ARTICLE
Journal of Cellular Biochemistry 116:81–90 (2015)

Journal of Cellular Biochemistry

Apoptotic Effects of Two COX-2 Inhibitors on Breast Adenocarcinoma Cells Through COX-2 Independent Pathway

Mahnaz Norouzi,[1] Shaghayegh Norouzi,[1] Mohsen Amini,[2] Amir Amanzadeh,[3] Saeed Irian,[1] and Mona Salimi[4]*

[1]*Department of Cellular and Molecular Biology, Faculty of Science, Kharazmi University, Tehran, Iran*
[2]*Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*
[3]*National Cell Bank of Iran, Pasteur Institute of Iran, Tehran, Iran*
[4]*Department of Physiology and Pharmacology, Pasteur Institute of Iran, Tehran, Iran*

ABSTRACT

Recently, much effort has been directed toward the search for compounds that influence apoptosis and to understand their mechanisms of action. Cyclooxygenase (COX)-2 inhibitors may induce apoptosis through the COX-2-independent mechanism via a mitochondrial pathway. In view of the reported antiproliferative activities of two COX-2 inhibitor derivatives (**1**, **2**) in breast cancer cells (MCF-7), the present study was undertaken to evaluate the potential of these compounds to induce apoptosis and unravel the associated mechanisms. The apoptotic activities of the two compounds were assessed using flow cytometry, fluorescence microscope, and Western blot analysis. Compounds **1** and **2**-treated MCF-7 cells revealed the apoptotic cell death, as confirmed by the changes in nuclear morphology and the increased annexin-V/PI staining. Elevation of Bax to Bcl-2 ratio and activation of caspase-3 were found to be associated with the initiation of apoptosis induced by compound **1**. Further investigation showed that compounds **1** and **2** inhibited NF-κB, FHC, and ERK activation, while no dramatic change was revealed in c-Myc and EGR-1 levels. Our data suggest that induction of apoptosis by compounds **1** and **2** is not associated with COX-2 expression and occurs through the NF-κB pathway, which sequentially inhibits P-ERK and FHC expression. J. Cell. Biochem. 116: 81–90, 2015.

KEY WORDS: COX-2; APOPTOSIS; CANCER; MCF-7; NF-κB

Breast cancer is the most prevalent form of cancer in women worldwide, and current chemotherapy is unable to obtain clinical responses in patients with highly invasive metastatic disease. There is an essential need for more effective approaches to prevention and treatment of breast cancer [Boyle, 2012]. Breast cell homeostasis needs a balance between cell proliferation and apoptosis [Simstein et al., 2003]. In other words, breast cancer occurs due to decreased rates of apoptosis [Igeny and Krammer, 2002]. Recently, considerable attention has been assigned to apoptotic cell death and the role of this process in the lethal effects of the diverse anti-neoplastic agents [Yang et al., 2006]. Many novel chemotherapeutic agents were designed to target molecules that play decisive roles in the execution of one or more cell death pathways. However, a further in depth understanding of the signaling pathways involved in cell death induced by chemotherapy is still required [Ricci and Zong, 2006].

Various experimental and clinical studies propose that selective cyclooxygenase (COX)-2 inhibitors, a subclass of nonsteroidal anti-inflammatory drugs (NSAIDs), have the potential to be used as anti-cancer agents [Thun et al., 2002]. Celecoxib as the first cyclooxygenase- 2 selective nonsteroidal inhibitor has been approved for the treatment of adult arthritis [Harris et al., 2000]. Celecoxib also exhibited potent chemopreventive activity in chemical carcinogen-induced colon, bladder, and breast cancers [Jendrossek, 2013]. Numerous mechanisms, including apoptosis, may be ascribed to the observed effects of NSAIDs against breast cancer [Cotterchio et al.,

The authors declare that there is no conflict of interest.
Grant sponsor: Pasteur Institute of Iran and Tehran University of Mecical Sciences (TUMS).
*Correspondence to: Mona Salimi, Department of Physiology and Pharmacology, Pasteur Institute of Iran, Tehran, P.O. Box 13164, Iran. E-mail: salimimona@pasteur.ac.ir
Manuscript Received: 30 April 2014; Manuscript Accepted: 15 August 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 20 August 2014
DOI 10.1002/jcb.24944 • © 2014 Wiley Periodicals, Inc.

2001]. In cellular systems, celecoxib mainly induces mitochondrial apoptosis through a COX-2-independent inhibitory pathway [Jendrossek, 2013]. Celecoxib has a 1, 2-di-aryl heterocyclic structure and should be an ideal lead compound for developing novel derivatives with potent apoptosis-inducing activity [Liu et al., 2004; Du et al., 2011; Norouzi et al., 2013]. Previous studies performed by Zarghi et al. [2012] presented the triaryl heterocycles as more potent COX-2 inhibitors which better fit in the COX-2 active site and show a wide range of activities such as cytotoxic effects. We have recently reported that two COX-2 inhibitor derivatives (**1**, **2**) (Fig. 1) showed significant biological features such as antiproliferative activity with considerable IC_{50} values (6.5 and 10.1 μM) in breast adenocarcinoma (MCF-7) cell line after 24 h treatment [Miralinaghi et al., 2013].

In this study, the effect of compounds **1** and **2** on induction of apoptosis was assessed on MCF-7 cells. The molecular mechanism was further clarified in MCF-7 cell line with the following traditional parameters: (1) observing of typical morphological changes, containing chromatin condensation under fluorescence microscope; (2) analysis of cell death by means of flow cytometry using annexin-V/PI staining; (3) activation of caspases-3; and (4) expression of two Bcl-2 family members (Bax and Bcl-2) using Western blot analysis. Moreover, we studied whether the anti-carcinogenic effects of compounds **1** and **2** are mediated through modulation of COX-2, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), ferritin heavy chain (FHC), early growth response protein 1 (EGR-1), c-Myc, and extra cellular signal-regulated kinase (ERK).

MATERIALS AND METHODS

CHEMICALS

The breast adenocarcinoma MCF-7 cell line (National Cell bank of Pasteur Institute of Iran, C135) was used as an in vitro model for studying the anticancer properties of the chemical compounds. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (Rockville, IN). Polyclonal anti-caspase-3 (1:500), anti-Bcl-2 (1:500), anti-Bax (1:500), anti-COX-2 (1:1,000), anti-GAPDH (1:1,000) antibodies and monoclonal anti-ERK (1:1,000), anti-Phospho ERK (1:1,000), anti-FHC (1:100), and anti-EGR-1 (1:200) antibodies were purchased from Abcam (Cambridge MA). Anti-rabbit IgG horseradish peroxidase (HRP) antibody (1:5,000) was obtained from Cell Signaling Technology (Beverly, MA). Annexin-V-FLUOS and NF-κB assays were performed with commercial kits purchased, respectively, from Roche Applied Science (USA) and from Active Motif Europe (Belgium). ECL advance Western blotting detection kit was prepared from General Electric Health Care Life Sciences (Buckinghamshire, UK). Compounds **1** and **2** were synthesized in the organic chemistry laboratory at the faculty of pharmacy of Tehran University of Medical Sciences. All other chemicals were in high purity and prepared from Merck (Darmstadt, Germany), Sigma–Aldrich (St Louis, MO).

CELL CULTURE AND TREATMENTS

The MCF-7 cells were cultured and routinely maintained in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 inside a CO_2 incubator. The MCF-7 cells were treated with compounds **1** and **2** at the IC_{50} concentrations (6.5 and 10.1 μM) for 8 and 16 h.

