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Research Article

NAPROXEN INHIBITS THE PROLIFERATION AND MIGRATION OF BREAST CANCER CELLS (MCF-7) THROUGH BCL-2 DOWNREGULATION, CASPASE ACTIVATION, BAX UPREGULATION, AND GENERATION OF EXCESS REACTIVE OXYGEN SPECIES (ROS)

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ABSTRACT: This study explored the antiproliferative activity of naproxen at the molecular level in human breast cancer cell lines, specifically MCF-7. Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed medications worldwide. Like other NSAIDs, naproxen has anti-inflammatory, analgesic, and antipyretic properties. The cytotoxic effects of naproxen on MCF-7 breast cancer cells were assessed using MTT cytotoxicity and lactate dehydrogenase (LDH) leakage assays. Additionally, quantitative reverse transcription PCR (qRT-PCR) was employed to evaluate the expression of apoptosis-related genes, including p53, caspases-3, 8, and 9, Bax, and Bcl 2. The anti-migratory effects of naproxen on breast cancer cells were investigated using a scratch assay. The H2DCFDA (H2-DCF, DCF) cellular assay was used to estimate the intracellular generation of reactive oxygen species (ROS) in breast cancer cells. Results indicated a significant decrease in the viability of MCF-7 cells, as highlighted by both the MTT cytotoxicity and LDH release assays. Compared to the untreated control cells, there was an overexpression of p53 and increased activities of caspases 3, 8, and 9. Bax was significantly upregulated, while Bcl-2 showed significant downregulation. Naproxen also significantly inhibited the migration of MCF-7 cells, as evidenced by the increased distance between the opposing edges of the scratch. In comparison, naproxen enhanced the intracellular generation of ROS in breast cancer cells. Based on these findings, it can be concluded that naproxen exhibits both antiproliferative and anti-migratory activities in the human breast cancer cell line MCF-7. Therefore, naproxen may be considered a promising candidate as an anti-cancer agent, especially when used in combination with other breast cancer therapies.

Keywords: Naproxen, Breast cancer cells, Cytotoxicity, Anti-proliferative, Anti-migratory, Gene expression.

INTRODUCTION

Naproxen, chemically known as (S)-6-methoxy-?-methyl-1,2-naphthaleneacetic acid, is a commonly prescribed non-steroidal anti-inflammatory drug (NSAID) [1, 2, 3]. It has analgesic, anti-inflammatory, and antipyretic properties, effectively relieving pain and treating various inflammatory conditions. Common uses for naproxen include managing arthritis, tendinitis, spondylitis, bursitis, and gout [4, 5, 6].

Non-steroidal anti-inflammatory drugs (NSAIDs) have gained significant attention across preclinical, clinical, and epidemiological research as potential agents for use in combination with other chemotherapeutics in the prevention and treatment of cancer. This represents an example of drug repositioning, where existing medications are utilized for new applications, ultimately saving time and costs compared to developing new drugs [7, 8]. Breast

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cancer is the second leading cause of cancer-related deaths. Epidemiological studies examining the link between NSAID use and breast cancer risk have produced conflicting results. While some studies have indicated an inverse relationship between NSAID use and breast cancer risk, others have found no association at all [9].

The antiproliferative effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on chemically induced cancers have been demonstrated in both experimental animal models and cancer cell lines [10, 11]. One of the primary mechanisms through which NSAIDs function as anti-proliferative (anti-neoplastic) agents is by inhibiting cyclooxygenase (COX), which leads to a reduced synthesis of prostaglandins [12,13]. COX isozymes are crucial for the production of prostaglandins, and overexpression of COX-2 and prostaglandin E_2 (PGE_2) has been observed in various malignant tumors [14, 15, 16]. This finding is a precancerous marker because COX enhances cancer cells' resistance to apoptosis, while PGE_2 promotes tumorigenesis as a mitogen [17]. Additionally, PGE_2 can alter the tumor microenvironment in ways that facilitate the proliferation, invasion, and metastasis of cancer cells. Experimental models of induced tumors have also revealed some COX-independent anti-neoplastic mechanisms of NSAIDs, including the inhibition of tumor growth (tumor regression), reduction of angiogenesis, and enhancement of cellular immunity [18, 19, 20].

