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Novel oxalamide derivatives for COXs expression and breast cancer: design, synthesis, biological evaluation, and docking studies

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Abstract: In the present study, new oxalamide-based compounds were designed from thalidomide and synthesized easily and with high yields (from 69% up to 93%) by a two-step method. The antiproliferative effects of synthesized **6a-d** and **7a-d** compounds on (ER+) MCF-7 and (ER-) MDA-MB-231 breast cancer cell line and human fibroblast WI-38 healthy cell line were investigated by the MTT method. The results showed that compound **7d** was the most potent candidate against both MCF-7 and MDA-MB-231 cell lines with IC_{50} = 4.72 µM and 6.37 µM, respectively. To investigate whether antiproliferative effect of the compounds on breast cancer cell lines is dependent on COXs, expressions of COX-1/2 on the MCF-7 cell line were investigated by the Western-Blot technique. Among synthesized compounds, compound **7d** increased the expression of both COX-1 and COX-2. The inhibition potential of compounds on COX-1/2 enzymes was investigated by molecular docking compared to inhibitor co-ligand celecoxib in crystal structures of COX-1 (PDB ID: 3KK6) and COX-2 (PDB ID: 3LN1). Docking results indeed showed that compound **7d** had a higher binding affinity for both COX-1 and COX-2 active sites. Consequently, the novel oxalamide-based compounds presented here may be important candidate molecules for the development of new COX-dependent antiproliferative agents.

Keywords: Oxalamides; antiproliferation; western blotting; COXs expression; molecular docking.

1. Introduction

Cancer is the second most deadly disease in the world among deaths with known causes. Among the cancer types, breast cancer can be seen in both men and women, but it is more common in women. Breast cancer, which is caused by the uncontrolled proliferation of breast cells, continues to be the most frequently diagnosed disease type in women after skin cancer.[1]

It is seen that the possibility of improvement in mortality rates increases with the use of chemotherapy agents, which is one of the important ways in the treatment of breast cancer. However,

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due to the serious side effects that occur with the use of chemotherapy agents, it is necessary to develop new agents that can offer inexpensive and simple treatment methods with fewer side effects in the treatment of breast cancer. For this reason, investigating the effects of drugs that are simpler and have fewer side effects on breast cancer can offer a cheaper treatment approach. In recent years, significant progress has been made in investigating the effects of promising, widely available, and inexpensive non-steroidal drugs (NSAIDs) on breast cancer. NSAIDs with analgesic, anti-inflammatory, antithrombotic, and antipyretic effects inhibit the production of prostaglandins that play a role in cellular adhesion, growth, and differentiation by inhibiting cyclooxygenase (COX) enzymes.[2,3]

Among the COX enzymes, three isoforms isolated from various animal cells, especially COX-1 and COX-2 are the most abundant. Although COX-1 is defined as constitutively expressed, COX-2 is mitogen-inducible, recently studies have shown that although both isozymes are frequently involved in the specified pathological and physiological conditions, in some cases they play a separate role.[4,5] Experimental studies have shown that COX-1 is a therapeutic target for cancer, with epidemiological results showing that low doses and regular use of COX-1 inhibitors reduce colon cancer incidence and mortality.[6-8] A similar situation has shown that COX-2 is a promising therapeutic target for cancer by determining the effect of carcinogenesis and angiogenesis on various tumor cells (prostate, colon, and breast cancer) by overexpression.[9] However, overdose using of COX-1 and COX-2 inhibitor drugs cause serious side effects such as cardiovascular system disorders and so, have restricted the use of these drugs or even removed them from the market.[10] Therefore, the design of structurally different new synthetic compounds for COX inhibitors and their research on various types of cancer remains current.

