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Original Article

Caspase Activation, Upregulation of Bax and P53, Downregulation of Bcl-2 and Enhanced Generation of Reactive Oxygen Species Mediate the Anti-proliferative and Anti-migratory Activity of Mefenamic Acid in Breast Cancer Cells

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ABSTRACT

Background: Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are among the most prescribed pharmaceuticals worldwide. Similar to other NSAIDs, mefenamic acid possesses anti-inflammatory, analgesic and antipyretic properties. The present study was carried out to further elucidate the antiproliferative activity of mefenamic acid at the molecular level in the human breast cancer cell line (MCF-7). **Materials and Methods:** The cytotoxic effect of mefenamic acid on breast cancer cells (MCF-7) was assessed using MTT cytotoxicity and Lactate Dehydrogenase (LDH) leakage assays. qRT-PCR analysis was employed to evaluate the expression of the apoptosis-related genes encoding p53, caspases-3, -8 and-9, Bax and Bcl-2. The anti-migratory effect of mefenamic acid on breast cancer cells was evaluated by using the scratch assay. H2DCFDA (H2-DCF, DCF) cellular assay was employed to estimate the intracellular generation of Reactive Oxygen Species (ROS) in breast cancer cells. **Results:** A significant decrease in the viability (survival rate) of MCF-7 cells was revealed by MTT cytotoxicity and LDH release assays. Compared to the untreated control cells, there were over-expression of p53, caspases-3, -8 and-9 activities. Bax was significantly upregulated and conversely, Bcl-2 showed significant downregulation. Mefenamic acid significantly inhibited the migration of MCF-7 cells as evidenced by the relatively increased distance between the opposing scratch edges. Comparatively, mefenamic acid enhanced the intracellular generation of ROS in breast cancer cells. **Conclusion:** Based on the present findings, it was concluded that mefenamic acid can exert anti-proliferative and anti-migratory activity in the human breast cancer cell line. Mefenamic acid could be considered a promising anti-cancer agent in combination with other therapies in the treatment of breast cancer.

Keywords: Antimigratory, Antiproliferative, Breast cancer cells, Cytotoxicity, Gene expression, Mefenamic acid.

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INTRODUCTION

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) have been originally synthesized as anti-inflammatory drugs to replace the use of steroidal drugs.[1] This group of drugs is ranked among the most prescribed pharmaceuticals worldwide.[2] The chemical structure of NSAIDs encompasses a carboxylic group (hydrophilic) and an aromatic group (lipophilic) and their chemical categories involve N-arylanthranilic acids, pyrazolone, oxicams and coxibs.[3]

Mefenamic acid (C_{15} H_{15} NO_2) (a derivative of N-arylanthranilic acid) is a member of NSAIDs; it belongs to the organic compounds under the class "aminobenzoic acids". Mefenamic acid, similar to other NSAIDs, is widely used because of its anti-inflammatory, analgesic and antipyretic pharmacological properties.[4] It is used to relieve pain mild to moderate pain as in cases of headache, dental pain, menstrual cramps, post-operative pain and dysmenorrhea,

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as well as the pain associated with the inflammatory processes in some disease conditions such as osteoarthritis and rheumatoid arthritis.

Mefenamic acid shares a common mechanism of action with other NSAIDs; this mechanism relies on the inhibition of synthesis and/or activity of substances that stimulate pain sensation. This mechanism is accomplished through inhibition of the activity and expression of Cyclooxygenase 2 (COX-2), which is crucial for the synthesis of prostaglandins (pain-stimulating substances) through its reaction with prostaglandin synthase.[5]

Cancer cells are known to undergo genetic changes and mutations (mutant cells) and their paramount feature is uncontrolled, uncoordinated and continued proliferation. Mutations are raised as an eventual consequence of the activation of proto-oncogenes or alternatively inhibition of tumour suppressor genes.[6] The uncontrolled *in vivo* proliferation of cancer cells results in the development of growths (tumours) with gradual progression and invasion of the surrounding tissues and may be metastasis to remote sites.

Cancer is one of the most common global diseases; it accounts for millions of deaths. According to expectations and prediction studies, the resultant mortality rate might be considerably increased during the next 20 years.[7] Breast cancer is ranked among the outstanding global medical concerns; its incidence is expected to exceed 20 million/year by 2025 as suggested by demographic studies. The predisposing risk factors that are known to increase susceptibility to developing breast cancer include genetic factors and older age.[8]

Many forms of inflammatory processes are associated with the development of cancer and the progression of both inflammation and cancer bears similarities. It is worth mentioning that COX-2, through accelerating synthesis of prostaglandins, maintains the inflammatory reactions associated proliferation, progression and dissemination of cancer. Moreover, prostaglandin E (2) promotes tumour growth through binding with specific cell receptors and subsequently initiates the signalling of cancer cell proliferation migration and angiogenesis. This explains why cancer cells exhibit overexpression of COX-2 (a trigger for cancer growth), which is essential for the synthesis of prostaglandins.[9] It is interesting to note that NSAIDs through the same mechanism (COX inhibition) can counteract the inflammatory processes and on the other side inhibit the initiation and growth of tumours. Moreover, it has been concluded that NSAIDs have higher antiproliferative activity in COX-2-expressing cancer cells compared to the non-expressing cells.[8]