Naproxen, like other nonsteroidal anti-inflammatory drugs (NSAIDs), exerts its effects by inhibiting the activity of cyclooxygenase (COX) enzymes, which in turn reduces the synthesis of prostaglandins [21, 22, 23]. Previous studies have also explored the antineoplastic properties and chemopreventive role of naproxen in experimental models of chemically induced tumors [24, 25, 26].

However, the antiproliferative effect of naproxen on human breast cancer cell lines has been reported in a limited number of studies. Consequently, this study aimed to clarify the molecular mechanisms behind the antiproliferative effects of naproxen on human breast cancer cell lines.

MATERIALS AND METHODS

MTT cytotoxicity assay

Naproxen was procured from Sigma-Aldrich (product No. PHR 1040) (Darmstadt, Germany). The cytotoxic effect of naproxen on MCF-7 cells was evaluated using the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay [27]. This assay measures the viability and survival rate of cancer cells exposed to naproxen, based on the principle that only viable and metabolically active cells can reduce MTT to purple formazan, which is the target of measurement. MCF-7 cells (1×10^6 cells/mL) were pre-incubated for 3 hours at 37°C and 5% CO_2 with 1 µg/mL actinomycin C in culture medium. Following this, the cultured cells were seeded at a concentration of 5×10^4 cells/well into a 24-well tissue culture microplate, with 100 µL of culture medium containing naproxen at various concentrations (50, 100, 200, 300, 400, and 500 µg/mL). The incubation lasted for 24 hours under the same conditions (37°C, 5% CO_2). Subsequently, 10 µL of MTT labeling reagent (final concentration of 5 mg/mL MTT in 1× PBS) was added to each well, and the microplate was incubated for an additional 4 hours in a humidified atmosphere (37°C, 5% CO_2). To dissolve the reduced MTT (purple formazan), 100 µL of acidified isopropanol solution was added to each well, and the microplate was incubated overnight in a humidified atmosphere. The complete solubilization of the purple formazan was verified; the darker the solution, the greater the number of viable, metabolically active cells. The formazan product was quantified by measuring absorbance at a wavelength of 570 nm using a microplate reader (BioTek Instruments Inc., USA). The IC50 (the dose sufficient to inhibit cell growth in 50% of treated cells) of naproxen was calculated based on a dose-response curve using software (OriginPro).

Lactate dehydrogenase (LDH) leakage assay

The LDH leakage (cytotoxicity) assay is a colorimetric method that provides a reliable way to determine cytotoxicity [28]. This assay was used to assess the cytotoxicity of naproxen in MCF-7 cells. The underlying principle is that LDH is a cytosolic enzyme released into the cell culture medium when the cell plasma membrane is damaged. MCF-7 cells were seeded at a concentration of 5×10^4 cells/well in a 24-well microplate and incubated for 24 hours. The cultured cells were then treated with naproxen (final concentration of 50 µg/mL) and incubated for an additional 48 hours. The LDH leakage was measured in the cell-free supernatant using an enzyme kit containing NADH and the LDH substrate sodium pyruvate (LDH mixture). The decrease in NADH levels was determined by spectrophotometry and was considered proportional to the LDH activity in the supernatant. A 100 µL aliquot of the obtained

supernatant was transferred to a 96-well microplate, and 100 µL of an LDH detection solution prepared from an LDH kit (Sigma-Aldrich, USA) was added. The reaction was allowed to incubate for 30 minutes to develop color, and the enzyme product was quantified by measuring absorbance at 490 nm using a microplate reader (BioTek Instruments Inc., USA).

qRT-PCR analysis

Quantitative real-time reverse transcription PCR (qRT-PCR) allows for the enumeration of mRNA copies of specific genes, facilitating the quantification of gene expression levels [29]. The analysis, performed as previously described, was used to evaluate the expression of apoptosis-related genes encoding p53, caspases-3, -8, and -9, Bax, and BCL-2. MCF-7 cells (5×10^4 cells/well) treated with naproxen (final concentration of 0.5 IC50, where IC50 = 123.5 µg/mL) were incubated for 24 hours. QIAzol Lysis Reagent (Qiagen, Cat. No. 79306, USA) was added, and the total RNA content was estimated from the cell lysates. cDNA synthesis was achieved using oligo dT primers and the Superscript II Reverse Transcriptase (cDNA Synthesis Kit, Applied Biosystems, Thermo Fisher Scientific, Lithuania). The polymerase chain reaction (PCR) was performed using a Rotor-Gene machine, following the manufacturer's (Prime Q, China) instructions and utilizing primers specified for the relevant genes (Table 1). Gene expression levels were assessed using GAPDH as the internal reference, applying the equation:

$2^{(-\Delta\Delta C_T)}$ (in other words, it doubles with every reduction of a single cycle in ΔC_T values).