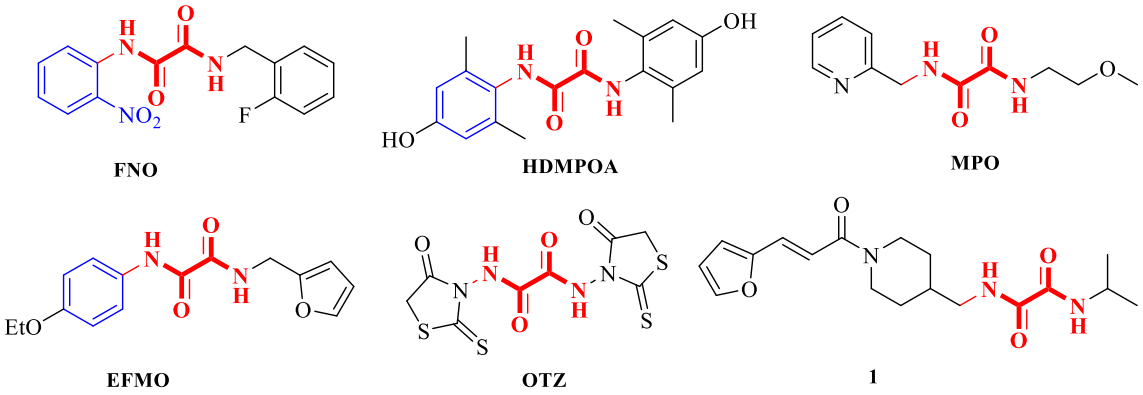


Figure 1. Some commercially available and biologically active oxalamide compounds

More well-known heterocyclic compounds (such as pyrazole, indole, etc.) come to the fore in the development of new COX inhibitors and related anti-inflammatory compounds. Recently, however, more active compounds with versatile pharmacological activities can be achieved by combining heterocyclic compounds with the oxalamide functional group. For example, compounds developed by combining the pyrazole ring with oxalamide have been found to inhibit neuroinflammation, exhibit an anti-neuroblastoma effect, selective COX-2 inhibition, and anti-inflammatory effect.[11,12] From this point of view, many oxalamide derivative compounds have been discovered to have various biochemical and physiological effects and are commercially available. For example, FNO has been shown to inhibit the cell cycle and apoptotic effect in cancer cells, inhibit the production of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and is a selective COX-2 inhibitor.[13] HDMPOA is known to inhibit the production of pro-inflammatory cytokines and chemokines, the formation of reactive oxygen species, as well as inhibition of COX-2 and LOX enzymes.[14] MPO has anti-inflammatory effects by inhibiting the activity of the nuclear factor kappa B (NF-κB) pathway, which is effective in the regulation of immune responses, and in high concentration, it causes apoptosis in tumor cells.[15] Finally, it was discovered that EFMO[16] inhibits the activity of COX-2 and activation of NF-κB, OTZ[17] is a potential drug candidate with its anti-inflammatory and analgesic properties in addition to its anticonvulsant properties, and compound **1** is a COX inhibitor and ion channel modulator (Figure 1).[18]

Many new molecules with various pharmacological activities (such as TNF-a regulator, peptidase inhibitor, cell differentiation inducer, NF-κB inhibitor) have been designed by substituting molecules with the diisopropyl group derived from the thalidomide molecule (Fig. 2). In addition, highly satisfactory COX inhibition results were obtained by replacing EWG and EDG, which were substituted for the aromatic group in the thalidomide molecule, and their positions (compound **2**).[19] Also, recently thalidomide inhibited COX-1 quite strongly compared to reference drug aspirin, and suppressing COX-2 with lipopolysaccharide-induced expression suggests that thalidomide is an excellent COXs inhibitor.[20,21]