The association between the use of NSAIDs and the risk of breast cancer has been focused in a considerable number of epidemiological studies.[10] The results of these studies are contradictory, while some studies reported a decreased risk of breast cancer associated with the regular use of NSAIDs, the other studies failed to prove such a correlation. Some researchers reported an inverse correlation between the use of NSAIDs and the incidence of breast cancer. Moreover, it was concluded that the correlation between the regular use of NSAIDs and breast cancer risk is not dependent on the hormone receptor status of the tumour.[11]

NSAIDs have been reported to enhance the sensitivity and responsiveness of cancer cells to chemotherapeutic agents, i.e., overcome the anticancer drug resistance, through inhibition of Aldo-Keto Reductase 1C (AKR 1Cs). In this regard, the prognosis rate was reportedly increased when a combined therapy of mefenamic acid and a chemotherapeutic agent was applied to counteract the resistance to antineoplastic drugs. Furthermore, mefenamic acid was found to enhance the sensitization of cancer cells to ionizing radiation (radiosensitizing effect). This implies a synergistic effect of mefenamic acid with ionizing radiation to induce cancer cell death.[12]

In addition to their therapeutic use as analgesics in the treatment of cancer, some NSAIDs, as potent COX-2 inhibitors, are used as antiproliferative (antineoplastic) agents. NSAIDs can be employed as a part of the therapeutic protocol of cancers to constitute synergism with other chemotherapeutic agents. This synergism is beneficial to reduce the required doses of the chemotherapeutic agents and in turn, minimizes their hazardous side effects.

The anti-proliferative activity of NSAIDs has been investigated in cancer cell lines as well as in clinical trials. Collectively, this activity was attributed to the suppression or blocking of the signaling pathways that account for the survival and proliferation of cancer cells. The inhibitory effect of NSAIDs on COX-2 expression is a good example of these pathways.

It is worth mentioning that NSAIDs have been shown to exert their anti-profliferative effect against both COX-2-expressing and non-expressing cancer cells.[13] This approves that the antiproliferative activity of NSAIDs is not restricted to COX inhibition (COX-independent). The main COX-independent pathways include caspase activation and modulation of the activities of Bcl-2, Nuclear Factor kappa B (NF-kB) and Mitogen-Activated Protein (MAP)-kinase. According to the targeted biomolecules, some of these COX-independent pathways are operated through upregulation mechanisms and others through downregulation ones.[14] In this regard, NSAIDs inhibit activation of NF-kB that accounts for the upregulation of antiapoptotic proteins and thus promote initiation of apoptosis and reduce proliferation of cancer cells. On the other hand, NSAIDs activate MAP-kinase that regulates cell survival and, in this way, initiate apoptosis.[7] These mechanisms were revealed in cancer cell lines as well as in experimental models that focused on the effect of NSAIDs on the regression of tumour size, reducing angiogenesis and inhibiting the invasiveness of tumour cells. Inhibition of platelet-derived growth factor is

another COX-independent mechanism of NSAIDs to suppress tumour growth. NSAIDs can exert their suppressive effect on the proliferation and survival of cancer cells through modulation of the gene expression related to tumorigenesis. This is exemplified by the upregulation of p53 gene expression and down-regulation of c-MYC expression.[11]

The antiproliferative (anti-cancer) property of NSAIDs has been focused in a considerable number of studies. The antiproliferative activity of mefenamic acid in breast cancer cells has been reported in a limited number of published studies. However, the reported findings are contradictory, while some researchers concluded a relevant activity of mefenamic acid; others failed to approve the selective cytotoxic effects of mefenamic acid on breast cancer cells.[14]

Therefore, the present study was conducted for more elucidation of the tested antiproliferative activity of mefenamic acid at the molecular level in a breast cancer cell line.

MATERIALS AND METHODS

Type of sampling and reasons for selection

The human breast cancer cell line (MCF-7) was selected as the investigated cell line in the present study. This cell line was chosen to evaluate the antiproliferative and anti-migratory activity of mefenamic acid in breast cancer cells.

Inclusion criteria

All breast cancer cells treated with mefenamic acid were included in the present study.

Exclusion criteria

No exclusion criteria were applied in the current study. In other words, all mefenamic acid-treated cells were included in the conducted assays. Any exclusion criteria can alter in different ways the accuracy of the performed analysis.

Mefenamic acid

Mefenamic acid (2-[(2,3-Dimethylphenyl)amino]benzoic acid,*N*-(2,3-Xylyl) anthranilic acid). ($C_{15}H_{15}NO_2$) (MW 241.9) (MFCD 00051721) was procured from Sigma-Aldrich (Darmstadt, Germany).