EFFECT OF COMPOUNDS ON CELL MORPHOLOGY

The treated and negative-control cells were stained by DAPI (Diamidine phenyl dihydrochloride, Roche Applied Science, Indianapolis) and their morphology was observed under a Zeiss fluorescence microscope (Zeiss, Germany) and photomicrographs were taken with an Olympus digital camera (Tokyo, Japan).

IDENTIFICATION OF APOPTOSIS BY ANNEXIN-V/PI STAINING

After treatment, 10^6 cells were washed in PBS and resuspended in 100 μL of the annexin-V-FLUOS labeling solution containing 2 μL annexin-V-FLUOS labeling agent, 2 μL propidium iodide (PI) solution, and 1 mL incubation buffer to achieve a concentration of 10^6 cells/mL. Following incubation at 37 °C for 15 min, cells were analyzed by flow cytometry. Annexin-V binds to those cells that

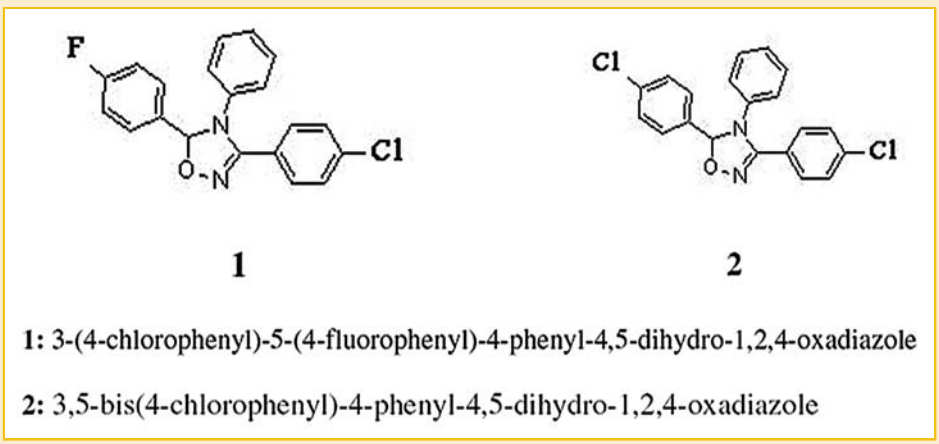

Fig. 1. Chemical structures of compounds 1 and 2.

express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those that have a compromised cell membrane [Fang et al., 2000]. This allows for the discrimination of live cells (unstained with either fluorochrome or PI) from apoptotic cells (stained with annexin-V) and necrotic cells (stained with PI).

WESTERN BLOT ANALYSIS

Western analyses of caspase-3, Bcl-2, Bax, COX-2, ERK, P-ERK, FHC, and EGR-1 proteins, were performed using specific antibodies as described previously. The MCF-7 cells were treated with compounds **1** and **2** at their IC_{50} concentrations for 8 and 16 h. The proteins of distinctively treated cells were collected and lysed in lysis buffer (Tris 62.5 mM [pH 6.8], DTT 50 mM, SDS 10%, glycerol 10%, and bromophenol blue 0.25% [w/v]) in the presence of protease inhibitor. Eighty micrograms of protein were separated by 12 or 15% SDS-PAGE and electroblotted to a PVDF membrane using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). After blocking with 1% casein, the membranes were incubated overnight at 4 °C with the primary antibodies, followed by labeling with the secondary antibody. Protein bands were visualized using the ECL advance Western blotting detection kit. GAPDH (glyceraldehyde phosphate dehydrogenase) was used as the endogenous control and the control cells were cultured in the complete medium without complexes (control cells were only treated with DMSO using the highest amount into the experiments).

ELISA-BASED TransAM ASSAY

Following appropriate treatment (with compounds **1** and **2**) of MCF-7 cells and 16 h incubation period, the NF-κB translocation into the nucleus was quantified using an ELISA-based TransAM p65 kit (TransAM NF-κB p65 Transcriptor Factor Assay kit) according to the manufacturer's protocol. In brief, the whole cell proteins were extracted using the lysis buffer provided in the kit and quantified by a classical Bradford protein assay. For TransAM assays, 20 μg of nuclear protein extract per well was added to a 96-well plate coated with the immobilized oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) and left to incubate for 1 h at room temperature with mild agitation. p65 proteins present in nuclear extracts will bind specifically to this sequence. Next, anti-p65antibody (100 μL, at a 1:1000 dilution) was added to each well for 1 h followed by 100 μL of horseradish peroxidase (HRP)-conjugated antibody (1:1000 dilution) for yet another 1 h. After adding 100 μL of developing solution for up to 5 min, colorimetric reaction was stopped and the NF-κB p65 translocation activity was determined by reading absorbance on a spectrophotometer at 450 nm with a correction wavelength of 630 nm.

SEMIQUANTITATIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from MCF-7 cells using RNX-Plus (CinnaGen, Iran). All gene segments were amplified and verified by semiquantitative RT-PCR. cDNAs were reverse-transcribed from 1 μg total RNA using RevertAid first strand cDNA synthesis Kit (Thermo, Lithuonia), following the manufacturer's protocol. The PCR primer sequences were shown in Table I. The products obtained from PCR amplification were separated and photographed in 1% agarose gel. Densitometry was made using the Image J software and the ratio of target to control PCR products was determined by dividing the densitometric volume of the target band by that of the GAPDH band.

TABLE I. The Primer Sequences for Semi-quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Primer name	Primer sequence	Size
c-Myc	Forward: 5′-TGGTGCTCCATGAGGAGACA-3′	20-mers
	Reverse: 5′-GTGTTTCAACTGTTCTCGTC-3′	20-mers
GAPDH	Forward: 5′-GAGCCCGCAGCCTCCCGCTT-3′	20-mers
	Reverse: 5′-CCCGCGGCCATCACGCCACAG-3	21-mers

STATISTICAL ANALYSIS

All data are represented as arithmetic mean ± S.E. of at least triplicate determinations, and statistical comparisons were based on ANOVA followed by the Tukey's post test. $P < 0.05$ was considered to be significant.

RESULTS

INDUCTION OF APOPTOSIS IN MCF-7 CELLS BY COMPOUNDS 1 AND 2

In order to examine whether compounds **1** and **2** showed cytotoxicity to MCF-7 cells through inducing apoptosis, DAPI staining assay was used to observe morphological changes of the MCF-7 cells after treatment with compound **1** (6.5 μM) and compound **2** (10.1 μM) for 8 and 16 h. Morphologic evidence of apoptosis is usually assessed as the chromatin condensation and nuclear fragmentation. Compound **1**-treated cells were found to have condensed and fragmented nuclei after 8 h (Fig. 2C) and 16 h (Fig. 2G) incubation, whereas compound **2**-treated MCF-7 cells indicated the morphologic alterations after 16 h treatment (Fig. 2H). However, no remarkable alteration observed after 8 h incubation of MCF-7 cells with compound **2** (Fig. 2D). To further confirm and quantify apoptosis in MCF-7 cells induced by compounds **1** and **2**, the cells were stained with annexin-V/PI and analyzed by flow cytometry. In the dual parameter fluorescent dot plots, the cells in the early (annexin V+/PI−, in the lower right quadrant) and late (annexin V+/PI+, in the upper right quadrant) stage apoptosis were counted (Fig. 3). As shown in Table II, 1.41% of cells were in annexin V+/PI− and 0.28% of cells were in annexin V+/PI+ in untreated cells. After treatment with compound **1** at 6.5 μM (IC_{50}) and compound **2** at 10.1 μM (IC_{50}) for 16 h, the population of cells indicated a shift from viable cells to early and late stage apoptosis. The percentage of both early and late apoptotic cells increased to 3.28, 1.34, and 3.57%, 0.84 for compounds **1** and **2**, respectively. In addition, the ability of compound **1** to induce MCF-7 cellular necrosis was more than compound **2**. These results are in line with our morphological data suggesting an apoptotic pathway induced by compounds **1** and **2** after 16 h treatment.