Scratch wound assay

The scratch wound assay involves creating a gap in a confluent monolayer of cells to mimic a wound, after which cell migration is evaluated in the presence of the substance being tested. This assay was conducted as previously described [29]. To achieve the required confluency of 70-80% with MCF-7 cells, they were cultured in a 12-well microplate. A fine scratch was made in the monolayer using a 10 µL pipette tip. Detached cells were removed by washing with PBS, and the culture medium was replaced with fresh media containing naproxen at a concentration of 10 µg/mL, considered non-cytotoxic. Sequential imaging was performed at 0 hours, 24 hours, and 48 hours using a Leica MC-170 HD camera (Leica, Germany) attached to a phase-contrast inverted microscope. The images obtained were analyzed using ImageJ software (NIH, USA). The cell migration ratios from the scratch edges were calculated using the following equation:

Relative Migration Ratio = (0 h Distance - 24 h Distance) / 0 h Distance [30].

H2DCFDA cellular ROS assay

The generation of reactive oxygen species (ROS) in MCF-7 cells treated with naproxen was assessed using the H2DCFDA (2,7-dichlorodihydrofluorescein di acetate) cellular ROS assay. This assay employs a cell-permeable fluorescent dye. The dye chemically reduces the non-fluorescent H2DCFDA to the highly fluorescent 2,7-dichlorofluorescein (DCF) inside the cells, which occurs through the oxidation and cleavage of its acetate groups. The assay used the Invitrogen ROS indicator (Thermo Fisher Scientific, USA). MCF-7 cells, treated with naproxen at its IC50, were seeded in a 96-well microplate at a density of 5×10^4 cells per well and incubated at 37°C for 24 hours. The culture medium was then replaced in the dark with 100 µL of diluted H2DCFDA fluorescent dye (1 µL of dye added to 99 µL of MEME) for a 2-hour re-incubation. The absorbance was measured at 495 nm (excitation) and 510 nm (emission) using a microplate reader (Gen5, BioTek Cytation 5, USA). The relative generation of ROS was calculated using the equation:

Relative ROS Generation (%) = [Optical Density (OD) of MCF-7 cells exposed to naproxen / OD of control untreated cells] × 100.

RESULTS AND DISCUSSION

MTT cytotoxicity assay

The cytotoxicity of naproxen was assessed using the MTT assay, which determined the IC50 (50% inhibitory concentration) to be 271.01 µg/ml. The viability curve indicated an inverse dose-dependent relationship between the tested concentrations of naproxen and the percentage of viable treated cells (Fig. 1).

LDH leakage assay

The results from the lactate dehydrogenase (LDH) assay were consistent with those from the MTT cytotoxicity assay. There was a significantly higher release of LDH from breast cancer cells treated with the IC50 dose of naproxen compared to untreated control cells (Fig. 2).

Table 1 The sequences of the primers (F for forward and R for reverse) and the target genes: 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'

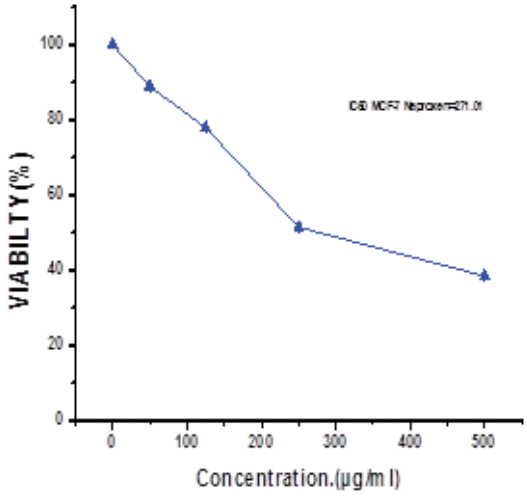

Fig. 1. MTT assay cytotoxicity curve. [Cytotoxicity induced by naproxen in MCF-7 cells was found to be dose-dependent. IC50 of naproxen = 271.01 µg / ml].