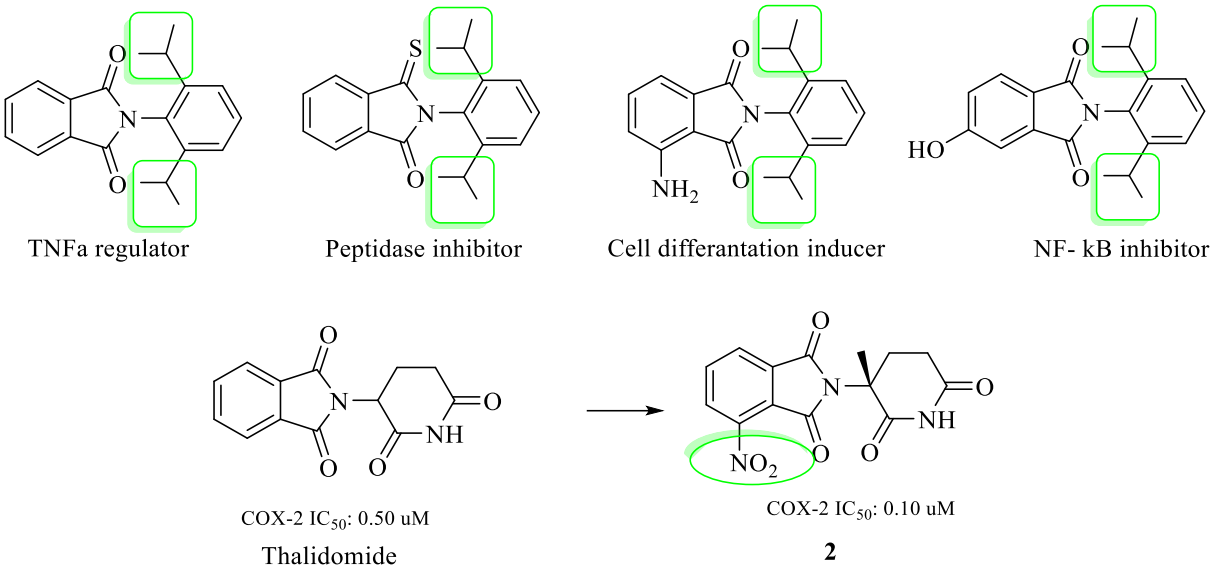


Figure 2. Some biologically active compounds derived from thalidomide

The fact that many derivatives developed with thalidomide and its analogs have many pharmacological activities such as androgen receptor antagonists, peptidase, glucosidase, and phosphorylase inhibitors indicate that thalidomide and its structural functions are multi-targeted drug.[22] In the structure-activity relationship of thalidomide derivatives, it was found that they have common essential pharmacophoric features.[23] These features include: i) hydrophobic domain, ii) phthalimide moiety, iii) glutarimide moiety (Fig. 3A). In this study, the thalidomide-like compounds designed based on the structural pharmacophoric properties of thalidomide; i) various EDG and EWG groups substituted in hydrophobic domains, ii) oxalamide linker mimicking phthalimide, iii) alicyclic and aliphatic amide functional groups added instead of glutarimide moiety (Fig. 3B), that to have better thalidomide-like activities.