WESTERN BLOTTING ANALYSIS ON APOPTOSIS SIGNALING PATHWAY

Western blotting was used in order to analyze the proteins related to apoptosis induced by compounds **1** and **2** in MCF-7 cells. As shown

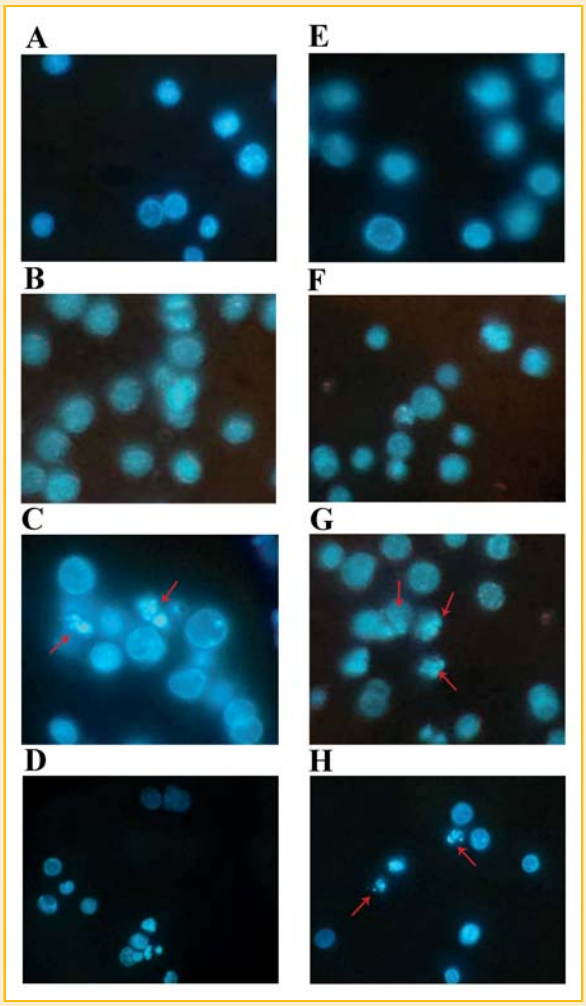

Fig. 2. Apoptotic morphological changes in MCF-7 cells. Control cells (A); cells treated with DMSO for 8 h (B); cells treated with compound 1 at 6.5 μM for 8 h (C); cells treated with compound 2 at 10.1 μM for 8 h (D); control cells (E); cells treated with DMSO for 16 h (F); cells treated with compound 1 at 6.5 μM for 16 h (G); cells treated with compound 2 at 10.1 μM for 16 h (H). Cells were stained by DAPI and observed under Zeiss fluorescent 100 × 10 microscopy.

in Fig. 4, the proteolytic cleavage of caspase-3 was observed in compound 1-treated MCF-7 cells after 8 h, whereas compound 2 demonstrated no cleavage of caspase-3 protein in these cells. Similar proteolytic cleavage pattern was shown in a time-dependent manner when the cells were treated with compounds 1 and 2 for 16 h at the IC_{50} concentrations (Fig. 4).

To further investigate the molecular mechanism of apoptosis induced by the two compounds, the expression of Bcl-2 and Bax were measured by Western blot analysis. As indicated in Fig. 5A, the MCF-7 cell line was highly expressed in Bcl-2. Treatment with compound 1 could suppress the Bcl-2 expression and up-regulate Bax expression in a time-dependent manner (Fig. 5A). A statistically significant increase ($P < 0.05$) of the Bax/Bcl-2 ratio was found in MCF-7 treated with compound 1 (6.5 μM) for 8 h, whereas no considerable increase was observed after 8 h exposure of MCF-7 cells to compound 2 (Fig. 5B). A similar pattern was observed when MCF-7 cells were treated with compound 1 (6.5 μM) for 16 h. Based on the obtained results, the ratio of Bax/Bcl-2 increased significantly ($P < 0.01$) after 16 h treatment, although no remarkable increase was shown after treatment of cells with compound 2 (Fig. 5C). The Bax-to-Bcl-2 ratio suggests that induction of apoptosis by compound 1 after 16 h may be due to a decrease in Bcl-2 and an increase in Bax level.

Collectively, the data on Western blot analysis proposes that the molecular mechanism of apoptosis induced by compound 1 is different from that compound 2.

EFFECT OF COMPOUNDS 1 AND 2 ON COX-2 EXPRESSION

It is well known that the tumor-inhibitory efficacy of selective COX-2 inhibitors is not necessarily related to their COX-inhibitory potential. COX-2 inhibitors can exert the cancer-preventive capacity in in vitro or in vivo models having rare or even deficiency of COX-2 expression, for example, celecoxib inhibited the growth of various cancer cell lines that are COX-2-deficient [Maier et al., 2004]. Here, we showed that both compounds 1 and 2 as two COX-2 inhibitors had no significant effect on expression of COX-2 by 16 h treatment in MCF-7cells (Fig. 6).

NF-κB INHIBITION BY COMPOUNDS 1 AND 2

NF-κB activation is essential in the apoptosis process. In this regard, it has been shown that a deficiency in NF-κB activity leads to apoptosis (Sheikh and Huang, 2003). Thus, development of compounds that target NF-κB is proposed as an approach for treatment of cancer. In this study, we examined the effects of compounds 1 and 2 on NF-κB p65 transcriptional activity. As shown in Fig. 7 and 16 h after treatment of the cells with 6.5 μM of compound 1, there was an evident and significant ($P < 0.01$) decrease in NF-κB activation as measured by its nuclear translocation. The inhibition was also noticeable after treatment of compound 2 at 10.1 μM for 16 h. In other words, the basal level of NF-κB p65 subunit was 100% in nuclei of MCF-7 cells. Addition of compounds 1 and 2 at their IC_{50} concentrations led to a significant decrease in the concentration of nuclear p65 subunit after 16 h.