MTT cytotoxicity assay

MTT(3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for evaluation of the cytotoxic effect of mefenamic acid on MCF-7 cells. Accordingly, the viability and survival rate of cancer cells exposed to mefenamic acid can be determined. The assay is based on the finding that only viable and metabolically active cells can reduce MTT into purple formazan, which is the measurement target. MCF-7 cells were preincubated at a concentration of 1×10^6 cells/mL in culture medium (with 1 ug/mL actinomycin C1) for 3 hr at 37°C and 5% CO_2. The cultured cells were then seeded at a concentration of 5×10^4/well into a microplate (tissue culture grade, 24 wells, flat bottom) in 100 µL culture medium containing mefenamic acid at different concentrations (50, 100, 200, 300, 400 and 500 µg/mL) and incubated for 24 hr at 37C and 5% CO_2. Thereafter, 10 µL of MTT labelling reagent (final concentration 5 mg/mL MTT in 1x PBS) was added to each well and the microplate was then incubated for 4 hr in a humidified atmosphere (37°C, 5% Co_2). Acidified isopropanol solution (100 µL) was then added to each well for more solubilization of the reduced MTT (purple formazan) and then the microplate was incubated overnight in a humidified atmosphere. The complete solubilization of the purple formazan was checked, the darker the solution, the greater the number of viable, metabolically active cells. Quantification of the formazan product was made by measuring absorbance at 570 nm using a microplate reader (Bio Tek instruments Inc., USA). Calculation of IC_{50} (the dose sufficient to induce inhibition of cell growth in 50% of treated cells) of mefenamic acid was based on a dose-response curve using software (OrignPro software).

Lactate Dehydrogenase (LDH) leakage assay

LDH leakage (cytotoxicity) assay is a colourimetric assay that provides a reliable method for determining cytotoxicity. The assay was used for the determination of the cytotoxic effect of mefenamic acid on MCF-7 cells. The assay idea is based on the finding that LDH is a cytosolic enzyme that is released into the cell culture medium upon damage to the cell plasma membrane. The assay was performed according to the method previously described. At a concentration of $5\times10^{4/}$well, MCF-7 cells were seeded in a microplate (24-well) and incubated for 24 hr. The cultured cells were then treated with mefenamic acid (final concentration, 50 µg/mL) and incubated for 48 hr. The leakage of LDH was determined in the cell-free supernatant of the cell culture using an enzyme kit containing NADH and the LDH substrate sodium pyruvate (LDH mixture). The rate of NADH decrease was determined by spectrophotometry and considered proportional to the activity of LDH in the supernatant. The obtained supernatant (100 µL) was transferred to a microplate (96-well) and 100 MI LDH detection solution prepared from an LDH kit (Sigma-Aldrich, USA) was added. The reaction was left for 30 min incubation for colour development and quantification of the enzyme product was made by measuring absorbance at 490 nm using a microplate reader (Bio Tek Instruments Inc., USA).

qRT-PCR analysis

Quantitative Real-Time reverse transcription PCR (qRT-PCR) allows for enumeration of the number of mRNA copies of the gene. It combines the effects of reverse transcription or real-time PCR and quantitative PCR to amplify and detect specific targets, one of its applications is quantifying gene expression levels. The analysis was performed to evaluate the expression of the genes

(apoptosis-related genes) encoding p53, caspases-3, -8 and-9, Bax and Bcl-2. MCF-7 cells (5×10^4 cells/well) treated with mefenamic acid (final concentration 0.5 IC_{50}, IC_{50}=123.5 µg/mL) were incubated for 24 hr. Then, the QIAzol Lysis Reagent (Qiagen, Cat. No. 79306, USA) was added and the total RNA content was estimated in the cell lysates. Accordingly, synthesis of cDNA was done by employing the oligo dT primers and using the Superscript II Reverse Transcriptase cDNA Synthesis Kit (Applied Biosystems, Thermo Fisher Scientific, Lithuania). Instructions from the manufacturer were followed to perform a Polymerase Chain Reaction (PCR) in a Rotor-Gene machine (PrimeQ, China) with the use of primers specific to the relevant genes (Table 1). Expression of mRNA relevant to each gene was evaluated, using GAPDH as the internal reference, according to the equation: Fold change=$2^{-\Delta\Delta CT}$.

Scratch assay

Scratch wound assay is based on the creation of a gap in confluent monolayer, mimicking a wound and subsequently, cell migration is evaluated in the presence of the tested substance. The required confluence (70-80%) of MCF-7 cells was attained by culturing in a 12-well microplate. A fine scratch was made in the monolayer using a 10 uL pipette tip. Detached cells were removed by washing with PBS and the culture medium was replaced by a fresh one. Mefenamic acid was added at a concentration of 10 µg/mL (non-cytotoxic concentration). Sequential imaging was then done (0 hr, 24 hr and 48 hr) by employing a Leica MC-170 HD camera (Leica, Germany) attached to a phase contrast inverted microscope. The obtained images were analyzed using Image J software (NIH, USA). The following equation was applied to calculate the cell migration ratios from the scratch edges: Relative migration ratio=0 hr distance (between the opposing scratch edges)-24 hr distance/0 hr distance.

