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Original Article

Effect of flurbiprofen derivative (SGK597) on cell proliferation and apoptosis of breast cancer cell lines

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ABSTRACT

Background and Aims: The incidence of breast cancer is increasing day by day, especially in women. The search for new drugs against breast cancer is the focus of attention in research. Breast cancer and prostate cancer have remarkable biological similarities. Therefore, the 4-(4-chlorophenyl)-3-(1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl)-5-((4-fluorobenzyl)thio)-4H-1,2,4-triazole (SGK597) compound that is suppressing cell proliferation in prostate cancer, was studied in MCF-7 breast cancer and MCF-10A mammary epithelial cell lines.
Methods: The WST-8 method was used to determine cell viability and cytotoxicity of SGK597 in MCF-7 and MCF10-A cell lines. The JC-1 test was applied to determine changes in mitochondrial membrane potential. The protein expression levels of Bax, Bcl-2, and c-PARP associated with apoptosis were determined using Western blot analysis.
Results: After 24 and 48 hours of incubation of SGK597, the IC50 values were 28.74 µM and 17.28 µM for MCF-7; 65.9 µM and 50.5 µM for MCF-10A, respectively. Mitochondrial membrane potential showed a tendency toward depolarization in MCF-7 cells as a result of increasing concentration of SGK597, while the same tendency was not seen for MCF-10A. As a result of western blot experiments, no increase in the Bax/Bcl-2 ratio and c-PARP expression level was observed, indicating no apoptosis.
Conclusion: It was observed that the compound SGK597 suppressed MCF-7 cell proliferation. These results indicate that SGK597 may be a candidate compound for use as an anticancer agent.
Keywords: Apoptosis, breast cancer, flurbiprofen, thioether, triazole

INTRODUCTION

Breast cancer is the most common cancer among women. More than 1.5 million women worldwide are diagnosed with breast cancer each year. The main risk factors for breast cancer in women can be listed as age, family history, and BRCA1 or BRCA2 gene mutations that are thought to be associated with breast cancer (Becker, 2015; Sun et al., 2017). The increase in the recurrence of cancer cases and the serious side effects of chemotherapeutic agents show that there is always a need to develop alternative anticancer drugs (Ali et al., 2012). While traditional chemotherapeutic drugs mostly directly target the DNA of cancer cells, recently designed new anticancer drugs target proteins with abnormal expression in cancer cells (Meegan & O'Boyle, 2019).

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The relationship between chronic inflammation and cancer is known. Therefore, it is thought that drugs that inhibit inflammation may be useful in the treatment or prevention of cancer. Inflammatory stimuli rupture arachidonic acid from phospholipids in the cell membrane via the enzyme phospholipase A2. Two isoforms of the cyclooxygenase (COX) enzyme, COX-1 and COX-2, catalyze the synthesis of various forms of prostaglandin (PG) from arachidonic acid. PGs are known mediators of inflammation. Studies in human breast cancer cell lines have shown that overexpression of COX-2 plays an important role in the pathogenesis of breast cancer. Non-steroidal anti-inflammatory drugs (NSAIDs) have the ability to inhibit the COX enzyme and, accordingly, the synthesis of COX products. (±) (R,S)-Flurbiprofen is one of the non-selective COX inhibitor NSAIDs. By inhibiting the COX activity of the enzyme PG synthetase, flurbiprofen inhibits the production of PG. (McCormick & Moon, 1983). Studies have reported that flurbiprofen and its structural derivatives (Aydın et al., 2013; Çıkla et al., 2013) exhibit anticancer activity. When flurbiprofen was added to chemotherapy in patients with metastatic breast cancer, it was observed that these patients responded better to the treatment (Powles, Alexander, & Millar, 1978). In addition, triazole (Küçükgüzel & Çıkla-Süzgün, 2015; Çıkla-Süzgün & Küçükgüzel, 2021) and thioether derivatives (Çoruh, Çevik, Yelekçi, Djikic, & Küçükgüzel, 2017; Birgül et al., 2020; Han & Küçükgüzel, 2022) have been reported to have anticancer effects. The SGK597 compound is a flurbiprofen triazole-thioether, as in Figure 1, with the chemical structure 4-(4-chlorophenyl)-3-(1-(2-fluoro-[1,1'-biphenyl]-4yl)ethyl)-5-((4-fluorobenzyl)thio)-4H-1,2,4-triazole. This compound was synthesized by Yılmaz et al (2020). Especially MetAP-2, one of the two cytoplasmic methionine aminopeptidases in mammalian cells and known to have high expression in cancer cells, is the target protein for the SGK597 compound. It has been shown that the compound SGK597 inhibits androgen receptor-negative prostate cancer PC-3 and DU-145 and androgen receptor-positive prostate cancer LNCaP cell proliferation with IC_{50} values of 27.1, 6.9 and 106.7 µM, respectively, after 24 hours of incubation (Yılmaz et al., 2020).

