

T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

MASTER OF SCIENCE THESIS

ELUCIDATION OF ANTI-INFLAMMATORY AND ANTICANCER PROPERTIES OF NOVEL NIMESULIDE DERIVATIVES

Adem ÖZLEYEN

Biomolecular Sciences

ÇANAKKALE

T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

MASTER OF SCIENCE THESIS

ELUCIDATION OF ANTI-INFLAMMATORY AND ANTICANCER PROPERTIES OF NOVEL NIMESULIDE DERIVATIVES

Adem ÖZLEYEN

Biomolecular Sciences

Date of Defense: **18/08/2020**

Thesis Supervisor:

Assoc. Prof. Dr. Tuğba TÜMER

ÇANAKKALE

The study entitled **"Elucidation of Anti-inflammatory and Anticancer Properties of Novel Nimesulide Derivatives"**, submitted by **Adem ÖZLEYEN** under supervision of Assoc. Prof. Dr. Tuğba TÜMER was defended on **18/08/2020.** This thesis has been approved as **Master of Science** in **Biomolecular Sciences** of Çanakkale Onsekiz Mart University School of Graduate Studies by the Examining Committee Members.

EXAMINING COMMITTEE

……

Assoc. Prof. Dr. Tuğba TÜMER
Supervisor

……

Prof. Dr. Kemal Melih TAŞKIN
Member

……

Prof. Dr. Şevki ARSLAN
Member

Assoc. Prof. Dr. Pelin KANTEN
Director
School of Graduate Studies

Order No:……

This study has been supported by Çanakkale Onsekiz Mart University Scientific Research Project Coordination Unit. Project Number: FYL-2019-3123.

PLAGIARISM DECLARATION PAGE

I declare that all the information and results presented in visual, audio, and written form are obtained by myself observing the academic and ethical rules and, all other results and information indicated in the thesis but not specific to this study are cited.

Adem ÖZLEYEN

ACKNOWLEDGEMENTS

"Tell me and I forget. Teach me and I remember. Involve me and I learn" B. Franklin

My sincere gratitude goes first to my supervisor, Assoc. Prof. Dr. Tuğba TÜMER, who involved me in her lovely laboratory team and precious scientific projects. Her office door was always open whenever I had problems with my research, writing, or life. Besides her scientific knowledge, her philosophy of life also is a source of inspiration for me.

I would like to thank each of my instructors who gave me valuable knowledge throughout my education life.

"Alone we can do so little; together we can do so much" H. Keller

I wish to thank Dr. Tuğba Güngör and Prof. Dr. Mehmet Ay for their efforts to design, synthesize, and characterize the compounds to be investigated. Dear Prof. Feray Köçkar and Assist. Prof. Dr. Esra Tokay opened the door of their research laboratory for a part of apoptosis-related studies in Balıkesir University. For SEM analyses, Prof. Dr. Şevki Arslan gave us a lead to complete related experiments. Without their bits of help, this study could not have reached its goal.

"Other things may change us, but we start and end with the family" A. Brandt

They kept me going on my way and gave me the support/motivation that they had no in their time. This journey would not have been possible if I did not have my mom Ayten ÖZLEYEN, my dad Cemal ÖZLEYEN, and my siblings Atilla & Azize. Further, I special thanks to my little nephews Kağan and Atalay who always made noises when I tried to concentrate on my studies.

It was great sharing the same lab bench, crying, and laughing at common things with my lab family Begüm, Berkay, Elif, Gizem. I had lots of unforgettable moments with each member of the TBT Lab team. Besides, I would like to thank Ayşe, Berfin, Ece, Gözde, Kübra, Raşit who did not withhold their friendship and smiling face in the same lab. I present my thanks and love to my dear friends Ahmet, Ebru, Hatice, Mehtap, and Melis who supported me spiritually during my thesis period.

For their financial supports, I would like to thank The Scientific and Technological Research Council of Turkey (Project No: 117Z398) and COMU Scientific Research Projects Coordination Unit (Project No: FYL-2019-3123).

Adem ÖZLEYEN

Çanakkale, August 2020

ABBREVIATIONS

AA	Arachidonic Acid
BCA	Bicinchoninic Acid
COX	Cyclooxygenase
DAAID	Dual-Acting Anti-Inflammatory Drug
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DOX	Doxorubicin
ELISA	Enzyme-Linked ImmunoSorbent Assay
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HMDS	Hexamethyldisilazane
iNOS	Inducible Nitric Oxide Synthase
LOX	Lipoxygenase
LPS	Lipopolysaccharides
LTB_4	Leukotriene B_4
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NDs	Nimesulide Derivatives
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSAID	Nonsteroidal Anti-Inflammatory Drug
PBS	Phosphate-Buffered Saline
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGHS	Prostaglandin H Synthase
PI	Propidium Iodide
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RPMI-1640	Roswell Park Memorial Institute Medium
SAR	Structure-Activity Relationship

SEM	Scanning Electron Microscope
SRB	Sulforhodamine B
TxA	Thromboxanes

ÖZET

YENİ NİMESULİD TÜREVLERİNİN ANTİ-İNFLAMATUAR VE ANTİKANSER ÖZELLİKLERİNİN AYDINLATILMASI

Adem ÖZLEYEN
Çanakkale Onsekiz Mart Üniversitesi
Lisansüstü Eğitim Enstitüsü
Biyomoleküler Bilimler Anabilim Dalı Yüksek Lisans Tezi
Danışman : Doç. Dr. Tuğba TÜMER
18/08/2020, 67

İnflamasyon, canlı organizmaların patojenler, hasarlı hücreler veya kötü yaşam koşulları gibi zararlı uyaranlara karşı doğal bir bağışıklık tepkisidir. Uzun süreli inflamasyon artrit, diyabet, astım gibi hastalıkların ortaya çıkması ile ilişkilidir. Bu nedenle, kronik inflamasyonun inhibisyonu çeşitli hastalıklar için alternatif bir terapötik ve önleyici strateji olabilir. Steroid olmayan anti-inflamatuar ilaçlar (NSAID'ler), güçlü analjezik ve anti-inflamatuar etkileri nedeniyle ilaç endüstrisinde en çok reçete edilen ilaç sınıfıdır. Etki mekanizmaları, siklooksijenaz (COX) enzim-aracılı prostaglandin üretiminin inhibisyonuna dayanır. Bununla birlikte, NSAID'lerin uzun süreli kullanımı kardiyovasküler, idrar ve gastrointestinal problem riskini artırır. Bu olumsuz etkilerin altında yatan sebep, istenmeyen COX-1 enzim inhibisyonudur. Daha ileri çalışmalar, COX-2 enzimini inhibe ederken COX-1 enzimini sınırlı bir oranda inhibe eden öncelikli COX inhibitörlerinin, geleneksel NSAID'lere kıyasla güvenli terapötikler olabileceğini göstermiştir. İkincil neden, araşidonik asit metabolizmasında homeostazın lipooksijenaz (LOX) yollarına doğru bozulmasıdır. 5-LOX, inflamatuar yanıtta rol oynayan başka bir enzimdir. 5-LOX yolağının aktivasyonu, gastrointestinala zarar veren aşırı miktarda lökotrien üretimine yol açar.

Nimesulid, anti-inflamatuar, analjezik ve antipiretik etkilere sahip kanıtlanmış öncelikli COX-2 inhibitörlerden birisidir. Nimesulidin terapötik etkisi, pro-inflamatuar prostaglandin ve sitokin üretimlerinin inhibisyonuna dayanır. Bu farmakolojik etkilerin dışında, nimesulid reaktif oksijen türleri (ROS), nitrik oksit (NO) ve lökotrien B_4 (LTB_4) seviyelerini de azaltır. Ayrıca, nimesulid kanser hücrelerinin çoğalmasını, serebral iskemiyi ve NSAID'lerin neden olduğu ülseri inhibe eder. Bu çalışmada amid/sülfonamid

içeren on yedi adet yeni nimesulid türevlerinin anti-inflamatuar ve antikanser etkilerinin aydınlatılması amaçlanmıştır.

Bu amaçla bütün bileşikler COX-2/COX-1 enzim aktivitesi üzerine etkileri açısından taranmıştır. Buna göre, **N8** ve **N10** bileşikleri, referans ilaç nimesulid ile karşılaştırıldığında COX-2 enzimi üzerinde daha iyi seçicilik göstermiştir. Ayrıca, hem **N8** hem de **N10**, LPS ile uyarılan COX-2 mRNA ekspresyon seviyesini seçici olarak düşürürken, COX-1 seviyesi makrofajlarda stabil kalmıştır. Makrofaj hücrelerinde hem PGE_2 salımı hem de nitrik oksit üretimi **N8** ve **N10** uygulama grubunda önemli ölçüde baskılanmıştır. Ayrıca bu iki bileşik, IL-lβ ve TNF-α seviyesini istatistiksel olarak anlamlı ölçüde düşürmüştür. Türevlerin antikanser özelliklerini belirlemek için yapılan tarama çalışmalarına göre, **N17** bileşiği kolon (IC_{50}: 9.24 µM) ve meme (IC_{50}: 11.35 µM) kanser hücresi hatlarında önemli sitotok özellik sergiledi. Özetle, bu sonuçlar **N8** ve **N10** bileşiklerinin umut verici anti-inflamatuar ajanlar olabileceğini, sitotoksik **N17** bileşiği ise daha sonraki potansiyel antikanser ilaç gelişimi öncüsü olarak çalışılabileceğini göstermektedir.

Anahtar sözcükler: Nimesulid, Antikanser, Anti-inflamatuvar, Öncelikli COX İnhibitörü

ABSTRACT

ELUCIDATION OF ANTI-INFLAMMATORY AND ANTICANCER PROPERTIES OF NOVEL NIMESULIDE DERIVATIVES

Adem ÖZLEYEN

Çanakkale Onsekiz Mart University

School of Graduate Studies

Master of Science Thesis in Biomolecular Sciences

Advisor : Assoc. Prof. Dr. Tuğba TÜMER

18/08/2020, 67

The inflammation indicates the innate immune response of organisms against detrimental stimuli including a variety of pathogens, damaged cells, or even worse living conditions. Prolonged-inflammation is associated with the occurrence of diseases such as arthritis, diabetes, asthma. Thus, inhibition of chronic inflammation could be an alternative therapeutic and preventive strategy for several diseases. Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most prescribed drug class in the pharmaceutical industry for their strong analgesic and anti-inflammatory effects. Their action mechanism is based on the inhibition of cyclooxygenase enzymes (COXs)-mediated prostaglandin production. Prolonged use of NSAIDs may promote the risk of cardiovascular, urinary, and gastrointestinal problems. The underlying reason for these adverse effects is the undesirable COX-1 enzyme inhibition. Further studies demonstrated that preferential COX inhibitors, which inhibit the COX-2 enzyme eloquently and COX-1 with at a limited rate, might be safe therapeutics as compared to the non-selective classical NSAIDs. The second reason is the deterioration of homeostasis in arachidonic acid metabolism towards lipoxygenases (LOX) pathways. 5-LOX is another enzyme playing a role in the inflammatory response. Over-activation of 5-LOX leads to an excess amount of gastro-damaging leukotrienes.

Nimesulide is one of the proven preferential COX-2 inhibitors which have anti-inflammatory analgesic and antipyretic effects. The therapeutic action of nimesulide is based on the inhibition of pro-inflammatory prostaglandin and cytokine productions. Apart from these pharmacological effects, nimesulide reduces the level of reactive oxygen species (ROS), nitric oxide (NO), and leukotriene B_4 (LTB_4). Besides, nimesulide inhibits

cancer cell proliferation, cerebral ischemia, and NSAIDs-induced ulcer. In this study, the anti-inflammatory and anticancer effects of seventeen novel amide/sulfonamide containing nimesulide derivatives were aimed to be elucidated.

With this aim, each compound was tested for its selective COX-2/COX-1 enzyme inhibitory properties. Accordingly the compounds **N8** and **N10** showed better selectivity on the COX-2 enzyme as compared to the reference drug nimesulide. Moreover, both **N8** and **N10** selectively reduced the LPS-stimulated COX-2 mRNA expression while the level of COX-1 remained stable in macrophages. Both PGE_2 release and nitric oxide production in macrophage cells were significantly suppressed in the **N8** and **N10** treatment group. Furthermore, these two compounds reduced the mRNA level of IL-1β and TNF-α statistically significant. Screening efforts to identify anticancer properties of derivatives resulted in compound **N17** with considerable cytotoxicity in the colon (IC_{50}: 9.24 µM) and breast (IC_{50}:11.35 µM) cancer cell lines. To sum up, these findings suggest that **N8** and **N10** could be promising agents with anti-inflammatory properties while cytotoxic **N17** might be studied as a lead for subsequent potential anticancer drug development.

Keywords: Nimesulide, Anticancer, Anti-inflammatory, Preferential COX Inhibitor

TABLE OF CONTENTS

Page No

THESIS DEFENSE EXAM RESULT FORM ii
PLAGIARISM DECLARATION PAGE iii
ACKNOWLEDGEMENTS iv
ABBREVIATIONS v
ÖZET vii
ABSTRACT ix
LIST OF FIGURES xiii
LIST OF TABLES xv
CHAPTER 1
INTRODUCTION 1
1.1. COXs As Biological Target of NSAIDs 3
1.2. Understanding the Reasons For Adverse Effects of NSAIDs 4
1.3. Traditional NSAIDs and Novel Dual Acting Anti-inflammatory Drugs (DAAIDs) .. 8
1.4. Nimesulide: A Preferential COX-2 Inhibitor 9
1.4.1. Chemical Features of Nimesulide 9
1.4.2. The Explanations on The Bioactivity of Nimesulide 10
1.5. The Origin of Novel Nimesulide Derivatives (NDs) 12
1.6. The Aim of The Study 15
CHAPTER 2
PREVIOUS STUDIES 16
CHAPTER 3
MATERIALS AND METHODS 19
3.1. Materials 19
3.1.1. Chemicals, Kits, and Essential Items 19
3.1.2. Equipment Used for Analysis 20
3.1.3. Cell Lines and Novel NDs 21
3.2. Methods 22
3.2.1. Enzyme Inhibition Studies 22
3.2.2. Stimulation of THP-1 Monocytes by PMA Treatment 23
3.2.3. The Effect of NDs on LPS-induced PGE_2 and LTB_4 Release 23
3.2.4. Inhibitory Effect of NDs on Nitric Oxide Release 24
3.2.5. Gene Expression Analyses at mRNA Level 24

3.2.6. Evaluation of Protein Expression Levels By SDS-PAGE and Western Blot Analysis 25
3.2.7. Screening of Anticancer Effects of NDs by Cytotoxicity Assays 26
3.2.8. Scratch Wound Healing Assay In Cancer Cell Model..... 27
3.2.9. Flow Cytometry Analysis 28
3.2.10. Imaging of Ultrastructural Changes by Scanning Electron Microscope Analysis 29
3.2.11. Statistical Analysis..... 30
CHAPTER 4
RESULTS AND DISCUSSION 31
4.1. Results 31
4.1.1. COX-1/2 Inhibitory Activities of NDs 31
4.1.2. THP-1 Differentiation by PMA Treatment 33
4.1.3.Determination The Effects of Lead NDs on COX-1/2 Expression Level..... 34
4.1.4. The Effects of Lead NDs on LPS-induced PGE_2 Production 35
4.1.5. The Effects of Lead NDs on 5-LOX Enzyme Activity, mRNA Expression Level, and LTB_4 Release 35
4.1.6. Activation of Macrophages by LPS Treatment to Induce NO Release and Determination The Effects of Lead NDs on NO Production and iNOS Expression.... 36
4.1.7. The Effects of Lead NDs on IL-1β and TNF-α mRNA Expression Levels..... 38
4.1.8. Time Optimization Studies for Screening of Anticancer Properties 38
4.1.9. Screening of Anticancer Effects of NDs by MTT Assay..... 39
4.1.10. Determination of IC_{50} Values of N17 by SRB Assay..... 41
4.1.11. Assessment of Anti-proliferation/migration Properties of N17 42
4.1.12. The Effects of N17 on Apoptosis Related Protein Expression Level..... 43
4.1.14. The Effects of N17 on Annexin V Staining Apoptosis Test 46
4.2. Discussion..... 47
CHAPTER 5
CONCLUSION..... 56
REFERENCES..... 58
BIOGRAPHY I

LIST OF FIGURES

Page No

Figure 1.The milestones in the developments of aspirin or non-aspirin NSAIDs in chronological order. 2
Figure 2.The synthesis of prostaglandin species from membrane-bound arachidonate via COX-1/COX-2 isoform catalysis. 5
Figure 3.The possible fates of arachidonic acid and the connections between COX and LOX pathways. 7
Figure 4.The structure of selective COX-2 inhibitor (celecoxib), 5-LOX inhibitor (zileuton), and dual inhibitor (licofelone). 9
Figure 5.Classification of NSAIDs according to their chemical properties (K. Rainsford, 2007). 10
Figure 6.The structures of novel NDs to be investigated in the study. 14
Figure 7.Step by step qPCR analysis: from cell culture to the gene expression analysis. 24
Figure 8.Experimental flow chart of (A) MTT and (B) SRB assays. 27
Figure 9.An illustration of the results of Guava Nexin. Q1 indicates live cells; Q2 indicates early apoptotic cells; Q3 indicates late apoptotic cells; Q4 indicates necrotic cells. 28
Figure 10.The working principle of a scanning electron microscope. 29
Figure 11.Screening of inhibitory effects of NDs on the COX-2 enzyme at 1 μM dose. As positive control nimesulide (NIM) and celecoxib (CLB) were examined at the same doses. C: Control. ***$p < 0.001$. 32
Figure 12.The comparison of inhibitory effects of NDs on COX-1/2 enzymes at 10 and 1 μM doses, respectively. 32
Figure 13.Differentiation of THP-1 monocyte cells into macrophage cells by PMA treatment. (A) Suspended monocytic cells (left side) and differentiated adherent macrophages (right side). (B) Experimental stages from cell differentiation to further analyses. 33
Figure 14.Effects of **N8** and **N10** on COX-1/2 mRNA expression levels in LPS-stimulated THP-1 cells. Celecoxib (100 μM): Positive control. C: Control. *,♦$p< 0.02$, **$p< 0.005$, ***, ♦♦♦$p < 0.001$. 34
Figure 15.Effects of **N8** and **N10** on PGE_2 release in LPS-stimulated THP-1 cells. Celecoxib (100 μM): Positive control. C: Control. ***$p < 0.001$. 35
Figure 16.Effects of **N8** and **N10** on 5-LOX (A) enzyme activity, (B) mRNA expression level, and (C) LTB_4 release. NDGA (100 μM): Positive control. C: Control. **$p< 0.005$, ***$p < 0.001$. 36
Figure 17Relative NO change in LPS-challenged human macrophage cells (A). The effect of **N8** and **N10** on NO production in LPS-treated murine macrophage cells (B). Nim (20 μM): Reference compound.1400W (20 μM): Positive control. C: Control. ***$p < 0.001$. 37
Figure 18.Effects of **N8** and **N10** on iNOS mRNA expression levels in LPS-stimulated RAW264.7 cells. C: Control. ***$p < 0.001$ 37
Figure 19.Effects of **N8** and **N10** on (A) IL-1β and (B) TNF-α mRNA expression levels in LPS-stimulated RAW264.7 cells. C: Control. ***$p < 0.001$. 38
Figure 20.The effect of FDA-approved doxorubicin on cell viability of HT-29 cell line at the end of the 24, 48, and 72 hours. The results were evaluated by (A) MTT and (B) SRB assays. C: Control. **$p< 0.005$, ***$p < 0.001$ 39
Figure 21.The cytotoxic effect of NDs on (A) HT-29 colon cancer, (B) PC-3 prostate cancer, (C) MCF-7 breast cancer, and (D) HUVEC healthy cell lines at 100 μM

dose. C: Control. DOX: Doxorubicin. *p< 0.02, **p< 0.005, ***p < 0.001. 40
Figure 22.The healing process of artificial wounds in the MCF-7 cell line throughout 48-hours incubation in the absence and presence of test compounds. 42
Figure 23.The effects of **N17** on Bax/Bcl-2 expression levels in MCF-7 breast cancer cells. Cells were treated with 10 and 25 µM of the compound **N17**.C: Control.***p < 0.001. 43
Figure 24.The effects of **N17** on Bax and Caspase-3 expression levels in HT-29 colon cancer cells. Cells were treated with 10, 25, and 50 µM of the compound **N17**. C: Control. ***p < 0.001. 44
Figure 25.The ultrastructural changes of treated HT-29 cells were displayed by SEM analysis. The treatment of (B, D) N17 and (C, E) reference compound DOX for 48 h led to the loss of normal morphology and normal cell size as compared to the (A, D) untreated cells. Magnifications: 500x for A,B, C; 1,500x for D,E, and F micrographs. 45
Figure 26.The effects of **N17** on cell viability and apoptosis of cancer cells. Bottom left indicates live cells; bottom right indicates early apoptotic cells; upper right indicates late apoptotic cells; upper left indicates necrotic cells. 46
Figure 27.Proposed molecular mechanism showing how NO molecule contributes to the COX activity and inflammation. 50
Figure 28.The proposed hallmark features of cancer by Hanahan and Weinberg (Ediriweera, Tennekoon, & Samarakoon, 2019). 52
Figure 29.The effects of the compound **N17** on expression levels of apoptotic proteins. .. 55
Figure 30.The biological activities of compounds **N8**, **N10,** and **N17**..... 57

LIST OF TABLES

Page No

Table 1.The comparison of the features of human COX isoenzymes 4
Table 2.Determined IC_{50} scores of NSAIDs for COX enzymes by whole blood assay and their selectivity index. The data were taken from the paper published by Rao & Knaus, 2008. 6
Table 3.List of chemicals, kits, and essential items 19
Table 4.List of equipment and their intended purposes 20
Table 5.List of cell lines and their optimized culture conditions 22
Table 6.The IC_{50} concentrations of **N17** and doxorubicin in HT-29 colon cancer, PC-3 prostate cancer, MCF-7 breast cancer, and HUVEC healthy cell lines. 41

CHAPTER 1
INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most prescribed drug class for ones suffering from pain, fever, and inflammation. Daily, over 30 million aspirin or non-aspirin NSAIDs are being consumed consciously or unconsciously (Bjarnason et al., 2018; Emery, 2001). Currently, Aspirin™ has been a widely utilized pharmaceutical for 123 years and is appointed as the progenitor of NSAIDs (Otterness, 1995). The origin of aspirin or non-aspirin NSAIDs is based on serendipitous discoveries in ancient times (Figure 1). In the journey to the past, it could be seen that Sumerians used to apply the salicylate-containing willow leaves (Salix species) to treat the inflammatory rheumatic disease (Montinari, Minelli, & De Caterina, 2019). The importance of the willow leaf could be also noted in the Ebers Papyrus (1534 B.C.) against inflammatory symptoms (Bryan & Smith, 1974). Years later, the Greek physician Hippocrates (400 B.C.) suggested the use of leaves and bark extracts of the Salix plants for the relief of pain and fewer. Moreover, a number of the report recorded by Pliny the Elder (23 CE), Dioscorides (40 CE), and Galen (129 CE) regarding the medicinal potency of these substances have been transmitted from generation to generation (Jeffreys, 2008). Those pre-record paved the way for the clinical application of willow bark extract on 50 patients with agues or fever in the 17th century. Accordingly, the results of trials were presented by Edward Stone who is the first author exhibiting the healing potency of willow bark as an antipyretic in a scientific manner (Stone, 1764). Owing to the developments in the fields of chemistry, the isolation of the active ingredient of willow called “salicin” was carried out by German pharmacologist Joseph Buchner in the mid-late 19th century (John R Vane & Botting, 1992). A few years later, Italian chemists, Raffaele Piria found a way to extract the salicylic acid, which is the primary compound for the synthesis of aspirin from salicin, and purified it (Piria, 1838).

In 1958, German chemist Hermann Kolbe and his assistant Rudolf Wilhelm Schmitt achieved a significant breakthrough in the chemical synthesis of salicylic acid (Sneader, 2005). As a result of its production with industrial scale, synthetic salicylates were examined by several clinical trials in the same years. The effectiveness of the compound encouraged further studies for the development of salicylate derivatives with diminished adverse effects. In the Bayer's lab (1897), pharmaceutical chemist Felix Hoffmann discovered the reaction conditions yielding acetylsalicylic acid (Zündorf, 1997). This pure,

stable, and cheap compound was patented by Bayer AG and manufactured in tablet form named Aspirin™. Although years have passed, approximately 100 billion standard aspirin tablets are still being marketed each year due to its potent anti-inflammatory, antipyretic, and antiplatelet therapeutic actions (K. Rainsford, 2004). Both clinical outcomes and the success of the aspirin in the pharmaceutical industry led to the development of further samples of NSAIDs. Eventually, drugs such as phenylbutazone, indomethacin, ibuprofen hit the markets in the pre-prostaglandin era (Otterness, 1995).

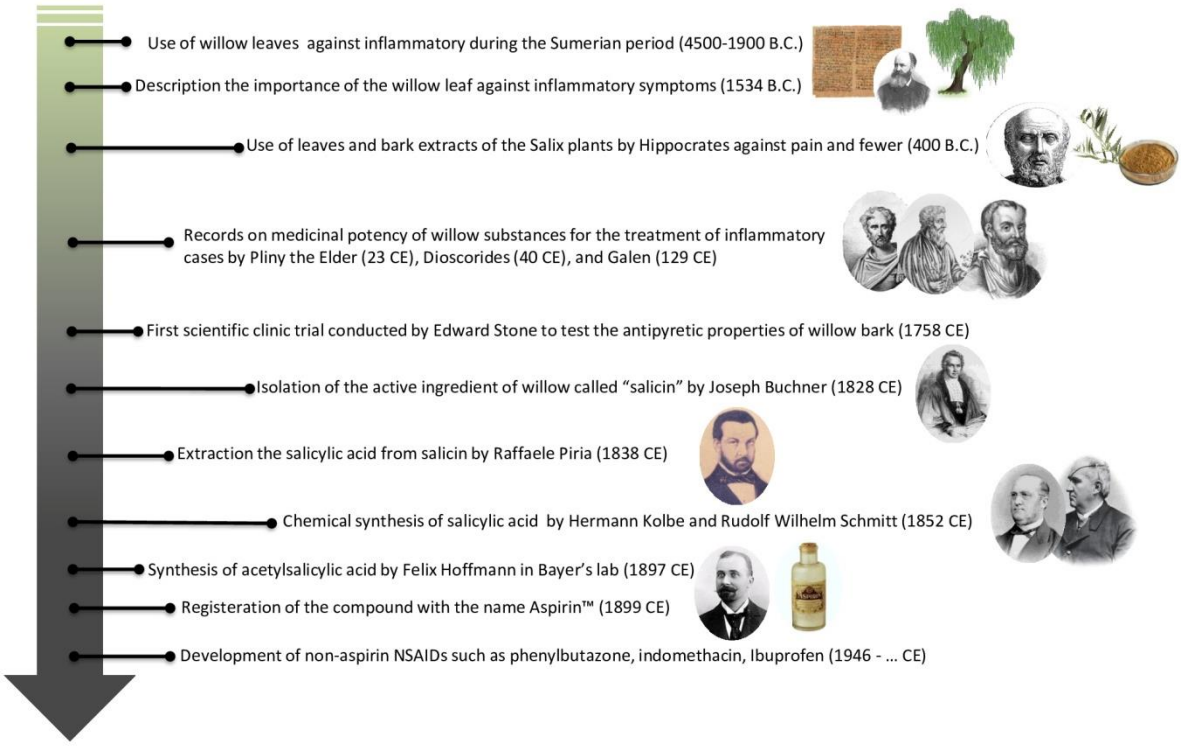

Figure 1. The milestones in the developments of aspirin or non-aspirin NSAIDs in chronological order.