H_2DCFDA (H_2-DCF, DCF) cellular ROS assay

The assay was employed to determine and assess the generation of excess Reactive Oxygen Species (ROS) in MCF-7 cells treated with mefenamic acid. The assay is dependent on the fluorescent dye 2,7-Dichlorohydroflurescin Diacetate (H_2 DCFDA) (known as dichlorofluores cin diacetate), which is a cell-permeant reagent. Chemically, this dye is the reduced form of fluorescein that is used as a fluorometric detector of intracellular generation of ROS. The detection mechanism is based on the cleavage of the acetate groups of the fluorescent dye by the intracellular oxidation, the nonfluorescent H_2DCFDA is converted to the highly fluorescent 2,7-Dichlorofluorescein (DCF). The assay was done using ROS indicator (Invitrogen, ThermoFisher Scientific, USA). Using a 96-well microplate, MCF-7 cells treated with mefenamic acid IC_{50} were seeded in a microplate ($5x10^4$/ well) and incubated at 37°C for 24 hr. The culture medium was then replaced in the dark by 100 µL diluted H_2DCFDA fluorescent dye (1 µL dye added to 99 µL MEME) and the microplate was re-incubated for 2 hr. Absorbance was assessed at 495 nm (excitation) and 510 nm (emission) using a microplate reader (Microplate Reader-Gen 5, Bio Tek Citation 5, USA). The relative ROS generation was measured according to the equation:

Relative ROS generation (%)=Optical Density (OD) of MCF-7 cells exposed to mefenamic acid/OD of control untreated cellsx100.

RESULTS

MTT cytotoxicity assay

The cytotoxicity assessed by MTT assay revealed that IC_{50} (123.5 µg/mL) of mefenamic acid reduced the viability of treated cancer cells by 56%, i.e. there was only a 44% survival rate among treated breast cancer cells compared to the viability of control untreated MCF-7 cells. The viability curve used to determine the IC_{50} of mefenamic acid showed an inverse dose-dependent pattern between the tested concentrations of mefenamic acid and the viability % of treated cells (Figure 1).

Table 1: Primer sequence (F, forward and R, reverse) and targets (GAPDH, p53, Bax, Bcl-2 and Caspases-3, -8 and-9).

Target	Primer sequence
GADPH	F: 5'-GGT ATC GTG GAA GGA CTC ATG AC-3' (23 mer). R: 5'-ATG CCA GTG AGC TTC CCG TTC AGC-(24 mer).
Bax	F: 5'-GGA TGC GTC CAC CAA GAA G-3' (19 mer). R: 5'-CCT CTG CAG CTC CAT GTT AC-3' (20 mer).
Bcl-2	F: 5'-GTC GAT GAC TGA GTA CCT GAA C-3' (22 mer). R: 5'-GCC AGG AGA AAT CAA ACA GAC G-3' (22 mer).
Caspase-3	F: 5'-CTG GTT TTC GGT GGG TGT G-3' (19 mer). R: 5'-ACG GCA GGC CTG AAT AAT GAA (21 mer).
Caspase-8	F: 5'-CTG GTC TGA AGG CTG GTT GT-3' (20 mer). R: 5'-CAG GCT CAG GAA CTT GAG GG-3' (20 mer).
Caspase-9	F: 5'-CAG GCC CCA TAT GAT CGA GG-3' (20 mer). R: 5'-TCG ACA ACT TTG CTG CTT GC-3' (20 mer).
p53	F: 5' CCCAGCCAAAGAAGAAACCA 3' R: 5' TTCCAAGGCCTCATTCAGCT 3'

LDH leakage assay

The results of the Lactate Dehydrogenase (LDH) assay were in parallel with those of the MTT cytotoxicity assay. There was a significantly higher release of LDH from mefenamic acid-treated cells (IC_{50} dose) compared to that of control untreated breast cancer cells (Figure 2).

qRT-PCR analysis

Significant overexpression (measured in fold change) of p53, caspases-3, -8 and-9 were detected in cancer cells treated with Mefenamic acid compared to control untreated MCF-7 cells. The analysis also revealed significantly upregulated Bax (apoptotic) and downregulated Bcl-2 (antiapoptotic) in the treated breast cancer cells (Figure 3).

Scratch assay

Mefenamic acid significantly inhibited migration of MCF-7 cells compared to the control untreated cancer cells at 24 and 48 hr as evidenced by the measured distance between the opposing edges of the scratch (Figure 4).

ROS assay

A significant increase in the generated ROS was recorded in MCF-7 cells exposed to an IC_{50} dose of mefenamic acid compared to the untreated control cancer cells (Figure 5).

DISCUSSION

The present study focused on mefenamic acid, a commonly used NSAID, to test its effects on the viability, proliferation and migration of human breast cancer cells.