COMPOUNDS 1 AND 2 INFLUENCE FHC AND P-ERK EXPRESSION IN MCF-7 CELLS

Since a deficiency in NF-κB activity leads to apoptosis via FHC expression, we evaluated whether compounds 1 and 2 can regulate FHC expression (Pham et al., 2004]. Protein expression of FHC was analyzed by Western blot. Interestingly, administration of compounds 1 and 2 to MCF-7 cells resulted in a decrease in FHC expression, which was significant ($P < 0.05$) after 16 h of treatment (Fig. 8A). Furthermore, evidence showed that NF-κB activation was involved with phosphorylated ERK, suggesting that ERK has a role in NF-κB activation. Consequently, we investigated the effects of these compounds on the active form of ERK. Similar behavior was observed in P-ERK expression. As shown in Fig. 8B, a significant ($P < 0.05$) decrease of P-ERK expression was observed with compounds 1 and 2 compared with DMSO-treated cells. These results demonstrated that NF-κB expression was inhibited by

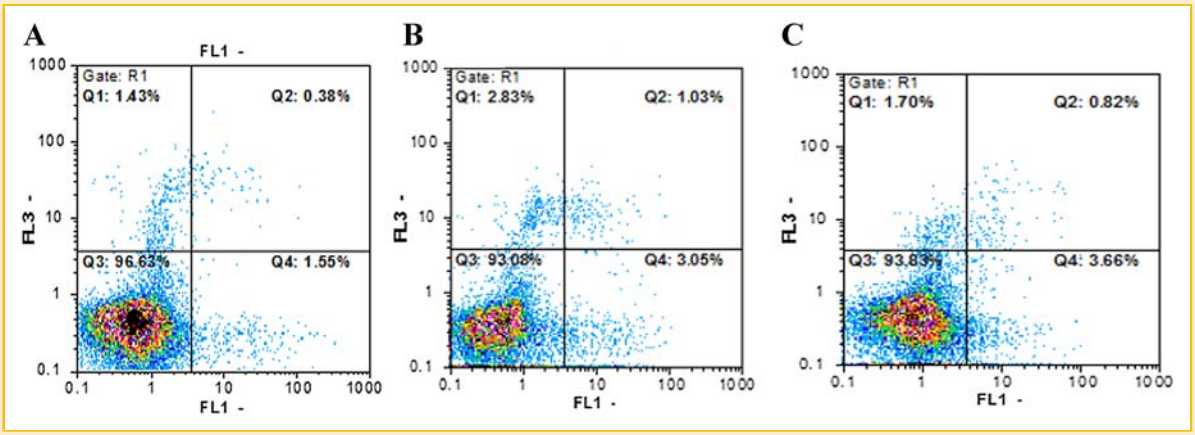

Fig. 3. Flowcytometric analysis of PI-annexin-V to quantify complexes-induced apoptosis in MCF-7 cells. Dot plot of MCF-7 cells for 16 h (A). Dot plot of MCF-7 cells with compound 1 treatment at 6.5 μM for 16 h (B). Dot plot of MCF-7 cells with compound 2 treatment at 10.1 μM for 16 h (C). The results shown are representative of three independent experiments. Quadrant 3, living cells An−/PI−; quadrant 4, early apoptotic cells An+/PI−; quadrant 2, late apoptotic cells An+/PI+; quadrant 1, necrotic cells An−/PI+.

compound 1 and 2 through a decrease in ERK phosphorylation and FHC expression.

c-Myc AND EGR-1 EXPRESSION DO NOT UNDERGO ALTERATIONS DURING TREATMENT OF COMPOUNDS 1 AND 2

To determine whether compounds 1 and 2 regulate c-Myc through Egr-1 expression, we examined the effects of compounds 1 and 2 on the gene transcription of an early gene, c-myc. Semi-quantitative RT-PCR analysis revealed the expression of c-Myc mRNA in MCF-7 cells (Fig. 9A); however, the results showed no change in c-Myc mRNA expression in cells treated with compounds 1 and 2 at their IC_{50} concentrations at 16 h (Fig. 9A). Similar to the expression of c-Myc, expression of EGR-1 was unchanged by compounds 1 and 2 at 16 h. In other words, in untreated cells and in those treated with compounds 1 and 2 for 16 h, the EGR-1 levels were similar (Fig. 9B).

DISCUSSION

This study presents data showing that two COX-2 inhibitor derivatives (1, 2) reduce in vitro proliferation of MCF-7 cells by inducing apoptosis and downregulating the expression of FHC, NF-κB, and P-ERk proteins. Following treatment with compounds 1 and 2 for 16 h, MCF-7 cells exhibited apoptotic bodies, while in cells treated for only 8 h, minor morphological changes were observed (Fig. 2). These results prompted us to perform the annexin-V experiment at 16 h. The data obtained from annexin-V/PI analysis support the morphological changes showing that compounds 1 and 2 induced apoptosis at early and late stages for 16 h. Interestingly, the increase in the percentage of late apoptotic cells was more pronounced in cell populations treated with compound 1 than in those treated with compound 2. These results are further confirmed by the increase of proteolytic cleavage of apoptosis inducers (caspase-3) in a time-dependent manner for compound 1. We found that compound 1 caused the cleavage of 35 kDa caspase-3 to generate 17 kDa fragment after 8 and 16 h treatment, indicating compound 1-induced apoptosis via caspase-3 activation. In contrast, compound 2 could not cleave caspase-3 suggesting apoptosis through an independent caspase-3 pathway [Salimi et al., 2014].

Process of apoptosis involves two distinct pathways. One pathway is the death-receptor pathway and the other is the mitochondrial pathway, which has been considered a notable mediator of cell apoptosis in mammals. In the mitochondrial pathway, Bcl-2 family members undertake the responsibility of regulation of apoptosis in

TABLE II. Percentage of MCF-7 Cells in Each State after Treatment with Compounds 1 and 2[a]

Compound	Vital cells (%) An−/PI−	Early apoptosis (%) An+/PI−	Late apoptosis (%) An+/PI+	Necrosis (%) An−/PI+
1	92.98 ± 0.34	3.28 ± 0.26	1.34 ± 0.39	2.4 ± 0.5
2	93.74 ± 0.70	3.57 ± 0.14	0.84 ± 0.05	1.85 ± 0.18
Control	96.45 ± 0.65	1.41 ± 0.33	0.28 ± 0.09	1.86 ± 0.5

[a]All experiments performed in triplicate at their IC_{50} concentrations after 16 h incubation.

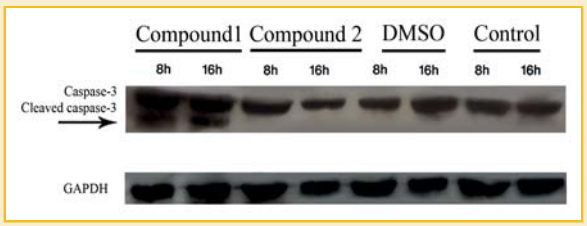

Fig. 4. Effect of compounds 1 and 2 on induction of apoptosis. MCF7 cells were treated with compound 1 (6.5 μM) and compound 2 (10.1 μM) for 8 h and 16 h. Cleaved caspase-3 level in total cell lysates was determined by Western blot analysis. GAPDH was used as loading control.

