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# A Novel Phenothiazine Derivative's Anti-Proliferative Action on the Colon Cancer Cell Line and Its Immunomodulatory Activity on LPS Activated Mammalian Macrophages

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**Background: In recent years, agents with anti-cancer, immunomodulatory and immunostimulatory potential have gained great importance. In this study, the cytotoxic and anti-prolifative activity of a new 10-[4-(4-chlorobutoxy)butyl]-10H-phenothiazine (OZF2) phenothiazine derivative against a colon cancer cell line as well as its antiinflammatory effects on lipopolysaccharide (LPS)-activated macrophage cell line (RAW 264.7) were examined.**

**Methodology: An N-substituted phenothiazine derivative named 10-[4-(4-chlorobutoxy)butyl]-10H-phenothiazine (OZF2) was synthesized and its molecular structure was confirmed by $^{1}$H Nuclear Magnetic Resonance (NMR) and $^{13}$C NMR. The molecule contains electronegative oxygen and chlorine atoms in the long alkyl chain attached to the nitrogen atom in the phenothiazine ring. The anti-cancer activity of the OZF2 phenothiazine derivative was evaluated with the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) test on the colon cancer cell line. The anti-inflammatory activity of OZF2 was tested by measuring the levels of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-$\alpha$) and interleukin-6 (IL-6) released from macrophage cells in the presence and absence of LPS and/or OFZ2 using enzyme-linked immunosorbent assay (ELISA). Quantitative real time polymerase chain reaction (q-RT-PCR) analysis was performed to evaluate expression levels of these cytokines also in presence and absence of LPS and/or OFZ2.**

**Results: OFZ2 had anti-proliferative activity on the colon cancer cell line and anti-inflammatory activity on the mammalian macrophages at subtoxic concentrations. It did not stimulate IL-6 and TNF-$\alpha$ cytokines in the absence of LPS in ELISA. But when stimulated with LPS, a significant decrease in the production level of the IL-6 cytokines was observed, while the TNF-$\alpha$ production was not affected. No change in the gene expression of *TNF-$\alpha$* was observed, whereas *IL-6* gene expression significantly decreased in the presence of OZF2.**

**Conclusions: The anti-proliferative activity of OZF2 against colon cancer cells makes of it a potential drug candidate for colon cancer research and treatment. It also has the potential to be an anti-inflammatory drug agent that can suppress the production of IL-6-based autoimmune and inflammatory diseases.**

**Keywords:** inflammation; phenothiazine; cytokines; macrophages; anti-proliferation

## Introduction

Cancer development has been associated with inappropriate activity of the immune system responses [1]. Cancer-related inflammation contributes to the emergence, development, vascular formation, and progression of cancer. Studies have shown that cancer cells create their own tumor microenvironment where various dendritic cells, macrophages, Natural killer (NK), eozonophils, mast cells and immune mediators can be found. Stimulation of cells in the tumor microenvironment can support the growth and propogation of cancer cell via the secretion of mediators such as various enzymes, cytokines, and chemokines. This can further result in angiogenesis and cancer metastasis, as well as as resistance against microenvironment cancer treatment methods [2–5].

Macrophages are key players in cancer microenvironment. They eliminate invading pathogens and dead cells resulting from inflammation and apoptosis, and are involved in antigen presentation and tissue shaping during embryonic development [6–8]. In addition, macrophages are associated with cell proliferation, metastasis and vascularization in cancer [6–8]. Because of these properties, they are targets for treatment of cancers caused by inflammation [9].

Tumor necrosis factor alpha (TNF-$\alpha$) and interleukin-6 (IL-6), which are secreted by different immune system

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cells but mainly by macrophages, are among the most important proinflammatory cytokines in the tumor microenvironment [10,11]. TNF-$\alpha$ plays a role in preventing infections, stimulating acute and chronic inflammatory reactions, apoptosis and tumor necrosis [12,13]. The IL-6 cytokine plays a role in the acute phase reactions such as inflammation and prevention of inflammation for wound healing [14]. Defects in IL-6 secretion have been associated with various diseases such as autoimmune disorders and Alzheimer's disease. High levels of IL-6 secretion have been observed in various cancer types including colon cancer [14–17], hence the importance of measuring the levels of these cytokines in cancer.

One of the main challenges in cancer treatment is that therapeutic agents could have too many side effects and do not show the same effect in every cancer type. Phenothiazine derivatives (PTZ) are known for their use in the treatment of many diseases and have different biological activities such as anti-tuberculosis [18], anti-histamine drugs acting on the nerve center (antiemetic) [19,20], anesthetic, anti-microbial [21], anti-fungal [22], anti-viral [23], anti-parkinsonian [24], anti-inflammatory and anti-cancer [25,26]. Studies on different cancer cell lines have revealed the role of these derivatives in DNA repair, cell proliferation, and metastasis inhibition [27]. In addition, its use in chemo sensors, probes, solar cells, LEDs, fluorescents, and modifiable optical materials and devices is developing [28]. These properties of phenothiazine derivatives are obtained by adding amino alkyl to the N10 position in the substitution pattern or sometimes by adding new molecules to replace the carbon molecule in the benzene ring [29,30].