The mystery behind the action mechanism of aspirin and a group of NSAIDs was satisfactorily unraveled for the first time by the efforts of John R. Vane and his colleagues in 1971 (Piper & Vane, 1969; John Robert Vane, 1971). In the study, it was demonstrated that those compounds inhibit the biosynthesis of prostaglandin (PG) in a dose-dependent manner (John Robert Vane, 1971). Additionally, subsequent studies conducted by Vane and Salvador Moncada enabled the discovery of prostacyclin. For these findings related to the PGs and bioactivity of related substances, Vane was chosen as the winner of the Nobel Prize in Physiology or Medicine 1982. Finally, the puzzle was completed with the identification of the bio target of aspirin and NSAIDs by Hemler et al. demonstrating the

principal enzyme in the synthesis of PG named cyclooxygenase (COX) (Hemler & Lands, 1976). Indeed, with advances in molecular biology, the classification and evaluation process of NSAIDs have been reshaped towards the concept of understanding their biological target(s) instead of chemical and structural similarities. Additionally, the molecular mechanisms for the adverse effects of NSAIDs have been revealed.

1.1. COXs As Biological Target of NSAIDs

The basic action mode of NSAIDs is based on repressing the release of PGs and thromboxane (TxA) by inhibiting the action of COX enzymes. When Hemler et al. identified the constitutive COX enzyme, also termed prostaglandin H synthase (PGHS) in 1976 for the first time, it was not known the existence of COX isoforms. In the late 1980s–early 1990s, a second isoform of the enzyme (COX-2) was identified, isolated, and cloned (Kujubu, Fletcher, Varnum, Lim, & Herschman, 1991). Both COX isoforms share a common role in arachidonic acid (AA) metabolism in which bioactive PG species are synthesized from arachidonate. They have not only similarities in the function, but the comparative analysis of these genes also revealed almost 60-65% amino-acid identity with each other in the same species and 85-90% similarity among different species (Yokoyama, Takai, & Tanabe, 1988). Another common feature of COXs is their subcellular locations: they are found mainly on the membranes of the luminal side of the endoplasmic reticulum (ER), besides, COX-2 is also found on the membrane of the nucleus (Chandrasekharan & Simmons, 2004).

The main differences between these isoenzymes are their expression levels and tissues in which they are expressed in the body. The COX-1 is constitutively expressed in various tissues at low levels for the maintenance of the synthesis of physiologically important prostanoids which are essential for the gastrointestinal cytoprotection, regulation of renal blood flow, improved organ perfusion as well as a healthy pregnancy (Bertolini, Ottani, & Sandrini, 2001). In contrast, the expression of COX-2 is induced and up-regulated in cells (mainly monocytes, macrophages, synovial microvessel endothelial cells, chondrocytes, and osteoblasts) by several stimulants including bacterial endotoxins, pro-inflammatory cytokines, and growth factors (Berenbaum et al., 1996; Debrumfernandes et al., 1994; Szczepanski, Moatter, Carley, & Gerritsen, 1994). Sustained activation of COX-2 is associated with the exaggerated production of inflammation and pain-related PGs.

Table 1
The comparison of the features of human COX isoenzymes

Feature	COX-1	COX-2
Chromosome	9q32-q33.3	1q25.2-q25.3
Gene Size	22 kb	8 kb
mRNA Length	2.8-3 kb	4-4.5 kb
Protein Lenght	599 amino acids	604 amino acids
Protein Molecular Weight	70 kDa	70-72 kDa
Expression	Constitutive	Inducible
Subcellular Localization	ER	Nuclear membrane; ER
Substrate	AA, γ-linolenic acid	AA, γ-linolenic acid, α-linolenic acid, eicosapentenoic acid
Cofactors	Heme	Heme
K_m for AA	~5 μM	~5 μM

1.2. Understanding the Reasons For Adverse Effects of NSAIDs

The production of the PGs and TxAs depends on the presence of AA in the site of action. When an inflammatory signal comes to the cell, the enzymes called secretory and cytoplasmic Phospholipases A2, which balance the eicosanoid levels, are activated to convert the membrane-bound arachidonate into free AA (Figure 2). The release of AA induces COX pathways in which these molecules are oxygenated by COXs to yield prostaglandin G_2. Immediately after, this molecule is catalyzed by peroxidase into prostaglandin H_2 which is a substrate for the cell-specific enzymes to generate bioactive PG species PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane A_2 (TxA_2) as shown in Figure 2 (Smith & Langenbach, 2001).

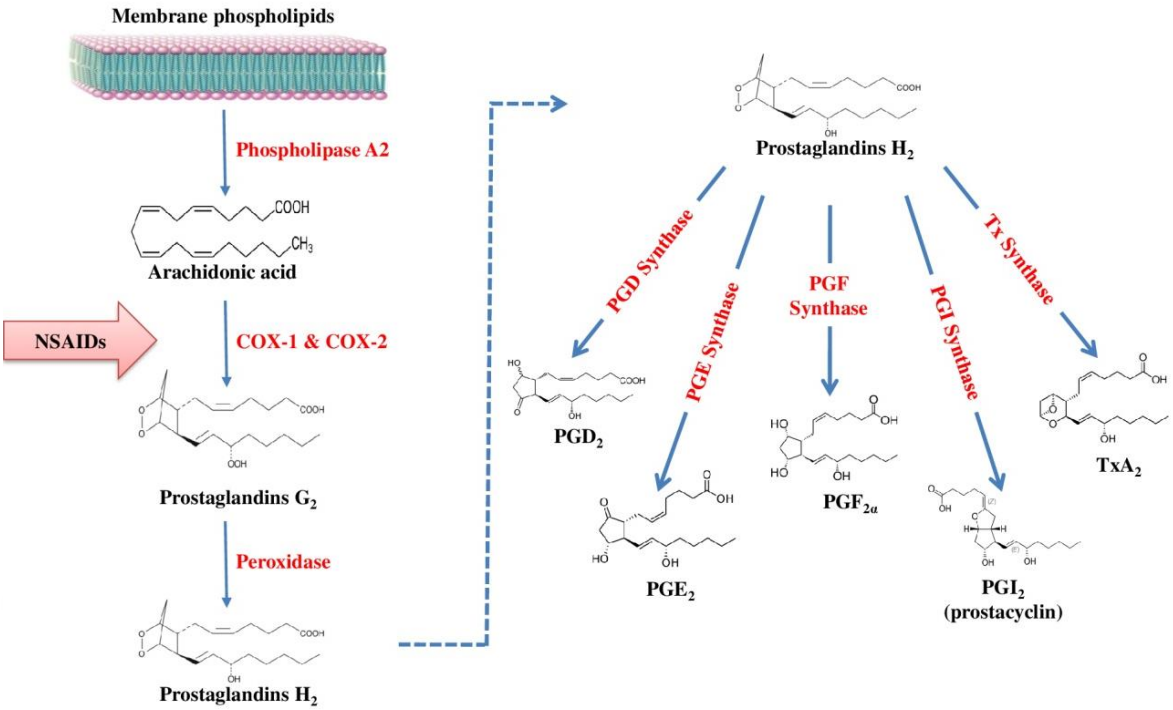

Figure 2. The synthesis of prostaglandin species from membrane-bound arachidonate via COX-1/COX-2 isoform catalysis.

COXs are key enzymes that catalyze the oxidation of AA to precursors in PGs metabolism and are considered as a "rate-limiting" factor (Park, Pillinger, & Abramson, 2006). However, undesired inhibition of COX-1 during long term NSAIDs therapy leads to a dramatic reduction in the gastroprotective PGs levels resulting in severe adverse effects including stomach erosions, bleeding, and perforation in gastrointestinal tracts – so much so that it is estimated that 15.3 people pass away per 100,000 NSAID users in Europe (Lanas et al., 2006). This emerging concerns prompt researchers to develop an alternative approach to treat and prevent inflammation-based diseases. Selective COX-2 inhibition was thought to be a promising strategy; the inhibition of inducible COX prevents the release of excess amount of pro-inflammatory PGE_2 while spare the gastroprotective prostaglandin synthesis mediated by constitutive COX. The elucidation of the structures of COXs was one of the most important milestones for the discorvery of selective COX-2 inhibitors (Chandrasekharan & Simmons, 2004). These studies showed that despite structural similarities in the active sites of both enzymes that interact with the inhibitors, a replacement of isoleucine with valine in the COX-1 enzyme (at 434th amino acid position) makes the size of the active site in COX-2 larger allowing the formation of electrostatic interactions (Gierse et al., 1996).

The drugs (Table 2) with high selectivity on COX-2 exhibit more favorable safety profiles as compared to the classical NSAIDs. The most popular selective COX-2 inhibitors, the class of ***cox***-inh**ib**it; coxibs (celecoxib, etoricoxib, parecoxib, rofecoxib, valdecoxib, lumiracoxib), have been preferred to be utilized in clinics. Despite the early success of these drugs after marketing, many cardiovascular problems have been reported with huge concern. Further studies explained the underlying reason as the imbalance in the synthesis of prothrombotic TxA_2 (mainly mediated by COX-1) and antithrombotic PGI_2 (mainly mediated by COX-2) (K. Rainsford, 2007). Clinical data suggest that NSAIDs with COX-2 selectivity have lost the cardio-friendly properties of classical NSAIDs. Accordingly, the drugs rofecoxib and valdecoxib were withdrawn from marketing due to side effects in the cardiovascular system. For the same occasion, etoricoxib is not approved in several countries. Moreover, another drug in the coxib group named lumiracoxib was withdrawn from marketing because of severe liver toxicity (Rao & Knaus, 2008). Apart from others, celecoxib is allowed to remain in pharmacies with related labels highlighting the potential adverse actions.

Table 2.
Determined IC_{50} scores of NSAIDs for COX enzymes by whole blood assay and their selectivity index. The data were taken from the paper published by Rao & Knaus, 2008.

Drug	Trade Name	Whole blood assay IC_{50} (µM)		Selectivity Index
		COX-1	COX-2	COX-1 /COX-2
Aspirin	Aspirin® Ecotrin®	1.7	> 100	0.017
Diclofenac	Voltaren®	0.075	0.038	1.97
Ibuprofen	Advil® Motrin®	7.6	7.2	1.05
Indomethacin	Indocin®	0.013	1.0	0.013
Ketoprofen	Orudis®	0.047	2.9	0.016
Naproxen	Naprosyn® Aleve®	9.3	28	0.33
Fluorbiprofen	Fluorbiprofen® Ansaid®	0.075	5.5	0.013
Paracetamol	Tylenol®	> 100	49	> 2.04
Meloxicam	Mobic®	5.7	2.1	2.7
Nimesulide	Mesulid®	10	1.9	5.26
Celecoxib	Celebrex®	6.7	0.87	7.7
Valdecoxib	Bextra®	26	0.87	29.8
Rofecoxib	Vioxx®	19	0.53	35.8
Etoricoxib	Arcoxia®	116	1.1	105.4
Lumiracoxib	Prexige®	67	0.13	515

Not only COXs but also lipoxygenases (LOXs) are key enzymes in the regulation of inflammation as illustrated in Figure 3. While COXs convert the AA into cell-specific PGs species, in the LOX pathway, the same substrate is catalyzed for the biosynthesis of leukotrienes (Natarajan & Nadler, 2004). LOXs are responsible for the insertion of the O_2 molecule in a specific site of AA. There are mainly four types of LOXs: 5-, 8-, 12- and 15-LOX, wherein the number indicates the position of the insertion site (Brash, 1999). Under the inflammatory condition, 5-LOX catalyzes the oxidation reaction of AA into 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and this molecule is metabolized into leukotriene A_4 (LTA_4). One of the fate of LTA_4 is to be hydrolyzed into dihydroxy acid leukotriene B_4 (LTB_4) which causes a release of excess amount ROS and stimulation of the NF-κB pathway (Lee et al., 2007; Okamoto, Saeki, Sumimoto, Yamasaki, & Yokomizo, 2010). Additionally, the produced LTB_4 triggers the macrophage activation as well as the expression of IL-1. It is then this cytokine stimulates the release of AA that leads to sustained activation of COX and LOX pathways. Moreover, the catalysis products of 5-LOX were reported as a risk factor for several inflammatory diseases such as asthma, ulcerative colitis, rhinitis, and even cancer formation (Y. Zhao, Wang, Wang, Zhang, & Ye, 2012).

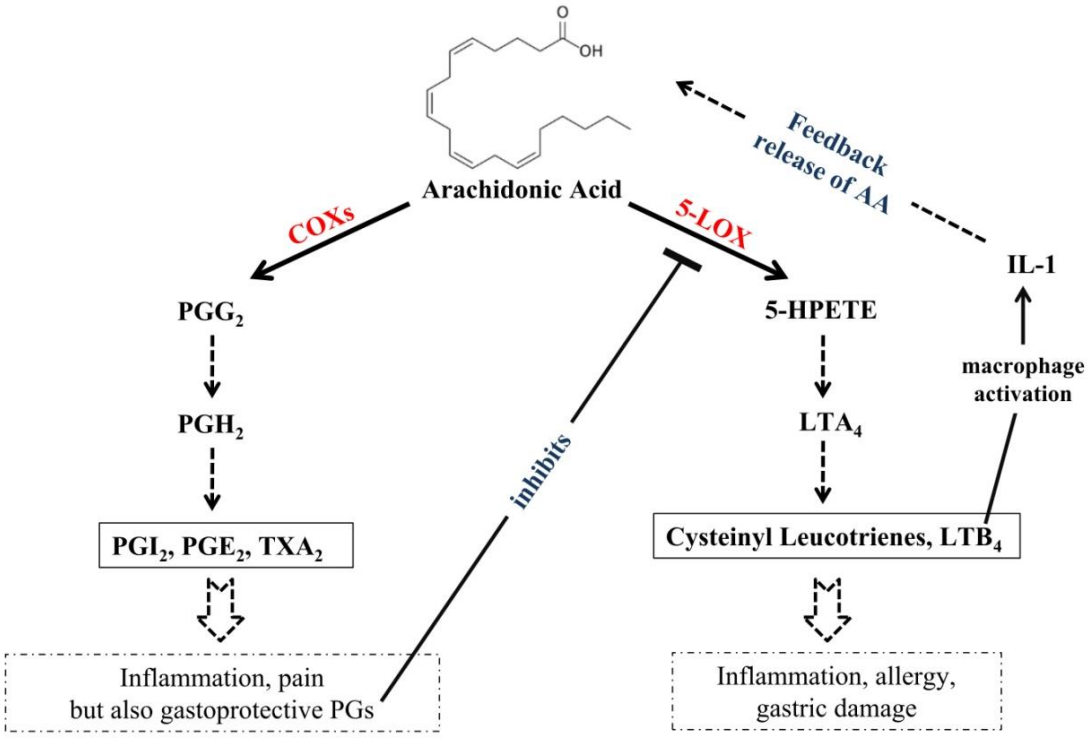

Figure 3. The possible fates of arachidonic acid and the connections between COX and LOX pathways.

1.3. Traditional NSAIDs and Novel Dual Acting Anti-inflammatory Drugs (DAAIDs)

According to the findings of the study conducted by Nickerson and Medvedeff in 1996, the concomitant use of separate inhibitors of leukotriene synthesis and COX provided a significant amelioration in animal models with arthritis (Nickerson-Nutter & Medvedeff, 1996). These observations usher in a new age in the name of a therapeutic approach against inflammation-based disorders. Further, it is postulated that the inhibition of COX pathways by NSAIDs may shift the direction of AA metabolism towards the 5-LOX pathway which means the over-production of pro-inflammatory leukotrienes (Figure 3). It could also be considered the other way around; the inhibition of 5-LOX may change the direction of metabolism towards the COX pathway (Hudson, Balsitis, Everitt, & Hawkey, 1993; K. Rainsford, 1987, 1993). Taken together, recent attempts involve the design of novel drugs that inhibit both COXs and 5-LOX activity. Such drugs are called "dual-acting anti-inflammatory drugs (DAAIDs)" display the mode of action similar to classical NSAIDs while reducing the risk of adverse events (Bertolini et al., 2001; Martel-Pelletier, Lajeunesse, Reboul, & Pelletier, 2003).

In clinical applications, celecoxib (Celebrex®) is commonly utilized to heal several symptoms of arthritis pain and to reduce precancerous polyps in the colon due to its selective COX-2 (IC_{50}: 0.82 μM) inhibitory property (Table 2). On the other hand, in recent years, many potent selective 5-LOX inhibitors have been synthesized and examined. Among them, the most well-known small molecule with its selective inhibitory effect on 5-LOX (IC_{50}: 3.7 μM) is zileuton (Zyflo®) while sparing the 12-or 15-LOX activities for the production of anti-inflammatory lipoxins (Singh & Pooja, 2013). It is being used to reduce the pathogenesis of asthma by interruption of the biosynthesis of leukotrienes at clinics. These two compounds have some characteristics in common; they do not act as DAAIDs and accordingly they lead to some adverse effects. Apart from these, the compound named licofelone was developed by the partnerships of Merckle GmbH and EuroAlliance pharmaceutical companies (Kulkarni & Pal Singh, 2007). It possesses dual-acting effects on both COX-2 (IC_{50}: 0.21 μM) and 5-LOX (IC_{50}: 0.18 μM), separately. It is the only DAAIDs that is under evaluation process in phase trials and its healing effect on osteoarthritis being examined (Kulkarni & Pal Singh, 2007). In brief, the pharmaceutical industry requires to develop more compounds with DAAI effects.

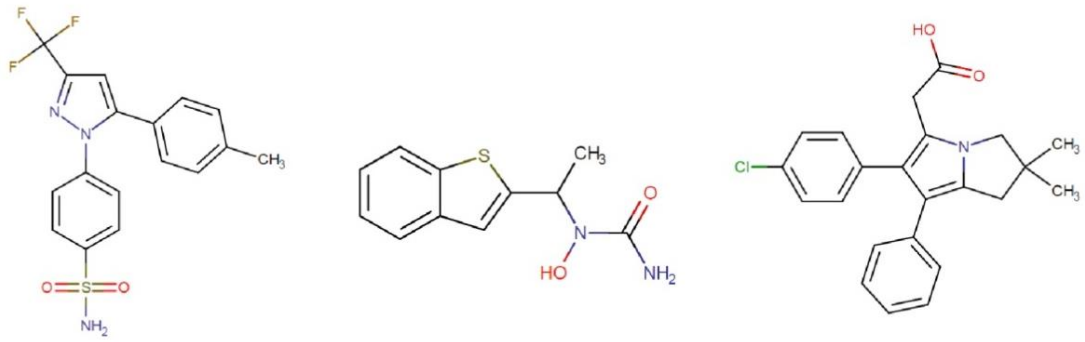

Figure 4. The structure of selective COX-2 inhibitor (celecoxib), 5-LOX inhibitor (zileuton), and dual inhibitor (licofelone).

1.4. Nimesulide: A Preferential COX-2 Inhibitor

As an alternative to the coxib compounds, nimesulide is one of the most commonly used preferential anti-inflammatory agents having analgesic and antipyretic effects (T. D. Warner et al., 1999). The drug was discovered in 1971 by medicinal-organic chemist Dr. George Moore, pharmacologist Dr. Karl Swingle, and medicinal chemist Dr. Robert Scherrer. It was then licensed and manufactured at an industrial scale for the first time in 1985, Italy (Caiazzo, Ialenti, & Cicala, 2019; Swingle, Bell, & Moore, 1985). The action mechanism of the drug was understood during the period of the 1990s: it is based on the preferential inhibition of the COX-2 enzyme. Unlike coxibs, nimesulide is around ≥5 fold more potent an inhibitor of COX-2 than of constitutive COX. Thus, nimesulide does not lead to gastric damage (like other NSAIDs inhibiting gastroprotective PGs synthesis) and severe cardiovascular problem (like other selective COX-2 inhibitors inhibiting the biosynthesis of vascular prostacyclin) (T. Warner, 2001).

1.4.1. Chemical Features of Nimesulide

As a chemical identity, nimesulide (IUPAC Name: *N*-(4-nitro-2-phenoxy phenyl) methane sulphonanilide) was the first acidic sulfonamide drug to be developed as a clinically effective anti-inflammatory agent (Figure 5) (K.D. Rainsford, 2005). Unlike other traditional NSAIDs, nimesulide does not include a carboxylic acid group in its skeletal, but methane sulfonamide moiety. In the pre-prostaglandin era, the anti-inflammatory properties of nimesulide were expressed by its anti-oxidant capacity. This radical scavenger trait of the compound is explained by the presence of nitro- and sulphonamides instead of a carboxyl group. Additionally, the preferential interaction of

COX-2 and nimesulide is due to the presence of the methanesulfonamide group allowing strong interactions.

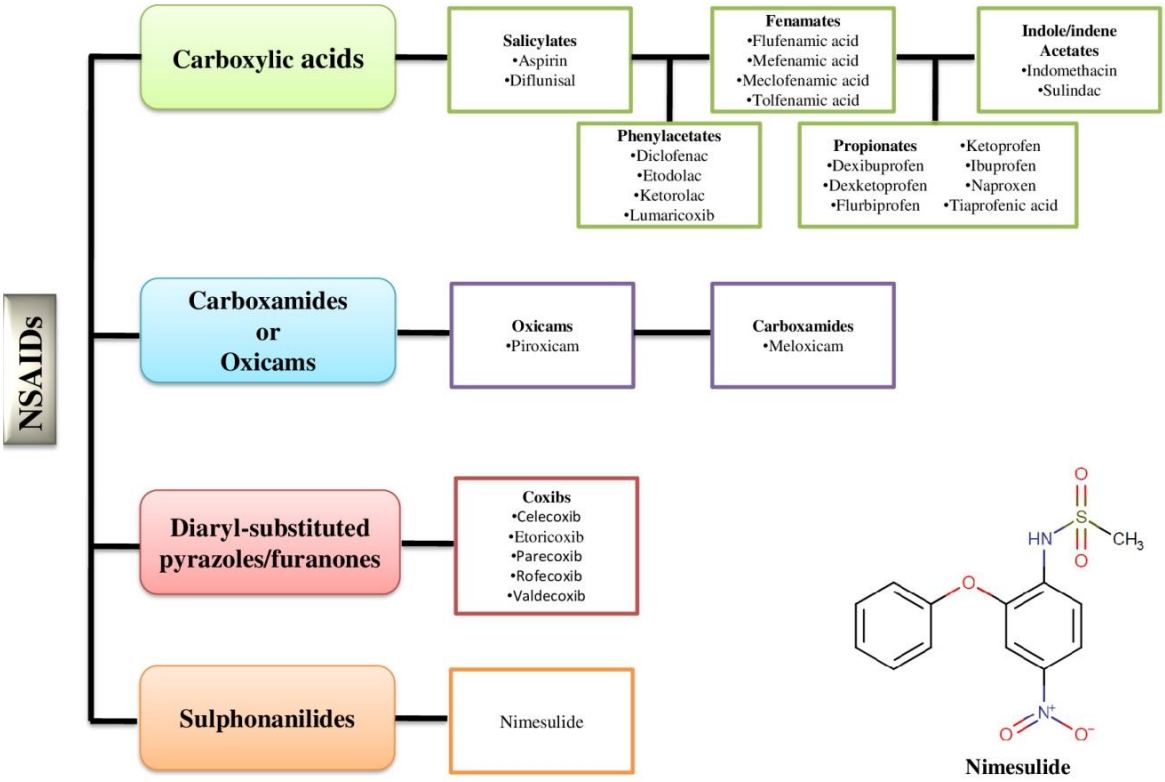

Figure 5. Classification of NSAIDs according to their chemical properties (K. Rainsford, 2007).

1.4.2. The Explanations on The Bioactivity of Nimesulide

Not only COX-2 dependent activities but also lots of multi-factorial COX-2 independent modes of actions of nimesulide have been studied. According to the observations, the antipyretic properties of nimesulide is based on the inhibition of COX-2 activity and pro-inflammatory cytokine productions. Apart from this effect, preferential inhibition of COX-2 also provides the nimesulide to exhibit the analgesic activity. According to the commentary written by K. D. Rainsford and his colleagues, nimesulide also exhibits the analgesic effect by inhibition of reactive oxygen species (ROS), nitric oxide (NO), histamine, and elastase releases. It also interferes with the activities of the nitric oxide synthases (NOS) and metalloproteinase (K. Rainsford & Nimesulide, 2006). Moreover, it reduces the rate of monocyte death, chemotaxis as well as trans-endothelial migration. Besides, in the study of Werner et al (2006), unlike classical NSAID indomethacin, nimesulide diminished the fever in animal models stimulated by

macrophage inflammatory protein-1α, $PGF_{2\alpha}$, corticotrophin-releasing factor, or endothelin-1, separately (Werner, Souza, & Zampronio, 2006). Additionally, Tool and Verhoeven (1995) also demonstrated that nimesulide dose-dependently inhibited both biosyntheses of platelet-activating factor and LTB_4 by regulating intracellular cAMP level in the activated neutrophils (Tool & Verhoeven, 1995). Moreover, in another study conducted by Gómez-Gaviro et al. (2002), the anti-L-selectin activities of currently available NSAIDs were investigated and discussed in detail. L-selectin is constitutively expressed in leukocytes to mediate the adhesion to vascular endothelial. The shedding of L-selectin is triggered when the cells are activated in response to stress conditions. According to the results, nimesulide led to an induction in the L-selectin shedding which, however, lower than the effects of other NSAIDs including the diclofenac, aceclofenac, flufenamic, and mefenamic acids; equal to the moderate releasers such as aspirin, indomethacin, flurbiprofen, and ketoprofen, and higher than phenylbutazone and oxicams (Gómez-Gaviro et al., 2002). Taken together all these actions, nimesulide is proven to be effective in the treatment of the acute pain states, osteoarthritis symptoms, and primary dysmenorrhoea. In the term of drug safety, nimesulide possesses a high gastrointestinal safety profile as compared to the traditional NSAIDs. It demonstrates rare undesired adverse actions in the renal and cardiovascular sites as compared to the coxib group (Bjarnason et al., 2018; H Suleyman et al., 2002; Süleyman, Akçay, & Altinkaynak, 2002). Accordingly, nimesulide has an exceptional market ranking in several countries from Europe, Asia, and Latin America, however, it has never been approved to be used in the USA, Japan, UK, Germany, and Canada due to the vigorous liver damages (Caiazzo et al., 2019). In Turkey, both tablet and gel forms of nimesulide are still used for the treatments of acute pain and primary dysmenorrhea. However, the identification of new actions for the chemical entity of nimesulide, especially in uncontrolled cell proliferation has taken the attention on this multitarget biologically active pharmacophore (Catarro, Serrano, Ramos, Silvestre, & Almeida, 2019). In this aspect, recent studies presented shreds of evidence for the potent role of nimesulide in reducing the number of villus and cyripta of the intestine, as well as inhibition of cancer cell proliferation (Catarro et al., 2019; Kaushal & Sanyal, 2007). According to the epidemiological data, COX-2 is overexpressed in the cancer sites of the colon, prostate, breast, skin, and lung as compared to the healthy tissues (Khan & Lee, 2011). The findings in the literature indicate that induction in the COX-2 expression is associated with prostaglandin-mediated tumor growth (Botha, Robinson, Ramchurren, Reddi, & Norman, 1986; Morgan, 1997). Accordingly, targeting of COX-2 activity is

considered as a significant therapeutic strategy against cancer. In recent years, phase II and III studies have been taken place on cancer patients for the concomitant use of NSAIDs with chemotherapy (ClinicalTrials.gov Identifier: NCT02429427 and NCT01238120). As a preferential inhibitor of COX-2, chemopreventive activities of nimesulide have been investigated in both animal and cell-based models. The mechanisms of its action are said to be based on the dual inhibitions of cell proliferation via COX inhibition and ROS production. Even the potential use of nimesulide-hyaluronic acid bioconjugate (CA102N) on patients with advanced or metastatic colorectal cancer has been evaluated in the clinical trial (ClinicalTrials.gov Identifier: NCT03616574). Following the entrance of CA102N in the CD44-expressing tumor cells, it is subjected to enzymatic degradation to set free COX-2 inhibitor and cytotoxic agent nimesulide. Subsequently, it interferes with cell signaling pathways for the inhibition of cancer cell proliferation and stimulation of the cell cycle arrest and apoptosis. In another trial (ClinicalTrials.gov Identifier: NCT01500577), the chemopreventive effects of nimesulide on subjects at high risk for breast cancer have been examined.