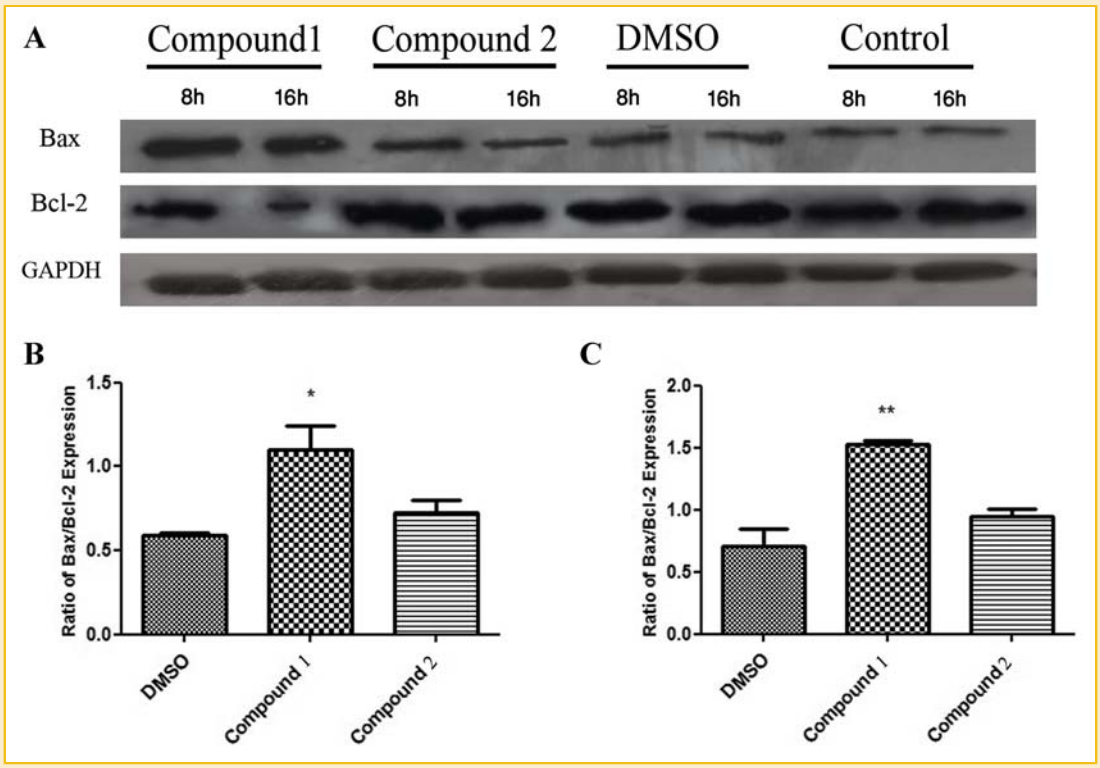

Fig. 5. Effect of compounds 1 and 2 on the levels of Bax and Bcl-2 protein expression in MCF-7 cancer cell line. Bax and Bcl-2 protein levels were assayed by Western blotting (A). Bax/Bcl-2 ratio was measured after 8 h (B) and 16 h (C) treatment. Differences between the Bax/Bcl-2 ratio in treated and their respective control cells were determined by one-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with control without treatment.

various conditions, containing the anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein [Hengartner, 2000]. Since estrogen positive MCF-7 cells demonstrate a high level of Bcl-2 expression, discovering agents with the ability to reduce Bcl-2 expression would be of great importance, especially in resistance to chemotherapy. A statistically significant increase of Bax/Bcl-2 ratio was found in MCF-7 cells treated with compound **1** (6.5 μM) for 8 and 16 h, although no increase exhibited after treatment of the cells with compound **2**. Our results in Bax-to-Bcl-2 ratio increment were in agreement with the results obtained from caspase proteolytic activity suggesting mitochondrial apoptosis induced by compound **1** in a time dependent manner. Therefore, our findings of apoptotic activity of compound **1** will help to drive its mechanism. However, compound **2** may produce an effect via a pathway other than mitochondrial pathway to induce apoptosis. In order to understand the upstream factors involved in induction of apoptosis by compounds **1** and **2** at 16 h, further extensive studies were performed in MCF-7 cells.

Selective cyclooxygenase (COX)-2 inhibitors have revealed encouraging results in the treatment of cancer in experimental and clinical studies [Chun and Surh, 2004]. The effects of selective COX-2 inhibitors on cancerous cells are as follows: inhibition of cell proliferation, induction of apoptosis, and reduction of cell motility and adhesion [Ding et al., 2000]. The mechanism by which COX-2 inhibitors amplify the action of apoptosis is, however, not well defined and it is suggested that mechanisms other than suppression of the COX-2 expression may be involved. In this regard, celecoxib as a selective COX-2 inhibitor with proapoptotic effects that do not critically depend on COX-2 [Jendrossek, 2013]. In the present study, no alteration in COX-2 expression is observed in MCF-7 cells exposed to compounds **1** and **2** after 16 h (Fig. 6).

The COX-2 independent pathway involves the inhibition of ERK and NF-κB [Dovizio et al., 2012]. NF-κB has been recently described as a COX-independent molecular target for functions of NSAIDs and COX-2 inhibitors, such as aspirin, indomethacin, and celecoxib [Takada et al., 2004]. NF-κB is considered as an ideal target for anti-cancer drug development, since it has been shown to block apoptosis and lead to proliferation. Its activation causes resistance to chemotherapeutic agents and constitutive expression of NF-κB induces proliferation in tumor cells [Nakanishi and Toi, 2005]. NF-κB has been shown to adjust the expression of a number of genes, the products of which are involved in tumorigenesis [Aggarwal, 2004; Aggarwal et al., 2004], such as anti-apoptotic genes (e.g., Bcl-2). Furthermore, inflammation is suggested to be an important element of cancer, and NF-κB activation is critical in the inflammatory process [Cai et al., 2013]. Thus, discovering of compounds that target NF-κB is recommended as an approach for the treatment of cancer. Interestingly, our results revealed that compounds **1** and **2** significantly downregulated NF-κB activation in MCF-7 cells after 16 h incubation (Fig. 7). Given the fact that the carcinogenic and inflammatory regulatory effects of many anti-cancer drugs are mediated by NF-κB [Subhashini et al., 2005], suppression of NF-κB

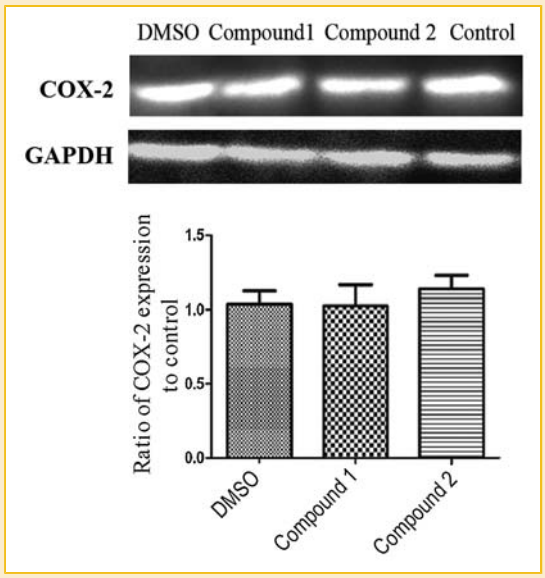

Fig. 6. Western blot analysis of COX-2 protein expression as ratio to control in MCF-7 cells treated with compounds 1 (6.5 μM) and 2 (10.1 μM) after 16 h incubation. Data are representative of three individual experiments (n = 3) that were performed for each individual group.

activation by compounds 1 and 2 is proposed as a mechanism for the anti-cancer effects of these two compounds in the MCF-7 cells.

Ferritin is a highly conserved protein and plays an important role in intracellular iron storage [Recalcati et al., 2008]. The molecule is created by ferritin heavy chains (FHC) and ferritin light chains (FLC) which assemble to form a 24-subunit protein [Torti and Torti, 2002]. FHC is identified to have a role in different pathological processes including inflammation [Kwak et al., 1995] and cancer [Boult et al., 2008]. It has been shown that the activation of NF-κB/p65 is adequate to upregulate FHC expression (Pham et al., 2004). In turn, FHC is responsible for iron sequestration, leading to suppression of apoptosis by the inhibition of ROS [Bubici et al., 2006]. Herein, FHC expression was found to be significantly ($P < 0.05$) decreased in MCF-7 cells treated with compounds 1 and 2 compared to control cells (Fig. 8A). These observations are in line with the earlier reported findings [Roperto et al., 2010] and suggest that FHC downregulation, mediated by NF-κB, may be one of the molecular keys whereby compounds 1 and 2 induce apoptosis.