In this study, we synthesized a new 10-[4-(4-chlorobutoxy)butyl]-10H-phenothiazine (OZF2) phenothiazine derivative shown in Fig. 1, then examined its anticancer effects on the colon cancer cell line, one of the cancer types with the highest incidence [1]. The secretion by lipopolysaccharide (LPS) activated macrophage of TNF-$\alpha$ and IL-6 and their expression levels cytokines cells were measured. Our results suggest anti-cancer and immunomodulatory role for this novel phenothiazine derivative.

## Experimental Section

### *Synthesis of OZF2*

All reagents and solvents were of reagent grade quality and obtained from commercial suppliers. Phenothiazine (CAS: 92-84-2, $P_{Code}$:102246495, product of Hong Kong, China), Bis(4-chlorobutyl)ether (CAS: 6334-96-9, $P_{Code}$:1003212982, product of Panjiva, Japan) were purchased from Sigma-Aldrich. Methanol (CAS-No: 67-56-1), chloroform (CAS-No: 67-66-3), ethyl acetate (CAS-No: 141-78-6) and NaOH (CAS-No: 1310-73-2) were received from Merck (Darmstadt, Germany).

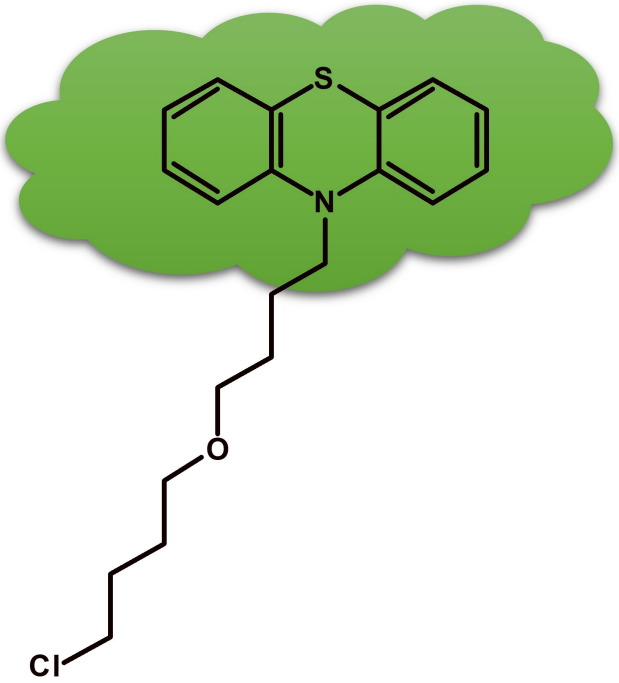


**Fig. 1. The structure of synthesized phenothiazine derivatives 10-[4-(4-chlorobutoxy)butyl]-10H-phenothiazine (OZF2).**

Phenothiazine derivative synthesis was done according to the protocols that were already establihsed in the literature with some minor modifications (Fig. 2) [31]. NaOH (0.184 mol) and bis(4-chlorobutyl)ether (0.048 mol) were slowly added to a solution of phenothiazine (0.022 mol) in dry dimehylsulfoxide (DMSO) (50 mL). The solution mixture was stirred at room temperature for 24 hours. The reaction was then poured into water and extracted with EtAc. The organic phase was dried over anhydrous $MgSO_4$. The crude product was purified via silica gel chromatography using chloroform/methanol (10:0.1; v/v) as the eluent to give the desired compound as dark red oil.

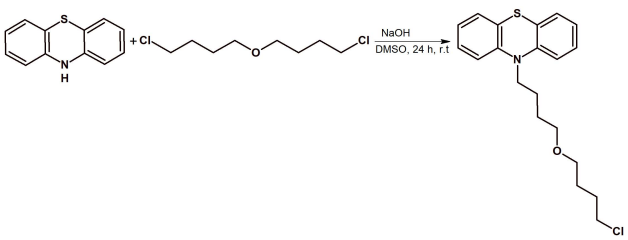


**Fig. 2. Synthesis of 10-[4-(4-chlorobutoxy)butyl]-10H-phenothiazine (OZF2).**

### *Cell Culture*

Caco-2 (ATCC HTB-37) cells stored at –80 °C were thawed in a water bath and grown in 10% Fetal Bovine Serum (FBS) 1% antibiotic [100 µg/mL] and Roswell Park

