNA repair enzyme, by cleaving it. Considering the anti-apoptotic effects of COX enzymes, the inhibition of COX and in particular COX-2 can be accounted for the cytotoxicity of DEW, DDW separately and their combination with celecoxib or indomethacin. The most potent COX inhibition was achieved by combination of celecoxib/DEW. These results were in line with the MTT cytotoxicity findings. Since the cytotoxic and apoptotic effects of NSAIDs may not be exclusively mediated by a COX-2– dependent pathway (18, 62), the changes in some other apoptosis-related proteins including MAPKs, Bcl2 and caspase activity were also investigated. In this regard, DEW increased the cytotoxicity of celecoxib and indomethacin greater than DDW, which was in consistent with slight increasing of JNK and p38 proteins and inactivation of ERK/MAPK signaling pathway. MAPKs including ERK1/2, p38, and JNK are crucial enzymes, which have many important regulatory roles in the proliferation and apoptosis of the cells (63, 64). In general, although JNK and p38 pathways are activated by stress stimuli and are involved in apoptosis, ERK phosphorylation and subsequent activation is in response to growth factors (65) and has generally been associated with anti-apoptotic effects; therefore, inactivation of ERK has been shown to be necessary for the cytotoxic-induced apoptosis (66).

Besides, the apoptotic pathway was activated by combination of DEW as well as DDW with both celecoxib and indomethacin. Combination of DEW with celecoxib and indomethacin increased pro-apoptotic Bax protein expression. This effect was also observed in combination of DDW with indomethacin. Additionally, DEW/indomethacin could decrease expression of the anti-apoptotic protein Bcl2 greater than DEW/celecoxib. The impact of bcl-2 and bax expression on the response to chemotherapy has been supported by the laboratory and the clinical data (15, 16). As reported, NSAIDs may exert their anti-carcinogenic effects in various cancer cell lines through the induction of apoptosis. PGE2 can inhibit apoptosis by inducing the expression of anti-apoptotic proteins such as Bcl-2 and inhibition of pro-apoptotic proteins like Bax (67). Bcl-2, as a representative of anti-apoptotic proteins, and Bax that is widely described as a pro-apoptotic factor, are involved in the late signaling phase of programmed cell death presenting opposite functions. A high level of Bcl-2 expression prevents cells from apoptosis caused by cytotoxic factors or cellular stress. Bax-associated proteins appear to be dominant inhibitors of Bcl-2 action; they promote apoptosis via mitochondrial membrane damage facilitating the release of other apoptotic mediators, especially cytochrome C, resulting in caspase cascade activation followed by cell death (68). It has been denoted that expression of the pro-apoptotic protein Bax increases caspases activity (69); therefore, the cytotoxicity of DEW and DDW and their combination with celecoxib and indomethacin was also related to apoptotic pathways since caspase-3 was activated in these treated cells (15).

Conclusion

Our study underscores that both COX inhibitors and water with various D content (DEW and DDW) as monotherapy could activate some mechanisms involved in apoptosis. Moreover, combination of DEW with celecoxib and indomethacin can be effective against NSCLC by influence on some cell signaling pathways and may become candidates for chemotherapy.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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