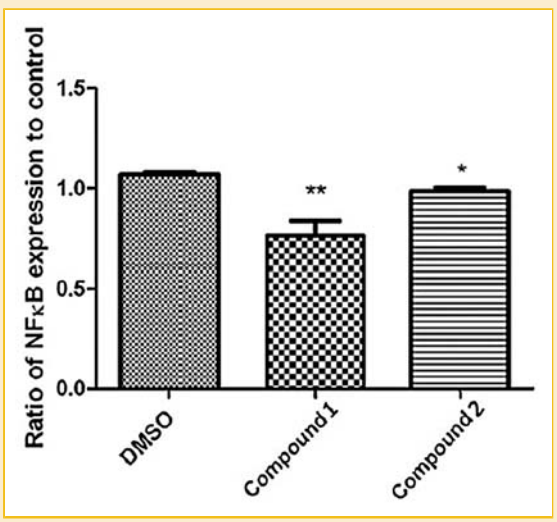

Fig. 7. MCF-7 cells were treated with compound 1 (6.5 μM) and compound 2 (10.1 μM) for 16 h. Nuclear proteins were extracted and NF-κB activity determined as the ratio to control cells using kit. Mean values ± standard error (n = 3) are shown. *$P < 0.05$, **$P < 0.01$ compared with DMSO-treated cells.

In addition to apoptotic genes, proto-oncogen, and oncogen genes such as c-Myc and ERK can also induce anti-apoptosis signaling and augment the uncontrolled proliferation of cancer cells [Khan and Bisen, 2013]. ERK2/ERK1 are two isoforms of extracellular signal-regulated kinases (ERK) that are classified with the family of mitogen-activated protein kinases (MAPKs). ERK activation regulates various cell responses, such as proliferation, migration, differentiation, and death [Murphy and Blenis, 2006]. Several studies link the oncogenic potential of ERK to increased cell survival through enhancing the activity of anti-apoptotic proteins such as Bcl-2 and suppressing of pro-apoptotic proteins such as Bax [Balmanno and Cook, 2009]. Based on the previous studies [Cagnol and Chambard, 2010], phosphorylation of MAPKs is completely inhibited when NF-κB is repressed, indicating that NF-κB is upstream of MAPKs in the presence of anticancer drugs such as paclitaxel. In our study, P-ERK expression was reduced significantly ($P < 0.05$) by compounds 1 and 2 in MCF-7 cells after 16 h of incubation (Fig. 8B). These data support our NF-κB results suggesting that NF-κB is probably upstream of MAPKs in compound 1 and 2 signaling, which has been previously reported as a mechanism of paclitaxel.

Alterations in the level of c-Myc expression are connected with many malignancies found in humans. In fact, c-Myc affects a variety of cellular functions such as cell cycle regulation, apoptosis, differentiation, cell adhesion, and tumorigenesis [Hoffman et al., 2002]. The exact role of the c-Myc protein, and in particular the mechanism by which it induces cell proliferation in normal and cancer cells is not clear. Studies by Caffrey et al. [2002] revealed that COX-2 inhibitors cause apoptosis without disruption of the c-Myc proto-oncogene. It has also been shown that COX-2 inhibitors are able to downregulate c-Myc and initiate this alteration efficiently. Thus, both c-Myc-dependent and independent mechanisms are suggested for COX-2 inhibitors [Sobolewski et al., 2011].

Several immediate-early genes including c-Myc contribute to cell proliferation. Additionally, zinc finger transcription factors, which belong to the early growth response family of proteins, involve in many processes, including proliferation, apoptosis, and tumorigenesis [Gottfried et al., 2013]. In this regard, Egr-1 as the best-characterized family member controls the expression of a number of target genes. Interestingly, some of the genes have been shown to be targets of c-Myc [Collins et al., 2008]. Furthermore, it was found that activated c-Myc induced the expression of Egr-1 protein suggesting that Egr1 expression is regulated by endogenous c-Myc [Boone and Hann, 2011]. Some experiments showed that celecoxib could increase c-Myc expression level and assist in reducing the tumor progression and develop the apoptotic rate [Boone et al., 2010].

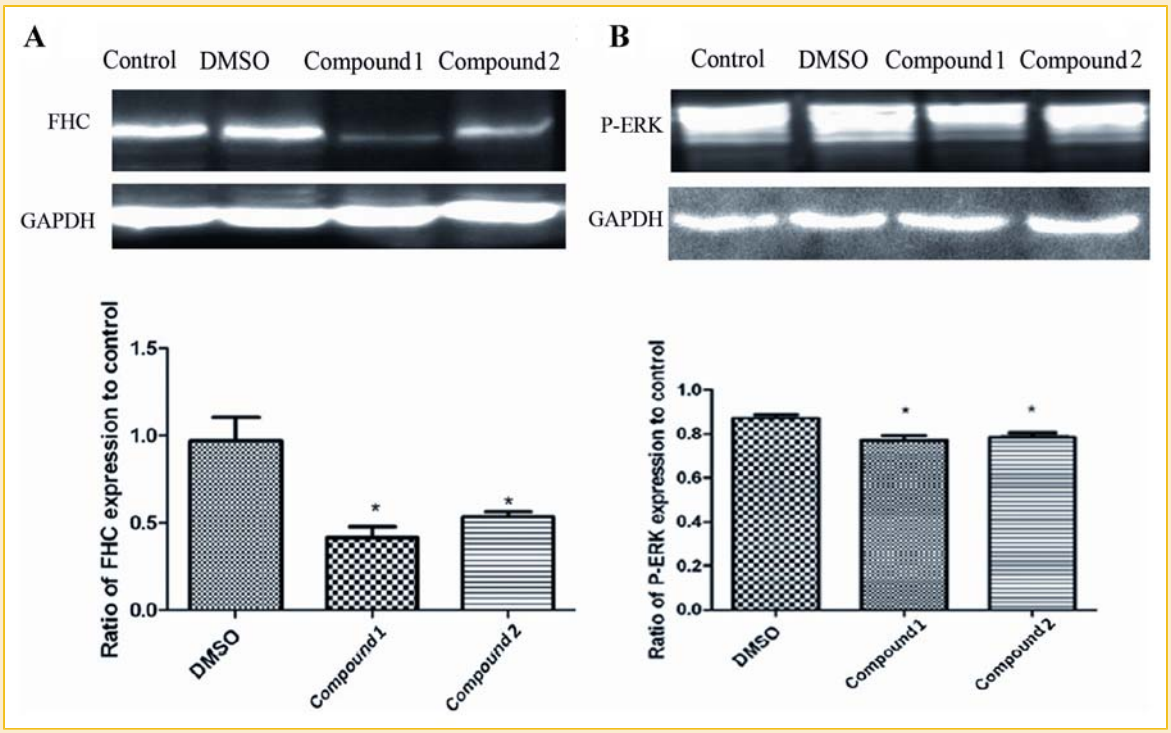

Fig. 8. Western Blot analysis of FHC (A) and P-ERK (B) expression in MCF-7 cells in the presence of compounds 1 (6.5 μM) and 2 (10.1 μM). The experiment was carried out in three independent experiments in triplicate. Data are given as mean ± SE. * $P < 0.05$ vs. DMSO-treated cells.