1.5. The Origin of Novel Nimesulide Derivatives (NDs)

The pharmacological profile of nimesulide is a source of inspiration for researchers to develop novel NDs with improved anti-inflammatory, antipyretic, analgesic, and anticancer activities as well as diminished adverse actions (Caiazzo et al., 2019; Catarro et al., 2019; K. Rainsford & Nimesulide, 2006). At the beginning of this study, there was a strong collaboration between the molecular biologist and chemist colleagues for the structural design of novel compounds. After discussions on the subject, the novel NDs with amide, sulfonamide, and nitro moieties have been decided to be investigated (Figure 6). Literature data reveals that the candidates containing sulfonamide as a pharmacophore are very frequently studied molecules in the medicinal chemistry (Meanwell, 2011; C. Zhao, Rakesh, Ravidar, Fang, & Qin, 2019). In the organic compounds, sulfonamide moiety displays several therapeutic activities such as antiviral, antidiabetic, antimalarial, anticancer as well as an anti-inflammatory (C. Zhao et al., 2019). Apart from molecules in the experimental stage, the highly marketed anti-inflammatory small molecules such as SC-558 and celecoxib contain sulfonamide moiety which is the major determinant for COX-2 selectivity. Additionally, both structure-activity relationship (SAR) and experimental analyses indicate that generation of amide containing molecules might be a strategy for increasing the selectivity of compounds towards COX-2 (Kalgutkar, Marnett,

Crews, Remmel, & Marnett, 2000). Currently, the number of approved amide containing drugs is more than fifty and used as a therapeutics against mainly tuberculosis, pain, fever, and cancer. Unlike others, nimesulide is the sole NSAID containing the nitro group. While nitro-containing compounds are considered as structurally unsafe molecules, some physiologically active drugs are having this group in the marketing process such as nilutamide (non-steroidal antiandrogen), nitrendipine (antihypertensive), nitrazepam (anticonvulsant, hypnotic) and nitromide (antibacterial). (Hida et al., 2000; Renard, Julémont, Leval, & Pirotte, 2006). Considering all this information, seventeen novel NDs were structurally designed before synthesis and activity processes (Figure 6).

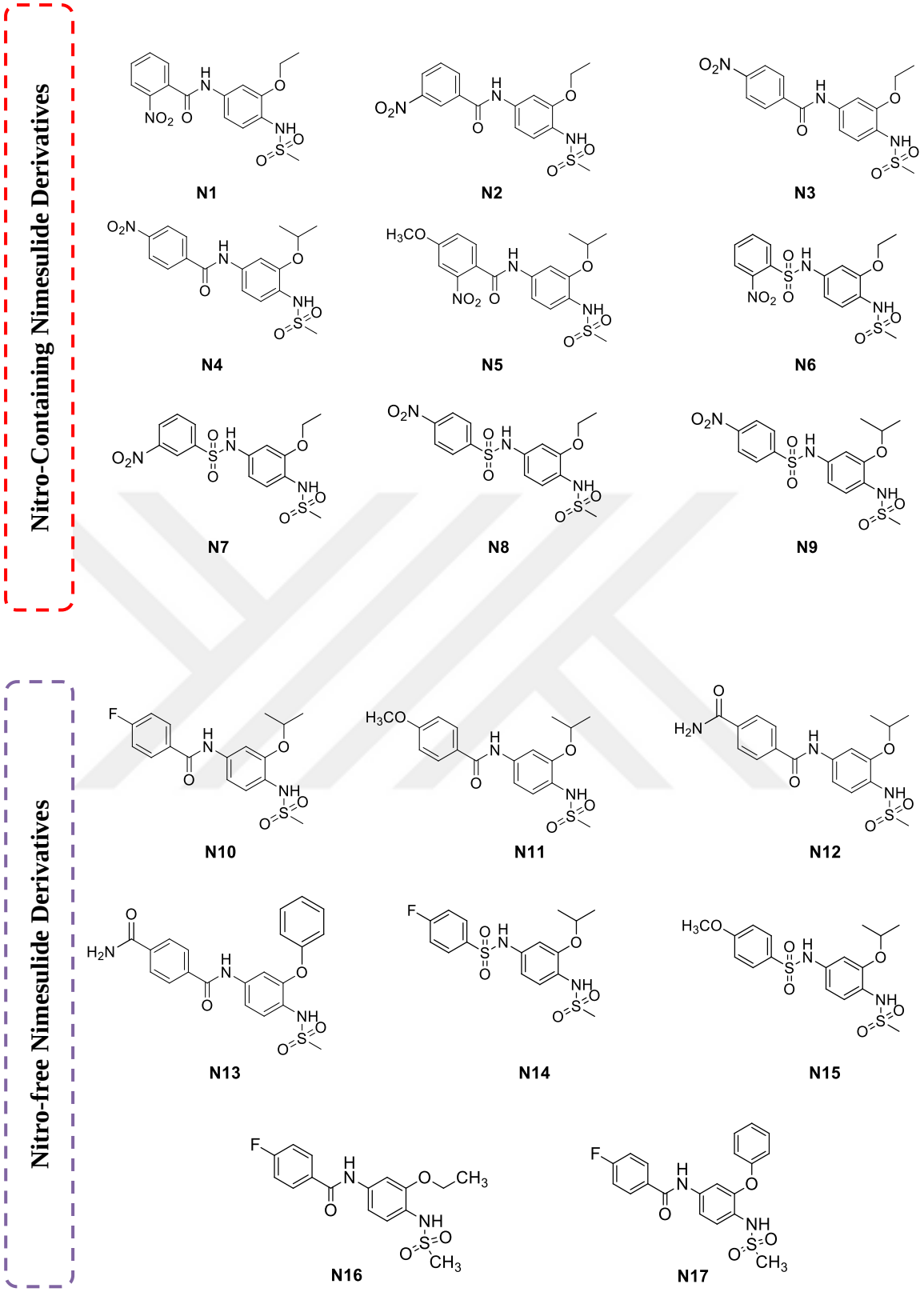

Figure 6. The structures of novel NDs to be investigated in the study.

1.6. The Aim of The Study

This study aims to investigate the therapeutic potentials of seventeen novel NDs against inflammation and cancer by enzyme and cell-based in vitro assays. Besides, the mechanism of action for the lead compounds was elucidated at the molecular level. All of the compounds have been synthesized by Dr. Tuğba Güngör with the supervision of Prof.Dr. Mehmet Ay in the scope of the TUBİTAK project (117Z398). The biological activities for these compounds were identified for the first time in this thesis study. The proposed compounds were screened for their potential inhibitory properties on the COX-2 enzyme by using COX (human) inhibitor screening assay kit. The compounds displaying inhibitory effect for COX-2 were also examined on the COX-1 enzyme to indicate selectivity. Moreover, the compounds inhibiting preferentially the COX-2 were examined for the 5-LOX enzyme to highlight whether they are a dual-acting or not. These compounds with preferential inhibitory effect on COX-2 were then tested in THP-1 human macrophage cells to determine the effects on lipopolysaccharides (LPS) induced PGE_2 and LTB_4 releases. Additionally, the effects of compounds on NO production were displayed in the macrophage cells.

In the meantime, all compounds were investigated for their anticancer effects. Colon, breast, prostate cancer cell lines were selected for cancer model as well as healthy epithelial cell line (HUVEC) were used to determine the selective cytotoxicity of compounds.

In the scope of the proposed thesis;

a- The selective toxicity of compounds was determined by cytotoxicity tests on each cell line.

b- The compounds having toxicity were analyzed in the terms of anti-proliferation/migration ability, cell cycle distribution, and apoptotic effect by SRB assay, cell culture wound closure assay, cell cycle analysis, and Annexin apoptosis detection method, respectively.

c- The mechanisms of proposed compounds that increase the apoptosis of cells were investigated at the protein level using the Western Blot technique, respectively.

d- The apoptotic and ultrastructural changes in cancer cell lines were imaged under the scanning electron microscope.

CHAPTER 2
PREVIOUS STUDIES

Until the beginning of this study, there was no evidence related to the biological activities of these novel compounds (Figure 6) in literature. However, since the structure of nimesulide could be easily modified to yield new molecules, the number of researches has attempted to synthesize and investigate the biological activities of novel nimesulide derived compounds and analogs in the last decade. To summarize them;

In the study of Su et al., (2010), the aromatase suppressor activity of novel nimesulide derivatives were examined in the aromatase transfected MCF-7 and T47D breast cancer cell lines. The results showed that the aromatase activity was inhibited by compounds A and B treatments in these cells in a dose-dependent manner. Although the compounds interfere with the aromatase activity in the sub-micromole concentration, there was no statically significant change in the transcriptional level of aromatase at even high concentrations. The findings suggest that these compounds decrease the activity via post-transcriptional modification of aromatase but not transcriptional regulation. Besides, the compounds dose-dependently diminished testosterone-stimulated cell proliferation. Moreover, as compared to compound A, compound B significantly inhibited the long-term estrogen deprived cell proliferation in the transfected MCF-7. Results indicate that compound B might be a lead for further potent therapeutics to be used against breast cancer with aromatase inhibitor-resistance (Su, Cai, Hong, & Chen, 2010).

Similarly, Zhong et al., (2016) elucidated the potential use of an analog of nimesulide called JCC76 against aromatase inhibitor-insensitive breast cancer cells. JCC76 significantly reduced the proliferation of AI resistance breast cancer cells with an IC_{50} of 2.8 μM. Furthermore, several parameters were investigated to determine the action mechanism of the drug as anticancer properties. Although JCC76 did not lead cell arrest, it induced apoptosis in this cell line by downregulation of the proteins such as pAKT, BCL-2, and pBad. JCC76 treatment also increased the release of cytochrome-c from mitochondria. Animal studies also supported the cell-based experimental results. JCC76 reduced the growth of tumor size in mice. The suppression in the tumor growth was said to be an induction of apoptosis mediated by cleaved caspase-3 (Zhong et al., 2011).

In another study, several nimesulide derivatives were synthesized and characterized by the introduction of a 5-LOX pharmacophore into nimesulide. The inhibitory properties of compounds on both COX and LOX enzymes were then analyzed by enzyme-based

assays. According to the inhibition scores, among compounds, **20f** were found to be the best selective COX-2 inhibitor. It also decreased the activity of 5-LOX effectively. Additionally, compounds **20e** was also found to be overall dual enzyme inhibitors. The structure-activity relationship studies indicate that the presence of the sulfonamide linkage and polar substitutive moiety in the structures of **20e** and **20f** gave them strong inhibitory properties on COX and LOX (Li et al., 2011).

Pal et al., (2011), synthesized a group of novel dihydroquinoline derivatives from nimesulide. Phosphodiesterase type 4 (PDE4) is an enzyme responsible for the hydrolysis of cAMP to AMP in a variety of cells. A high level of cAMP is associated with the diminished inflammatory response of living cells to the stress. Thus, the inhibition of PDE4B, one of the isoforms of PDE4, is considered as a therapeutic strategy against inflammatory and immunological diseases to decrease the inflammatory cell function by increasing the intracellular cAMP concentration. Based on this hypothesis, PDE4B inhibitory properties of compounds were screened at 30 µM by performing enzyme inhibition assay. Few compounds showed moderate inhibitory action on the enzyme and molecular modeling studies were performed to determine the interactions with PDE4B protein. This work paved the way to design, synthesize, and evaluate further novel nimesulide-derived PDE4B inhibitors (Pal et al., 2011). Accordingly, two years later, the same research group focused on elucidating the biological activities of 1,2,3-triazole derivatives derived from nimesulide as potential inhibitors of PDE4B. As compared to their first attempt, some of these new molecules exhibited better inhibitory properties on PDE4B with >50% inhibition score at 30 µM. Among compounds, **3a** and **3d** (IC_{50}: 21–22 µg/mL) were found as promising cytotoxic agents in the colon cancer cells. These results propose that 1,2,3-triazole based PDE4 inhibitors could be a potential lead template for the development of novel anticancer drugs (Mareddy et al., 2013). Besides, in their following study, a new class of hybrid molecules containing nitro-free nimesulide and the 1,2,3-triazole moieties was synthesized. Their anticancer effects were determined in the A549, HepG2, HeLa, and DU145 cancer cell lines. The compounds **3f** and **3g** demonstrated anti-proliferative properties in each cell line (IC_{50}: ~6–10 µM) while no significant effects on the healthy cell line. Additionally, both compounds inhibited PDE4 activity by 62.8 and 67.5 % at 10 µM, respectively. As the parent compound, nimesulide was investigated and it showed 52% inhibition at the same dose (Mareddy et al., 2017).

In the study of Yamamoto et al., (2015), isomeric methoxy analogs of nimesulide at the ortho-, meta- or para-position on the phenyl ring were designed and synthesized for the

development of a brain COX-2 imaging radiotracer. Their COX inhibitory properties were determined by enzyme inhibition assay and results were supported by molecular docking studies. According to the findings, the compounds having methoxy group at para-position inhibited the COX-2 activity effectively and selectively (IC_{50}: 2.31 µM for COX-2 and >100 µM for COX-1), however, the methoxy substituents at the ortho-, meta-position was associated with loss of COX-2 inhibitory activity (Yamamoto et al., 2015). Just a year later, the same research group examined isomeric iodinated derivatives of nimesulide by a similar research question. In this study, the para-iodo-substituted analog was found to have a more potent inhibitory effect on COX-2 (IC_{50}: 0.47 µM for COX-2 and >100 µM for COX-1) than the parent compound nimesulide (Yamamoto et al., 2016). Taken together, para-methoxy and para-iodo analogs of nimesulide might be developed as imaging agents.

In the recent study performed by Liu et al., (2020) the applicability of Co-NMS, a derivative of nimesulide modified with cobalt carbonyl complex, on malignant glioma cells was explored. In the zebrafish model, 5 µM of Co-NMS did not lead to neurotoxicity in a meaningful manner and this non-toxic dose of Co-NMS was studied in further steps. The simultaneous treatment of Co-NMS and irradiation significantly reduced the cell proliferation and clonogenic capability of human U251 glioma cells. Moreover, co-treatment of Co-NMS and irradiation stimulated the G2/M arrest and apoptosis as compared to the cells which subjected to the irradiation alone treatment. In glioma cells, Co-NMS treatment disrupted the mitochondrial function by interfering membrane potential, mitochondrial fusion, and redox homeostasis. All of these data suggest that Co-NMS might be a promising therapeutic which can be used in glioblastoma patients due to its radiosensitizing effects (Liu et al., 2020).

In conclusion, nimesulide-derived molecules have been examined in different models to highlight its potential use in the pharmaceutical industry.

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals, Kits, and Essential Items

The chemicals, kits, and essential items for given studies were listed in Table 3.

Table 3
List of chemicals, kits, and essential items

	# Code	Item
Cell Culture Studies	12800017	Dulbecco's Modified Eagle Medium (DMEM)
	R6504	Roswell Park Memorial Institute Medium (RPMI-1640)
	10500064	Fetal Bovine Serum, heat inactivated
	35050061	GlutaMAX™ Supplement
	15140122	Penicillin-Streptomycin (10,000 U/mL)
	PBP01	Dulbecco's phosphate-buffered saline
	25200056	Trypsin-EDTA (0.25%), phenol red
	L6529	Lipopolysaccharides from Escherichia coli O55:B5
	BML-PE160-0001	Phorbol 12-myristate 13-acetate (PMA)
	T6146	Trypan Blue
	S1402	Sulforhodamine B sodium salt
	M5655	MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)
	SC-358801	Dimethyl Sulfoxide (DMSO)
	D2975000	Doxorubicin Hydrochloride
Kits	EHPGE2	Prostaglandin E_2 Human Competitive ELISA Kit
	EHLTB4	Leukotriene B_4 Multispecies Competitive ELISA Kit
	12183018A	PureLink™ RNA Mini Kit
	4368814	High-Capacity cDNA Reverse Transcription Kit
	23225	Pierce™ BCA Protein Assay Kit
	Q10210	Qubit™ RNA BR Assay Kit
	4500-0450	Guava Nexin Reagent for Flow Cytometry
	701230	COX (human) Inhibitor Screening Assay Kit
RNA and Protein Quantification Studies	34580	SuperSignalTM West Pico PLUS Chemiluminescent
	88018	Nitrocellulose Membrane, 0.45 µm
	88620	Western Blotting Filter Paper, Extra Thick
	sc-2324	Blotto, Non-Fat Dry Milk
	26619	Prestained Protein Ladde
	4444557	TaqMan™ Fast Advanced Master Mix
		TaqMan Probes -
		COX-1, COX-2, iNOS, TNF-α, IL-1β
	sc-56053	Caspase-3 Antibody (31A1067)
	sc-20067	Bax Antibody (2D2)
	sc-7382	Bcl-2 Antibody (C-2)
	sc-516102-CM	m-IgGκ BP-HRP (CM)
SEM Analysis	-	Coverslips
	P1851	Piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES)
	40191	Hexamethyldisilazane

3.1.2. Equipment Used for Analysis

A variety of laboratory equipment in the research lab of Molecular Biology and Genetics Department at the Çanakkale Onskiz Mart University (COMU) was used and maintained according to the user's manuals for the thesis studies. Analyses involving scanning electron microscopy were carried out in Advanced Technology Application and Research Center (ILTAM) at Pamukkale University. The analysis of cell cycle arrest and a part of apoptosis-related studies by using a flow cytometer were carried out in Balıkesir University, Department of Molecular Biology and Genetics in the Laboratory of Prof. Feray Köçkar. All types of equipment and their intended purposes were summarized in Table 4.

Table 4
List of equipment and their intended purposes

Brand	**Model**	**Equipment**	**Aim**
Esco	CCL-170T-8	Cell Culture CO_2 Incubator	Providing a sterile and proper environment for cell culture
Esco	Class II BSC	Biological Safety Cabinets	Providing a safe environment for cell culture studies
BioRad	T100™	Thermal Cycler	Synthesis of the complementary DNA
Thermo Fisher Scientific	Applied Biosystems™ 7500	Real-Time PCR Systems	Providing quantitative real-time PCR applications for the specific gene(s)
BioRad	-	Vertical Electrophoresis System	Running protein samples under electrical current
Thermo Fisher Scientific	-	Wet Electroblotting System	Blotting protein samples on the surface of the nitrocellulose membrane
Hettich	MIKRO 200R	Refrigerated Micro Centrifuge	Centrifugation of samples at refrigerated mode
Hettich	Universal 320R	Standart Centrifuge	Centrifugation of samples
BioSan	TS-100	Thermo–Shaker	Preparation of samples for protein, enzyme, and ELISA analysis
TECAN	Infinite® 200 PRO	Microplate Reader	Absorbance measurement for colorimetric methods
TECAN	NanoQuant Plate™	Microplate Reader	Quantification the concentration of nucleic acids

Table 4 (continued)

LI-COR Biosciences	C-DiGit	Blot Scanner	Imaging the membranes for chemiluminescent Western blot analysis
GEMINI FESEM	-	Scanning Electron Microscopy	Imaging the ultrastructural changes on cells by high magnification capability
Major Science	MW-23	Waver Shaker	Shaking the samples during staining or washing process
Scientific Industries	Genie 2	Vortex	Mixing applications to make samples completely homogeneous
HANNA Instruments	HI-2211	pH Meter	Precise measurement and adjustment of the pH of buffers
Izmak	-	Ice Machine	Production of ice to save protein, RNA, and cDNA samples

3.1.3. Cell Lines and Novel NDs

In this thesis, the evaluation of biological activities was taken place by both cell-based and cell-free experimental set-ups. When considering cell culture analyses, a group of cell line including THP-1, RAW 264.7, HT-29, MCF7, PC-3, and HUVEC was maintained at 37 ºC in humidified air containing and 5% CO_2, stocked up, and stored in deep freeze (-80 ºC) and liquid nitrogen storage tank. Optimized medium conditions for each cell line were listed in Table 5.

The novel NDs were designed, synthesized, and chemically characterized by using sequential multistep organic reactions by Dr. Tuğba Güngör and Prof. Dr. Mehmet Ay in the Laboratory of Natural Products and Drug Research at the Department of Chemistry, COMU.

Table 5.
List of cell lines and their optimized culture conditions

Cell Line	Type Species	Disease Model	Cell Media Condition
THP-1	Monocyte Homo sapiens	Acute monocytic leukemia	RPMI-1640 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 10% FBS
RAW264.7	Macrophage Mus musculus	Abelson murine leukemia virus-induced tumor	DMEM modified with 100 U/mL penicillin, 100 µg/mL streptomycin, 10% FBS
HT-29	Epithelial Homo sapiens	Colorectal adenocarcinoma	
MCF7	Epithelial Homo sapiens	Breast adenocarcinoma	
PC-3	Epithelial Homo sapiens	Prostatic adenocarcinoma	
HUVEC	Endothelial Homo sapiens	Normal	

3.2. Methods

Once all synthesized NDs were received, the screening of their anti-inflammatory activities' was started with enzyme-based analysis. The compounds selectively inhibiting recombinant COX-2 were further studied in the cell-based analysis including release and expression levels of pro-inflammatory factors by using ELISA assays and qPCR method. Moreover, the anticancer properties of novel compounds were determined in cancer cell models by cytotoxicity tests, and the mechanism of actions of the most potent compound was clarified by Western blot, qPCR, cellular proliferation, cell cycle arrest as well as apoptosis-related analyses.

3.2.1. Enzyme Inhibition Studies

Cyclooxygenase (COX) Inhibition Assay

The inhibitory effect of the novel compounds on COXs was investigated according to the instructions given for the COX (human) inhibitor screening assay kit (701230, Cayman Chemical). Briefly, COX-2 enzyme aliquots were incubated with 1 µM dose of compounds at 37 ^{o}C for 10 minutes. In reaction tubes, AA (100 µM final concentration) was added as a substrate to initiate the enzymatic reaction and stannous chloride solution to block enzyme catalysis. The compounds, which demonstrated an inhibitory effect, were

also tested on the aliquots of COX-1 at 10 µM dose with the same experimental steps. Enzymes were treated with nimesulide and celecoxib as positive controls as well as incubated with vehicle only for negative control.

Lipoxygenase (LOX) Inhibitor Screening Assay

Similar to enzyme studies with COX, the inhibitory effect of NDs on the 5-LOX enzyme was also demonstrated according to the standard protocol of assay kit (760700, Cayman Chemical). Basically, inhibitor wells were loaded with 90 µL of enzyme aliquots and 10 µL of inhibitor solutions in duplicate, respectively. To detect 100% activity, the enzyme was subjected to a vehicle only. After pre-incubation, the enzyme activity in all wells was initiated by adding 10 µL linoleic acid and the plate was then placed to the orbital shaker for ten minutes. To end-up enzyme catalysis, the plate was subjected to 100 µL of the chromogen solution for 60 minutes. In the final step, absorbance was read at 490 nm by using a plate reader.

3.2.2. Stimulation of THP-1 Monocytes by PMA Treatment

To investigate the anti-inflammatory effects of NDs, THP-1 human monocytes were differentiated to macrophage-like cells by PMA treatment. For this aim, suspended THP-1 cells were seeded at a density of $1x10^6$ cells/mL and subjected to 10 ng/mL of PMA to induce differentiation for 24h incubation. Then, cells were maintained in a PMA-free fresh medium for another 24 hours for the resting stage before treatment.

3.2.3. The Effect of NDs on LPS-induced PGE_2 and LTB_4 Release

To evaluate the effects of NDs on LPS stimulated PGE_2 and LTB_4 production, commercially available human-specific ELISA kits were utilized. For this purpose, differentiated cells were treated with compounds and LPS (1 µg/mL) simultaneously for 24 hours. Then, supernatants were saved for the quantification of PGE_2 and LTB_4 concentrations. The ELISA process was carried out according to the manufacturer's instructions of kits. The concentration of PGE_2 and LTB_4 were normalized to the total protein content of cells assayed by the BCA Protein Assay Kit.

3.2.4. Inhibitory Effect of NDs on Nitric Oxide Release

Macrophages release the nitric oxide (NO) molecules to the cell media under stress conditions. An analytical method called Griess assay was carried out to investigate the effect of NDs on LPS-induced NO release as previously detailed (Kapche et al., 2017). Briefly, RAW 264.7 macrophages were plated at a density of $5x10^4$ cell/mL in 24- well plate. Once cells adhere, they were incubated in red phenol-free media for 24 hours in the presence of LPS (1 µg/mL) and NDs. Then, the amount of nitrite present in the supernatants was quantified with the Griess method and the total protein concentration of cells was determined by the BCA assay for the normalization.