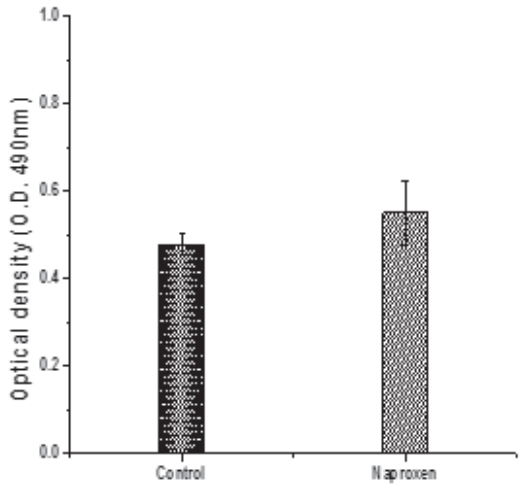

Fig. 2. Lactate dehydrogenase (LDH) leakage assay. [MCF-7 cells treated with naproxen exhibited a significant release of LDH compared to the control untreated cells].

qRT-PCR analysis

The qRT-PCR analysis revealed a significant overexpression (measured in fold change) of p53 and caspases (3, 8, and 9) in MCF-7 cells treated with naproxen compared to control cells. Notably, the overexpression of caspase-8 was more pronounced than that of caspases-3 and -9. Additionally, there was a significant upregulation of Bax (an apoptotic marker) and a downregulation of Bcl-2 (an anti-apoptotic marker) in the treated breast cancer cells (Fig. 3). The downregulation of Bcl-2 in naproxen-treated cells was particularly striking compared to that in untreated control cells.

Scratch assay

Naproxen significantly inhibited the migration of

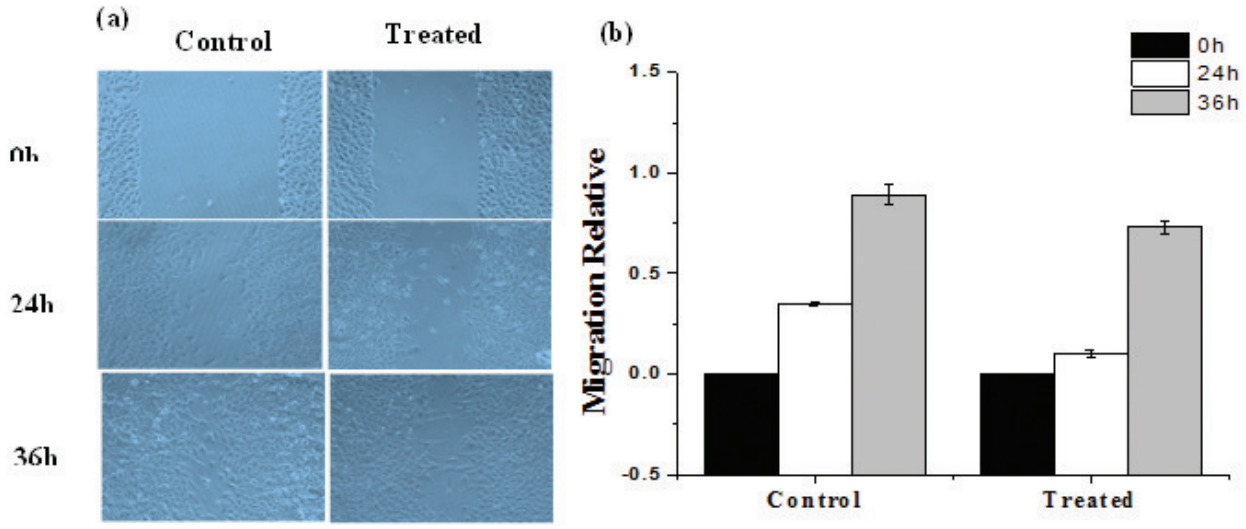

Fig. 3. qRT-PCR analysis. [MCF-7 cells treated with naproxen exhibited significant overexpression (measured in fold change) of p53, caspases 3, 8, and 9 compared to control untreated MCF-7 cells. The analysis also revealed significantly up-regulated Bax (apoptotic) and down-regulated BCL-2 (antiapoptotic) in the treated breast cancer cells].