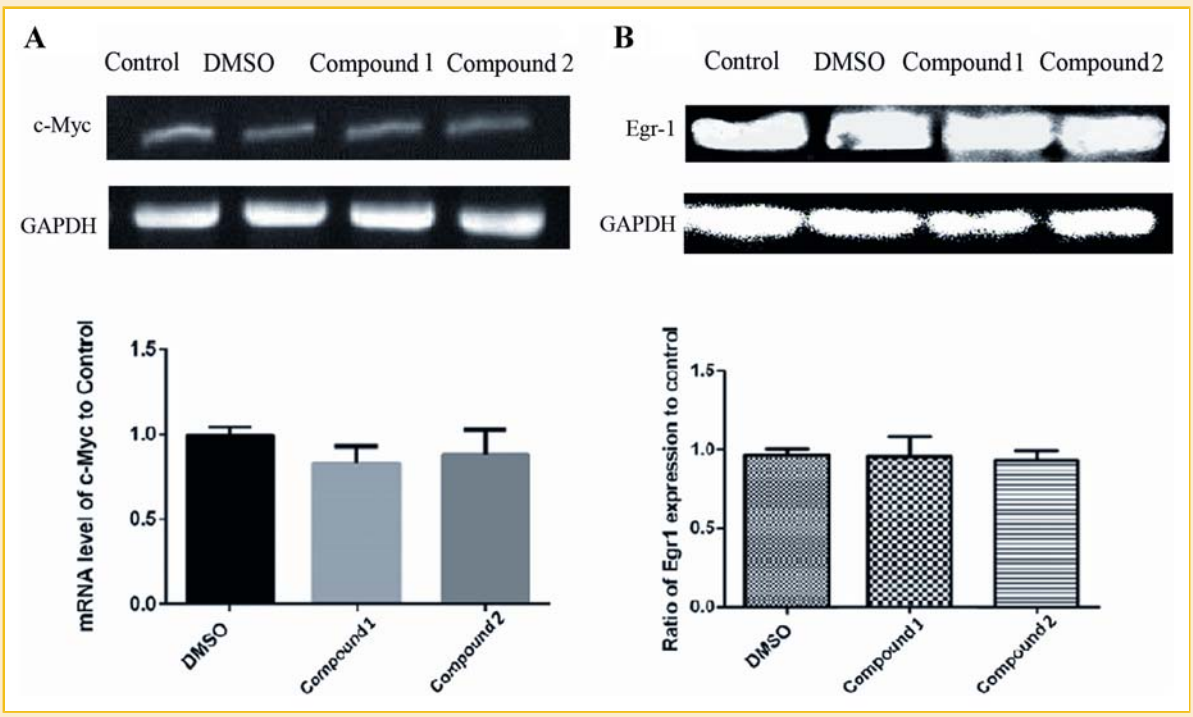

Fig. 9. Effects of compounds 1 (6.5 μM) and 2 (10.1 μM) on c-Myc mRNA levels (A) and EGR-1 expression (B) in MCF-7 cells after 16 h treatment. Expression of GAPDH was used as an internal control. The figure shown is representative of three independent experiments.

These results prompted us to determine Egr-1 protein expression and c-Myc mRNA level in MCF-7 cells treated with compounds **1** and **2** as the novel celecoxib derivatives. Our data indicated that neither compounds could alter c-Myc mRNA levels. These results are in consistent with the reported effects of the selective COX-2 inhibitor (NS-398) which did not change c-Myc mRNA levels in colon mucosa as compared with control [Vaish et al., 2013]. Given that compounds **1** and **2** have also no effect on Egr-1 protein expression, we propose an independent c-Myc/Egr-1 mechanism in apoptosis triggered by the two compounds in MCF-7 cells. However, further research on the true nature of their mechanisms needs to be performed.

CONCLUSION

In summary, we show that compounds **1** and **2**, two novel derivatives of COX-2 inhibitors, activate apoptosis in human breast adenocarcinoma cell line, MCF-7, through different pathways. The mitochondrial pathway and regulation of NF-κB, FHC, and P-ERK expressions are probably involved in MCF-7 induced cell apoptosis by compound **1**. Since these two compounds could not alter the expression of COX-2, the observed effects are likely to be associated with a COX-2 independent pathway. However, the more detailed molecular pathways remain to be clarified. These observations should serve as a reference for further studies to develop compounds **1** and **2** as two chemotherapeutic agents against breast cancer.

ACKNOWLEDGMENTS

This study was financially supported by Pasteur Institute of Iran and Tehran University of Mecical Sciences (TUMS).

REFERENCES

Aggarwal BB. 2004. Nuclear factor-kappa B: the enemy within. Cancer Cell 6:203–208.

Aggarwal BB, Takada Y, Shishodia S, Gutierrez AM, Oommen OV, Ichikawa H, Baba Y, Kumar A. 2004. Nuclear transcription factor NF-kappa B: role in biology and medicine. Indian J Exp Biol 42:341–353.

Balmanno K, Cook SJ. 2009. Tumour cell survival signalling by the ERK1/2 pathway. Cell Death Differ 16:368–377.

Boone DN, Hann SR. 2011. The Myc-ARF-Egr1 pathway unleashing the apoptotic power of c-Myc. Cell Cycle 13:2043–2044.

Boone DN, Qi Y, Li Z, Hann SR. 2010. Egr1 mediates p53-independent c-Myc–induced apoptosis via a noncanonical ARF-dependent transcriptional mechanism. Proc Natl Acad Sci USA 108:632–637.

Boult J, Roberts K, Brookes MJ, Hughes S, Bury JP, Cross SS, Anderson GJ, Spychal R, Iqbal T, Tselepis C. 2008. Overexpression of cellular iron import proteins is associated with malignant progression of esophageal adenocarcinoma. Clin Cancer Res 14:379–387.

Boyle P. 2012. Triple-negative breast cancer: epidemiological considerations and recommendations. Ann Oncol 23:7–12.

Bubici C, Papa S, Pham CG, Zazzeroni F, Franzoso G. 2006. The NF-kB-mediated control of ROS and JNK signaling. Histol Histopathol 21:69–80.

Caffrey AS, Patton WC, Corselli JU, Swensen RE, King A, Chan PJ. 2002. Cyclooxygenase (COX)-2 and granulosa cell apoptosis in vitro. J Assist Reprod Genet 19:577–581.

Cagnol S, Chambard J. 2010. ERK and cell death: mechanisms of ERK-induced cell death apoptosis, autophagy and senescence. FEBS J 277:2–21.

Cai X, Lu W, Yang Y, Yang J, Ye J, Gu Z, Hu C, Wang X, Cao P. 2013. Digitoflavone inhibits IkB-α kinase and enhances apoptosis induced by TNF-α through downregulation of expression of Nuclear Factor kB-regulated gene products in human pancreatic cancer cells. PLoS One 8:e77126.

Chun KS, Surh YJ. 2004. Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. Biochem Pharmacol 68:1089–1100.

Collins S, Lutz MA, Zarek PE, Anders RA, Kersh GJ, Powell JD. 2008. Opposing regulation of T cell function by Egr-1/NAB2 and Egr-2/Egr-3. Eur J Immunol 38:528–536.