3.2.5. Gene Expression Analyses at mRNA Level

The effects of NDs on the expression of pro-inflammatory and apoptosis-related genes were investigated by the Quantitative Polymerase Chain Reaction (qPCR) technique. qPCR is one of the most reliable and useful methods in which specific fluorescently labeled probes are used as a reporter for the target genes. The fluorescence monitored by the machine during amplification is directly proportional to the level of the target gene. As shown in Figure 7, cell treatment, RNA isolation, and cDNA synthesis protocols were performed as previously summarized (Tumer et al., 2018). In the final products, the level of our target genes was determined by using TaqMan ® probes.

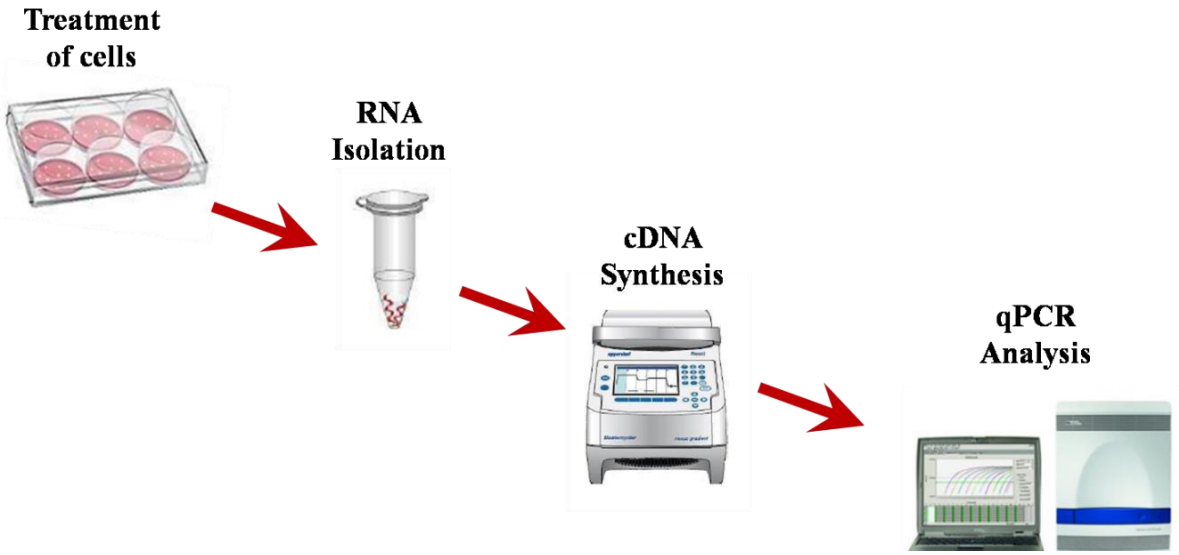

Figure 7. Step by step qPCR analysis: from cell culture to the gene expression analysis.

RNA Isolation with Trizol Reagent and cDNA Synthesis

The manufacturer instructions of the PureLink™ RNA Mini Kit were applied with slight modifications. Briefly, cells were plated with $1x10^6$and $4x10^5$ cells/mL densities, respectively. After appropriate cell culture treatments for 24h, cells were collected by Trizol reagent. Once the sample was centrifuged, the aqueous phase was subjected to further steps for isolation. The purity and amount of RNA samples were evaluated by NanoQuant Plate™.

cDNA synthesis was achieved by a series of reverse transcriptase reactions according to manufacturer's instructions of the High-Capacity cDNA Reverse Transcription Kit. The final products of PCR were stored -20 ^oC till used.

Assessment of the Expression Level of Target Genes by qPCR Technique

The effects of treatment on target genes were investigated at the mRNA level by qPCR analysis. As mentioned previously (Tumer et al., 2017), diluted cDNA samples were cycled in the presence of specific TaqMan ® probes and TaqMan™ Fast Advanced Master Mix under recommended conditions. As endogenous control, the level of β-Actin was examined to normalize the gene expression levels. The results were given as relative change according to the control group and were calculated by using the comparative $\Delta\Delta C_t$ method.

3.2.6. Evaluation of Protein Expression Levels By SDS-PAGE and Western Blot Analysis

The effects of NDs on protein expression levels were assigned by western blotting as previously described (Kapche et al., 2017). With the help of antibody labeling, immunoblotting provides the identification of a target protein level in the whole protein lysate. Briefly, cells were treated with compounds properly and the total protein samples were isolated from cells by using RIPA buffer. The concentration of total protein lysates was determined by the BCA assay. Equally loaded samples were run throughout the discontinuous buffer system including 4% stacking and 12% separating gel (Laemmli, 1970). By gel electrophoresis, proteins in the sample were forced to separate under an electrical current based on their sizes.

The gel was then further processed in an ice-cold transfer buffer (10% methanol, 192 mM glycine,25 mM Tris) and compacted to nitrocellulose membrane between two Whatman papers (Tovey, Ford, & Baldo, 1987). The prepared sandwich-like system was

placed to an electrophoretic transfer tank and filled with transfer buffer. Under constant electronic voltage (90 V), the migration of proteins from gel to the membrane was completed in 2h. To prevent non-specific bindings, the membrane was incubated in TBST solution containing 5% skimmed milk powder for an hour.

Ready to use membrane was incubated overnight with appropriate dilutions of primary antibody at 4°C. The non-binding primary antibody was removed by washing steps with TBST and the membrane was subjected to 2h incubation with the recommended dilution of horseradish peroxidase (HRP) -conjugated secondary antibody. The substrate of HRP was supplied by the reagent named SuperSignal™ West Pico PLUS Chemiluminescent. After 5 min. incubation of the membrane with the reagent at dark, the intensity of the chemiluminescence signal was scanned by the C-DiGit® Blot Scanner. The densitometric analysis was taken place by using Image Studio Digits Software 5.0 and the results were given as relative change.

3.2.7. Screening of Anticancer Effects of NDs by Cytotoxicity Assays

The potential anticancer effects of compounds were screened by two most commonly used cytotoxicity assays called MTT and Sulforhodamine B (SRB) on a group of cancer cell models including HT-29, MCF7, and PC-3 as well as a healthy cell line, HUVEC. MTT analyses were carried out as described in the study of Tumer et al. (2017) with slight modifications. As represented in Figure 8.A, cells in the exponential growth phase, were plated in 96-well plate at a density of $5x10^4$ cells/mL (100 µL per well). After adherence, cells were treated with nimesulide derivates and reference compound at 100 µM for 48 hours. End of the period, the MTT solution (0.1 mg/mL well concentration) was pipetted in the fresh media for 2 h. The precipitated formazan crystal was solubilized in dimethyl sulfoxide (DMSO) and absorbances were quantified by a microplate reader (Tecan Infinite® 200 PRO, Switzerland). The same seeding steps were also performed for SRB assay (Figure 8.B) and cells were maintained in the presence of the compounds at 0-500 µM doses for 48 hours incubation. At the end of the period, cell media was aspirated and the remaining cells were exposed to fixation with 10% (m/V) trichloroacetic acid (TCA) solution at 4 ^oC for an hour. Then, the cells were washed by utilizing dH_2O and air-dried. 100 µL of SRB dye (0.04 % m/V) was pipetted to wells for 30 min at RT. To prevent the margin of error, the unbound SRB residues were discarded by washing with a 1% (V/V) acetic acid solution. At the final stage, the protein-bound dye was dissolved with 200 µL of 10 mM of Tris base solution (pH:10.5), and absorbance was recorded by the microplate

reader. With the data we obtained, IC_{50} values of compounds for each cell line were evaluated by GraphPad Prism 5 software.

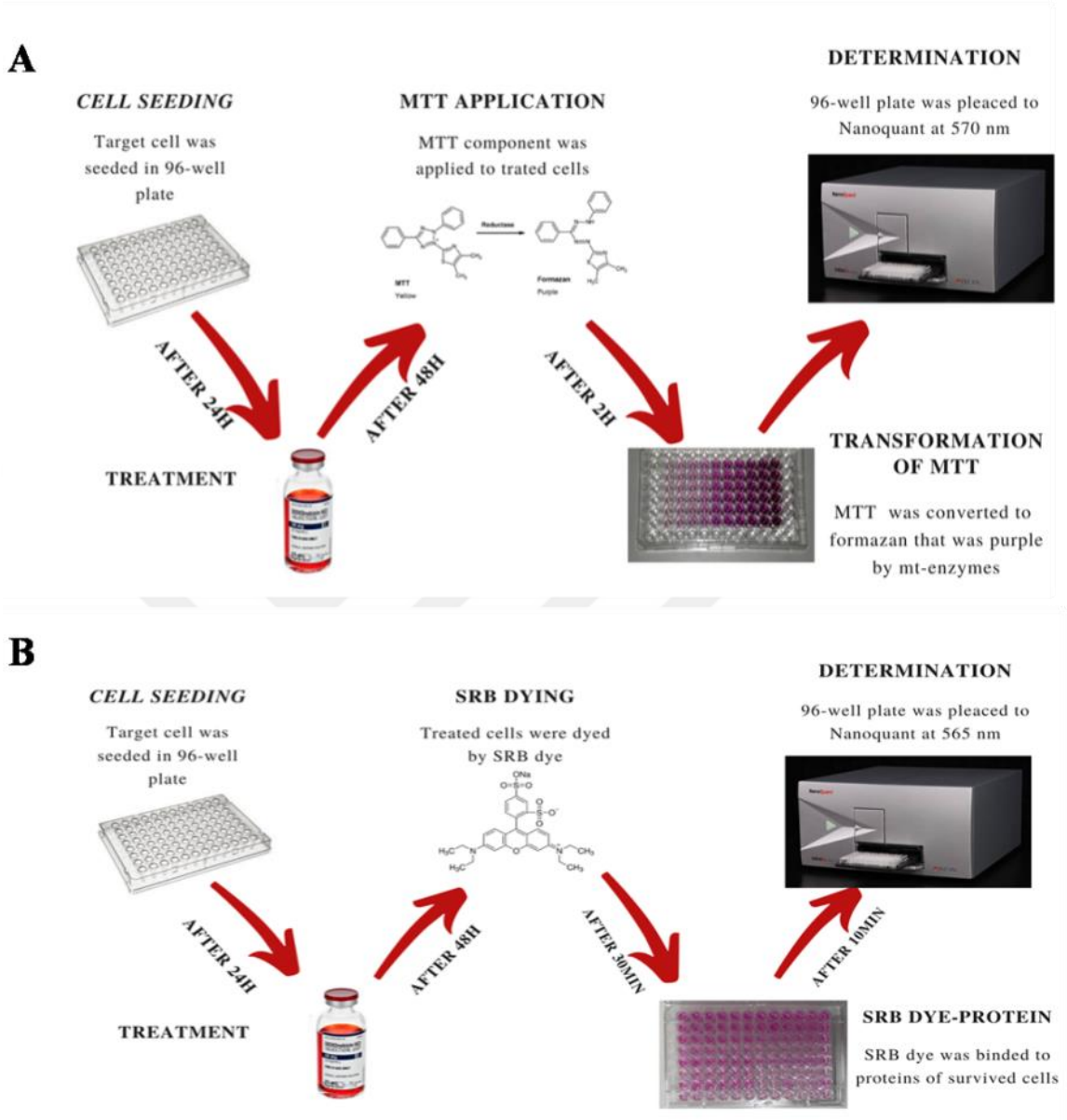

Figure 8. Experimental flow chart of (A) MTT and (B) SRB assays.

3.2.8. Scratch Wound Healing Assay In Cancer Cell Model

The scratch wound healing assay (or cell wound closure assay) was performed to determine the proliferation and migration ability of whole-cell masses in the presence and absence of test compounds. For analysis, MCF-7 cells were plated in a 24-well plate at $7x10^5$cells/mL density and incubated for 24 h for 100% confluence. The artificial linear wound was introduced into the wells by using sterile pipette tips (200 µL) and cellular residues were removed by PBS (1X) washing. After supplying fresh media, cells were treated with compounds and taken the 100X magnified photo under an inverted

microscope. The same imaging process was repeated at 12 and 24h of incubation. The distance of wounds was measured by using Image J software and the results were given as a relative healing percentage.

3.2.9. Flow Cytometry Analysis

Flow cytometry is an analytical technique that illustrates the physical and/or chemical characteristics of cells. In these studies, Annexin V analysis was performed to assign the percentage of apoptotic, necrotic as well as viable cells as shown in Figure 9. The method is based on the high affinity of Annexin V for the phosphatidylserine sites on the outer surface of the apoptotic cells. While Annexin V-PE conjugate labels the apoptotic cells, a fluorescent called7-amino actinomycin D (7-AAD) permeates selectively late-stage apoptotic and dead cells' membranes and interacts with DNA.

For analysis, colon cancer cells were incubated for 48 h. after appropriate treatment in 6 well plates. The cells were then washed with PBS (1X) and trypsinized. The collected cells were maintained in the media containing %1 BSA. In the next step, 100 µL of cell suspension was added 100 µL Guava Nexin reagent and incubated for 20 minutes in dark at RT. The prepared samples were investigated by the flow cytometer instrument.

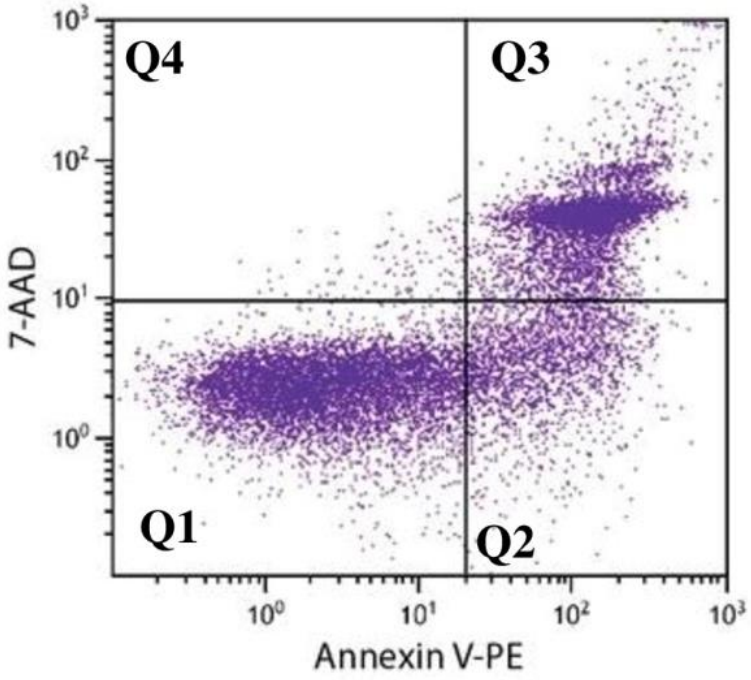

Figure 9. An illustration of the results of Guava Nexin. Q1 indicates live cells; Q2 indicates early apoptotic cells; Q3 indicates late apoptotic cells; Q4 indicates necrotic cells.

3.2.10. Imaging of Ultrastructural Changes by Scanning Electron Microscope Analysis

A scanning electron microscope (SEM) provides the scanning of a surface by releasing a stream of electrons with high-energy to create an image. Once the electron beam penetrates the analyte surface, it leads to the production of X-rays, secondary, and backscattered electrons as figured in Figure 10. These signals were monitored and recorded by a detector generating the magnified image on the screen.

By taking advantage of this technology, the apoptotic bodies and ultrastructural changes in cancer cell lines were examined. Briefly, the cells were plated on coverslips in culture plate and overnight incubated. Then, cells were treated with NDs at given doses. At the end of incubation, the cells were washed with PIPES buffer. The cell fixation was achieved in a 2.5% glutaraldehyde solution for 1 h at 4ºC. The fixed cells were maintained and dehydrated by a series of incubation in the ethanol bath (50–70–80–90–100–100%) for 7 min each. For the complete drying process, coverslips were subjected to HMDS for overnight incubation. In the last step, the slides were mounted on a metal stub and coated with conductive metal for the observing under FESEM found in ILTAM at Pamukkale University.

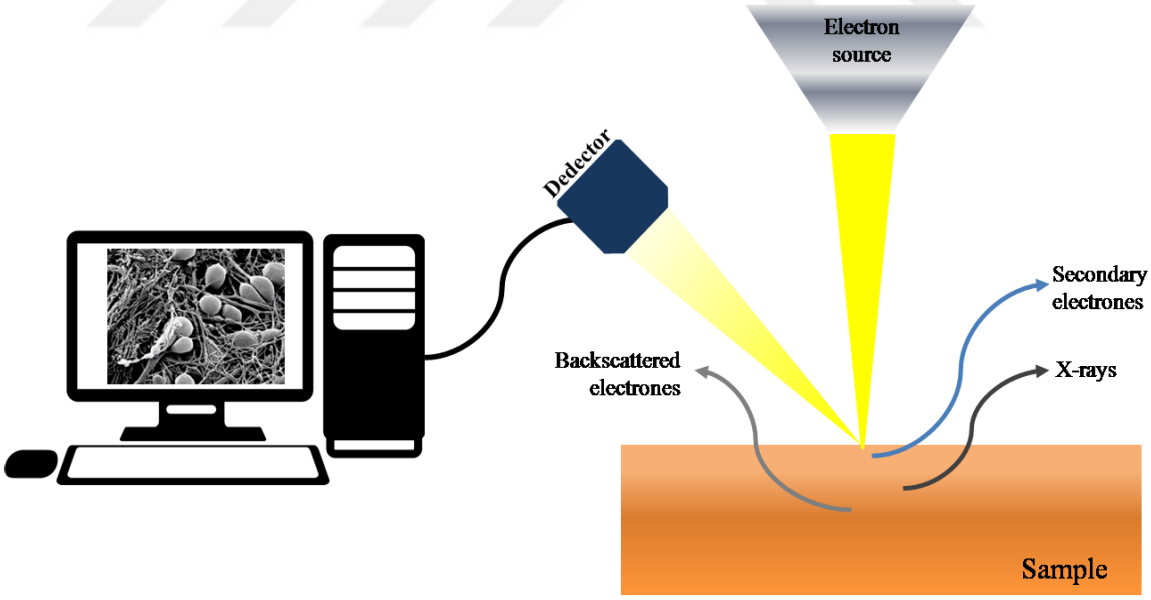

Figure 10. The working principle of a scanning electron microscope.

3.2.11. Statistical Analysis

Statistical analyses were performed using the Graphpad Prism 6 software to determine significant differences as previously described in Kapche et al. (2017) and Tumer et al. (2018). Data were obtained through technical and biological replicated experiments. Results were displayed as the standard error of mean ± (SEM).One-way analysis of variance (ANOVA) was used for comparison between control and treatment groups (*p< 0.02, **p< 0.005, ***p < 0.001).

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Results

As a research strategy, the compounds were tested in recombinant enzymes at the beginning of the study for the determination of their inhibitory properties. The compounds showing desired effects were then examined on cell models for inflammation-related analyses. Additionally, all compounds were screened in murine macrophage cells to demonstrate their effects on LPS-stimulated NO release. A panel of human-derived cancer cells was studied to elucidate the anticancer effects of NDs. All these studies including inflammation and cancer-related ones were performed along with the number of experimental optimizations in terms of several factors such as incubation time, doses, differentiation of certain cell lines, etc. which were also explained below in detail. Colon cancer cells were subjected to several doses of DOX throughout the time optimization studies and the best incubation period was selected for the cytotoxicity analyses. Moreover, other cancer-related studies were performed following the results of cytotoxicity analyses.

4.1.1. COX-1/2 Inhibitory Activities of NDs

As detailed in section 3.2.1, the inhibitory properties of NDs on COXs were screened in vitro enzyme-based analyses. At the beginning of the process, the aliquots of recombinant COX-2 were subjected to 1 µM of each ND. According to the results given in Figure 11, the compounds **N7**, **N9**, **N12**, **N13**, **N14**, **N15**, **N16**, and **N17** did not demonstrate the inhibitory properties on the COX-2 enzyme. It was also found that **N1**, **N2**, **N3**, **N4**, **N5**, and **N6** slightly decreased enzymatic activity but these changes were not statistically significant. In contrast to these findings, **N8** and **N10** decreased the COX-2 activity by 78 and 72.8 %, respectively. When considering positive controls, nimesulide reduced the activity by 12.1% whereas celecoxib showed a better inhibitory effect by 80.8% reduction as compared to the total enzyme activity.

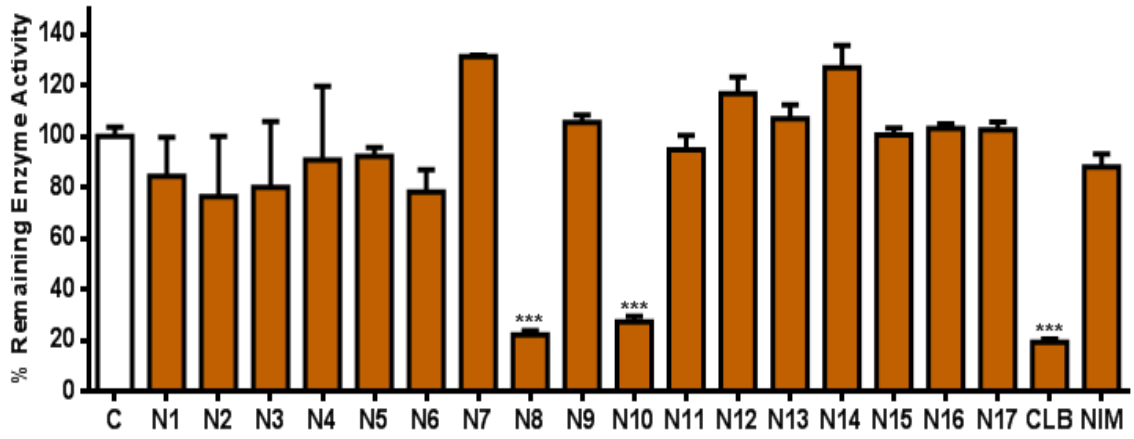

Figure 11. Screening of inhibitory effects of NDs on the COX-2 enzyme at 1 µM dose. As positive control nimesulide (NIM) and celecoxib (CLB) were examined at the same doses. C: Control. ***p < 0.001.

To decide if these inhibitory effects are COX-2 selective, the compounds demonstrating inhibitory effect were also tested on the COX-1 enzyme at a higher dose. As shown in Figure 12, even though all compounds exhibited COX-2 selectivity, both **N8** and **N10** indicated better selectivity on COX-2 than nimesulide. Moreover, their effects were found to be compatible with the celecoxib.

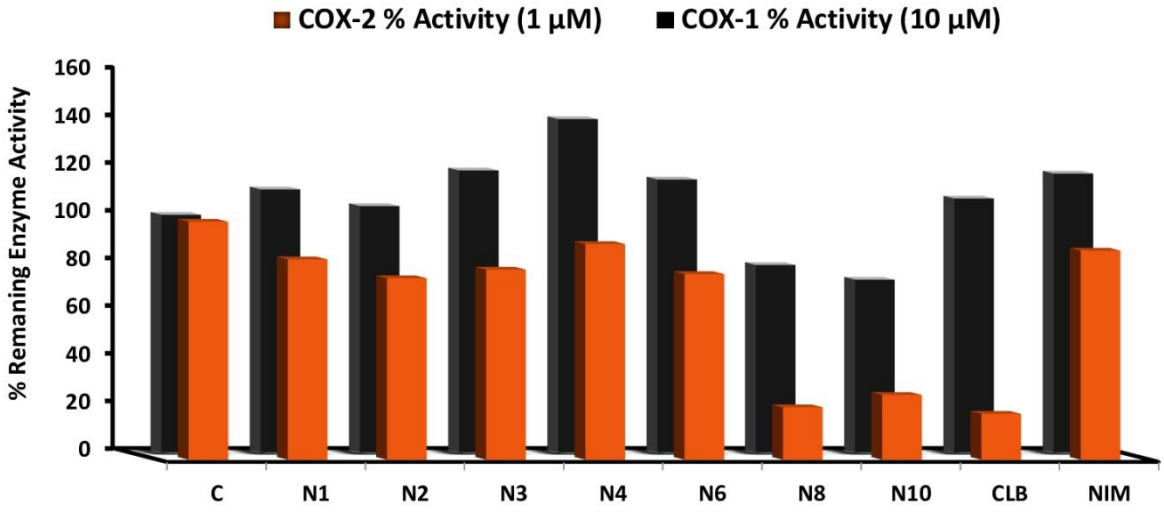

Figure 12. The comparison of inhibitory effects of NDs on COX-1/2 enzymes at 10 and 1 µM doses, respectively.

4.1.2. THP-1 Differentiation by PMA Treatment

To investigate the anti-inflammatory effects of lead compounds **N8** and **N10** through cell-based experiments, a common model THP-1 (human monocytic cell line) was adapted and maintained in our laboratory. For the preparation of the cells, 10 ng/mL of PMA stimulation was taken place for 24 hours followed by incubation in the PMA-free culture media for 24 hours resting stage. The morphological changes were then imaged with the aid of the inverted microscope by the 100X magnification factor.

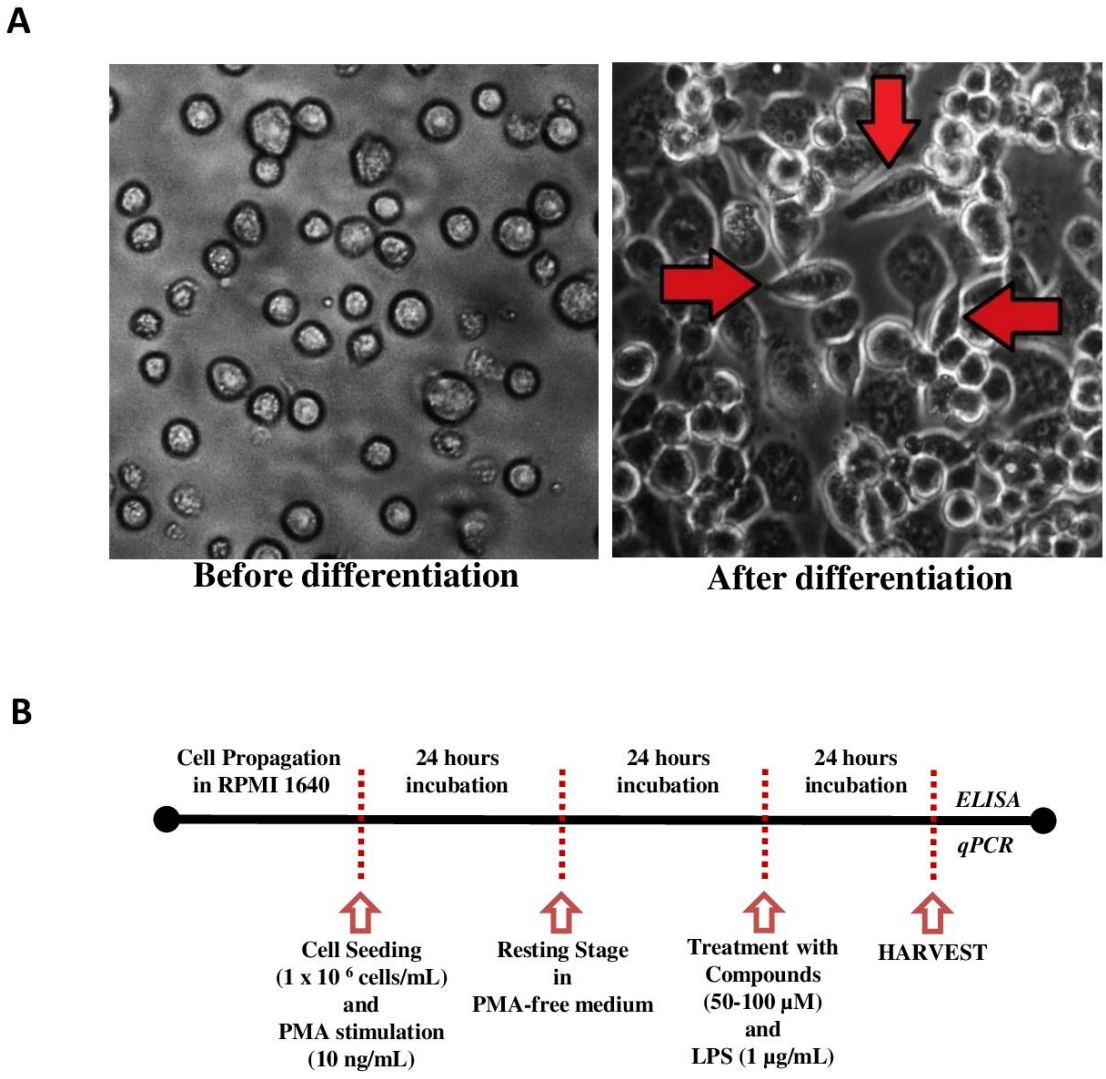

Figure 13. Differentiation of THP-1 monocyte cells into macrophage cells by PMA treatment. (A) Suspended monocytic cells (left side) and differentiated adherent macrophages (right side). (B) Experimental stages from cell differentiation to further analyses.