The antiproliferative activity of NSAIDs has been reported in cancer cell lines as well as in clinical trials.[15] In this regard, the basic activities of NSAIDs involve inhibition of cancer cell proliferation and enhancing apoptosis of these cells, as well as reducing tumour regression, angiogenesis and invasiveness. Significant regression of tumour size (inhibited tumour growth)

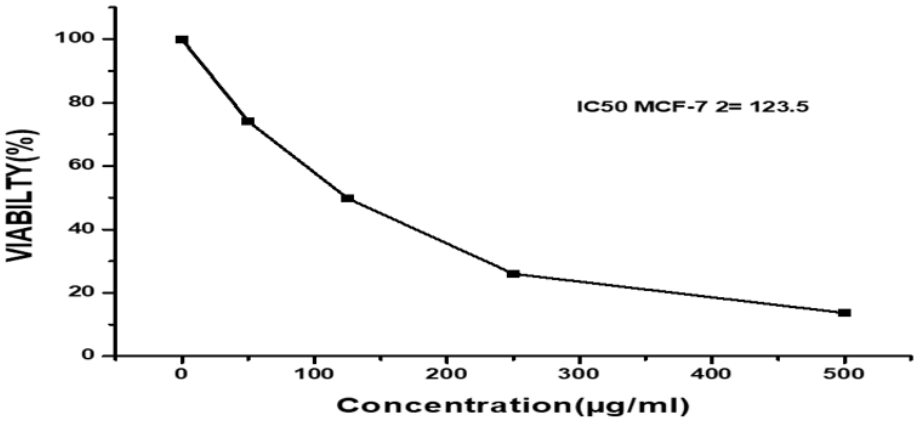

Figure 1: MTT assay cytotoxicity curve. Cytotoxicity induced by mefenamic acid in MCF-7 cells is dose-dependent. IC_{50} of mefenamic acid=123.5 µg/mL.

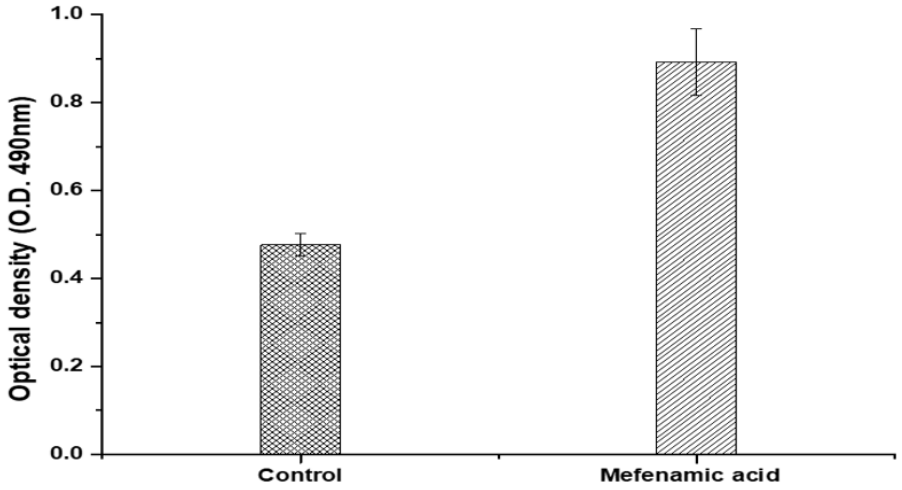

Figure 2: Lactate Dehydrogenase (LDH) leakage assay. MCF-7 cells treated with mefenamic acid exhibited significant release of LDH compared to the control untreated cells.

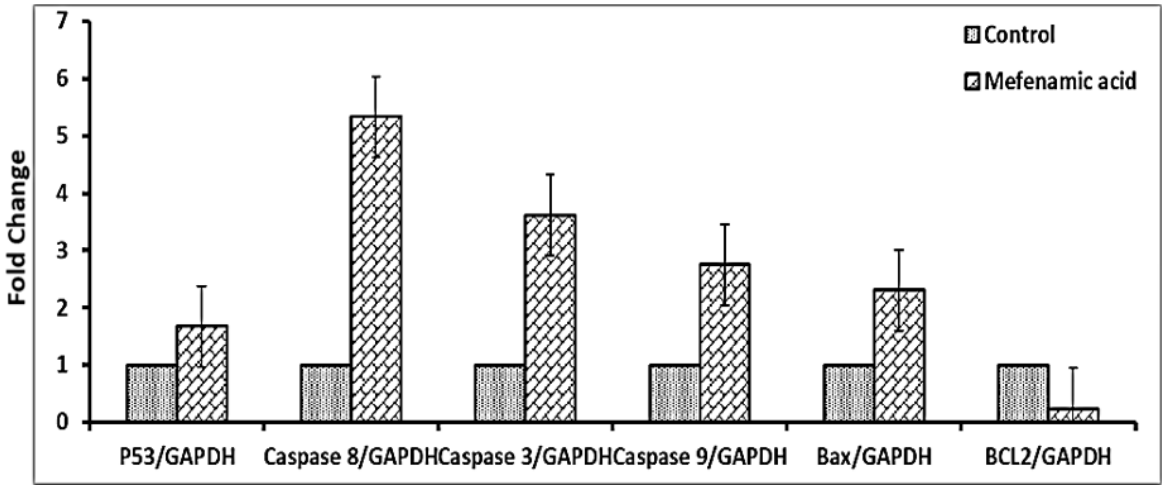

Figure 3: qRT-PCR analysis. MCF-7 cells treated with mefenamic acid exhibited significant over expression (measured in fold change) of p53, caspases-3, -8 and-9 compared to control untreated MCF-7 cells. The analysis also revealed significantly upregulated Bax (apoptotic) and downregulated Bcl-2 (antiapoptotic) in the treated breast cancer cells.