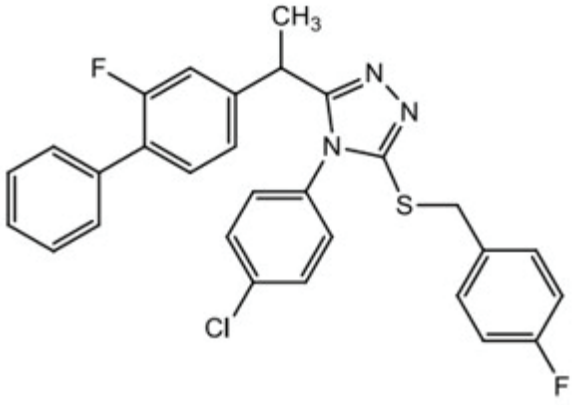

Figure 1. Chemical structure of Flurbiprofen derivative SGK597 (Yılmaz et al., 2020).

Prostate cancer is one of the most common invasive cancers in men. It is known that breast and prostate cancer have important biological similarities. There is clear evidence that BRCA1 and BRCA2 mutations carried by patients with a family history of breast cancer have a higher risk of prostate cancer. A family history of breast cancer in first-degree relatives has also been linked to prostate cancer in previous studies (Ren et al., 2019).

With the understanding of the pathophysiology of breast and prostate cancer, new treatment strategies have begun to be developed against breast and prostate cancer (Risbridger, Davis, Birrell, & Tilley, 2010). In an in vivo study, epigallocatechin gallate, which is abundant in green tea, inhibited the growth of human prostate and breast cancer cells and rapidly reduced their size (Liao, Umekita, Guo, Kokontis, & Hiipakka, 1995). Carboxyamidotriazole was discovered to be a selective inhibitor of breast and prostate cancer migration in vitro, and this compound is known to have entered phase III clinical trials in cancer patients (Bradke, Hall, Carper, & Plopper, 2008). Some cinnamic acid derivatives inhibited the growth of prostate and breast cancer cells by inducing apoptosis (Imai, Yokoe, Tsubuki, & Takahashi, 2019). In another in vitro study, a series of hybrid compounds based on tamoxifen, estrogen, and artemisinin were synthesized and some of these compounds were reported to have anticancer activity in human prostate and breast cancer cell lines (Fröhlich et al., 2020). Accordingly, it can be thought that compounds that may have anticancer effects against prostate cancer can also be used against breast cancer.

The aim of this study is to determine the cytotoxic and apoptotic effects of the SGK597 compound applied at different concentrations (0, 10, 25, 50, 75, 100 µM) and periods (24 and 48 h) in MCF-7 breast cancer and MCF-10A mammary epithelial cell lines using the WST-8 test, JC-1 mitochondrial membrane potential test, and Western blot.

MATERIAL AND METHODS

Cell culture

MCF-7 breast cancer cells in DMEM medium containing 10% FBS and 1% Pen/Strep; MCF-10A mammary epithelial cells in DMEM/F12 medium containing 5% horse serum, 0.02% EGF, 0.05% hydrocortisone, 0.01% cholera toxin, 0.1% insulin and 1% Pen/Strep were incubated at 37 °C, in an incubator containing 5% CO_2. The medium was changed three times a week.