Cotterchio M, Krieger N, Sloan M, Steinegart A. 2001. Non-steroidal anti-inflammatory drug use and breast cancer risk. Cancer Epidemiol Biomarkers Prev 10:1213–1217.

Ding XZ, Tong WG, Adrian TE. 2000. Blockade of cyclooxygenase-2 inhibits proliferation and induces apoptosis in human pancreatic cancer cells. Anticancer Res 20:2625–2631.

Dovizio M, Tacconelli S, Sostres C, Ricciotti E, Patrignani P. 2012. Mechanistic and pharmacological issues of asprin as an anticancer agent. Pharmaceuticals 5:1346–1371.

Du H, Li W, Wang Y, Chen S, Zhang Y. 2011. Celecoxib induces cell apoptosis coupled with up regulation of the expression of VEGF by a mechanism involving ER stress in human colorectal cancer cells. Oncol Rep 26:495–502.

Fang G, Kim CN, Perkins CL, Ramadevi N, Winton E, Wittmann S, Bhalla KN. 2000. CGP57148B (STI-571) induces differentiation and apoptosis and sensitizes Bcr-Abl – positive human leukemia cells to apoptosis due to antileukemic drugs. Blood 96:2246–2253.

Gottfried E, Lang S, Renner K, Bosserhoff A, Gronwald W, Rehli M, Einhell S, Gedig I, Singer K, Seilbeck A, Mackensen A, Grauer O, Hau P, Dettmer K, Andreesen R, Oefner P, Kreutz M. 2013. New aspects of an old drug diclofenac targets MYC and glucose metabolism in tumor cells. PLOS ONE 8:1–12.

Harris RE, Alshafie GA, Abou-Issa H, Seibert K. 2000. Chemoprevention of breast cancer in rats by celecoxib, a cyclooxygenase 2 inhibitor. Cancer Res 60:2101–2103.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407:770–776.

Hoffman B, Amanullah A, Shafarenko M, Liebermann D. 2002. The proto-oncogen c-myc in hematopoietic development and leukemogenesis. Oncogene 21:3414–3421.

Igeny FH, Krammer PH. 2002. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2:277–288.

Jendrossek V. 2013. Targeting apoptosis pathways by celecoxib in cancer. Cancer Lett 332:313–324.

Khan Z, Bisen P. 2013. Oncoapoptotic signaling and deregulated target genes in cancers: special reference to oral cancer. Biochim Biophys Acta 1836:123–145.

Kwak EL, Larochelle DA, Beaumont C, Torti SV, Torti FM. 1995. Role for NF-kappa B in the regulation of ferritin H by tumour necrosis factor-alpha. J Biol Chem 270:15285–15293.

Liu X, Yue P, Zhou Z, Khuri FR, Sun SY. 2004. Death receptor regulation and celecoxib-induced apoptosis in human lung cancer cells. J Natl Cancer Inst 96:1769–1780.

Maier TJ, Schilling K, Schmidt R, Geisslinger G, Grosch S. 2004. Cyclooxygenase-2 (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. Biochem Pharmacol 67:1469–1478.

Miralinaghi P, Salimi M, Amirhamzeh A, Norouzi M, Kandelousi HM, Shafiee A, Amini M. 2013. Synthesis, molecular docking study, and anti cancer activity of triaryl-1,2,4-oxadiazole. Med Chem Res 22:4253–4262.

Murphy LO, Blenis J. 2006. MAPK signal specificity: the right place at the right time. Trends Biochem Sci 31:268–275.

Nakanishi C, Toi M. 2005. Nuclear factor-κB inhibitors as sensitizers to anticancer drugs. Nat Rev Cancer 5:297–309.

Norouzi M, Irian S, Divsalar A, Salimi M. 2013. A study on the DNA cleavage morphological changes and cytotoxicity activities of the two synthetic COX-2 inhibitors. J Rep Pharm Sci 2:38–44.

Pham CG, Bubici C, Zazzeroni F, Papa S, Jones J, Alvarez K, Jayawardena S, De Smaele E, Cong R, Beaumont C, Torti FM, Torti SV, Franzoso G. 2004. Ferritin heavy chain upregulation by NF-kB inhibits TNFα-induced apoptosis by suppressing reactive oxygen species. Cell 119:529–542.

Recalcati S, Invernizzi P, Arosio P, Cairo G. 2008. New functions for an iron storage protein: the role of ferritin in immunity and autoimmunity. J Autoimmun 30:84–89.

Ricci MS, Zong WX. 2006. Chemotherapeutic approach for targeting cell death pathways. Oncologist 1:342–357.

Roperto S, Borzacchiello G, Brun R, Costanzo F, Faniello MC, Raso C, Rosati A, Russo V, Leonardi L, Saracino D, Turco MC, Urraro C, Roperto F. 2010. Ferritin heavy chain (FHC) is up-regulated in papillomavirus-associated urothelial tumours of the urinary bladder in cattle. J Comp Path 142:9–18.

Salimi M, Ardestaniyan MH, Mostafapour Kandelous, Saeidnia H, Gohari S, Amanzadeh AR, Sanati A, Sepahdar H, Ghorbani Z, Salimi S. 2014. Antiproliferative and apoptotic activities of the constituents of chloroform extract of *Juglans regia* leaves. Cell Prolif 47:172–179.

Sheikh MS, Huang Y. 2000. Death receptor activation complexes. Cell Cycle 6:550–552.

Simstein R, Burow M, Parker A, Weldon C, Beckman B. 2003. Apoptosis, chemoresistance and breast cancer: insights from the MCF-7 cell model. Exp Biol Med 228:995–1003.

Sobolewski C, Crella C, Dicato M, Diederich M. 2011. COX-2 inhibitors induce early c-Myc downregulation and lead to expression of differentiation markers in leukemia cells. Cell Cycle 17:2978–2993.

Subhashini J, Mahipal SVK, Reddanna P. 2005. Anti-proliferative and apoptotic effects of celecoxib on human chronic myeloid leukemia in vitro. Cancer Lett 224:31–43.

Takada Y, Bhardwaj A, Potdar P, Aggarwal BB. 2004. Nonsteroidal anti-inflammatory agents differ in their ability to suppress NF-κB activation, inhibition of expression of cyclooxygenase- 2 and cyclin D1, and abrogation of tumor cell proliferation. Oncogene 23:9247–9258.

Thun MJ, Henley SJ, Patrono C. 2002. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic and clinical issues. J Natl Cancer Inst 94:252–266.

Torti FM, Torti SV. 2002. Regulation of ferritin genes and protein. Blood 99:3505–3516.

Vaish V, Piplani H, Rana C, Vaiphei K, Sanyal SN. 2013. NSAIDs may regulate EGR-1-mediated induction of reactive oxygen species and non-steroidal anti-inflammatory drug-induced gene (NAG)-1 to initiate intrinsic pathway of apoptosis for the chemoprevention of colorectal cancer. Mol Cell Biochem 378:47–64.

Yang HL, Chen CS, Chang WH, Lu FJ, Lia YC, Chen CC, Hseu TH, Kuo CT, Hseu YC. 2006. Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by Antrodia camphorate. Cancer Lett 231:215–227.

Zarghi A, Arfaei S, Ghodsi R. 2012. Design and synthesis of new 2,4,5-triarylimidazole derivatives as selective cyclooxygenase (COX-2) inhibitors. Med Chem Res 21:1803–1181.