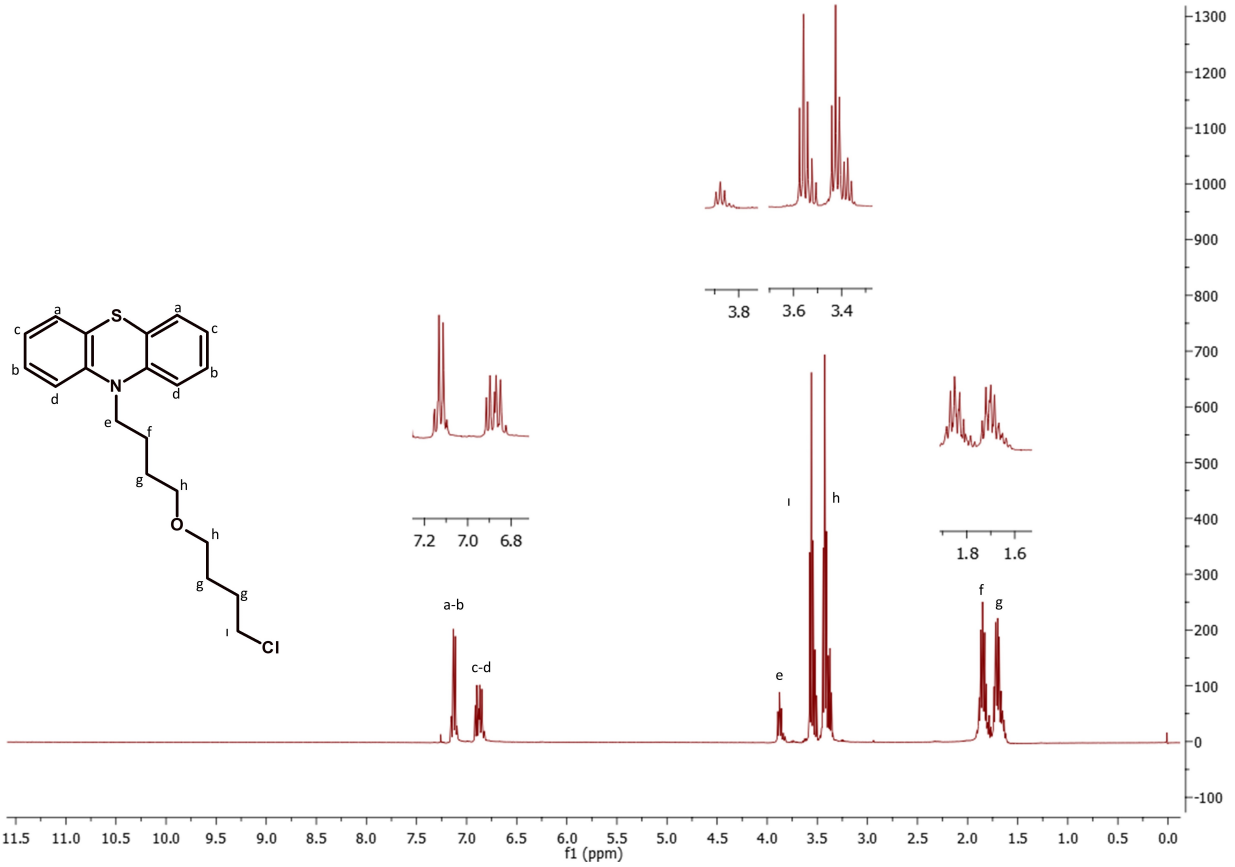


**Fig. 3.** $^{1}$**H Nuclear Magnetic Resonance (NMR) spectrum of 10-[4-(4-chlorobutoxy)butyl]-10H-phenothiazine (OZF2).**

Memorial Institute Media (RPMI) (SIGMA Aldrich R8758, Merck, Darmstadt, Germany). Cells were grown in 37 °C and 5% $CO_2$ humidified incubator [32]. The cell lines were mycoplasma free and STR analysis confirmed their respective identities.

### *Cell Count*

Cells were removed from the plate surface by gentle pipetting. Then, they were washed with 1 mL of PBS for cell counting. 10 µL of cells were added into 90 µL of trypan blue solution and live/dead cells were counted with a hemocytometer [32].

### *Cytotoxicity Analysis: MTT Assay*

A total of $10^5$/100 µL cells per well were added to 96-well plates and incubated for 12 hours at 5% $CO_2$ and 37 °C. After their adherence to the wells, various concentrations of phenothiazine derivative (1 µL, 2 µL, 4 µL, 8 µL and 10 µL) were added and then the samples were incubated for three different time points as 24 hours, 48 hours and 72 hours. These plates were made in triplicate and no phenothiazine derivative was added to the negative control for comparison. Afterwards, 10 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) solution was added. Then, the plate was incubated at 5% $CO_2$ and 37 °C for 3 hours and formazan crystals were dissolved with detergent reagent (SDS) and the absorbance was measured at 570 nm in the spectrophotometer device [33]. MTT kit was purchased from Roche (11465007001, Roche Diagnostics, Mannheim, Germany). Cell viability was calculated with the formula Cells viability % = (Total cells – Dead cells/Total cells) × 100.

### *Administration of Phenothiazine, LPS and Salicylic Acid (SA)*

Macrophage cell line RAW 264.7 (ATCC TIB-71) cells were added to 12-well Plates and stimulated for 24 hours with LPS. $10^6$ cells were added into each well. The experimental conditions were: negative control (just buffer), LPS (isolated from mg/mL Enzo Life Sciences, Salmonellaminnesota R595, Farmingdale, NY, USA) and SA, phenothiazine derivatives were added in different concentrations (0.5 µL, 1 µL, 3 µL) and phenothiazine in combination with LPS were added into the appropriate wells. Salicylic acid was used as a control because it is a molecule with anti-inflammatory activity [34,35]. The experiment was carried out as a triplicate. Afterwards the cell viabilities were counted by trypan blue staining and the supernatants were used for enzyme-linked immunosorbent assay (ELISA) [36].