As marked with red arrows in Figure 13.A, PMA treatment led to rising in the cytoplasmic volume of differentiated macrophages as compared to the monocytic cells. Moreover, the differentiation process was approved by the simple observation of cell adhesion and spreading. Taken together, 10 ng/mL of PMA stimulation was found to be an adequate treatment to differentiate these cells into stable macrophages. Differentiated cells were then subjected to compounds for qPCR and ELISA analyses.

4.1.3.Determination The Effects of Lead NDs on COX-1/2 Expression Level

For the assay, differentiated THP-1 cells were subjected to 50 and 100 µM of compounds **N8** and **N10** separately, followed by LPS (1 µg/mL) simultaneous stimulation. At the end of 24h incubation, RNA samples were isolated from cells and prepared for qPCR analysis as described in the part of 3.2.5. According to the results, LPS treatment induced the COX-2 mRNA expression level in a significant manner (Figure 14). The compound **N8** suppressed the COX-2 expression by 46.6 and 59.1% at 50 and 100 µM doses, respectively. On the other hand, the same doses of **N10** decreased the COX-2 expression level by 57.4 and 61.1%, respectively as compared to the control group. In contrast to these results, LPS stimulation did not exhibit the same dramatic increase in the expression of COX-1. Similarly, both **N8** and **N10** treatments did not lead to statistically significant change at even high concentrations. As a positive control, 100 µM of celecoxib increased the COX-1 expression level by 37.5% whereas it led to a reduction in COX-2 level by 60.4%.

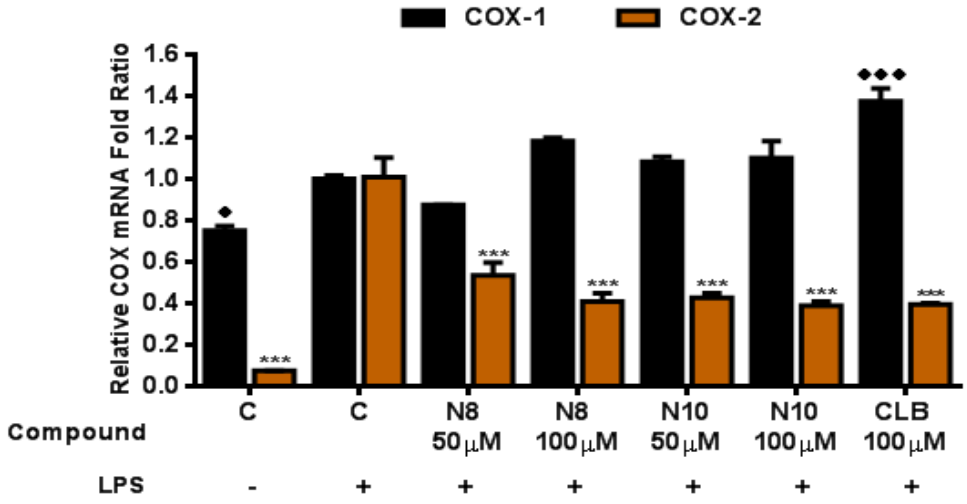

Figure 14. Effects of **N8** and **N10** on COX-1/2 mRNA expression levels in LPS-stimulated THP-1 cells. Celecoxib (100 µM): Positive control. C: Control. *,♦p< 0.02, **p< 0.005, ***, ♦♦♦p < 0.001.

4.1.4. The Effects of Lead NDs on LPS-induced PGE_2 Production

To examine the reducing effects of **N8** and **N10** on PGE_2 release, stable macrophages were maintained in the culture media containing the 50 or 100 µM doses of compounds and 1 µg/mL of LPS for 24 h. The amount of PGE_2 found in each collected cell media was normalized to the protein content separately. The results were expressed in Figure 15 as relative change. Accordingly, LPS stimulation promoted PGE_2 production almost twice as much in THP-1 cells. However, **N8** reduced the LPS-induced PGE_2 release by 77.5 and 44.0% at 50 and 100 µM treatment doses, respectively. At identical treatment, **N10** demonstrated 67.5 and 68.7% decrease in PGE_2 release as compared to the control group. The release of PGE_2 was diminished by 78.4% in the positive control group.

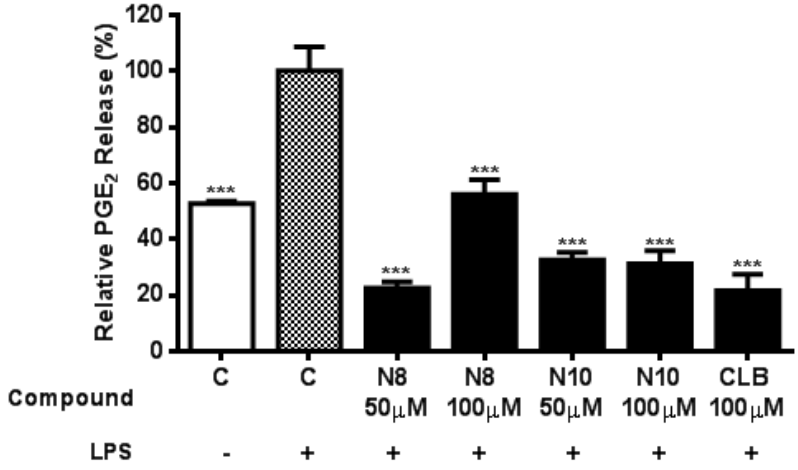

Figure 15. Effects of **N8** and **N10** on PGE_2 release in LPS-stimulated THP-1 cells. Celecoxib (100 µM): Positive control. C: Control. ***$p < 0.001$.

4.1.5. The Effects of Lead NDs on 5-LOX Enzyme Activity, mRNA Expression Level, and LTB_4 Release

To determine the dual-acting properties of **N8** and **N10**, they were tested in both enzyme and cell-based assays. As prescribed in section 3.2.1, the 5-LOX enzyme aliquots were subjected to compounds at 100 µM to assign the inhibitory properties of lead NDs. According to the results, both **N8** and **N10** did not exhibit an inhibitory effect on 5-LOX while positive control (NDGA) almost completely inhibited the enzyme at the same dose (Figure 16.A). Furthermore, the changes of 5-LOX mRNA expression level in NDs-treated THP-1 cells were evaluated by qPCR analysis. As shown in Figure 16.B, compound **N10** did not change the mRNA expression level and remained stable as compared to the control group. In **N8**-treated cells, the expression of 5-LOX was downregulated by 14 and 39% at

50 and 100 µM treatment doses, respectively. The ELISA assay demonstrated that both **N8** and **N10** were not found effective against LTB_4 release. The released LTB_4 levels remained almost the same in NDs-treated groups and non-treated cells.

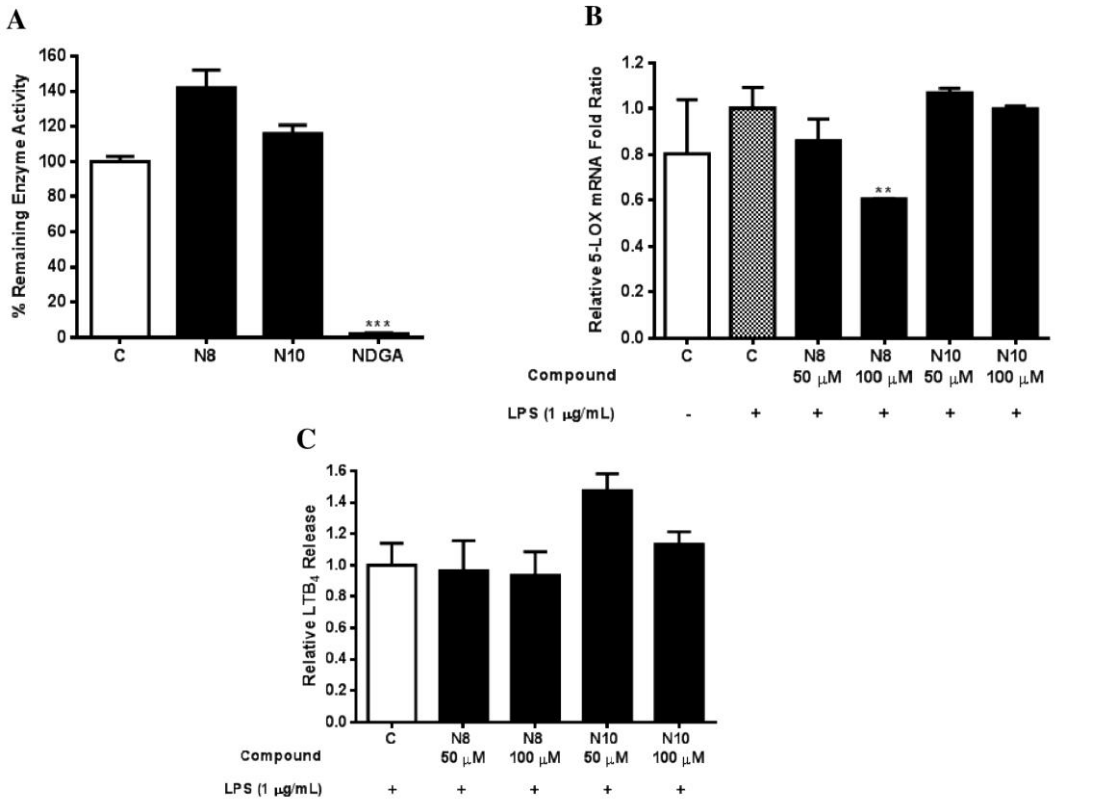

Figure 16. Effects of **N8** and **N10** on 5-LOX (A) enzyme activity, (B) mRNA expression level, and (C) LTB_4 release. NDGA (100 µM): Positive control. C: Control. **p< 0.005, ***p < 0.001.

4.1.6. Activation of Macrophages by LPS Treatment to Induce NO Release and Determination The Effects of Lead NDs on NO Production and iNOS Expression

It is expected that when macrophages exposed to LPS, the expression level of inducible NOS (iNOS) is triggered at a high rate results in exaggerated NO production (Kulabas et al., 2019). To induce the overproduction of NO, human and murine macrophage cells were stimulated by LPS treatment. According to findings shown in Figure 17.A, the difference between NO concentrations in the media of LPS (0.05, 1, and 10 µg/mL) treated THP-1cells and in the media of LPS-free macrophages was not statistically significant. Once murine macrophages were challenged with 1 µg/mL of LPS, the concentration of released NO was boosted almost 100 times. Further, 20 and 50 µM of **N8** suppressed the production of NO by 40.8 and 68.1 %, respectively. The same doses of the compound **N10** reduced the NO level by 43.1 and 46.6 % as compared to the control

group. Moreover, 20 µM of nimesulide did not diminish the level of NO however, 1400W decreased it by 62.5 % as a positive control (Figure 17.B).

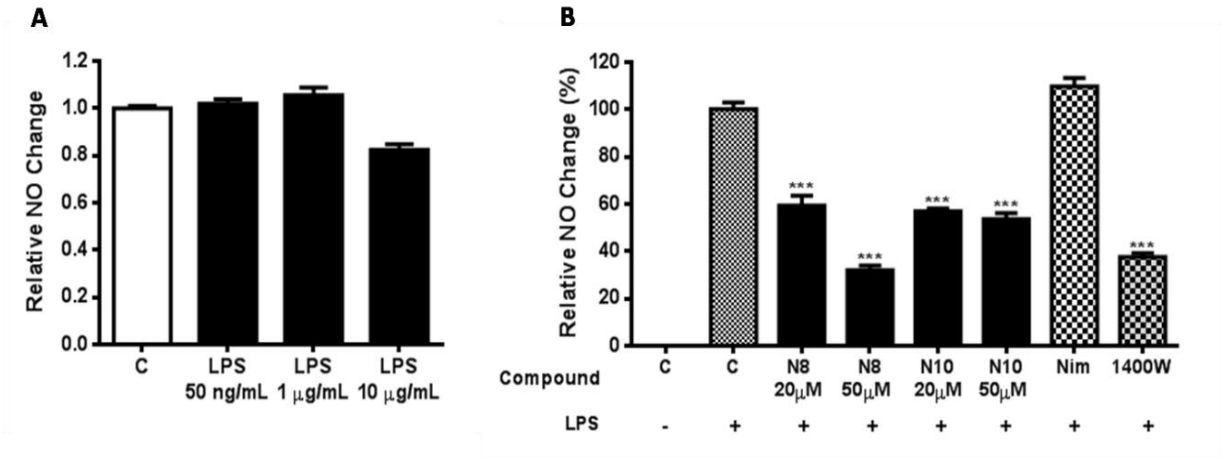

Figure 17. Relative NO change in LPS-challenged human macrophage cells (A). The effect of **N8** and **N10** on NO production in LPS-treated murine macrophage cells (B). Nim (20 µM): Reference compound.1400W (20 µM): Positive control. C: Control. ***p < 0.001.

The effects of N8 and N10 on the expression level of LPS-induced iNOS expression in murine macrophages were determined by qPCR analysis. As shown in graph 18, the expression level of iNOS at the mRNA level remained stable in the **N8** treated group as compared to the control group. But the iNOS expression was decreased by the **N10** treatment as a dose-dependent manner. **N10** down-regulated the expression by %40 at 20 µM dose and almost completely ended at 50 µM dose.

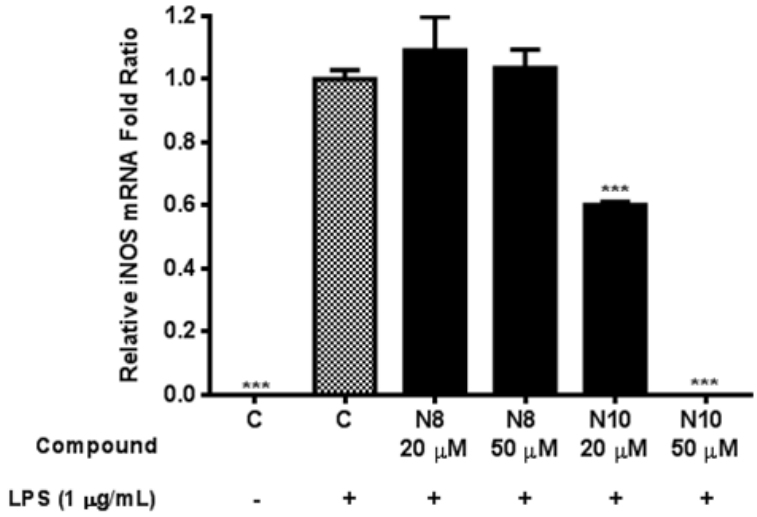

Figure 18. Effects of **N8** and **N10** on iNOS mRNA expression levels in LPS-stimulated RAW264.7 cells. C: Control. ***p < 0.001.

4.1.7. The Effects of Lead NDs on IL-1β and TNF-α mRNA Expression Levels

To evaluate the effects of **N8** and **N10** on the expression level of IL-1β and TNF-α, murine macrophages were stimulated with LPS and treated with lead NDs simultaneously for 24 hours. Then, the changes in the mRNA expression levels were screened by qPCR analysis. According to the results (Figure 19), 20 and 50 µM doses of **N8** led to a reduction in the mRNA expression level of IL-1β by 39 and 22%, respectively. Additionally, at the same doses, **N10** reduced the IL-1β mRNA level by 45 and 51% as compared to the control group. The expression of TNF-α was also changed by **N8** and **N10** treatment at 20 and 50 µM doses. The treatment of **N8** down-regulated the mRNA expressions of TNF-α by 46 and 22%, respectively. Additionally, **N10** reduced the LPS-induced TNF-α mRNA level by 44 and 57% at the same doses.

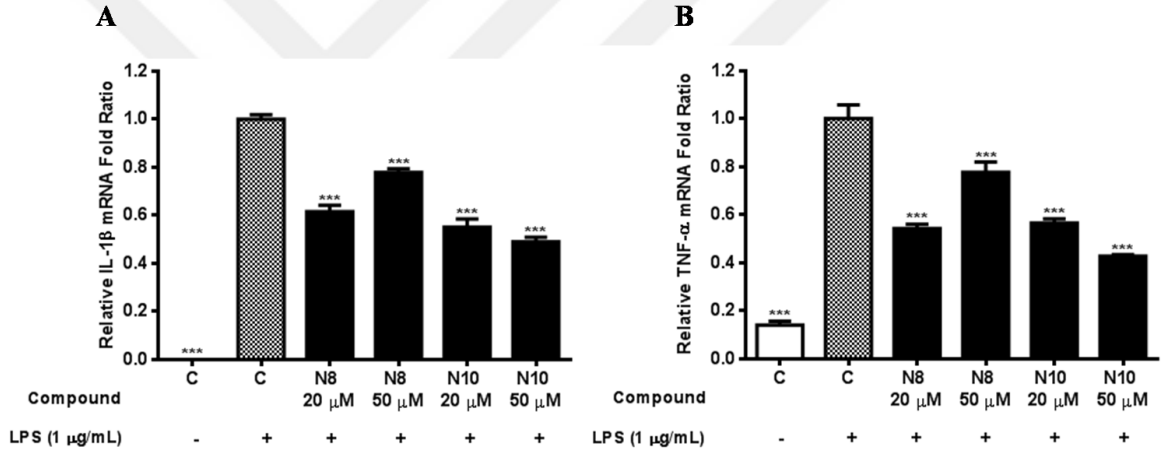

Figure 19. Effects of **N8** and **N10** on (A) IL-1β and (B) TNF-α mRNA expression levels in LPS-stimulated RAW264.7 cells. C: Control. ***$p < 0.001$.

4.1.8. Time Optimization Studies for Screening of Anticancer Properties

To decide the most proper incubation period for the evaluation of anticancer properties of compounds, HT-29 colon cancer cells were subjected to several doses of a reference compound named doxorubicin for 24, 48, and 72 hours. At the end of the given times, remaining cell viabilities were examined by both MTT and SRB assay separately, as detailed in the part 3.2.7. The findings shown in Figure 20 suggested that 48 hours incubation period would be the optimum time to show the potential anticancer effects of test compounds.

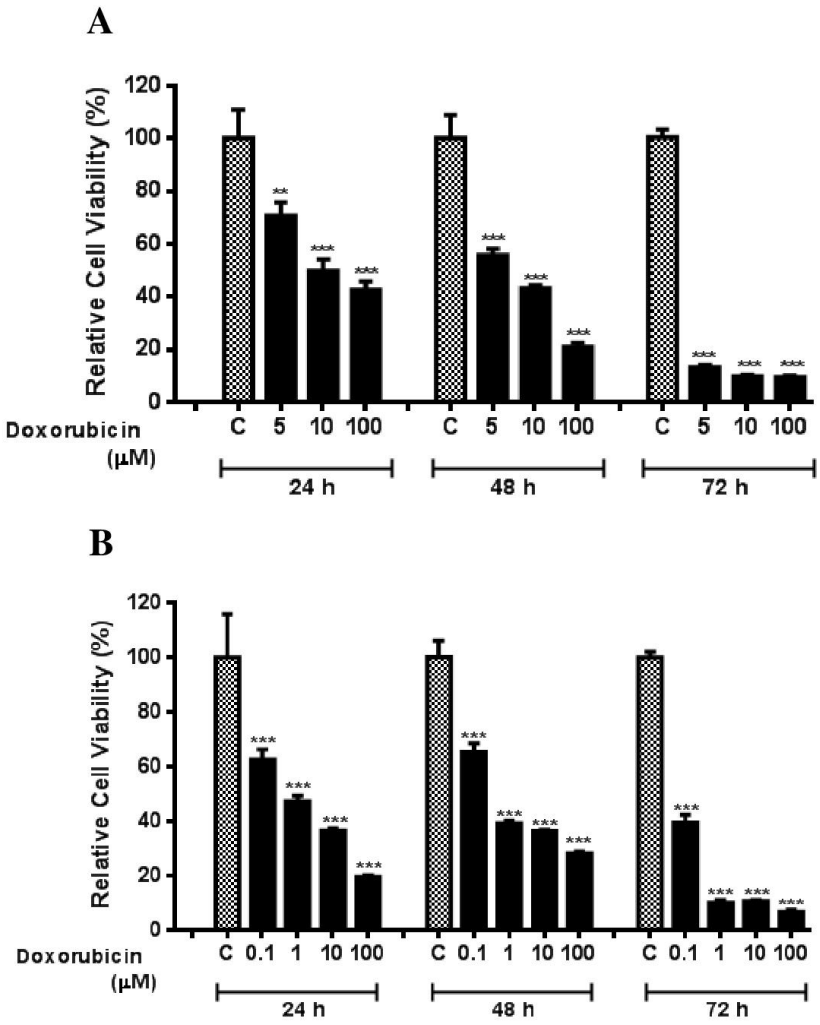

Figure 20. The effect of FDA-approved doxorubicin on cell viability of HT-29 cell line at the end of the 24, 48, and 72 hours. The results were evaluated by (A) MTT and (B) SRB assays. C: Control. **p< 0.005, ***p < 0.001.

4.1.9. Screening of Anticancer Effects of NDs by MTT Assay

The cytotoxicity of NDs was evaluated in a panel of cell models including colon, breast, and prostate cancer cells as well as a healthy endothelial cell line by colorimetric MTT assay. The assay is based on the enzymatic conversion of the yellowish MTT compound into the insoluble purple formazan in living cells. For these analyses, cells were incubated in the presence of 100 µM of each ND for 48 hours. The remaining cell viability was then quantified following the assay procedure and given as % survival in Figure 21.

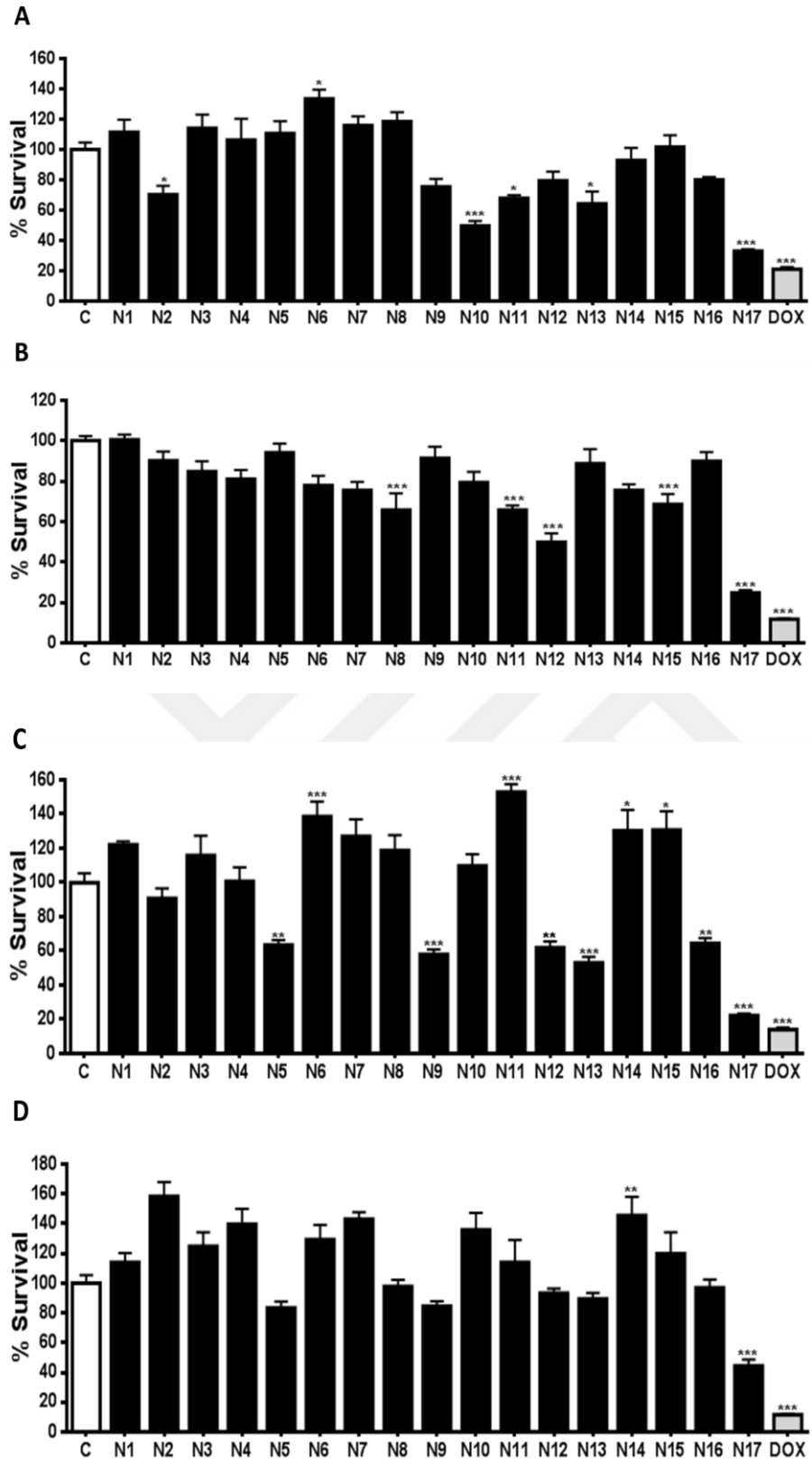

Figure 21. The cytotoxic effect of NDs on (A) HT-29 colon cancer, (B) PC-3 prostate cancer, (C) MCF-7 breast cancer, and (D) HUVEC healthy cell lines at 100 μM dose. C: Control. DOX: Doxorubicin. *p< 0.02, **p< 0.005, ***p < 0.001.