Preparation of SGK597 stock solution

96 mM stock solution was prepared by dissolving 50 mg of SGK597, supplied as a white powder, in 1 mL of DMSO. This stock was used in lower concentrations to be applied to cells later.

WST-8 colorimetric test

The effects of SGK597 on cell viability and cytotoxicity in MCF-7 and MCF-10A cell lines were investigated using a CCK-8 kit which is based on the WST-8 colorimetric change. Into 96-well plates, 1500 cells/well were seeded and different concentrations of SGK597 were applied to the treatment group for 24 and 48 hours. Subsequently, the CCK-8 kit was applied according to the manufacturer's protocol (Cell Counting Kit-8, KTC011001, Abbkine). A microplate reader was used to detect absorbance at 450 nm after 4 hours (Synergy H1, BioTek Instruments Inc., USA).

JC-1 mitochondrial membrane potential test

The JC-1 mitochondrial membrane potential test was carried out in MCF-7 and MCF-10A cell lines. The cells seeded in a 96-well black opaque plate and incubated with SGK597 in different concentrations (10, 25, 50, 75, 100 µM) for 48 hours were stained with JC-1 dye as recommended by the manufacturer (JC-1 Mitochondrial Membrane Assay Kit, 10009172, Cayman Chemical). The green/red fluorescence ratio was used to evaluate the apoptosis of the cells (Mega Tiber, Kocyigit Sevinc, Kilinc, & Orun, 2019).

Western blot

MCF-7 cell pellets were lysed with a lysis solution which was prepared for each sample using 8 µL of protease inhibitor cocktail, 2 µL of NaF, 190 µL of RIPA lysis buffer (RIPA Lysis Buffer System, sc-24948A, Santa Cruz). Fifty µg of protein from each sample was run for 2 hours in SDS-PAGE under 150 V and the transfer to the membrane was provided at 25 V for 2 hours. Blocking was performed with 5% BSA. The membranes were incubated with primary and secondary antibodies to β-actin, Bcl-2, Bax, and c-PARP which were dissolved in 1X TBS-T with 1% BSA. For the detection, a chemiluminescent substrate solution was used (WesternBright ECL HRP Substrate, Advansta). Protein quantification was performed using the chemiluminescence imaging system Biostep Celvin and TotalLab 1D software.

Statistics

For the WST-8 colorimetric test and JC-1 mitochondrial membrane potential results, one-way ANOVA analyses were followed by Dunnett's post-hoc tests. For western blot results, Kruskal Wallis analyses were followed by Dunn's post-hoc tests. The analyses were conducted using GraphPad Prism (version 8.0.1, GraphPad Software, CA, USA). The level of significance was accepted for $p < 0.05$.

RESULTS

Cell viability and cytotoxicity

The absorbance values for MCF-7 and MCF-10A cells after the WST-8 test are as in Figure 2 and Figure 3, respectively.

The IC_{50} values calculated after the analysis of absorbance values obtained from the WST-8 test for the incubation of MCF-7 and MCF-10A cells with SGK597 at different concentrations and periods are shown in Table 1.

Apoptosis

Mitochondria are well-known for their significance in the apoptotic process (Ly, Grubb, & Lawen, 2003). During apoptosis, the opening of mitochondrial permeability transition pores results

Table 1. IC_{50} values in µM for MCF-7 and MCF-10A cells after 24 and 48 hours of incubation with SGK597 compound.

SGK597 (µM)		
Incubation period	24h	48h
MCF-7	28.74	17.28
MCF-10A	65.9	50.5

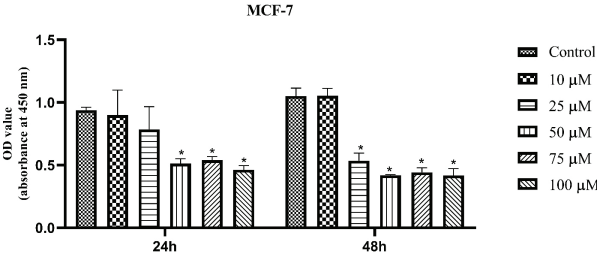

Figure 2. Bar chart of the absorbance values for MCF-7 cells after the incubation with SGK597 for 24 and 48 hours (n = 3) with standard deviation error bars. There was a statistically significant difference in the absorbance for the concentrations. Post-hoc comparisons using the Dunnett's test indicated that the mean score of the absorbance for 50, 75, 100 µM at 24 hours of incubation and 25, 50, 75, 100 µM at 48 hours of incubation was significantly different from the control group, *p < 0.05. OD: optical density.