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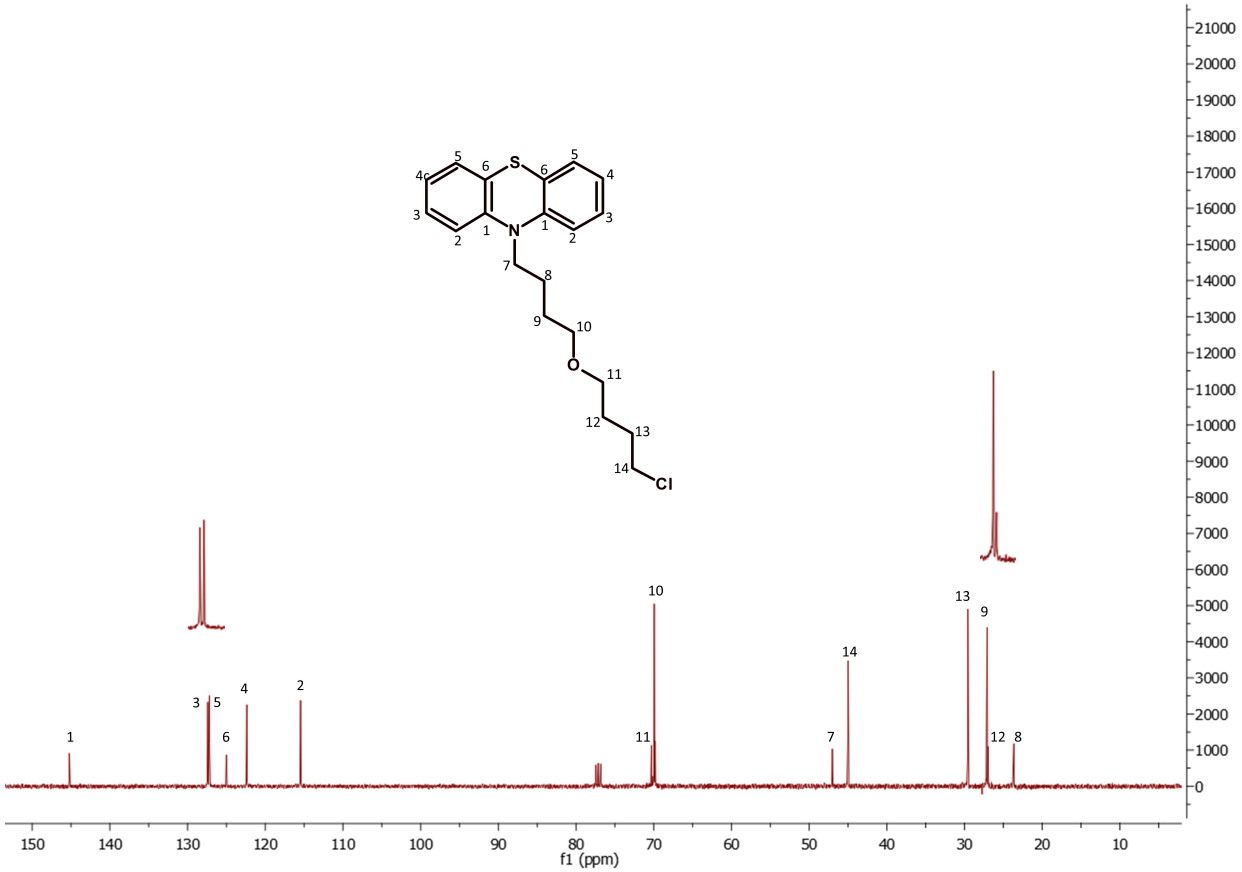


**Fig. 4.** $^{13}$**C NMR spectrum of 10-[4-(4-chlorobutoxy)butyl]-10H-phenothiazine (OZF2).**

*Enzyme-Linked Immunosorbent Assay (ELISA)*

Sandwich ELISA of TNF-$\alpha$ and IL-6 (BD Biosciences, San Jose, CA, USA for TNF-$\alpha$: AB_2869351, for IL-6: AB_2869049) was performed following the instructions in the user manual on the kit: Hamster mouse TNF-$\alpha$ and IL-6 antibodies (0.5 µg/mL in bicarbonate buffer pH: 9.5, 100 µL) were added to 27 wells of the 96 wells plate (Krackeler, Corning, NY, USA) and incubate for 24 hours at 4 °C. Plates were emptied and washed 3 times with Tween 20 PBS and then treated with blocking buffer (1% BSA PBS). Plates were subsequently washed again and 100 µL of samples was added to 27 wells in triplicate and incubated. Plates were washed again three times and TNF-$\alpha$ and IL-6 biotinylated human anti-mouse (0.5 µg/mL 10% FBS PBS) was added to each well. Then they were washed again and 50 µL of streptavidin HRP was added and left for 60 minutes. After washing three more times, 100 µL of TMB substrate was added and incubated in the dark for 10 minutes. Sulfuric acid (50 µL) was added to allow the development of blue color. A stop solution was added and a yellow color was developped. The measurement was then made at 450 nm [37].