According to results, **N10** decreased the viability of colon cancer cells by almost half as compared to the control group as shown in Figure 21.A. Additionally, in the same cell line, viability was reduced by 29, 32, and 36% with the **N2**, **N11**, and **N13** treatment respectively. However, the most cytotoxic effect was seen in the **N17**-treated group where the survival was detracted by 67% as compared to the non-treated cells. In the prostate cancer cells, the effect of compound **N11** was found almost identical as in the colon cancer cells by leading 34% cell death (Figure 21.B). The compound **N8** and **N15** demonstrated cytotoxicity in solely prostate cancer cells by decreasing the survival by 34 and 31% as compared to the control, respectively. While **N12** decreased the viability to almost half in the prostate cancer cells, the most effective score was seen in the **N17** treatment with 75% cell death. Further, the compound **N9** exhibited a moderate cytotoxic effect by decreasing the viability by 42% in the breast cancer cells (Figure 21.C). Additionally, **N5**, **N12**, **N13**, and **N16** demonstrated a similar effect but these were not as effective as **N17** treatment where the cell survival was reduced by 55.5%. NDs were also tested in a healthy cell line. The cell viability remained higher than 80% after each treatment with **N1-16** but not **N17** in these cells (Figure 21.D). Apart from NDs, cell viabilities of the colon, prostate, breast cancer cells, and healthy cell model were determined as 21, 12, 14, and 11 % after doxorubicin treatment, respectively.

4.1.10. Determination of IC_{50} Values of N17 by SRB Assay

Once the effectiveness of **N17** was approved against cancer cells, its IC_{50} values were also identified by SRB assay and shown in Table 6. For that purpose, cells were subjected to several doses of **N17** and reference compound doxorubicin for 48 h. The concentrations that able to lead to the death of half of the cells were then calculated by using GraphPad Prism 5 software.

Table 6.
The IC_{50} concentrations of **N17** and doxorubicin in HT-29 colon cancer, PC-3 prostate cancer, MCF-7 breast cancer, and HUVEC healthy cell lines.

Compound	HT-29	PC3	MCF-7	HUVEC
Doxorubicin	0.15 µM	16.86 µM	0.16 µM	0.10 µM
N17	9.24 µM	>100 µM	11.35 µM	>100 µM

4.1.11. Assessment of Anti-proliferation/migration Properties of N17

The anti-proliferation/migration effects of **N17** were shown in MCF-7 breast cancer cells by scratch wound healing assay. An artificial wound was introduced to the cells and treated with 10, 25, and 50 µM doses of **N17** as well as doxorubicin treatment, separately. The cell-free area between the cell masses was imaged by an inverted microscope and analyzed by using ImageJ software. Accordingly, the wound in the control group healed as a time-dependent manner and improved by 43.0 % after 48-hour incubation as compared to the initial state. Contrary to these results, doxorubicin obstructed the healing entirely and the wound remained stable during incubation. In other respects, **N17** treatment decelerated the healing process but did not stop recovery. In the 10, 25, and 50 µM treatment groups, the cell-free area was reduced by 30.3, 27.2, and 32.5 %, respectively as shown in Figure 22.

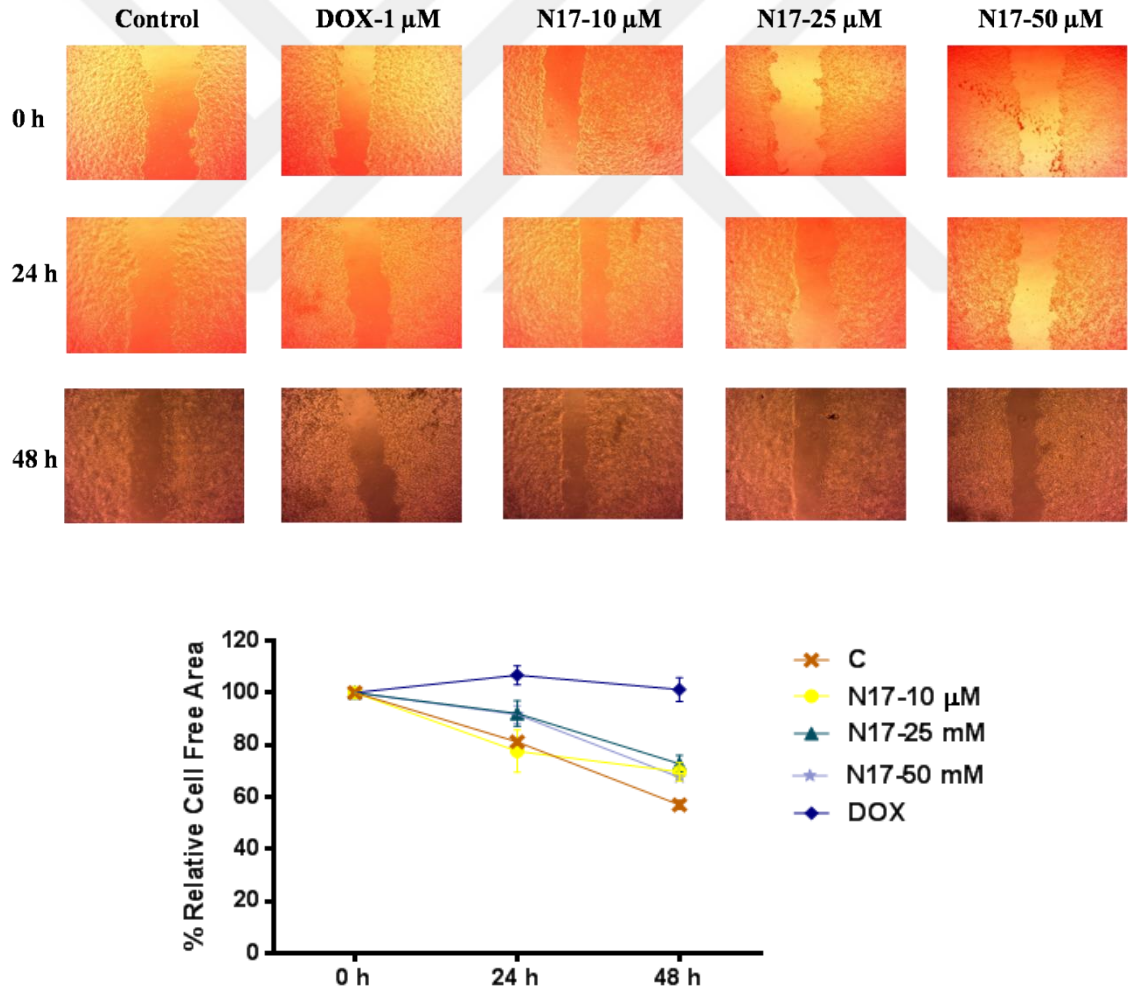

Figure 22. The healing process of artificial wounds in the MCF-7 cell line throughout 48-hours incubation in the absence and presence of test compounds.

4.1.12. The Effects of N17 on Apoptosis Related Protein Expression Level

The changes in the protein expression levels were determined by Western blot analyses. To show the effects of **N17**, colon, and breast cancer cells were subjected to several doses of the compound for 48 hours. At the end of the period, protein lysates were isolated by using RIPA solution and saved for further experimental stages including SDS-PAGE and blotting. In this analysis, the level of Caspase-3, Bax, Bcl-2, and as a housekeeping gene Actin-β was examined. According to the results, the Bax/Bcl-2 ratio was increased in the breast cancer cells by 10 and 25 µM of **N17** treatment by 16.5 and 69.0%, respectively as shown in Figure 23.

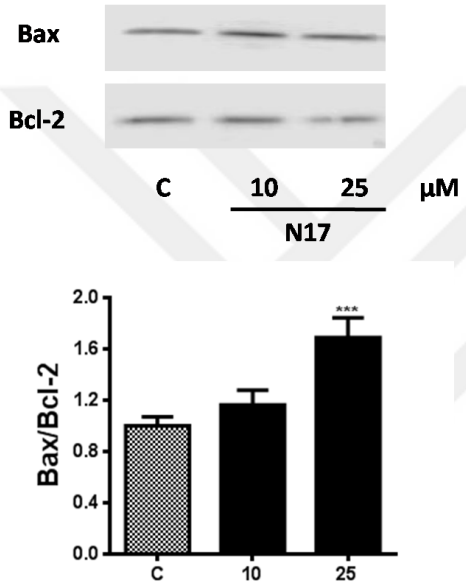

Figure 23. The effects of **N17** on Bax/Bcl-2 expression levels in MCF-7 breast cancer cells. Cells were treated with 10 and 25 µM of the compound **N17**.C: Control.***p < 0.001.

Moreover, **N17** enhanced the expression of active caspase-3 levels in colon cancer cells at 10, 25, and 50 µM by 31.3, 11.2, and 81.2%, respectively as compared to the control group. In the same cell line, the level of apoptotic Bax protein expression was induced by 11, 15, 23 fold with the treatment of **N17** at 10, 25, and 50 µM doses, respectively as compared to the non-treated control cells (Figure 24).

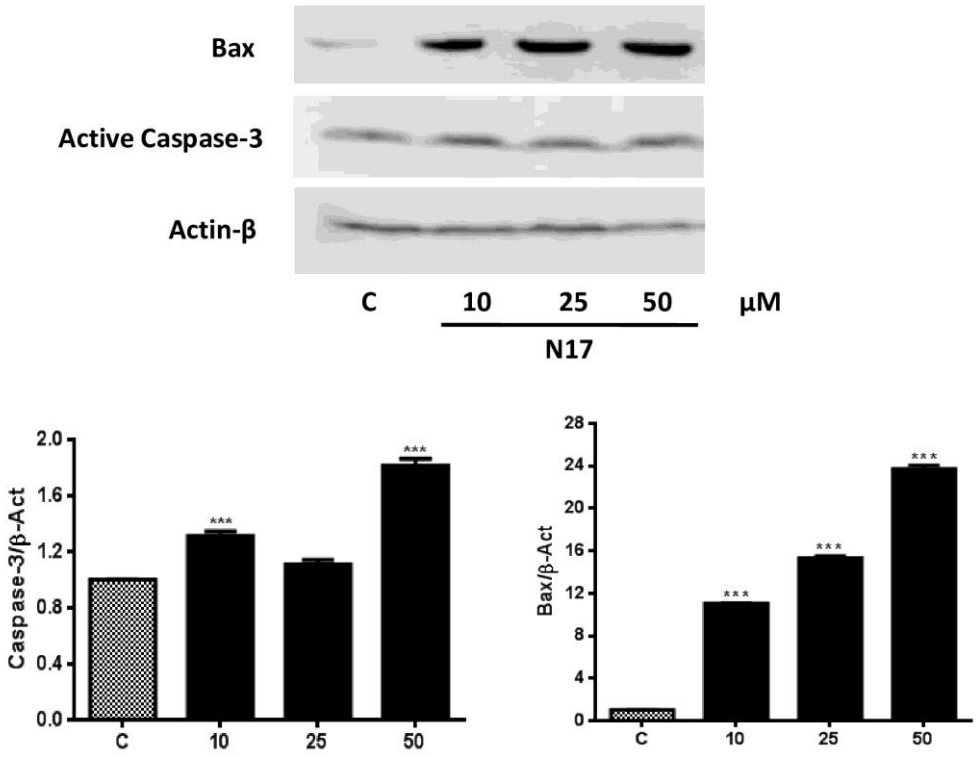

Figure 24. The effects of **N17** on Bax and Caspase-3 expression levels in HT-29 colon cancer cells. Cells were treated with 10, 25, and 50 μM of the compound **N17**. C: Control. ***$p < 0.001$.

4.1.13. The Detection of Morphological Changes in HT-29 Cells by SEM Analysis

As described in section 3.2.10, to investigate the ultrastructural changes of HT-29 cells, which were subjected to **N17** (25 μM) and reference compound DOX (1 μM), SEM analyses were performed. The treated cells appeared to be irregular because of the increasing number of blebs and apoptotic bodies. The control cells exhibited typical morphologies and kept intact. However, unlike non-treated cells, **N17** and DOX treated cells lost their pre-apoptotic structures by elongating and shrinkage (Figure 25).

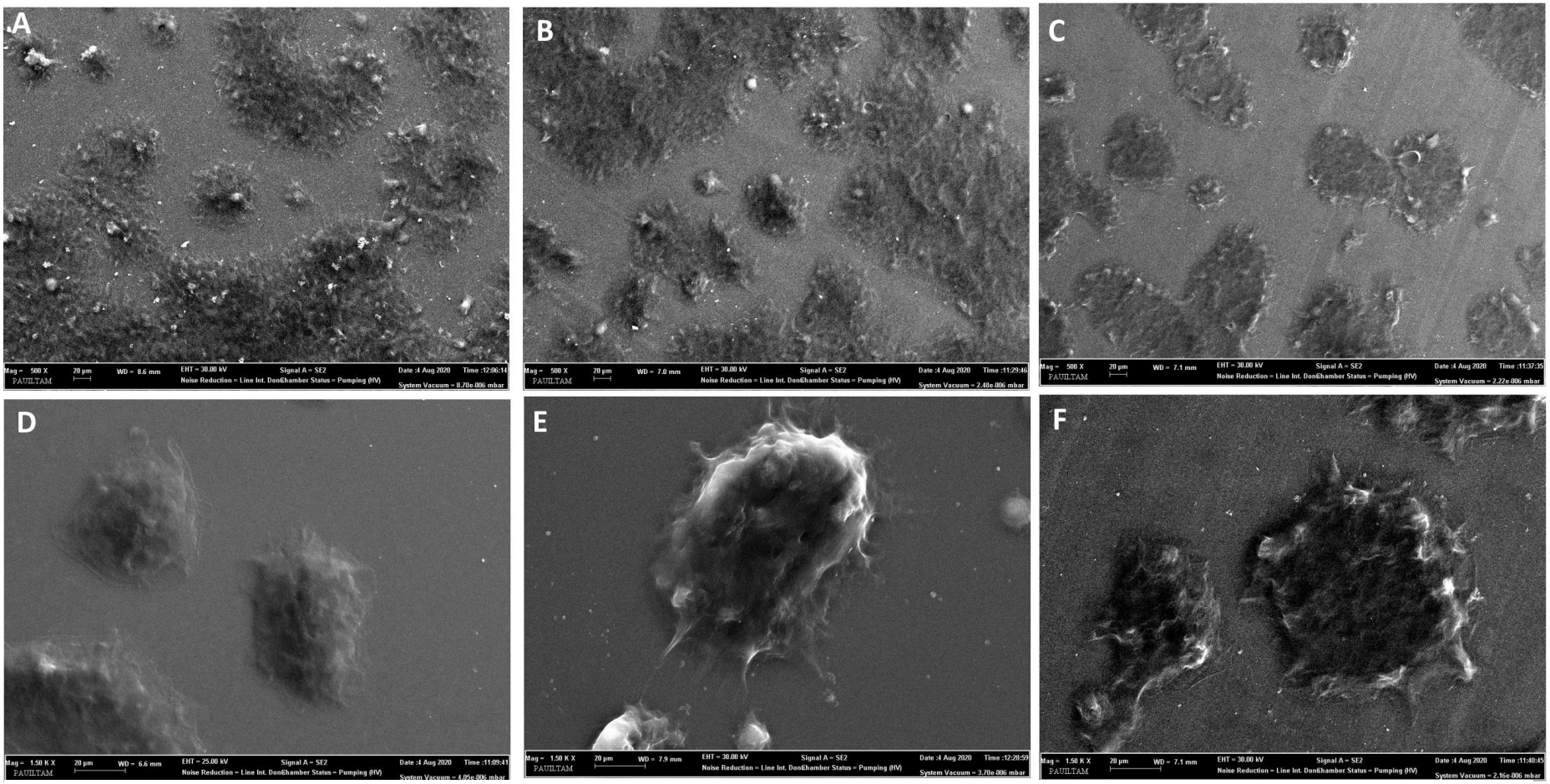

Figure 25. The ultrastructural changes of treated HT-29 cells were displayed by SEM analysis. The treatment of (B, D) N17 and (C, E) reference compound DOX for 48 h led to the loss of normal morphology and normal cell size as compared to the (A, D) untreated cells. Magnifications: 500x for A,B, C; 1,500x for D,E, and F micrographs.

4.1.14. The Effects of N17 on Annexin V Staining Apoptosis Test

To investigate the effect of **N17** on apoptotic cell percentage, flow cytometry analyses were performed in Balıkesir University. After appropriate cell treatment, cells were collected in fresh medium, and samples were transported Balıkesir in an ice-cooled isolated box. According to the results shown in Figure 26, the cell viabilities in the control group were found 58.02 and 64.11%, separately. However, in the same control group, the necrotic cell percentages were read 38.27 and 31.64%, undesirably. Moreover, the necrotic cell populations demonstrated similarity in the N17-treated cell groups. The underlying reason for the cells facing necrosis is thought to be inappropriate transportation conditions. In further studies, these experiments will be optimized for transportation conditions.

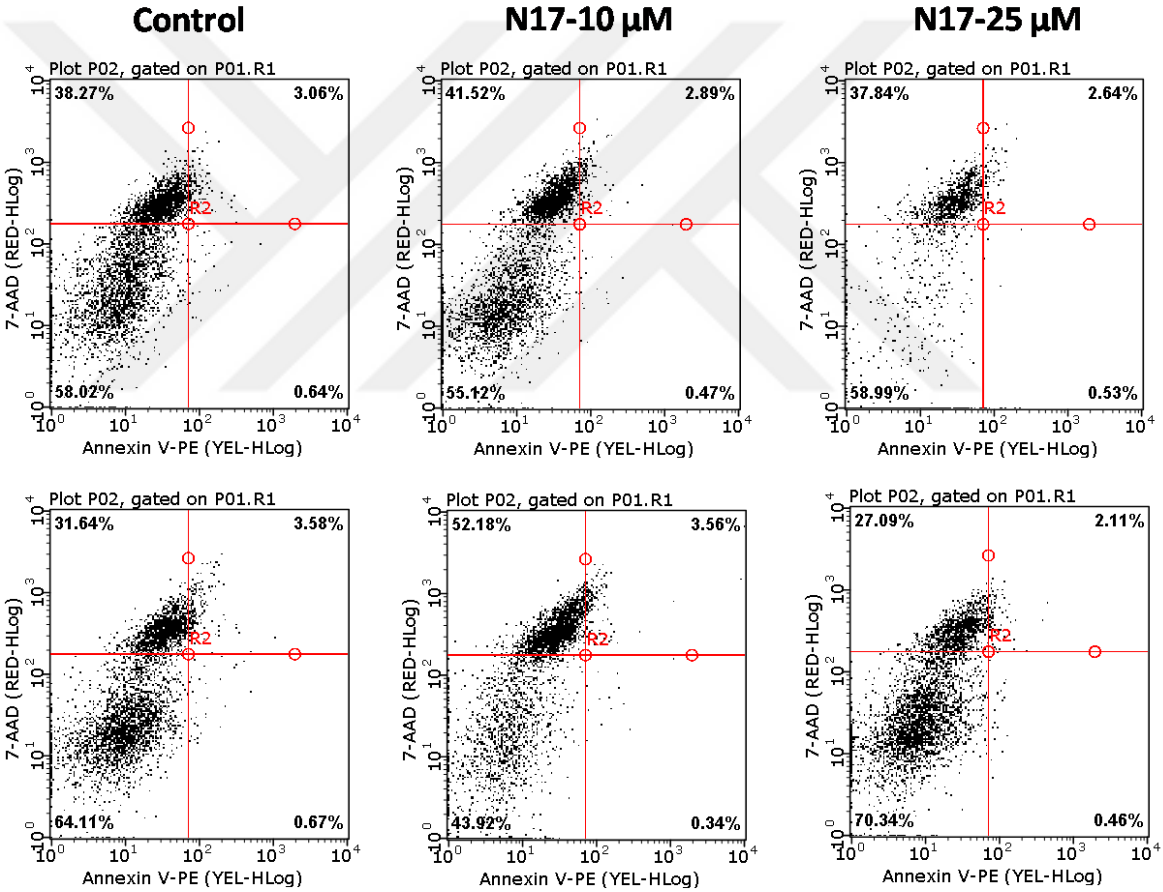

Figure 26. The effects of **N17** on cell viability and apoptosis of cancer cells. Bottom left indicates live cells; bottom right indicates early apoptotic cells; upper right indicates late apoptotic cells; upper left indicates necrotic cells.

4.2. Discussion

Inflammation is a kind of innate immune response to any stress conditions such as pathogens, injuries, or even unhealthy diets. Long-term and uncontrolled inflammatory conditions are associated with chronic inflammatory diseases involving arthritis, diabetes, asthma, and autoimmune problems (L. Chen et al., 2018). Classical NSAIDs are the frequently adopted medication method in clinics to reduce the risk factors in the inflammatory response (Curry, Cogar, & Cook, 2005). The particular biomolecular target of NSAIDs is COX enzymes and the drug actions' are based on the inhibition of COXs activities. Both isoenzymes COX-1/2 are the key enzymes found in the AA pathway and they convert the membrane-bounded AA into bioactive PGs (Ghosh, Chaki, Mandal, & Mandal, 2010). COX-1 is continuously transcribed for the yielding of PGs which are required for circulatory hemostasis. On the other hand, inducible isoform COX-2 is overexpressed in the presence of inflammatory and mitogenic stimuli in various tissues. The induction of COX-2 expression results in the excessive production of PGE_2 which leads to an exaggerated inflammatory response (Curry et al., 2005). For this reason, the administration of NSAIDs could be preventive against chronic inflammation and associated degenerative diseases. However, long term uses of classical NSAIDs demonstrate some drawbacks such as GI bleeding, peptic ulceration, and perforation owing to depletion of the of gastroprotective PGs (Van Ryn & Pairet, 1999; John R Vane & Botting, 1998).

To overcome these side effects, nowadays researchers put considerable efforts into the design and synthesis of the novel COX-2 targeted inhibitors as anti-inflammatory or analgesic agents. Thus, this kind of molecule is thought to have a relatively safer profile as compared to the classical NSAIDs by inhibiting the inducible isoform of PG synthase to suppress the chronic inflammation while constitutive COX still performs its physiological functions (Wright, 2002). Yet, this does not indicate that selective COX-2 inhibitors display a more potent gastro-friendly effect in gastric tissue. Additionally, further studies exhibited that several selective COX-2 inhibitors demonstrated severe cardiovascular problems. Consequently, drugs such as rofecoxib and valdecoxib were withdrawn from marketing (Brinker, Goldkind, Bonnel, & Beitz, 2004; Hernandez et al., 2004).

Nimesulide is an FDA-approved sulfonamide compound with prominent anti-inflammatory, analgesic as well as antipyretic effects. It does not only inhibits COX-2 activity but also has the multifactorial modes of actions including the inhibition of NO, ROS, cytokine releases (K. D. Rainsford, 2006). In the current study, the anti-

inflammatory properties of seventeen novel NDs were investigated through cell-free and cell-based experiments. Firstly, the inhibitory properties of NDs on both COX-1/2 were examined by using the human recombinant enzyme. According to the results given in section 4.1.1, compounds **N8** and **N10** inhibited the COX-2 enzyme preferentially at even low concentration. Additionally, the results indicated that both compounds displayed better inhibitory properties than nimesulide in which inhibitory scores were found compatible with the effects of celecoxib. Celecoxib is another proven NSAID with much better selectivity for COX-2 (H. Suleyman, Cadirci, Albayrak, & Halici, 2008). Thus, celecoxib was used as a control treatment in cell-based analysis in which the potential **N8** and **N10** compounds were investigated.

The second stage of the study was about the generation of an appropriate cell model to show the anti-inflammatory properties of chosen compounds. Accordingly, the human monocytic THP-1 cell line was propagated in cell culture conditions. When the cells reached enough density ($\geq 10^8$cells/mL), they were subjected to the PMA treatment to stimulate macrophage differentiation from monocytes as previously described in section 4.1.2. During PMA stimulation, the proportion of suspended cells were decreased and vanished at the end of the 24 hours. As shown in Figure 13, stable macrophages were able to adhere and spread on the surface of the cell culture plate with growing cytoplasmic volume.

Differentiated macrophages were treated with compounds **N8**, **N10,** and control celecoxib for further ELISA and real-time PCR analyses. To activate the cells, macrophages were stimulated with 1 µg/mL LPS simultaneously. According to results shown in Figure 14, given stress led to a remarkable increase in the mRNA level of COX-2 whereas the COX-1 expression level remained stable even LPS treatment. Both **N8** and **N10** suppressed the COX-2 mRNA expression level in a dose-dependent manner. Besides these compounds did not change the mRNA expression of COX-1. The effects of celecoxib on COX-2 mRNA expression were found almost identical with compounds at 100 µM dose however, the COX-1 expression level was induced by 37% as compared to the control. Moreover, the effects of compounds on PGE_2 release were assayed with the ELISA method. Macrophages were subjected to LPS to trigger the PGE_2 production after appropriate compound treatment. As illustrated in Figure 15, the concentration of released PGE_2 was almost doubled by LPS stimulation. However, both **N8** and **N10** diminished PGE_2 release significantly as the celecoxib did. Numerically, **N8** decreased the PGE_2 release by 77.5 and 44.0% at 50 and 100 µM treatment doses, respectively as well as **N10**

led to falling by 67.5 and 68.7%. All these results indicated that enzyme-based and cell-based analyses were considerably compatible and support each other.

However, unlike COX enzyme assays, the findings indicated that both **N8** and **N10** did not exhibit inhibitory effects on the 5-LOX enzyme. Moreover, these compounds did not reduce the LTB_4 release as compared to the control group. However, only **N8** at 100 µM downregulated the 5-LOX mRNA expression in a statistically significant manner. Considering all this, the compounds **N8** and **N10** are not dual-acting on both COX/LOX enzyme but act on the COX enzyme effectively.