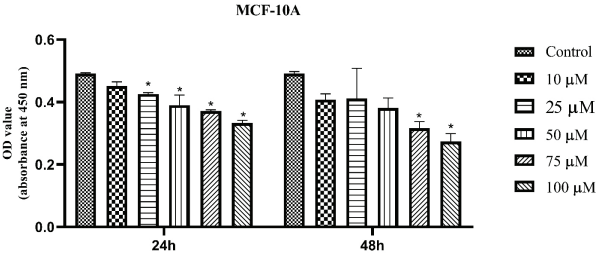

Figure 3. Bar chart of the absorbance values for MCF-10A cells after incubation with SGK597 for 24 and 48 hours (n = 3) with standard deviation error bars. There was a statistically significant difference in the absorbance for the concentrations. Post-hoc comparisons using the Dunnett's test indicated that the mean score of the absorbance for 25, 50, 75, 100 µM for 24 hours of incubation and 75 and 100 µM for 48 hours of incubation was significantly different from the control group, *p < 0.05. OD: optical density.

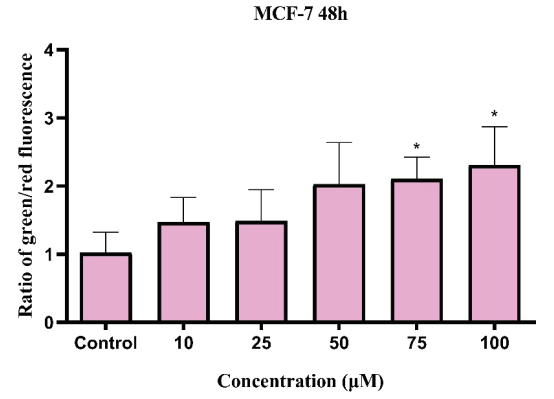

Figure 4. Bar chart of the ratio of green/red fluorescence for control and experimental groups in MCF-7 cells that were incubated with SGK597 for 48 hours (n = 3) with standard deviation error bars. For MCF-7 cells, there was a statistically significant difference in the ratio of green/red fluorescence for the concentrations: p = 0.036. Post-hoc comparisons using the Dunnett's test indicated that the mean score of the ratio of green/red fluorescence for 75 µM and 100 µM was significantly different from the control group, *p < 0.05.

in a loss of the electrochemical gradient and a decrease in mitochondrial membrane potential is expected. Green/red fluorescence ratios obtained from JC-1 mitochondrial membrane potential test results at 48 h incubation of MCF-7 and MCF-10A cells with different doses of SGK597 are shown in Figure 4 and Figure 5, respectively. Red fluorescence was obtained in healthy cells and green fluorescence in apoptotic cells.

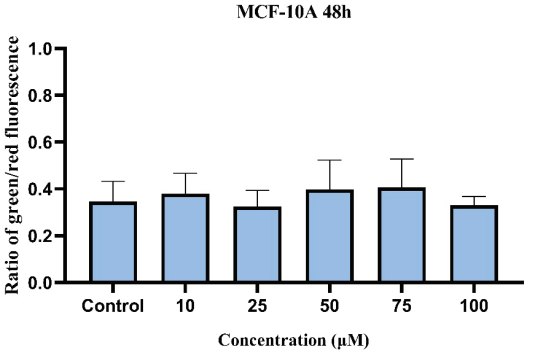

Figure 5. Bar chart of the ratio of green/red fluorescence for control and experimental groups in MCF-10A cells that were incubated with SGK597 for 48 hours (n = 3) with standard deviation error bars. For MCF-10A cells, there was no statistically significant difference in the ratio of green/red fluorescence for the concentrations, p = 0.822.