*q-RT-PCR Analysis*

Macrophage cell line RAW 264.7 cells were added to 12-well plates and stimulated for 24 hours. Cell concentration was $10^6$/mL and 1 mL of the cells were added into each well of the 12 well plates. The experimental conditions were: negative control, LPS (isolated from mg/mL Enzo Life Sciences, Salmonellaminnesota R595, USA) and SA, phenothiazine derivatives were added in different concentrations (0.5 µL, 1 µL, 3 µL) and OZF2 in combination with LPS were added into the appropriate wells. Salicylic acid was used as a control because it is a molecule with anti-inflammatory activity. The experiment was carried out as a triplicate. The mRNA extraction, generation of cDNA were done according to standard procedures. Quantitative real time polymerase chain reaction (q-RT-PCR) analysis was done to determine the expression levels of *TNF-$\alpha$* and *IL-6* genes. *$\beta$-actin* (murine genome) expression was used as relative control. SYBR green master mix (Thermofisher) was used to conduct q-RT-PCR by following the standard protocols. Primers were produced by Integrated DNA Technologies (Integrated DNA Technologies, Coralville, IA, USA) as listed in Table 1. RNA was extracted using the RNeasy Mini isolation kit (74106, Qiagen, Hilden, Germany) according to the manufacturer's instructions. By applying 1.5 µL of the extracted RNA, its absorbance was detected at

230 nm, 260 nm and 280 nm. The 260/280 ratio was used to determine RNA purity and nucleic acid concentrations in ng/µL, while the 260–230 nm ratio was used to identify potential contaminants absorbed at or below 230 nm. Only RNA samples with 260–280 nm and 260–230 nm ratios above 2.0 were used for cDNA synthesis. After verification of concentration and purity, RNA samples were diluted to a concentration of 250 ng/µL, aliquoted, and stored at –80 °C. All RNA samples were simultaneously reverse transcribed with the High Capacity Reverse Transcription kit (Applied Biosystems, 74106, Valencia, CA, USA) by applying 500 ng of total RNA per 1 µL MultiScribe™ Reverse Transcriptase enzyme in the kit and stored in aliquots at –80 °C. q-RT-PCR with 2x SsoFast was done in a 15 µL reaction volume. Eva Green Supermix (Bio-Rad, Cat# 1725201, Lot# 64294998, CA, USA) was used for q-RT-PCR, 40 cycles were repeated sequentially at 95 °C for 5 min, followed by 95 °C for 5 s and 51 °C for 20 s done. Real-time melting analysis was then performed on technical triplicates with the LighCycler480 II device (Roche Diagnostics, Mannheim, Germany) to confirm product specificity [38–40]. The $2^{-\Delta\Delta Ct}$ method, one of the more accepted methods for gene expression analysis, was used. $2^{-\Delta\Delta Ct}$ is a calculation method based on estimating DNA amounts by comparing them. The formula is as follows; $2^{-\Delta\Delta Ct}$ = (Ct-tumor – Cthousekeeping) – (Ctnormal – Cthousekeeping) [41].

**Table 1. Primers analyzed together with q-RT-PCR are given in the table.**

| Gene symbol | Forward (5′-3′) Reverse (5′-3′) |
|---|---|
| *Actb* (murine genome) | *TGGAATCCTGTGGCATCCATGAAAC*<br>*TAAAACGCAGCTCAGTAACAGTCCG* |
| *IL-6* (murine genome) | *TTCCTCTCTGCAAGAGACT*<br>*TGTATCTCTCTGAAGGACT* |
| *TNF-α* (murine genome) | *ATGAGCACAGAAAGCATGATC*<br>*TACAGGCTT GTCACTCGAATT* |

*IL-6*, interleukin-6; *TNF-α*, tumor necrosis factor alpha; q-RT-PCR, quantitative real time polymerase chain reaction.

### *Statistical Analysis*

GraphPad Software version-5 (GraphPad Software, Inc., San Diego, CA, USA) was used in the statsitical evaluations. Unpaired two-tailed student's *t*-test was used for all studies [40]. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

## Results

### *NMR Results of 10-[4-(4-chlorobutoxy)butyl]-10H-phenothiazine*

Carbon and hydrogen Nuclear Magnetic Resonance (NMR) results confirmed the structure and synthesis of the

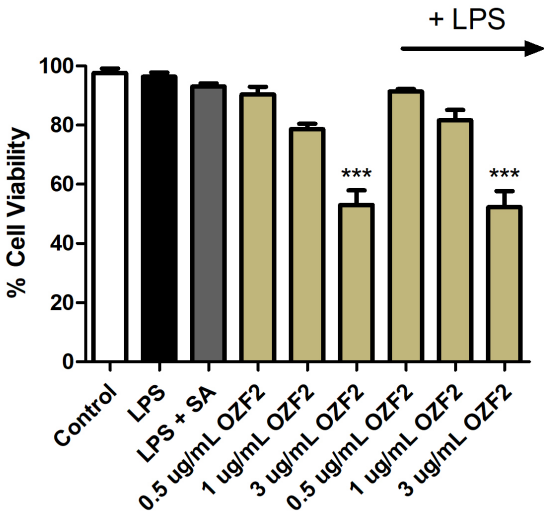


**Fig. 5. Trypan Blue staining of the cells after 24 hours of incubation.** 0.5, 1 and 3 µg/mL of OZF2 was added with or without 1 µg/mL of lipopolysaccharide (LPS) into the respective wells. 1 × $10^6$ cells/mL of RAW 264.7 mammalian macrophages were incubated overnight and then stimulated for 24 hours. 10 µg/mL of Salicylic acid was used as control. $***p < 0.001$ vs LPS, N = 3. SA, Salicylic Acid.