has been reported in laboratory animals administered with mefenamic acid. The synergistic effect of mefenamic acid with other cancer therapies has been documented; this is exemplified by the reported synergism between mefenamic acid and ionizing radiation in experimental animals showing colon cancer.[16] The positive impact of this anti-tumour synergism was attributed to the aggravated histopathological changes in tumour tissue due to the significantly enhanced immunoreactivity of caspase-3. Moreover, it has been demonstrated that mefenamic acid enhances the sensitivity to anti-cancer drugs, i.e. helps overcome anti-cancer drug resistance by modulating the biological function of AKR 1 C.

The current results showed the activity of caspases-3, -8 and-9 in breast cancer cells treated with Mefenamic acid. Caspases are cysteine proteases that are crucial for initiation and inducing apoptosis. The caspase cascade is initiated by proapoptotic signals and then the initiator caspases (caspases-2, -8,-9 and-10) activate the executioner ones (caspases-3, -6 and-7).[17] Executioner caspases account for the cleavage of nuclear proteins, plasma membrane proteins and mitochondrial proteins (massive proteolysis) and degradation of PARP with eventual apoptotic cell death. It has been shown that mefenamic acid can induce *in vivo* apoptosis in oral malignant Burkitt's lymphoma through the caspases-3 and-9 pathways (intrinsic apoptosis).[15]

In the presently studied breast cancer cells, mefenamic acid significantly enhanced the activity of caspases-3, -9 and-8, which implies initiation and activation of both intrinsic and extrinsic pathways of apoptosis. The increased activity of caspases-3 and-9 indicates the initiation of apoptotic events and triggering of proteolytic activity. In the case of caspase-8 activation, it directly causes cleavage and activation of caspase-3 (extrinsic pathway), caspase-8 may also activate caspase-9 which in turn activates caspase-3 (intrinsic pathway). Alternatively, caspase-8 may cause cleavage of Bcl-2 (antiapoptotic protein) or Bid with subsequent release of mitochondrial cytochrome-c and formation of apoptosomes (apoptotic activating protein-1, Apaf-1 bound to caspase-9) which in turn activate caspase-3.[18]

The antiproliferative effect of mefenamic acid has been shown in some cancer cell lines. Some studies emphasized activation of caspases, with the induced apoptosis and COX-2 inhibition as the main antiproliferative mechanism. The contribution of caspase activation was approved by the finding that caspase-3 inhibitors offer protection against apoptosis induced by mefenamic acid in the colon and hepatic cancer cells.[19]

On the other hand, some researchers focused on alternative mechanistic pathways that account for the anticarcinogenic effect of mefenamic acid. In this regard, mefenamic acid has been shown to suppress proliferation, invasiveness and angiogenesis of hepatocellular carcinoma cells by the mean of inhibitory effect on Platelet-Derived Growth Factor (PDGF) and COX-2, upregulation of P53 and tumour suppressor genes and down-regulation of vascular endothelial growth factor.[20] Mefenamic acid was found to inhibit calcium influx (uptake) in cancer cells and this was interpreted as a signal by mefenamic acid to initiate the events of apoptosis. It was also concluded that the anti-neoplastic property of mefenamic acid is achieved through potent inhibitory effects on the activities of Aldo-Keto Reductase (AKR) and COX-2.[21]

The present findings involve significant up-regulation of Bax (apoptotic) and down-regulation of Bcl-2 (antiapoptotic). NSAIDs interfere with the mechanisms that enable cancer cells to survive and proliferate; inhibition of cyclin and AKt expression is an example of these mechanisms to arrest the cell cycle and initiate apoptosis.[19] NSAIDs can modulate the expression of Bcl-2 family proteins that regulate apoptosis; this family comprises Bax, Bid, Bcl-2 and Bcl-X. NSAIDs inhibit Bcl-2 (antiapoptotic protein) expression and enhance Fas and Bax (apoptotic proteins) expression. Overexpression of Bcl-2 inhibits the