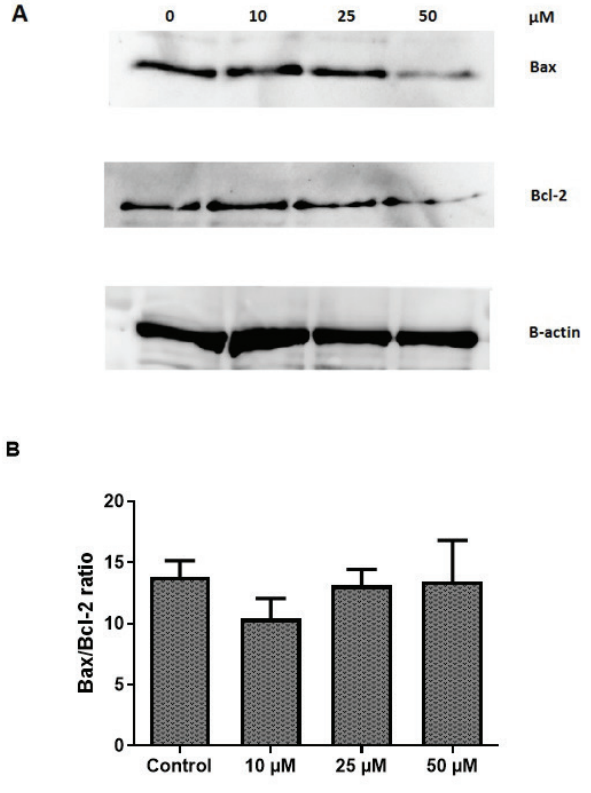

Figure 6. Western blot results of Bax/Bcl-2. (A) Bax/Bcl-2 protein expression with β -actin standard in MCF-7 cells that were incubated with SGK597 for 48 hours. (B) Bar chart of band intensities normalized to β-actin (n = 2) with standard deviation error bars. There was no statistically significant difference in Bax/Bcl-2 protein expression levels, p = 0.495.

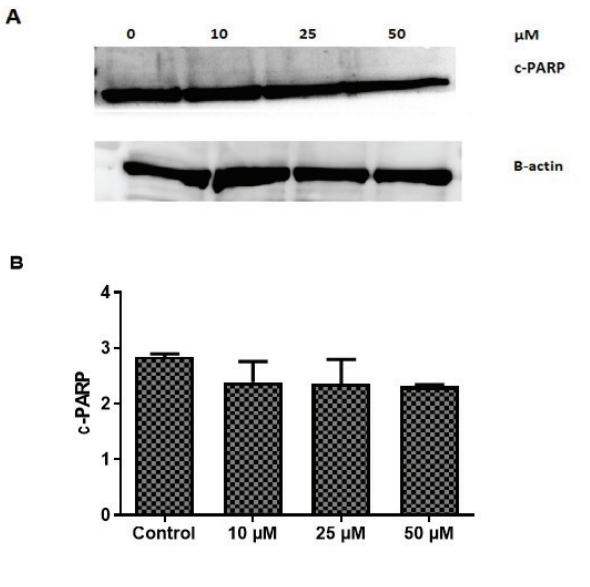

Figure 7. Western blot results of c-PARP. (A) c-PARP protein expression with β -actin standard in MCF-7 cells that were incubated with SGK597 for 48 hours. (B) Bar chart of band intensities normalized to β-actin (n = 2) with standard deviation error bars. There was no statistically significant difference in c-PARP protein expression levels, p = 0.343.