NO is another signaling molecule involving both physiological and pathological processes. This free diatomic molecule is produced and released by the stimulated several cell types including macrophages, monocytes, microglia, fibroblasts, lymphocytes, smooth muscle cells, neutrophils, eosinophils, Kupffer cells, epithelial cells, and various tumor cells on account of the catalytic action of the nitric oxide synthase (NOS). Mainly, three genes found in different chromosomes encode the NOS isoforms called endothelial NOS, neuronal NOS, and inducible NOS (iNOS) (Mollace, Muscoli, Masini, Cuzzocrea, & Salvemini, 2005). Apart from others, the expression of iNOS is triggered by various inflammatory stimuli such as bacterial LPS and cytokines (IL-1β, TNF-α, IFN-γ, and endotoxins) (Bonaiuto, McDonald, Rossi, & Cassatella, 1997; Hartlage-Rübsamen, Lemke, & Schliebs, 1999). Another main difference between isoforms is related to the quantity of released NO and their activity periods. While iNOS produces a large amount of NO in a prolonged manner, others generate the NO molecule with short-acting and small scale. Accordingly, sustained iNOS activation is found to be a risk factor for having severe inflammatory diseases and cancer by leading the excess amount of NO production which contributes to the production of reactive oxygen and nitrogen species extensively as well as to the activation of several inflammatory pathways. Also, a great number of scientific reports highlighted the “cross-talk” between NO and PGs biosynthesis pathways and put effort to clarify this connection (Mollace et al., 2005). In this regard, activated mouse macrophages were subjected to both selective/non-selective iNOS inhibitors. As expected, inhibitors decreased the level of released NO as well as there was a simultaneous reduction in PG levels Emery, 2001(Corbett et al., 1992; D Salvemini et al., 1993). Moreover, further studies demonstrated that these iNOS inhibitors do not inhibit the COX activity unlike classical NSAIDs (Connor et al., 1995; D Salvemini et al., 1993; Daniela Salvemini et al., 1995). All these findings suggested that endogenous NO exhibits a stimulatory action on COX activity and PGs production as shown in Figure 27. Therefore, the effects of the

compound **N8** and **N10** on LPS-stimulated NO production were also analyzed as detailed in section 3.2.4.

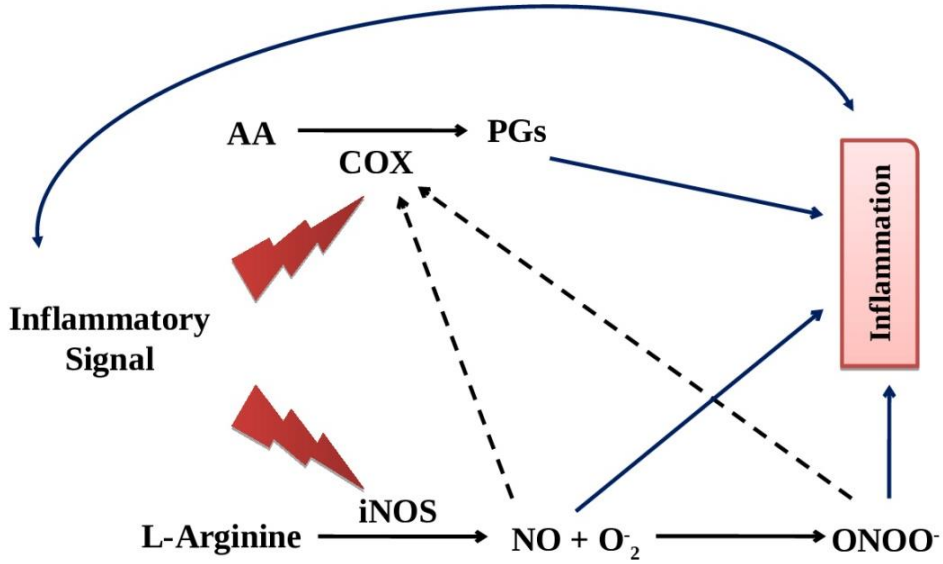

Figure 27. Proposed molecular mechanism showing how NO molecule contributes to the COX activity and inflammation.

To activate the NO release in human macrophages, differentiated THP-1 cells were treated with 0.05, 1, and 10 µg/mL doses of LPS, separately for 24 hours. The concentration of released NO was determined by an analytical method named Griess assay. The findings are shown in Figure 17.A, the release of NO was not triggered at even the highest LPS concentration and NO level remained constant in each treatment group as compared to the LPS-free cells. Interestingly, there are a variety of conflicting data regarding the ability of NO release by human macrophage models. In some studies, THP-1 human macrophages were utilized as a cell model to investigate the NO release (F. Chen, Kuhn, Gaydos, & DEMERS, 1996; Saad et al., 2011), however, a great number of studies reported that human macrophages cells do not give a response to LPS stimulation (Gross et al., 2014; van den Bogaart, Mens, Adams, Grobusch, & Schallig, 2017). Although iNOS is expressed at both mRNA and protein levels in human macrophages, they do not release NO due to lacking a complete mechanism required for NO biosynthesis in the cell culture (Bertholet, Tzeng, Felley-Bosco, & Mauel, 1999; van den Bogaart et al., 2017). Alternatively, RAW264.7 murine macrophage cells were studied to screen the reducing effects of **N8** and **N10** on LPS-induced NO release. As optimized in our previous studies (Kulabas et al., 2019; Tumer et al., 2018), murine macrophages were stimulated with 1 µg/mL LPS to induce NO production. According to results, **N8** decreased the NO level by

40.8 and 68.1 % at 20 and 50 µM doses, respectively. The compound **N10**, at the same doses, suppressed the NO level by 43.1 and 46.4% as compared to the control. While both compounds inhibited the release, nimesulide did not change the NO level significantly. Taken together, the compounds inhibiting PGE_2 release also decreased the NO production.

The proteins like IL-1β and TNF-α are the main biomolecules producing the pro-inflammatory cytokines under stress conditions. Overexpression of these molecules is associated with the release of exaggerated amounts cytokines that lead to the progression of several inflammatory diseases such as metabolic syndrome, cancer, type II diabetes (Tumer et al., 2018). Besides, coronavirus-19 is a worldwide epidemic that causes severe respiratory syndrome by promoting the cytokine storm. In the war against coronavirus and other inflammation-based diseases, the inhibition of pro-inflammatory proteins has been considered as a therapeutic strategy (Conti et al., 2020). To investigate the effects of **N8** and **N10** on the mRNA expression of IL-1β and TNF-α**,** murine macrophages were stimulated with LPS. Results demonstrated that **N8** downregulated the mRNA expression levels of IL-1β by % 39 and 22; TNF-α by % 46 and 22 at 20 and 50 µM doses, respectively. Additionally, **N10** decreased the LPS-induced IL-1β and TNF-α mRNA expression levels in a statistically significant manner. All of this data suggests that **N8** and **N10** could be potent anti-inflammatory agents.

Various epidemiological studies have attracted attention to the inverse relationship between the use of NSAIDs and the risk of development of some cancer types (Rayburn, Ezell, & Zhang, 2009). In recent years, phase II and III studies have been carried out on cancer patients for the concomitant use of NSAIDs with chemotherapy (ClinicalTrials.gov Identifier: NCT02429427 and NCT01238120). Nimesulide is a NSAIDs with a long term safe and effective usage and proven chemopreventive effects on subjects at high risk for breast cancer (ClinicalTrials.gov Identifier: NCT01500577). Considering these findings, anticancer properties of novel NDs were also investigated in cancer cell models. In vitro techniques/assays in our study were chosen concerning the hallmark features of cancer (Figure 28) proposed by Hanahan and Weinberg in the early 21st century (Hanahan & Weinberg, 2000, 2011).

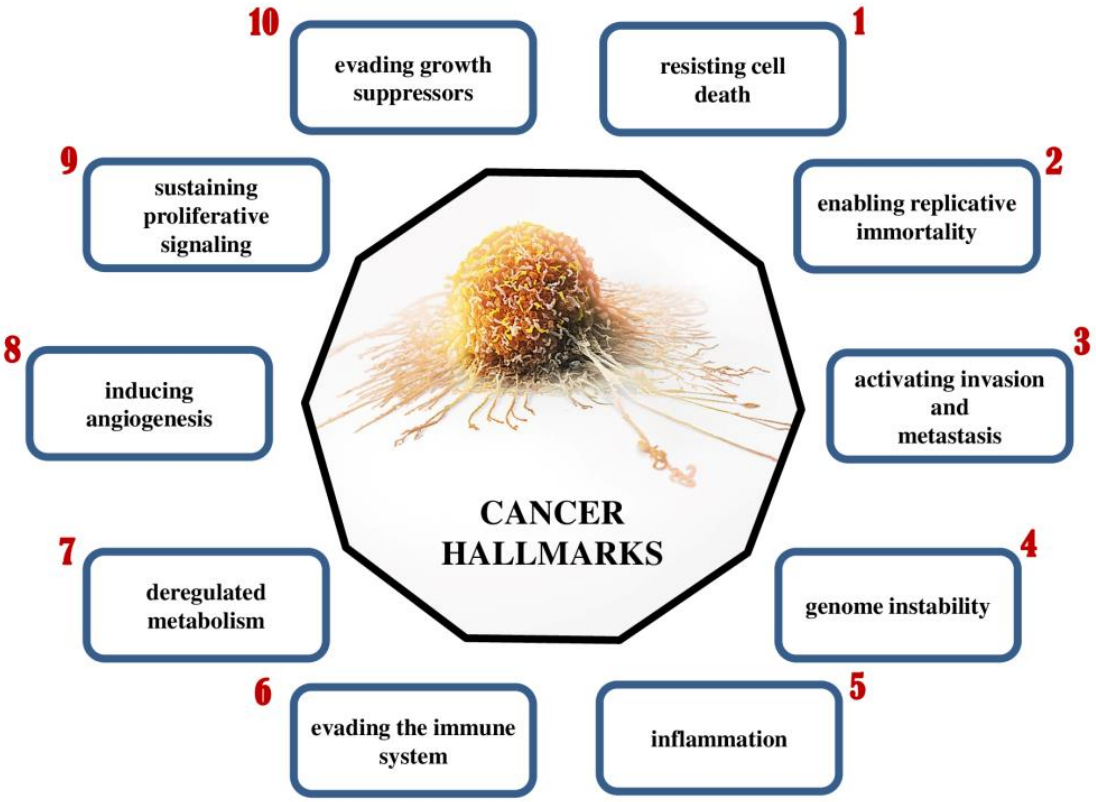

Figure 28. The proposed hallmark features of cancer by Hanahan and Weinberg (Ediriweera, Tennekoon, & Samarakoon, 2019).

In the first part of the analyses, all compounds were given to a panel of cancer cell and the cytotoxicity/antiproliferation effects of compounds were screened by MTT assay. Additionally, results for the lead compound were validated with SRB assay. MTT assay is one of the most used colorimetric assays in which the MTT compound is converted into insoluble and purple formazan by enzymes. SRB assay also is a colorimetric method that provides the quantification of the total cellular protein content of living cells by SRB staining. Although MTT is accepted as the 'gold standard' in numerous studies since the efforts of Mosmann in the 1980′s, recent studies discuss the assay parameters such as potential interferences, linearity, sensitivity, and reproducibility. SRB assay, in contrast, is independent of cell functionality and becoming a popular method due to its high-throughput nature (Rubinstein et al., 1990; Van Tonder, Joubert, & Cromarty, 2015). The National Cancer Institute utilizes SRB assay in its anticancer lead compound screening studies (Vichai & Kirtikara, 2006). Consequently, both MTT and SRB assays were performed to assign the lead compound and its effective doses for further studies.

The most proper incubation period was determined for both MTT and SRB assay as detailed in section 4.10. For this time optimization analysis, HT-29 colon cancer cells were treated with one of the most powerful anticancer drugs called doxorubicin. Due to the high response of breast and esophageal tumors, it has been particularly utilized in the clinical treatment of these carcinomas (Singal & Iliskovic, 1998). According to the results (Figure 20), both MTT and SRB demonstrated reproducible results for each given period. However, 48 h incubation was selected since the dose-dependent and effective pattern at that interval. The cells then were subjected to 100 µM of NDs and remaining cell viabilities were determined by MTT assay at the end of the 48 h incubation. As given in Figure 21, only one compound (**N17**) led to higher than 50% cell death in each cell line and it was selected as a lead compound for further analyses. In SRB assay, IC_{50} values of **N17** were calculated as 9.24 and 11.35 µM in the colon and breast cancer cells, respectively. Interestingly, in contrast to MTT results, IC_{50} values of **N17** were found higher than 100 µM in both prostate and healthy endothelial cells. Accordingly, compound **N17** was further investigated by other techniques to clarify the mechanism of actions.

To examine the anti-migration properties of **N17**, scratch wound healing assay was performed in MCF-7 breast cancer cells. The assay is based on monitoring the change in an area of the scratch which is proportional to the invasiveness of cancer cells by times. Attached cells were introduced to an artificial wound by sterile tips and treated with various doses of **N17** for 48 h. The state of scratches was recorded under a microscope every 24 h and the changes in the wound area were given as a percentage. According to the results (Figure 22), **N17** decelerated the healing process as compared to the control group which means **N17** exhibited the anti-migration properties in breast cancer cells.

Apoptosis is a well-organized death process in cells to kill themselves by activating metabolic and physiological responses (Kerr, Wyllie, & Currie, 1972). Generally, the molecules such as calcium, ceramide, and proteins including the Bcl-2 family, p53, caspases, and cytochrome-c play a role in the regulation of apoptosis. In cellular apoptosis, two major pathways have been well characterized named as extrinsic and intrinsic (Kroemer, Galluzzi, & Brenner, 2007; Smaili, Hsu, Youle, & Russell, 2000). Proper ligands should bind to cell membrane receptors such as TNF, TRAIL R2/DR5, DR3, and Fas to initiate the extrinsic pathway. The binding of ligands to the receptors leads to structural change which results in receptor oligomerization. This trimeric structure is then recruited adaptor proteins and pro-caspases to form a death-inducing signaling complex and it further transduces the apoptotic downstream cascades by the activation of caspases

(Curtin & Cotter, 2003; Spierings et al., 2004). Furthermore, such cellular signals including mainly DNA damage attenuated contact with the extracellular matrix, and lack of nutrient and growth factor in the cellular environment triggers the intrinsic pathway. When the pathway is initiated, Bid protein inactivates the anti-apoptotic Bcl-2 which is essential proteins for the integrity of the mitochondrial membrane (Spierings et al., 2004). As well, Bid stimulates the activation of Bax and Bak proteins leading to a significant change in mitochondrial membrane potential. Thus, facilitated the release of cytochrome c, second mitochondria-derived activator of caspase (Smac), endonuclease G (Endo-G), Ca^{++}, apoptosis-inducing factor (AIF) accelerates the apoptotic response. Briefly, AIF translocates into the nucleus to fragment the DNA and condense the chromatin. Similarly, when Endo-G is an apoptotic DNase and it causes DNA degradation. Smac inhibits the inhibitor of apoptosis protein (IAP) and gives full rein to caspases. Also, released Smac oligomerizes with apoptotic protease activating factor 1 and it can further recruit deoxyadenosine triphosphate to form a complex called apoptosome (Spierings et al., 2004). Then, the apoptosome provides the cleavage of procaspase-9 into the active state. When the initiator caspase-activated, the caspase-3 is also cleaved and the activation of effector caspase provokes the downstream endonucleases and other caspases to rekindle the apoptosis (Palmer, Greengrass, & Cavalla, 2000; Spierings et al., 2004).

Accordingly, the effects of the **N17** on the expression levels of the key proteins Bcl-2, Bax, and caspase-3 were investigated by western blot analyses, separately. According to the results, **N17** upregulated the apoptotic Bax protein expression level in both colon and breast cancer cells. On the other hand, the level of cleaved caspase-3 was induced in colon cancer cells by **N17** treatment. Also, **N17** treated colon cancer cells exhibited apoptotic structures including an increasing number of blebs and apoptotic bodies as compared to the control cells. These findings suggest that **N17** leads to cell death by triggering apoptosis (Figure 25).

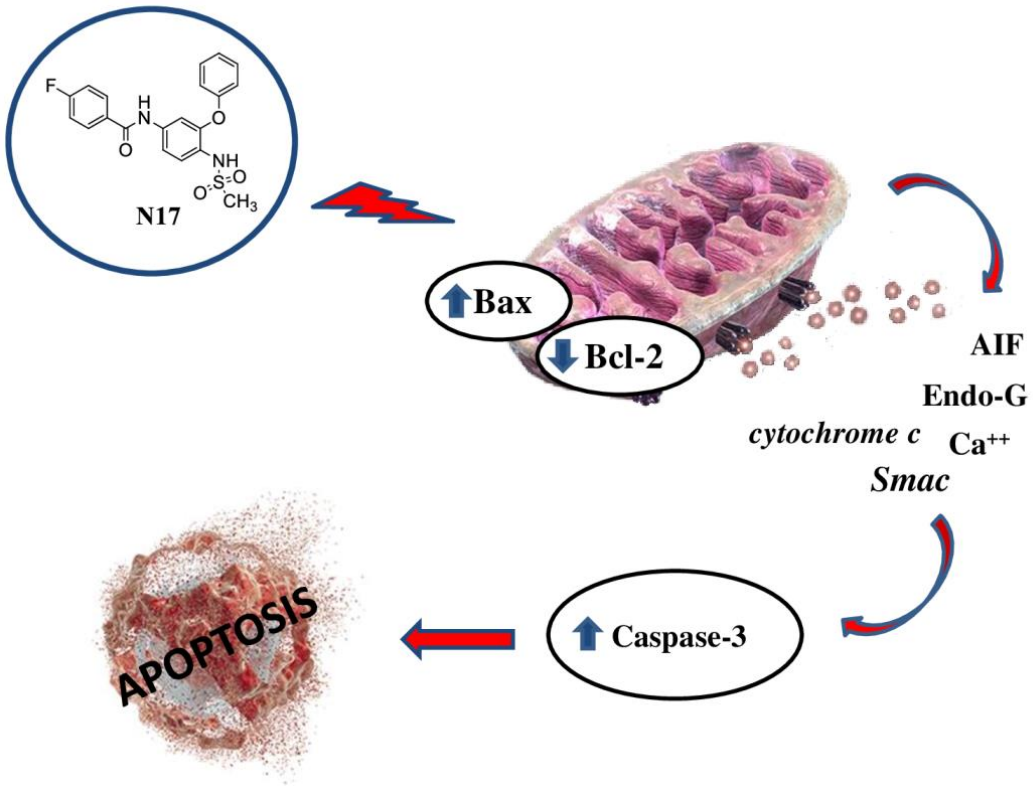

Figure 29. The effects of the compound **N17** on expression levels of apoptotic proteins.

CHAPTER 5
CONCLUSION

Nimesulide is a well-known NSAID with its preferential COX-2 inhibitory properties. Since 1985, it has been commercialized as an anti-inflammatory agent. Due to increased concerns about the use of nimesulide as therapeutic, numerous works have been proceeded to develop potent nimesulide-derived compounds with anti-inflammatory and anticancer properties. In this study, the anti-inflammatory properties of novel amide/sulfonamide containing NDs **(N1-N17)** were evaluated. According to the screening of COX-1/2 inhibitory properties of NDs, the compounds **N8** and **N10** demonstrated better selectivity on the COX-2 enzyme as compared to the parent compound nimesulide. Besides, both compounds downregulated the LPS-stimulated COX-2 expression at the mRNA level, however, the expression of COX-1 remained stable as compared to the control group. These compounds decreased the LPS-induced PGE_2 release in activated macrophages. While both **N8** and **N10** significantly suppressed the NO production, only **N10** alleviated the iNOS gene expression at the mRNA level. It could be suggested that **N8** might interfere with NO production by a post-transcriptional modification. Furthermore, the mRNA expression levels of IL-1β and TNF-α were diminished by **N8** and **N10** in the macrophage cell line. To elucidate the anticancer activities of NDs, a panel of cancer cell lines including colon, prostate, and breast was subjected to compounds and further investigated with cytotoxicity tests. Accordingly, compound **N17** exhibited significant cytotoxicity in the colon (IC_{50}: 9.24 µM) and breast (IC_{50}: 11.35 µM) cancer cell lines. Collectively, all these data suggest that compounds **N8** and **N10** could be promising anti-inflammatory agents while cytotoxic **N17** might be studied as a lead for subsequent potential anticancer drug development.

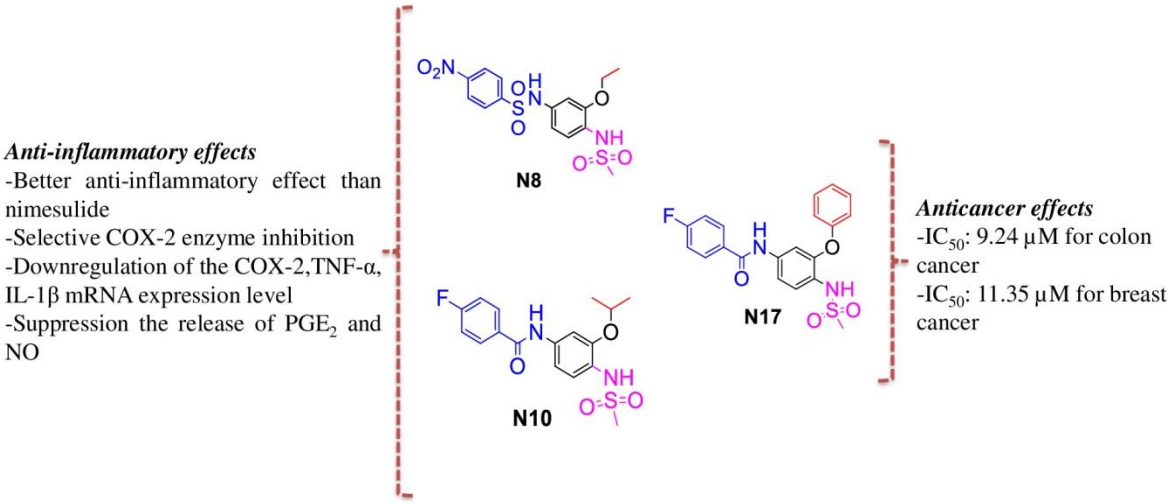

Figure 30. The biological activities of compounds **N8**, **N10,** and **N17**.

REFERENCES

Berenbaum, F., Jacques, C., Thomas, G., Corvol, M. T., Bériziat, G., & Masliah, J. (1996). Synergistic Effect of Interleukin-1β and Tumor Necrosis Factor α on PGE2Production by Articular Chondrocytes Does Not Involve PLA2Stimulation. *Experimental Cell Research, 222*(2), 379-384. doi: https://doi.org/10.1006/excr.1996.0047

Bertholet, S., Tzeng, E., Felley-Bosco, E., & Mauel, J. (1999). Expression of the inducible NO synthase in human monocytic U937 cells allows high output nitric oxide production. *Journal of leukocyte biology, 65*(1), 50-58.

Bertolini, A., Ottani, A., & Sandrini, M. (2001). Dual acting anti-inflammatory drugs: a reappraisal. *Pharmacological Research, 44*(6), 437-450.

Bjarnason, I., Scarpignato, C., Holmgren, E., Olszewski, M., Rainsford, K. D., & Lanas, A. (2018). Mechanisms of damage to the gastrointestinal tract from nonsteroidal anti-inflammatory drugs. *Gastroenterology, 154*(3), 500-514.

Bonaiuto, C., McDonald, P. P., Rossi, F., & Cassatella, M. A. (1997). Activation of nuclear factor-κB by β-amyloid peptides and interferon-γ in murine microglia. *Journal of neuroimmunology, 77*(1), 51-56.

Botha, J. H., Robinson, K. M., Ramchurren, N., Reddi, K., & Norman, R. J. (1986). Human Esophageal Carcinoma Cell Lines: Prostaglandin Production, Biological Properties, and Behavior in Nude Mice23. *JNCI: Journal of the National Cancer Institute, 76*(6), 1053-1056. doi: 10.1093/jnci/76.6.1053

Brash, A. R. (1999). Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. *Journal of Biological Chemistry, 274*(34), 23679-23682.

Brinker, A., Goldkind, L., Bonnel, R., & Beitz, J. (2004). Spontaneous reports of hypertension leading to hospitalisation in association with rofecoxib, celecoxib, nabumetone and oxaprozin. *Drugs & aging, 21*(7), 479-484.

Bryan, C. P., & Smith, G. E. (1974). *Ancient Egyptian medicine: the papyrus ebers* (Vol. 6): Ares Chicago.

Caiazzo, E., Ialenti, A., & Cicala, C. (2019). The relatively selective cyclooxygenase-2 inhibitor nimesulide: What's going on? *European Journal of Pharmacology, 848*, 105-111.

Catarro, M., Serrano, J. L., Ramos, S. S., Silvestre, S., & Almeida, P. (2019). Nimesulide analogues: From anti-inflammatory to antitumor agents. *Bioorganic Chemistry, 88*,

102966. doi: https://doi.org/10.1016/j.bioorg.2019.102966

Chandrasekharan, N., & Simmons, D. L. (2004). The cyclooxygenases. *Genome biology, 5*(9), 241.

Chen, F., Kuhn, D. C., Gaydos, L. J., & DEMERS, L. M. (1996). Induction of nitric oxide and nitric oxide synthase mRNA by silica and lipopolysaccharide in PMA-primed THP-1 cells. *Apmis, 104*(1-6), 176-182.

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., . . . Zhao, L. (2018). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget, 9*(6), 7204.

Connor, J. R., Manning, P. T., Settle, S. L., Moore, W. M., Jerome, G. M., Webber, R. K., . . . Currie, M. G. (1995). Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. *European Journal of Pharmacology, 273*(1), 15-24. doi: https://doi.org/10.1016/0014-2999(94)00672-T

Conti, P., Ronconi, G., Caraffa, A., Gallenga, C., Ross, R., Frydas, I., & Kritas, S. (2020). Induction of pro-inflammatory cytokines (IL-1 and IL-6) and lung inflammation by Coronavirus-19 (COVI-19 or SARS-CoV-2): anti-inflammatory strategies. *Journal of biological regulators and homeostatic agents, 34*(2). doi: 10.23812/conti-e

Corbett, J. A., Tilton, R. G., Chang, K., Hasan, K. S., Ido, Y., Wang, J. L., . . . McDaniel, M. L. (1992). Aminoguanidine, a Novel Inhibitor of Nitric Oxide Formation, Prevents Diabetic Vascular Dysfunction. *Diabetes, 41*(4), 552-556. doi: 10.2337/diab.41.4.552

Curry, S. L., Cogar, S. M., & Cook, J. L. (2005). Nonsteroidal antiinflammatory drugs: a review. *Journal of the American Animal Hospital Association, 41*(5), 298-309.

Curtin, J. F., & Cotter, T. G. (2003). Live and let die: regulatory mechanisms in Fas-mediated apoptosis. *Cellular signalling, 15*(11), 983-992.

Debrumfernandes, A. J., Laporte, S., Heroux, M., Lora, M., Patry, C., Menard, H. A., . . . Leduc, R. (1994). Expression of Prostaglandin-Endoperoxide Synthase 1 and Prostaglandin-Endoperoxide Synthase 2 in Human Osteoblasts. *Biochemical and Biophysical Research Communications, 198*(3), 955-960. doi: https://doi.org/10.1006/bbrc.1994.1136

Ediriweera, M. K., Tennekoon, K. H., & Samarakoon, S. R. (2019). In vitro assays and techniques utilized in anticancer drug discovery. *Journal of Applied Toxicology, 39*(1), 38-71.