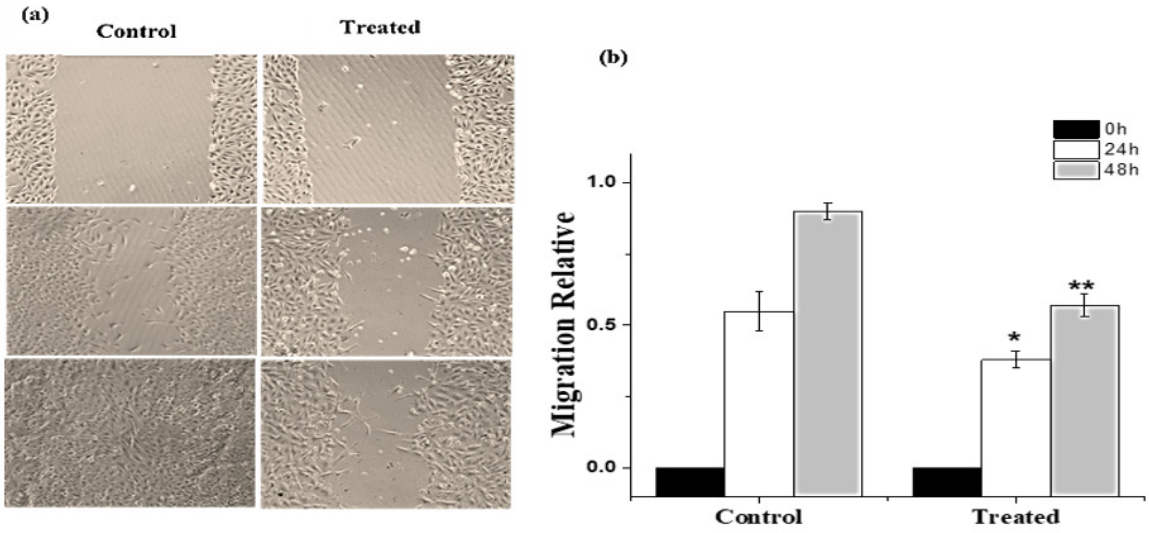

Figure 4: Effect of mefenamic acid on migration of MCF-7 cells. Migration of mefenamic acid-treated cells and control untreated cells were monitored at 0, 24 and 48 hr post-incubation. (a) Control untreated cells (left), (b) Treated cells (right). Measurements of three replicates were represented as mean±SD, *p<0.05, **p<0.01.

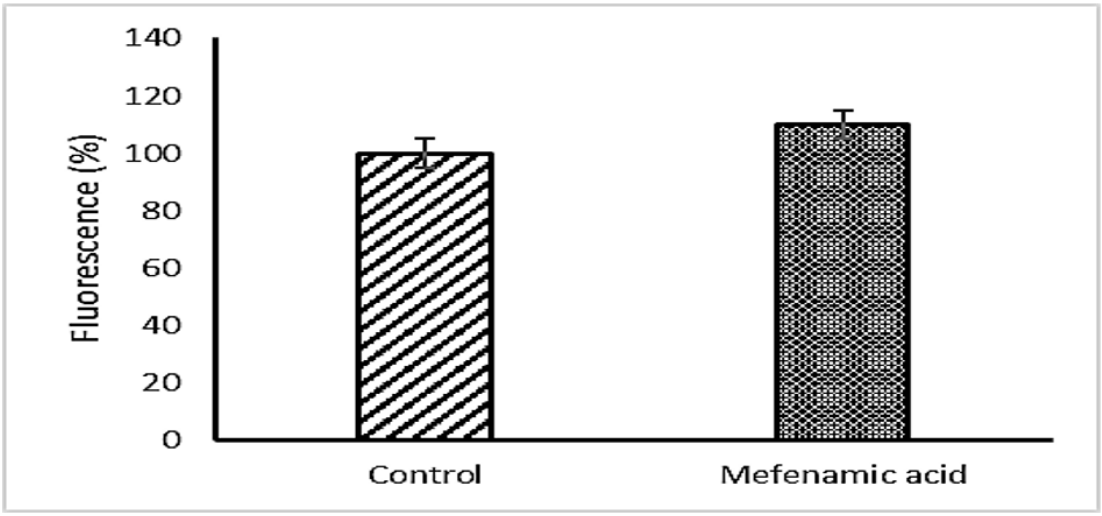

Figure 5: H_2DCFDA (H_2-DCF, DCF) cellular ROS assay. MCF-7 cells treated with mefenamic acid showed enhanced generation of ROS compared to the untreated control cells.

intrinsic pathway of apoptosis through inhibition of cytochrome c release from mitochondria.[20] Therefore, enhanced expression of Bcl-2 implies the maintaining of mitochondrial integrity and the inhibitory effect on apoptosis. Release of cytochrome c from the damaged mitochondria is preceded by translocation of Bax to mitochondria.[21] The present breast cancer cells treated with mefenamic acid exhibited inhibited Bcl-2 expression and enhanced Bax expression. Accordingly, it is logical to conclude that mitochondria represent a key proapoptotic station in the mefenamic acid-induced apoptotic cell death. This conclusion is supported by the previously reported mitochondrial changes, such as disrupted oxidative phosphorylation, inhibited electron transport chain and membrane transition associated NSAIDs-induced apoptosis.[22]

The present findings, as evidenced by MTT cytotoxicity and LDH leakage assays, demonstrated that the survival percentage of breast cancer cells treated with mefenamic acid was significantly decreased. Some studies reported up to 35% loss of viability in cancer cells treated with mefenamic acid. However, the presently estimated loss of viability in the treated cells reached 44%.