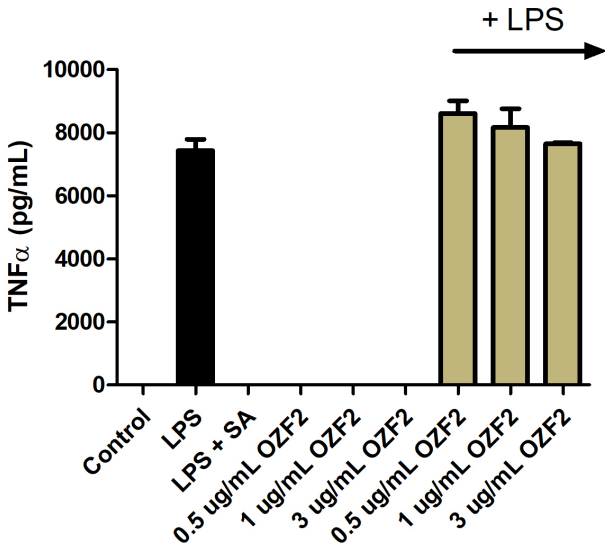


**Fig. 6. Enzyme-linked immunosorbent assay (ELISA) of TNF-α from the supernatants of macrophages, not stimulated or stimulated with 1 µg/mL of LPS after 24 hours of incubation.** 0.5, 1 and 3 µg/mL of OZF2 was added with or without 1 µg/mL of LPS into the respective wells. 1 × $10^6$ cells/mL of RAW 264.7 mammalian macrophages were incubated overnight and then stimulated for 24 hours. 10 µg/mL of Salicylic acid was used as control. N = 3.

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novel phenothiazine derivative (Figs. 3,4). The molecular structure of phenothiazine derivative was characterized by $^{1}$H NMR and $^{13}$C NMR. NMR spectra was recorded on a Mercuryplus-AS 400 MHz using $CDCl_3$. The results can be summarized as: $^{1}$H NMR (400 MHz, CDCl3, $\delta$ ppm): 7.12–6.85 (m, 8H, Ar-H), 3.88 (t, 2H, CH2), 3.56 (2H, CH2), 3.42 (4H, CH2), 1.85 (m, 2H, CH2), 1.70 (m, 6H, CH2). $^{13}$C NMR (50 MHz, CDCl3, $\delta$ ppm): 145.41, 127.49, 126.98, 125.07, 122.36, 115.35, 70.25, 69.81, 46.86, 44.75, 29.55, 26.96, 26.94, 23.46 ($C_{20}H_{24}NOCl$).

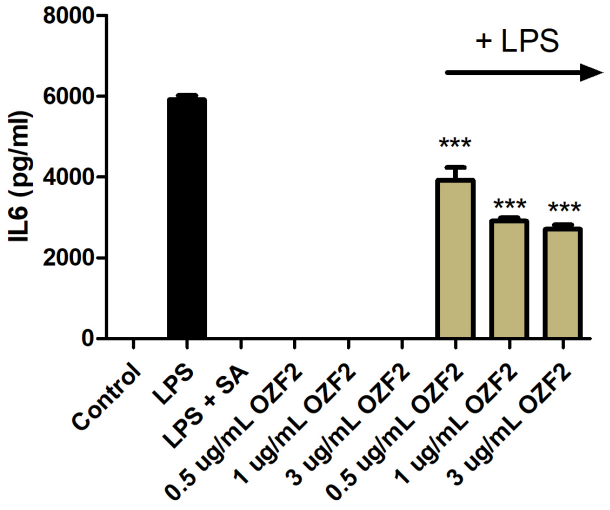


**Fig. 7. ELISA of IL-6 from the supernatants of macrophages, not stimulated or stimulated with 1 µg/mL of LPS after 24 hours of incubation.** 0.5, 1, and 3 µg/mL of OZF2 was added with or without 1 µg/mL of LPS into the respective wells. $1 \times 10^6$ cells/mL of RAW 264.7 mammalian macrophages were incubated overnight and then stimulated for 24 hours. 10 µg/mL of Salicylic acid was used as control. ***$p < 0.001$ vs LPS, N = 3.

## *OZF2 had Anti-Proliferative Activity*

OZF2 phenothiazine was applied at five different concentrations: 1, 2, 4, 8, 10 µg/mL and incubated for 24, 48, and 72 hours with caco-2 cells. The molecule caused a decrease in cell proliferation rate compared to the control group in direct proportion to the increasing different concentration (Table 2). Inhibition concentration ($IC_{50}$) values of OZF2 derivative on five different concentrations of colon cancer cell lines were analyzed at 24, 48, and 72 hour intervals. Inhibition concentrations were: 2.9990 µg/mL at the end of the 24th hour, 2.9384 at the end of the 48th hour and 2.9177 at the end of the 72nd hour (Table 2). The observed decrease in proliferation rate could be a promising indication for potential anti-cancer activity.