Emery, P. (2001). Cyclooxygenase-2: a major therapeutic advance? *The American Journal of Medicine, 110*(1, Supplement 1), S42-S45. doi: https://doi.org/10.1016/S0002-

9343(00)00649-5

Ghosh, N., Chaki, R., Mandal, V., & Mandal, S. C. (2010). COX-2 as a target for cancer chemotherapy. *Pharmacological reports, 62*(2), 233-244.

Gierse, J. K., McDonald, J. J., Hauser, S. D., Rangwala, S. H., Koboldt, C. M., & Seibert, K. (1996). A single amino acid difference between cyclooxygenase-1 (COX-1) and− 2 (COX-2) reverses the selectivity of COX-2 specific inhibitors. *Journal of Biological Chemistry, 271*(26), 15810-15814.

Gómez-Gaviro, M. a. V., González-Alvaro, I., Domínguez-Jiménez, C., Peschon, J., Black, R. A., Sánchez-Madrid, F., & Díaz-González, F. (2002). Structure-function relationship and role of tumor necrosis factor-α-converting enzyme in the down-regulation of L-selectin by non-steroidal anti-inflammatory drugs. *Journal of Biological Chemistry, 277*(41), 38212-38221.

Gross, T. J., Kremens, K., Powers, L. S., Brink, B., Knutson, T., Domann, F. E., . . . Monick, M. M. (2014). Epigenetic silencing of the human NOS2 gene: rethinking the role of nitric oxide in human macrophage inflammatory responses. *The Journal of Immunology, 192*(5), 2326-2338.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *cell, 144*(5), 646-674.

Hartlage-Rübsamen, M., Lemke, R., & Schliebs, R. (1999). Interleukin-1β, inducible nitric oxide synthase, and nuclear factor-κB are induced in morphologically distinct microglia after rat hippocampal lipopolysaccharide/interferon-γ injection. *Journal of neuroscience research, 57*(3), 388-398.

Hemler, M., & Lands, W. (1976). Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *Journal of Biological Chemistry, 251*(18), 5575-5579.

Hernandez, M. R., Tonda, R., Pino, M., Serradell, M., Arderiu, G., & Escolar, G. (2004). Evaluation of effects of rofecoxib on platelet function in an in vitro model of thrombosis with circulating human blood. *European journal of clinical investigation, 34*(4), 297-302.

Hida, T., Kozaki, K.-i., Muramatsu, H., Masuda, A., Shimizu, S., Mitsudomi, T., . . . Takahashi, T. (2000). Cyclooxygenase-2 inhibitor induces apoptosis and enhances cytotoxicity of various anticancer agents in non-small cell lung cancer cell lines. *Clinical Cancer Research, 6*(5), 2006-2011.

Hudson, N., Balsitis, M., Everitt, S., & Hawkey, C. (1993). Enhanced gastric mucosal leukotriene B4 synthesis in patients taking non-steroidal anti-inflammatory drugs. *Gut, 34*(6), 742-747.

Jeffreys, D. (2008). *Aspirin: the remarkable story of a wonder drug*: Bloomsbury Publishing USA.

Kalgutkar, A. S., Marnett, A. B., Crews, B. C., Remmel, R. P., & Marnett, L. J. (2000). Ester and amide derivatives of the nonsteroidal antiinflammatory drug, indomethacin, as selective cyclooxygenase-2 inhibitors. *Journal of medicinal chemistry, 43*(15), 2860-2870.

Kapche, D. W. F. G., Lekane, N. M., Kulabas, S. S., Ipek, H., Tok, T. T., Ngadjui, B. T., . . . Tumer, T. B. (2017). Aryl benzofuran derivatives from the stem bark of Calpocalyx dinklagei attenuate inflammation. *Phytochemistry, 141*, 70-79. doi: https://doi.org/10.1016/j.phytochem.2017.05.007

Kaushal, N., & Sanyal, S. N. (2007). Modulations in the intestinal disaccharide hydrolases and membrane dynamics: Effect of non-steroidal anti-inflammatory drugs aspirin and nimesulide. *Molecular and Cellular Biochemistry, 294*(1), 107-115. doi: 10.1007/s11010-006-9250-3

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. *British journal of cancer, 26*(4), 239-257.

Khan, M. N. A., & Lee, Y. S. (2011). Cyclooxygenase inhibitors: Scope of their use and development in cancer chemotherapy. *Medicinal Research Reviews, 31*(2), 161-201. doi: 10.1002/med.20182

Kroemer, G., Galluzzi, L., & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiological reviews, 87*(1), 99-163.

Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R. W., & Herschman, H. R. (1991). TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J Biol chem, 266*(20), 12866-12872.

Kulabas, S. S., Onder, F. C., Yılmaz, Y. B., Ozleyen, A., Durdagi, S., Sahin, K., . . . Tumer, T. B. (2019). In vitro and in silico studies of nitrobenzamide derivatives as potential anti-neuroinflammatory agents. *Journal of Biomolecular Structure and Dynamics*, 1-14.

Kulkarni, S., & Pal Singh, V. (2007). Licofelone-a novel analgesic and anti-inflammatory

agent. *Current Topics in Medicinal Chemistry, 7*(3), 251-263.

Lanas, A., García-Rodríguez, L.-A., Arroyo, M.-T., Gomollón, F., Feu, F., González-Pérez, A., . . . Santolaria, S. (2006). Risk of upper gastrointestinal ulcer bleeding associated with selective cyclo-oxygenase-2 inhibitors, traditional non-aspirin non-steroidal anti-inflammatory drugs, aspirin and combinations. *Gut, 55*(12), 1731-1738.

Lee, K. S., Kim, S. R., Park, H. S., Park, S. J., Min, K. H., Lee, K. Y., . . . Lee, Y. C. (2007). Cysteinyl leukotriene upregulates IL-11 expression in allergic airway disease of mice. *Journal of allergy and clinical immunology, 119*(1), 141-149.

Li, Y., Chen, S.-H., Ou, T.-M., Tan, J.-H., Li, D., Gu, L.-Q., & Huang, Z.-S. (2011). Syntheses and characterization of nimesulide derivatives for dual enzyme inhibitors of both cyclooxygenase-1/2 and 5-lipoxygenase. *Bioorganic & medicinal chemistry, 19*(6), 2074-2083.

Liu, Y., Zhang, T., Li, G., Li, S., Li, J., Zhao, Q., . . . Zhang, L. (2020). Radiosensitivity enhancement by Co-NMS-mediated mitochondrial impairment in glioblastoma. *Journal of Cellular Physiology*.

Mareddy, J., Nallapati, S. B., Anireddy, J., Devi, Y. P., Mangamoori, L. N., Kapavarapu, R., & Pal, S. (2013). Synthesis and biological evaluation of nimesulide based new class of triazole derivatives as potential PDE4B inhibitors against cancer cells. *Bioorganic & medicinal chemistry letters, 23*(24), 6721-6727.

Mareddy, J., Suresh, N., Kumar, C. G., Kapavarapu, R., Jayasree, A., & Pal, S. (2017). 1, 2, 3-Triazole-nimesulide hybrid: Their design, synthesis and evaluation as potential anticancer agents. *Bioorganic & Medicinal Chemistry Letters, 27*(3), 518-523.

Martel-Pelletier, J., Lajeunesse, D., Reboul, P., & Pelletier, J.-P. (2003). Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. *Annals of the rheumatic diseases, 62*(6), 501-509.

Meanwell, N. A. (2011). Synopsis of Some Recent Tactical Application of Bioisosteres in Drug Design. *Journal of Medicinal Chemistry, 54*(8), 2529-2591. doi: 10.1021/jm1013693

Mollace, V., Muscoli, C., Masini, E., Cuzzocrea, S., & Salvemini, D. (2005). Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. *Pharmacological reviews, 57*(2), 217-252.

Montinari, M. R., Minelli, S., & De Caterina, R. (2019). The first 3500 years of aspirin history from its roots–A concise summary. *Vascular pharmacology, 113*, 1-8.

Morgan, G. (1997). Deleterious effects of prostaglandin E2 in oesophageal carcinogenesis. *Medical Hypotheses, 48*(2), 177-181. doi: https://doi.org/10.1016/S0306-9877(97)90285-1

Natarajan, R., & Nadler, J. L. (2004). Lipid Inflammatory Mediators in Diabetic Vascular Disease. *Arteriosclerosis, Thrombosis, and Vascular Biology, 24*(9), 1542-1548. doi: doi:10.1161/01.ATV.0000133606.69732.4c

Nickerson-Nutter, C. L., & Medvedeff, E. D. (1996). The effect of leukotriene synthesis inhibitors in models of acute and chronic inflammation. *Arthritis & Rheumatism: Official Journal of the American College of Rheumatology, 39*(3), 515-521.

Okamoto, F., Saeki, K., Sumimoto, H., Yamasaki, S., & Yokomizo, T. (2010). Leukotriene B4 augments and restores FcγRs-dependent phagocytosis in macrophages. *Journal of Biological Chemistry, 285*(52), 41113-41121.

Otterness, I. G. (1995). The discovery of drugs to treat arthritis: a historical view *The Search for Anti-Inflammatory Drugs* (pp. 1-26): Springer.

Pal, S., Durgadas, S., Nallapati, S. B., Mukkanti, K., Kapavarapu, R., Meda, C. L. T., . . . Pal, M. (2011). Novel 1-alkynyl substituted 1, 2-dihydroquinoline derivatives from nimesulide (and their 2-oxo analogues): A new strategy to identify inhibitors of PDE4B. *Bioorganic & medicinal chemistry letters, 21*(21), 6573-6576.

Palmer, A. M., Greengrass, P. M., & Cavalla, D. (2000). The role of mitochondria in apoptosis. *Drug news & perspectives, 13*(6), 378-384.

Park, J. Y., Pillinger, M. H., & Abramson, S. B. (2006). Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. *Clinical immunology, 119*(3), 229-240.

Piper, P. J., & Vane, J. R. (1969). Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature, 223*(5201), 29-35.

Piria, R. (1838). Sur la composition de la salicine et quelques-unes de ses réactions. *CR Acad Sci, 6*, 620-624.

Rainsford, K. (1987). The effects of 5-lipoxygenase inhibitors and leukotriene antagonists on the development of gastric lesions induced by nonsteroidal antiinflammatory drugs in mice. *Agents and Actions, 21*(3-4), 316-319.

Rainsford, K. (1993). Leukotrienes in the pathogenesis of NSAID-induced gastric and intestinal mucosal damage. *Agents and Actions, 39*(1), C24-C26.

Rainsford, K. (2004). History and Development of the. *Aspirin and related drugs*, 11.

Rainsford, K. (2007). Anti-inflammatory drugs in the 21st century *Inflammation in the pathogenesis of chronic diseases* (pp. 3-27): Springer.

Rainsford, K., & Nimesulide, C. R. G. o. (2006). Nimesulide–a multifactorial approach to inflammation and pain: scientific and clinical consensus. *Current medical research and opinion, 22*(6), 1161-1170.

Rainsford, K. D. (2005). The discovery, development and novel actions of nimesulide. In K. D. Rainsford (Ed.), *Nimesulide — Actions and Uses* (pp. 1-49). Basel: Birkhäuser Basel.

Rainsford, K. D. (2006). Nimesulide – a multifactorial approach to inflammation and pain: scientific and clinical consensus. *Current Medical Research and Opinion, 22*(6), 1161-1170. doi: 10.1185/030079906X104849

Rao, P., & Knaus, E. E. (2008). Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. *Journal of Pharmacy & Pharmaceutical Sciences, 11*(2), 81s-110s.

Rayburn, E. R., Ezell, S. J., & Zhang, R. (2009). Anti-inflammatory agents for cancer therapy. *Molecular and cellular pharmacology, 1*(1), 29.

Renard, J.-F., Julémont, F., Leval, X. d., & Pirotte, B. (2006). The use of nimesulide and its analogues in cancer chemoprevention. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 6*(3), 233-237.

Rubinstein, L., Shoemaker, R., Paull, K., Simon, R., Tosini, S., Skehan, P., . . . Boyd, M. (1990). Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *JNCI: Journal of the National Cancer Institute, 82*(13), 1113-1117.

Saad, B., AbouAtta, B. S., Basha, W., Hmade, A., Kmail, A., Khasib, S., & Said, O. (2011). Hypericum triquetrifolium—derived factors downregulate the production levels of LPS-induced nitric oxide and tumor necrosis factor-α in THP-1 cells. *Evidence-Based Complementary and Alternative Medicine, 2011*.

Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., & Needleman, P. (1993). Nitric oxide activates cyclooxygenase enzymes. *Proceedings of the National Academy of Sciences, 90*(15), 7240-7244. doi: 10.1073/pnas.90.15.7240

Salvemini, D., Settle, S. L., Masferrer, J. L., Seibert, K., Currie, M. G., & Needleman, P. (1995). Regulation of prostaglandin production by nitric oxide; an in vivo analysis. *British Journal of Pharmacology, 114*(6), 1171-1178. doi: 10.1111/j.1476-5381.1995.tb13330.x

Singal, P., & Iliskovic, N. (1998). Adriamycin cardiomyopathy. *N Engl J Med, 339*, 900-

905.

Singh, P., & Pooja. (2013). N-1, C-3 substituted indoles as 5-LOX inhibitors—In vitro enzyme immunoaasay, mass spectral and molecular docking investigations. *Bioorganic & Medicinal Chemistry Letters, 23*(5), 1433-1437. doi: https://doi.org/10.1016/j.bmcl.2012.12.068

Smaili, S. S., Hsu, Y.-T., Youle, R. J., & Russell, J. T. (2000). Mitochondria in Ca2+ signaling and apoptosis. *Journal of bioenergetics and biomembranes, 32*(1), 35-46.

Smith, W. L., & Langenbach, R. (2001). Why there are two cyclooxygenase isozymes. *The Journal of clinical investigation, 107*(12), 1491-1495.

Sneader, W. (2005). *Drug discovery: a history*: John Wiley & Sons.

Spierings, D. C., de Vries, E. G., Vellenga, E., van den Heuvel, F. A., Koornstra, J. J., Wesseling, J., . . . de Jong, S. (2004). Tissue distribution of the death ligand TRAIL and its receptors. *Journal of Histochemistry & Cytochemistry, 52*(6), 821-831.

Stone, E. (1764). An Account of the Success of the Bark of the Willow in the Cure of Agues. In a Letter to the Right Honourable George Earl of Macclesfield, President of RS from the Rev. Mr. Edmund Stone, of Chipping-Norton in Oxfordshire. *New universal magazine: or, Miscellany of historical, philosophical, political and polite literature, 35*(242), 122-123.

Su, B., Cai, X., Hong, Y., & Chen, S. (2010). COX-2 inhibitor nimesulide analogs are aromatase suppressors in breast cancer cells. *The Journal of steroid biochemistry and molecular biology, 122*(4), 232-238.

Suleyman, H., Altinkaynak, K., Gocer, F., Maras, A., Akçay, F., Onuk, M. D., & Gepdiremen, A. (2002). Effect of nimesulide on the indomethacin-and ibuprofen-induced ulcer in rat gastric tissue. *Polish journal of pharmacology, 54*(3), 255-260.

Suleyman, H., Cadirci, E., Albayrak, A., & Halici, Z. (2008). Nimesulide is a Selective COX-2 Inhibitory, Atypical Non-Steroidal Anti-Inflammatory Drug. *Current Medicinal Chemistry, 15*(3), 278-283. doi: 10.2174/092986708783497247

Süleyman, H., Akçay, F., & Altinkaynak, K. (2002). The effect of nimesulide on the indomethacin-and ethanol-induced gastric ulcer in rats. *Pharmacological research, 45*(2), 155-158.

Swingle, K., Bell, R., & Moore, G. (1985). Anti-inflammatory activity of antioxidants. *Anti-inflammatory and anti-rheumatic drugs, 3*, 105-126.

Szczepanski, A., Moatter, T., Carley, W. W., & Gerritsen, M. E. (1994). Induction of cyclooxygenase ii in human synovial microvessel endothelial cells by interleukin-1.

Arthritis & Rheumatism, 37(4), 495-503. doi: 10.1002/art.1780370409

Tool, A., & Verhoeven, A. (1995). Inhibition of the production of platelet activating factor and of leukotriene B4 in activated neutrophils by nimesulide due to an elevation of intracellular cyclic adenosine monophosphate. *Arzneimittel-Forschung, 45*(10), 1110-1114.

Tumer, T. B., Yılmaz, B., Ozleyen, A., Kurt, B., Tok, T. T., Taskin, K. M., & Kulabas, S. S. (2018). GR24, a synthetic analog of Strigolactones, alleviates inflammation and promotes Nrf2 cytoprotective response: In vitro and in silico evidences. *Computational biology and Chemistry, 76*, 179-190.

van den Bogaart, E., Mens, P. F., Adams, E. R., Grobusch, M. P., & Schallig, H. D. (2017). Phagocytosis of hemozoin by RAW 264.7 cells, but not THP-1 cells, promotes infection by Leishmania donovani with a nitric oxide-independent mechanism. *Parasitology international, 66*(2), 196-206.

Van Ryn, J., & Pairet, M. (1999). Clinical experience with cyclooxygenase-2 inhibitors. *Inflammation Research, 48*(5), 247-254.

Van Tonder, A., Joubert, A. M., & Cromarty, A. D. (2015). Limitations of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC research notes, 8*(1), 47.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature new biology, 231*(25), 232-235.

Vane, J. R., & Botting, R. M. (1992). *Aspirin and other salicylates*: Chapman & Hall Medical.

Vane, J. R., & Botting, R. M. (1998). Mechanism of action of nonsteroidal anti-inflammatory drugs. *The American journal of medicine, 104*(3S1), 2S-8S.

Vichai, V., & Kirtikara, K. (2006). Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature protocols, 1*(3), 1112-1116.

Warner, T. (2001). Introduction to" Nimesulide: Beyond COX-2. *Clin. Exp. rheumatol, 19*(1), S1-S2.

Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J. A., & Vane, J. R. (1999). Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: A full in vitro analysis. *Proceedings of the National Academy of Sciences of the United States of America, 96*(13), 7563-7568. doi: 10.1073/pnas.96.13.7563

Werner, M. F., Souza, G. E., & Zampronio, A. R. (2006). Nimesulide-induced antipyresis in rats involves both cyclooxygenase-dependent and independent mechanisms. *European journal of pharmacology, 543*(1-3), 181-189.

Wright, J. M. (2002). The double-edged sword of COX-2 selective NSAIDs. *Cmaj, 167*(10), 1131-1137.

Yamamoto, Y., Arai, J., Hisa, T., Saito, Y., Mukai, T., Ohshima, T., . . . Yamamoto, F. (2016). Isomeric iodinated analogs of nimesulide: Synthesis, physicochemical characterization, cyclooxygenase-2 inhibitory activity, and transport across Caco-2 cells. *Bioorganic & medicinal chemistry, 24*(16), 3727-3733.

Yamamoto, Y., Hisa, T., Arai, J., Saito, Y., Yamamoto, F., Mukai, T., . . . Ohkubo, Y. (2015). Isomeric methoxy analogs of nimesulide for development of brain cyclooxygense-2 (COX-2)-targeted imaging agents: Synthesis, in vitro COX-2-inhibitory potency, and cellular transport properties. *Bioorganic & medicinal chemistry, 23*(21), 6807-6814.

Yokoyama, C., Takai, T., & Tanabe, T. (1988). Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. *FEBS letters, 231*(2), 347-351.

Zhao, C., Rakesh, K., Ravidar, L., Fang, W.-Y., & Qin, H.-L. (2019). Pharmaceutical and medicinal significance of sulfur (SVI)-Containing motifs for drug discovery: A critical review. *European journal of medicinal chemistry, 162*, 679-734.

Zhao, Y., Wang, W., Wang, Q., Zhang, X., & Ye, L. (2012). Lipid metabolism enzyme 5-LOX and its metabolite LTB4 are capable of activating transcription factor NF-κB in hepatoma cells. *Biochemical and biophysical research communications, 418*(4), 647-651.

Zhong, B., Cai, X., Yi, X., Zhou, A., Chen, S., & Su, B. (2011). In vitro and in vivo effects of a cyclooxygenase-2 inhibitor nimesulide analog JCC76 in aromatase inhibitors-insensitive breast cancer cells. *The Journal of steroid biochemistry and molecular biology, 126*(1-2), 10-18.

Zündorf, U. (1997). *Aspirin, 100 Years: The Future Has Just Begun*: Bayer AG, Consumer Care Business Group.

BIOGRAPHY

PERSONAL DETAILS

Name Surname: Adem ÖZLEYEN

Place of Birth: Manisa/Yunus Emre

Date of Birth: 07.02.1995

EDUCATION STATUS

Bachelor's Degree: Çanakkale Onsekiz Mart University Faculty of Arts and Sciences, Department of Molecular Biology and Genetics

Erasmus Programme: University of Ostrava, Department of Biology and Ecology

Master's Degree: Çanakkale Onsekiz Mart University School of Graduate Studies, Graduate Program of Biomolecular Sciences

Foreign Languages: English

ORCID iD: 0000-0003-0195-3383

SCIENTIFIC ACTIVITY

a. Publishing -SCI –Other

1. Kurt B, **Ozleyen A**, Antika G, Yılmaz YB, Tumer TB. **(2020).** ***ACS Chemical Neuroscience***. 11(4), 501-507. DOI: 10.1021/acschemneuro.9b00694
2. B. Salehi, **A. Ozleyen** , T. Tumer, ….., J.S. Rad. **(2019).** ***Biomolecules***. 9(11), 679. DOI: 10.3390/biom9110679
3. SS. Kulabas, FC. Onder, B. Yilmaz, **A. Ozleyen**, S. Durdagi, K. Sahin , M. Ay, TB Tumer. **(2019).** ***Journal of Biomolecular Structure & Dynamics*****.** 1-14. DOI: 10.1080/07391102.2019.1684368
4. Salehi, B., Sharopov, F., Boyunegmez Tumer, T., **Ozleyen, A.**, Rodríguez-Pérez, C., Ezzat, S. M., ...& Bostan, C. (2019). Molecules. 24(12), 2272. DOI: 10.3390/molecules24122272

5. Salehi, B., Tumer, T. B., **Ozleyen, A.**, Peron, G., Dall'Acqua, S., Rajkovic, J., ...& Milella, L. **(2019)**. ***Trends in Food Science & Technology***. 88, 260-273. DOI: 10.1016/j.tifs.2019.03.028
6. Salehi, B., Zakaria, Z. A., Gyawali, R., Ibrahim, S. A., Rajkovic, J., Shinwari, Z. K., Khan T., Sharifi-Rad ., **Ozleyen A.**, …, & Valussi, M. **(2019)**. ***Molecules***. 24(7), 1364. DOI: 10.3390/molecules24071364
7. Tumer, T. B., Yılmaz, B., **Ozleyen, A.**, Kurt, B., Tok, T. T., Taskin, K. M., & Kulabas, S. S. **(2018)**. ***Computational Biology and Chemistry***. 76, 179-190. DOI: 10.1016/j.compbiolchem.2018.07.014
8. Karav S., Talak E., Tuncer M., Ozleyen A. **(2017)**. ***International Journal of Agriculture Innovations and Research.*** 6(3), 2319-1473.

b. Notifications -International -National

1. Sungu MM, **Ozleyen A**, Tumer TB.: Comparison of Tetrazolium (MTT) and Sulforhodamine B (SRB) Protein Assays for Assessing in vitro Cell Viability. **8th International Drug Chemistry Conference**, *27 February-01 March 2020, Antalya, Turkey* (Honorable Mention Award for Poster Presentation) .
2. **Ozleyen, A**., Yılmaz, B. Y., Güngör, T., Tok, T. T., Ay M., Tümer T.: Mechanism of Action of Novel Nimesulide Derivatives as potential anti-inflammatory compounds with secondary anticancer effects. **7th International Bahçeşehir University (BAU) Drug Design Congress,** *19-21 December in Istanbul, Turkey* (The Second Runner Up Award for Poster Presentation)
3. Tumer TB, Kurt B, **Ozleyen A**, Turkdonmez E, Antika G, Yılmaz YB. Phytohormones Strigolactones: Their novel potential therapeutic activities in different chronic inflammation related disease conditions.**1st International Conference on Preventive Medicine**, *12-14 November 2019, Antalya, Turkey.*
4. Güngör, T., **Ozleyen, A**., Yılmaz, B. Y., Tok, T. T., Ay M., Tümer T.: Synthesis, Antiinflammatory and Anticancer Effects of Novel Amide Containing Compounds. **1st International Multidisciplinary Cancer Research Congress,***18-22 2019 September in Diyarbakır, Turkey.*
5. Begum Kurt, **Adem Ozleyen**, Elif Turkdonmez, Gizem Antika, Berkay Yilmaz, Tugba Boyunegmez Tumer. Neuroprotective Potential of Strigolactones on LPS-activated brain endothelial and microglial cells.**FENS Regional Meeting**, *10-13 July 2019,*

Belgrade, Serbia.

6. B. Yilmaz, FC. Onder, **A. Ozleyen** , T. Gungor, SS. Kulabas , S. Durdagi , M. Ay, TB Tumer. Nitrobenzamide Derivatives as iNOS Inhibitors AlsoPromotes Nrf2 Mediated Cytoprotective Response: inVitro and in Silico Approaches. **6thInternational Congress of theMolecular Biology Associationof Turkey,** *5-8 September 2018 in İzmir, Turkey.*
7. S. Karav, A. Bayraktar, G. Gırgın, **A. Ozleyen**, O. Limoncu, TB Tumer; Optimization of Protein Matrices for the efficient sorption of Polyphenols found in Fruit Juices. **International Conference on Agriculture, Forest, Food Sciences and Technologies,** *2-5 April 2018 in Çeşme-İzmir, Turkey.*

c. Projects

Project Title: Elucidation of Anti-inflammatory and Anticancer Properties of Novel Nimesulide Derivatives, ÇOMÜ-BAP Master's Thesis Project (Researcher, FYL-2019-3123), 2019-Ongoing.

Project Title: In Silico Design, Synthesis and Screening of New Generation Anti-inflammatory Molecules, Secondary Anticarcinogenic Properties and Elucidation of Mechanisms. TUBİTAK 1001 Project, (Scholarship Student, Project Number: 117Z398), 2018-Ongoing.

Project Title: Development and characterization of innovative protein matrix technologies in absorption of natural sources of anti-inflammatory bioactive phenolics.COMU BAP, (Research Student, Project Numbers: FBA-2017-1268), 2017-2018.

d. Scholarships

Scholarship Student - TUBİTAK 1001 Project - Project Number: 117Z398
Project Title: In Silico Design, Synthesis and Screening of New Generation Anti-inflammatory Molecules, Secondary Anticarcinogenic Properties and Elucidation of Mechanisms.

e. Certificates

7th International BAU Drug Design Congress, 19-21 December, , Certificate of Participation and Certificate of Reward

6th International Congress of the Molecular Biology Association of Turkey, 5-8 September, Certificate of Participation

CONTACT
E-mail address: ademozleyen@gmail.com or ademozleyen@stu.comu.edu.tr