DISCUSSION

It was previously determined that the flurbiprofen derivative SGK597 may have a cytotoxic effect against prostate cancer cells (Yılmaz et al., 2020). Based on previous studies, it is thought that agents showing beneficial effects against prostate cancer may also be effective against breast cancer. On this basis, we studied the cytotoxic effects of SGK597 in breast cancer cell lines. IC_{50} values were calculated by applying SGK597 to MCF-7 and MCF-10A cell lines for 24 and 48 hours. The IC_{50} value determined in prostate cancer cells by Dr. Kücükgüzel et al. was 27.1 µM after a 24-hour incubation period for SGK597. The fact that this value is close to the value determined for MCF-7 of 28.74 µM supports the idea that there may be a relationship between the two types of cancer. The IC_{50} value was much higher in MCF-10A epithelial cells. This suggests that SGK597 applied to MCF-7 will not cause a significant effect on MCF-10A in the same incubation period. However, the IC_{50} value is determined according to all cells that are dead or in the early or late apoptotic stage where cellular functions are interrupted. Therefore, the applied WST-8 test cannot show apoptosis. However, it gives an idea about the viability and metabolic activity of cells.

Although the induction of apoptosis was demonstrated by depolarization of the mitochondrial membrane potential, it was not demonstrated by the Bax/Bcl-2 ratio and c-PARP level. It is expected that the Bax/Bcl-2 ratio and c-PARP expression level will increase in cells undergoing apoptosis. According to the results obtained, the idea that SGK597 does not lead MCF-7 cells to apoptosis is strengthened. Apart from this, although there was no change in the apoptosis-related Bax/Bcl-2 ratio and c-PARP protein expression, the depolarization seen as a result of the JC-1 mitochondrial membrane potential test suggests that

there is some apoptotic stimulation in cells and that SGK597 has the potential to be used as an apoptotic agent in addition to suppressing proliferation. Western blotting is not very sensitive for quantitative evaluation and experiments need to be repeated at least three times. Further tests are needed for the exact determination of apoptotic cell death.

Studies with different proteins may show that the drug is effective on different pathways because the compound SGK597 was designed as a good MetAP-2 inhibitor. It should also be experimentally proven whether the MetAP-2 level changes in the MCF-7 cell line when SGK597 is applied. MetAPs are proteases responsible for removing methionine from the amino terminus of newly synthesized proteins. In eukaryotes, MetAP-1 and MetAP-2 are known to have MetAP activity. Current reports suggest that MetAP-2 plays an important role in the growth of different tumor types due to its role in angiogenesis. It has been reported that MetAP-2 may function as an oncogene. Therefore, this enzyme can be used against cancer cells by reducing its concentration. The TNP-470 compound, known to be a MetAP-2 inhibitor, is known to have entered human clinical trials in the treatment of metastatic breast cancer (Selvakumar et al., 2006; Selvakumar et al., 2009), and it has also been determined to inhibit the growth of MDA-MB-231 triple-negative breast cancer cells (Yamaoka et al., 1993).

In another study, human cervical cancer cell line (HeLa) and liver cancer cell line (HepG2) also showed cytotoxic, genotoxic, and apoptotic effects through the intracellular pathway after flurbiprofen treatment (Bakır et al., 2021). Also, the efficacy of flurbiprofen to suppress the growth of tumor cell lines derived from medulloblastoma and glioblastoma multiform was investigated, and it was revealed that flurbiprofen effectively inhibited the growth of various tumor cells in a dose-dependent manner (King & Khalili, 2001). Similarly, the proliferation suppressive effect of SGK597 was observed in our study. However, this observation needs to be supported by different methods to demonstrate apoptosis.

It is clear that studies on the effect of NSAIDs on breast cancer have led to conflicting results. It is not known whether these differences are due to drug design or a lack of understanding of the action mechanism of NSAIDs on the natural history of breast cancer.

In conclusion, the flurbiprofen derivative SGK597 compound showed cell proliferation suppressive properties in breast cancer cells as well as in prostate cancer cells. This study was a preliminary study for future studies.

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

Author Contributions: Conception/Design of Study- İ.A., P.M.T.; Data Acquisition- İ.A., P.M.T., S.K.S., O.O., Ö.Y., Ş.G.; Data Analysis/Interpretation- İ.A., P.M.T., S.K.S., O.O., Ö.Y., Ş.G.K.; Drafting Manuscript- İ.A., P.M.T.; Critical Revision of Manuscript- P.M.T., O.O., Ö.Y., S.G.K.; Final Approval and Accountability- İ.A., P.M.T., S.K.S., O.O., Ö.Y., Ş.G

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