As evidenced by the scratch assay, the present results showed the significant inhibitory effect of mefenamic acid on the migration of the treated breast cancer cells. The suppressive effect of NSAIDs on cancer cell migration and viability was related to their ability to downregulate Wnt/β-catenin signalling.[23]

The currently treated breast cancer cells exhibited a significant increase in the generated intracellular Reactive Oxygen Species (ROS). These reactive species include radical members such as superoxide anions, peroxyl radicals and hydroxyl radicals, as well

as nonradical members like hydrogen peroxide. In healthy cells, the generation of ROS occurs at a controlled rate as a natural consequence of aerobic energy metabolism; but under conditions of oxidative stress, ROS generation is dramatically increased. ROS exerts a deleterious oxidation effect on membrane lipids, proteins and nucleic acids.[24,25]

The anti-proliferative effects of NSAIDs may be mediated by the induced generation and accumulation of intracellular ROS and the associated oxidative stress. The resultant oxidative stress constitutes a proapoptotic event that acts synergistically with other proapoptotic factors. The accumulated ROS in mitochondria and the associated redox imbalance were found to be highly effective in releasing mitochondrial cytochrome c. This is followed by caspase activation and eventually apoptosis (redox-mediated apoptosis).[26-28] The extensive mitochondrial damage, because of oxidative stress, induced by NSAIDs disrupts cellular respiration in cancer cells.[29] Moreover, during oxidative stress, the biomolecules in cancer cells are oxidized and act as activators to MAP kinase and inhibitors to NF-kB and in this way, the events of apoptosis are accelerated.[24]

Because of the risk of metastasis, surgical intervention is avoided in patients with breast cancer,[21] and those patients are treated with chemotherapy, which exposes them to hazardous side effects. Moreover, some of these patients develop a varied degree of drug resistance. For these reasons, therapeutic agents possessing anti-cancer properties with no or minimal drug resistance become an urgent need. Furthermore, the use of therapeutic agents that target cancer cells only with no effects on normal cells contributes greatly to avoiding the side effects.[30] Some of the available NSAIDs, including mefenamic acid, might meet these requirements and be considered promising anti-neoplastic agents if employed in a synergism with other cancer therapies.[31]

CONCLUSION

The present findings support the conclusion that mefenamic acid can induce apoptotic cell death of breast cancer cells through both intrinsic and extrinsic apoptotic pathways. The presently estimated significant decrease in the survival rate of cancer cells approves that apoptotic cell death is an efficient activity of mefenamic acid to block the proliferation of cancer cells.

The revealed efficiency of mefenamic acid in the present study to induce cytotoxic effects in breast cancer cells reinforces their use as a synergistic therapeutic agent in association with other breast cancer therapies. This effective synergism serves to decrease the recommended doses of chemotherapeutic agents to avoid the risk of their side effects.

In conclusion, the present study might provide evidence that mefenamic acid is a promising anti-cancer agent (anti-neoplastic drug) in association with other therapies in the treatment of breast cancer.

OUTCOMES OF THE STUDY

The results of the present study give evidence that mefenamic acid can exert an antiproliferative and anti-migratory effect on human cancer cells.

THE RATIONALE OF THE STUDY

Given the results from the investigation of the treated cancer cell line, it is reasonable to conclude that mefenamic acid has antiproliferative and anti-migratory properties on the human breast cancer cell line under investigation.

LIMITATIONS OF THE STUDY

The current results provide evidence of the antiproliferative and antimigratory effects of mefenamic acid. However, further investigation is recommended to unveil more detailed molecular mechanisms through which mefenamic acid exerts its anticancer properties.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MTT: (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide); **LDH:** Lactate dehydrogenase; **qRT-PCR:** Quantitative real-time reverse transcription PCR.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The guidelines, to conduct experimental lab work, per the institutional and national regulations, stated by the "Research Ethics Committee" of Imam Mohammad Ibn Saud Islamic University (IMSIU) were accurately followed (LAB-2023-0157).

SUMMARY

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are among the most prescribed pharmaceuticals worldwide. The present study was carried out to further elucidate the antiproliferative activity of mefenamic acid at the molecular level in the human breast cancer cell line (MCF-7). The cytotoxic effect of mefenamic

acid on breast cancer cells (MCF-7) was assessed using MTT cytotoxicity and Lactate Dehydrogenase (LDH) leakage assays. qRT-PCR analysis was employed to evaluate the expression of the apoptosis-related genes encoding p53, caspases-3, -8 and-9, Bax and Bcl-2. The anti-migratory effect of mefenamic acid on breast cancer cells was evaluated by using the scratch assay. H2DCFDA (H2-DCF, DCF) cellular assay was employed to estimate the intracellular generation of reactive oxygen species (ROS) in breast cancer cells. The current results demonstrated a significant decrease in the viability (survival rate) of MCF-7 cells, over-expression of p53, caspases-3, -8 and-9 activities, a significant upregulation of Bax and significant downregulation of Blc-2. Mefenamic acid significantly inhibited the migration of MCF-7 cells and enhanced the intracellular generation of ROS in breast cancer cells.

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