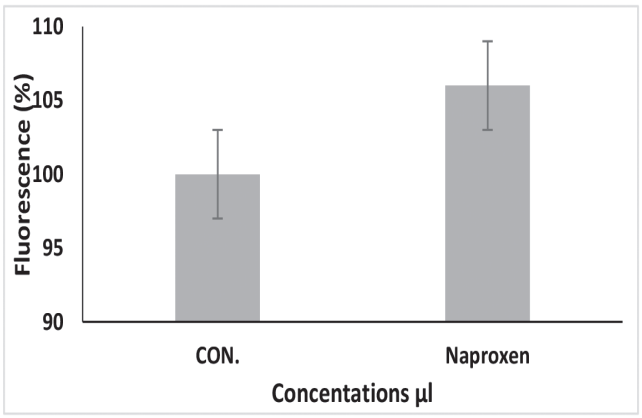

Fig. 4. Effect of naproxen on migration of MCF-7 cells. [Migration of naproxen-treated cells and control untreated cells were monitored at 0, 24, and 36 hr post-incubation. (a) Control untreated cells (left), (b) treated cells (right). The estimated three replicates were expressed as mean ± SD, *p < 0.05, **p < 0.01].

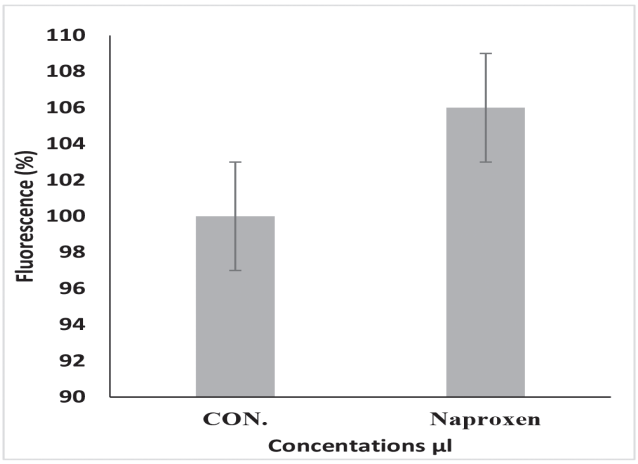

Fig. 5. H_2DCFDA (H_2-DCF, DCF) cellular ROS assay. [MCF-7 cells treated with naproxen showed enhanced generation of ROS compared to the untreated control cells].

MCF-7 cells compared to untreated control cancer cells at 24 and 48 hours, as demonstrated by the measured distance between the opposing edges of the scratch (Fig. 4).

ROS assay

A significant increase in reactive oxygen species (ROS) generation was observed in MCF-7 cells exposed to the IC50 dose of naproxen compared to untreated control cancer cells (Fig. 5).

Several *in vitro* studies have investigated the anti-cancer activity of nonsteroidal anti-inflammatory drugs (NSAIDs) through their ability to inhibit cancer cell proliferation. Additionally, NSAIDs may have synergistic anti-proliferative effects when used in combination with other chemotherapeutic agents [31, 32]. A significant portion of the anti-proliferative activity induced by NSAIDs is independent of cyclooxygenase (COX) inhibition. This is evidenced by their effects on cancer cells that do not express COX or when these cells are treated with prostaglandins.

The current findings from MTT and LDH release assays, which evaluate cytotoxicity, demonstrate a dose-

dependent decrease in the survival of cancer cells treated with naproxen. In addition to its inhibitory effects on COX-2 and the synthesis of prostaglandins, naproxen has been shown to have a COX-independent anti-proliferative effect on various cancer cell lines. This effect is mediated by cell cycle arrest, reduced Akt signaling, inhibition of phosphatidylinositol 3-kinase (PI3-K), suppression of cyclin D1, and activation of MAP kinase [33, 34, 35]. Furthermore, naproxen's anti-tumor activity has been observed in experimental rodent models, attributed to its inhibition of both COX and PI3-K. Naproxen, whether used alone or in combination with other agents, has demonstrated effective chemopreventive effects against colon, urinary bladder, and prostate cancer cells [36, 37, 38].