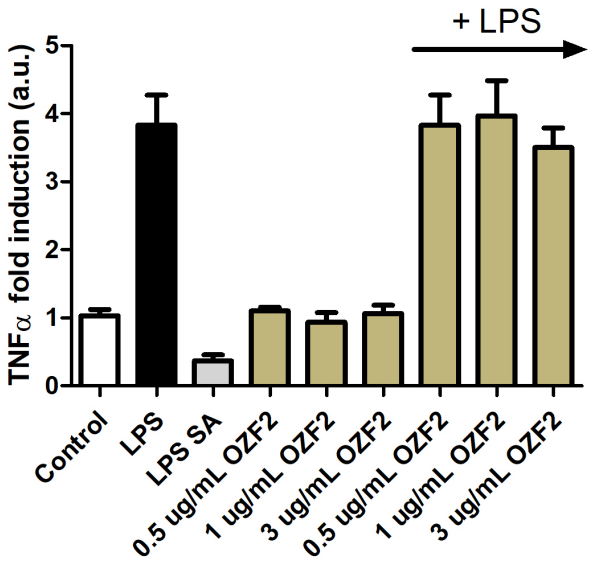


**Fig. 8. TNF-$\alpha$ q-RT-PCR for the macrophages that were not stimulated or stimulated with 1 µg/mL of LPS after 24 hours of incubation.** 0.5, 1, and 3 µg/mL of OZF2 was added together with or without 1 µg/mL of LPS into the respective wells. $1 \times 10^6$ cells/mL of RAW 264.7 mammalian macrophages were incubated overnight and then stimulated for 24 hours. 10 µg/mL of Salicylic acid was used as control. N = 3.

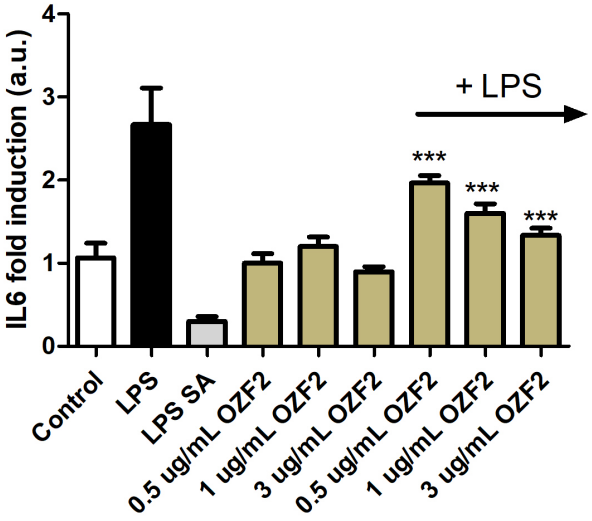


**Fig. 9. IL-6 q-RT-PCR for the macrophages that were not stimulated or stimulated with 1 µg/mL of LPS after 24 hours of incubation.** 0.5, 1, and 3 µg/mL of OZF2 was added with or without 1 µg/mL of LPS into the respective wells. $1 \times 10^6$ cells/mL of RAW 264.7 mammalian macrophages were incubated overnight and then stimulated for 24 hours. 10 µg/mL of Salicylic acid was used as control. ***$p < 0.001$ vs LPS, N = 3.

**Table 2. $IC_{50}$ values of OZF2 on colon caco-2 cells at different time points.**

| Hours | $IC_{50}$ (µg/mL ± SD) |
|---|---|
| 24 h | 2.9990 ± 0.2 |
| 48 h | 2.9384 ± 0.3 |
| 72 h | 2.9177 ± 0.1 |

$IC_{50}$, inhibition concentration.

### *The Derivative had Immunomodulatory Activities*

The OZF2 phenothiazine derivative caused a concentration-dependent decrease in viability of the mammalian macrophage cell line (Fig. 5). It was observed that the OZF2 did not stimulate the production of TNF-$\alpha$ and IL-6 cytokines from macrophage cells that were not treated with LPS (Figs. 6,7). This suggests the OZF2 phenothiazine derivative does not have immunostimulatory potential. Different concentrations of OZF2 did not positively or negatively affect the production level of TNF-$\alpha$ cytokine in cells treated with LPS (Fig. 6). With the increase of OZF2 concentration in the presence of LPS, a statistically significant decrease was observed in the production level of IL-6 cytokine compared to the only LPS treated group (Fig. 7). The molecule showed immunomodulatory activity in the mammalian macrophage cells as an anti-inflammatory agent for the IL-6 cytokine. When the gene expression levels of these cytokines were measured, there was no change in the expression of TNF-$\alpha$ gene, whereas IL-6 gene expression significantly decreased in the presence of OZF2 (Figs. 8,9). These results are in line with what was observed for the changes in the protein production levels (Figs. 6,7). OZF2 affected the gene expression level of IL-6 cytokine and exerted its immunomodulatory activity through the regulation of the gene expression.

## Discussion

Studying the anti-inflammatory and anti-proliferative activities of newly synthesized molecules is an active area of research. Phenothiazine derivatives are known for their many biological activities [18–27]. Today, more than 5000 derivatives of these molecules, which are widely known for their industrial and medical applications, have been synthesized. In the past, their use in the treatment of diseases in different fields and its anti-cancer, anti-apoptotic and anti-inflammatory activities on various cancer cells have been demonstrated [42–47].

Macrophage cells, which play an important role in inflammation, support the progression of cancer cells in the tumor microenvironment. Various cytokines released into the environment when macrophages are activated can stimulate the pathogenesis of cancer [2–5,48]. TNF-$\alpha$ cytokine, one of the most important of these cytokines, is thought to support the tumor growth [11]. Conversely, it could suppresse cancer, cause tumor necrosis and cytotoxicity in various types of cancer, and inhibit the growth of cancer cells. Therefore, its role is controversial and depends on the situation [49–51].

The high level of IL-6 cytokine release, which is another cytokine, is associated with different cancer types and colorectal cancer [52]. Positive results were obtained in clinical studies on the therapeutic use of IL-6 cytokine in various inflammatory diseases [53,54]. Its application in combination with drugs known to be used in chemotherapy in various cancer cells was tested. In addition, studies showing that the deficiency of this cytokine in colon cancer prevents metastasis in experimental animals are also suggesting its role in cancer development [55].

The reason why so many derivatives of these molecules can be synthesized with a broad range of biological activities is the positioning of the functional groups in the ring structures [56,57]. Since phenothiazine derivatives form the basis of many drugs, they are more advantageous and reliable compared to the discovery of new drugs that may pose a high cost and risk [58].

A novel phenothiazine derivative was synthesized in this study. We examined the anti-cancer activity of the OZF2 phenothiazine derivative on the colon cancer cell line by MTT assay and tested its potential for application in therapies targeting TNF-$\alpha$ and IL-6 cytokines in mammalian macrophage cells.

In anti-cancer activity experiments, with the increased concentration of our molecule, there was a notable decrease in the proliferation rate of colon cancer cells compared to the control groups. The derivative had potent $IC_{50}$ values. As expected, a decrease in the percentage of macrophage cell viability was observed in anti-inflammatory activity studies as well when the highest concentration of the molecule was used. Since no decrease in cell viability was observed in 0.5 µg/mg administered groups, it is recommended to be applied at this concentration for the immunomodulatory purposes. The OZF2 phenothiazine derivative did not affect the level of TNF-$\alpha$ cytokine production in LPS-stimulated macrophage cells, but caused a statistically significant decrease in the amount of IL-6 cytokine production. The fact that our molecule does not affect the level of TNF-$\alpha$ production but causes a decrease in the level of IL-6 cytokines provides advantages for cancer studies. TNF-$\alpha$ is mostly regarded as an anti-cancer cytokine whereas IL-6 is known to stimulate the cancer growth and angiogenesis for their dissemination to the body [59,60].

Moreover, when the gene expression levels were measured for TNF-$\alpha$ and IL-6, there was no change in the TNF-$\alpha$ expression level whereas IL-6 gene expression levels significantly dropped in the presence of OZF2 in LPS stimulated macrophages. These results suggest that OZF2 acted on the macrophages by changing the IL-6 gene expression

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levels. More studies will be conducted in the future to fully decipher the signalling pathways that might be altered in the presence of OZF2.

As a result, we propose a drug candidate that can be studied in cancer research and potentially used in cancer treatment, as this novel molecule shows anti-cancer activity on the colon cancer cells. Since the derivative was not affecting TNF-$\alpha$ levels while decreasing the IL-6 production, it has the potential to be used in cancer immunotherapy as well [61]. TNF-$\alpha$ is known to decrease the cancer cell viability whereas IL-6 has wound healing and angiogenic activities that support the tumor growth and propogation [62,63]. Having an anti-proliferative molecule that decreases the IL-6 levels without changing the TNF-$\alpha$ levels is advantageous for cancer treatment [64,65].

Furthermore, it has the potential to be offered to patients as an anti-inflammatory drug candidate that can suppress the production of IL-6 cytokine in autoimmune and inflammatory diseases that are heavily characterized by IL-6 cytokine production [66–68]. *In vitro* studies on phenothiazine derivatives have proven cytotoxicity, cell cycle regulatory, anti-proliferative and immunomodulatory activities in different cancer cells. Studies in the literature support our results [69–71].

In the future studies, a larger selection of cancer cell lines can be used, and the diversity of pro-inflammatory and anti-inflammatory cytokines for the anti-inflammatory studies can be increased. Moreover, the evaluation of the intracellular signaling pathways can be done to decipher the mechanism of its action in further details.

## Conclusions

In this study, a notable decrease in cell proliferation was observed as a result of OZF2 application on the colon cancer cell line. Experiments showed that, while OZF2 has anti-inflammatory and immunomodulatory activity in the presence of LPS, it does not have immunostimulatory potential when applied alone, since it does not affect the production level of cytokines. A wider range of cancer cell types should be used in future studies, and pro-inflammatory and immunomodulatory properties should be tested in anti-inflammatory studies. Anti-inflammatory cytokine diversity can be detailed by evaluating intracellular signaling pathways.

## Availability of Data and Materials

Data is available from the corresponding authors upon reasonable request.

## Author Contributions

ÖH conducted the chemistry experiments and drew the related figures. MŞA and FA conducted the molecular biology experiments and drew the related figures. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

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