Apoptotic cell death is regulated by specific genes related to apoptosis and their associated proteins, such as p53, p21, and c-myc. These proteins influence other effector proteins, including Bcl-2, cytochrome c, and caspases [27]. The current findings demonstrate that naproxen-induced apoptosis in breast cancer cells is linked to increased caspase activity, down-regulation of Bcl-2, and up-regulation of Bax. In the breast cancer cells treated with naproxen, there was an overexpression of both caspase-3 and caspase-8, with the increase in caspase-8 being particularly pronounced. This suggests that naproxen may promote and sustain the intrinsic pathway of apoptosis by activating caspase-8. Other studies have indicated that the enhanced cleavage of caspases-3 and -7 plays a crucial role in naproxen-induced apoptosis in various cancer cell lines [7]. Additionally, the cancer cells treated with naproxen exhibited overexpression of p53 in conjunction with the activated caspases. Previous research has also reported the activation of caspase-3 and an increase in p21 in response to naproxen-induced apoptosis in other cancer cell lines [27, 28, 29].

Moreover, other researchers have suggested that the increased cleavage of caspases 3 and 7 plays a crucial role in naproxen-induced apoptosis across various cancer cell lines. Additionally, the cancer cells treated with naproxen exhibited overexpression of p53 in conjunction with the activated caspases. Previous studies have also reported activated caspase - 3 and overexpression of p21 related to naproxen-induced apoptosis in other cancer cell lines.

Currently, in breast cancer cells treated with naproxen, there is an overexpression of Bax and p53, while Bcl-2 is down-regulated. Notably, the down-regulation of Bcl-2 in the naproxen-treated cells is particularly significant. This suggests that Bcl-2 is a major target of naproxen's action in triggering apoptosis. Bax and Bcl-2 are both pro-apoptotic proteins, and their expression levels (both up-regulation and down-regulation) play a crucial role in the process of apoptosis, highlighting their importance in the anti-cancer effects of chemotherapeutic agents. Bcl-2 overexpression can inhibit apoptosis by preventing the release of mitochondrial cytochrome c, whereas Bax facilitates this release. Some nonsteroidal anti-inflammatory drugs (NSAIDs) only induce Bax overexpression without affecting Bcl-2 levels. However, the findings of this study indicate that naproxen can modulate the expression of both Bcl-2 and Bax. The overexpression of p53 in the investigated cells suggests that the p53-mediated apoptotic pathway is involved in the anti-cancer activity of naproxen.

Currently, naproxen has been shown to delay the migration of breast cancer cells. This was evidenced by the failure of these cells to cover the gap between scratch edges in *in vitro* studies. Some researchers have proposed that non-steroidal anti-inflammatory drugs (NSAIDs) may inhibit the metastasis of cancer cells. The anti-migratory effect of naproxen may be linked to its significant suppression of prostaglandin synthesis due to cyclooxygenase (COX) inhibition. Prostaglandin E_2, in particular, has been identified as playing a crucial role in the invasiveness of cancer cells. This suggests that naproxen could potentially inhibit the invasion and metastasis of cancer cells *in vivo*, although this hypothesis requires confirmation through *in vivo* investigations in experimental models.

Based on the current results, naproxen appears to initiate early pro-apoptotic events by modulating both Bax and Bcl-2, which is followed by the activation of caspase-3 and caspase-8. Notably, the findings indicate that naproxen activates caspase-8 more prominently in breast cancer cells, suggesting that its mechanism of action may involve the extrinsic pathway of apoptosis [37]. Furthermore, evidence suggests that Bcl-2 is a primary target of naproxen's anti-proliferative activity. Overall, the apoptotic pathway induced by naproxen includes the modulation of Bcl-2, Bax, p53, and caspases, all of which are key proteins associated with apoptosis-related genes. Additionally, naproxen's ability to enhance the generation of reactive oxygen species (ROS) contributes to its anti-proliferative activity by reducing the viability of breast cancer cells.

CONCLUSION

In conclusion, the anti-proliferative and anti-migratory effects of naproxen may be due to apoptosis

triggered by activated pro-apoptotic factors, including Bcl-2, Bax, and p53, as well as the executioner caspases that facilitate proteolytic activity. Additionally, increased generation of reactive oxygen species (ROS) contributes to naproxen's anti-proliferative effects.

Based on the current *in vitro* findings, naproxen shows promise as a therapeutic agent when used in combination with other chemotherapeutics for treating breast cancer. Future experimental studies will investigate naproxen's additional anti-proliferative mechanisms in breast cancer cell lines.

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