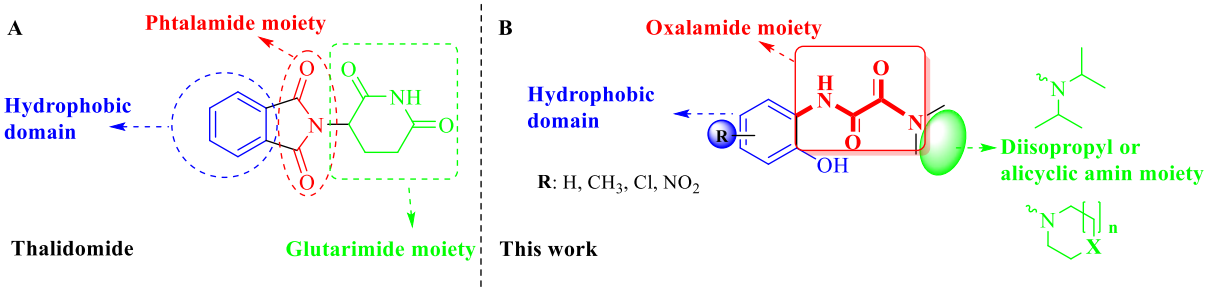


Figure 3. Pharmacophoric regions of thalidomide and our targeting compounds

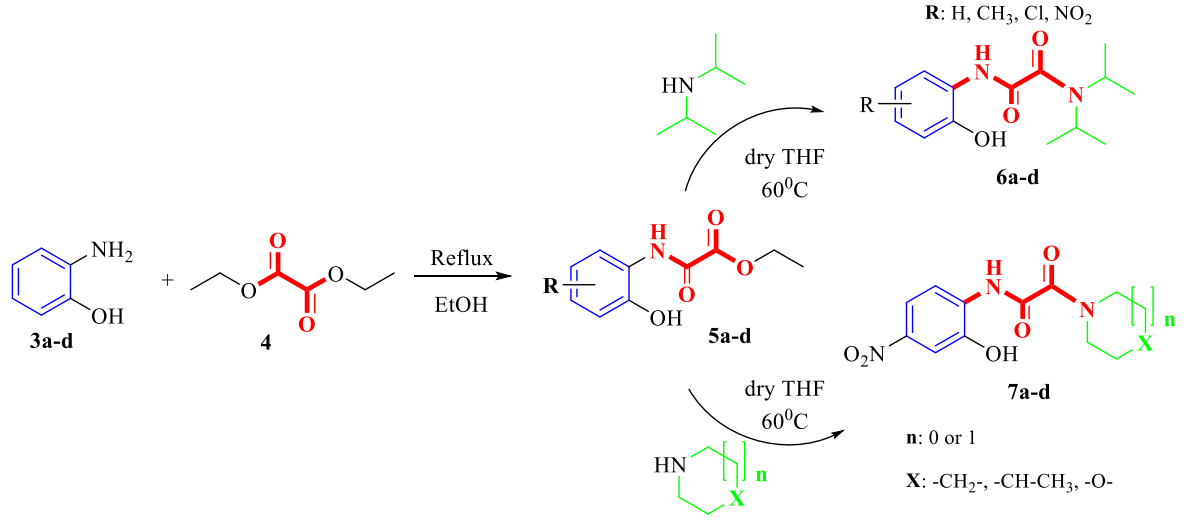
In our previous studies, we reported being immunomodulatory by regulating TNF-α and IL-6 production of alicyclic oxalamide derivative compounds, which we created inspired by the structure of thalidomide.[24,25] Based on our previous results, these oxalamide derivatives have differential effects on the production of the TNF-α and IL6 proinflammatory cytokines by the lipopolysaccharide-stimulated macrophages. In addition, many molecules with oxalamide functional groups have been designed by various research groups and their different pharmacological activities have been investigated. For example, *in vitro* activity of indole-based oxalamide derivatives with an IC_{50} of 21.80 µM in HeLa cells was determined.[26] Quizanolin-based thiazolinone diamide derivative was found to have 9 µM in vitro activity in MCF-7 cells.[27] It has also been reported that oxalamide complexes can create DNA-binding properties.[28]

As part of our ongoing study, based on the pharmacological properties of the oxalamide group in the literature, we replaced the phthalimide group in thalidomide with the oxalamide functional group. We also designed compounds to investigate the effect of various electron-withdrawing and donor substituents in the hydrophobic domain. Finally, we designed new oxalamide derivatives (Figure 3) by imitating the glutarimide part (piperidine dione structure) in thalidomide with diisopropyl and alicyclic amines (pyrrolidine, piperidine, morpholine, and 4-methylpiperidine). In this study, we investigated the *in vitro* antiproliferative activities, COX-1/2 expressions, and *in silico* COX-1/2 inhibitory potentials of the compounds **6a-d**, **7a-d**.

2. Experimental

2.1. Material and Methods

Chemicals and all the solvents, used in this study, were purchased locally from Aldrich, (Germany) and Acros (Germany) Chemical. Melting points of the compounds were recorded on an Electrothermal-9200 digital melting points apparatus and are uncorrected. The ^{1}H- NMR spectra were recorded in d_6-DMSO 400 MHz and ^{13}C- NMR spectra were recorded in d_6-DMSO 400 MHz on Bruker NMR spectrometer. Chemical shifts are reported in parts per million relatives to internal standard tetramethylsilane. Elemental analysis was performed on a Leco 932 CHNS instrument (St. Joseph. MI. USA) and the results were within ± 0.4% of the theoretical values.



Scheme 1. General synthesis procedure

2.2. Chemistry

*2.2.1. General Synthesis of Compounds **3a-d***

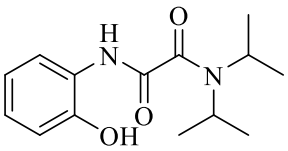
The synthetic strategy for the preparation of the target compounds is depicted in Scheme 1. To a stirred solution of 2-aminophenol derivatives **3a-d** (5 mmol) in ethanol, excess diethyl oxalate **4** (7.5 mmol) was added at room temperature. The reaction mixtures were refluxed for 6 hours, and reaction

completion was controlled with the TLC method. The reaction mixtures were cooled to room temperature, solid particles filtered, and the intermediates **5a-d** were used after crystallization in ethanol.[29]

*2.2.2. General Procedure for oxalamide derivatives **6a-d**, **7a-d***

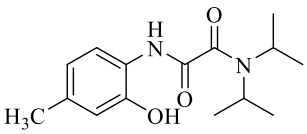
The obtained intermediates **5a-d** (0.5 mmol) were dissolved in 7 ml dry THF (tetrahydrofuran) by heating and 1.2 mmol of the corresponding secondary amine (diisopropylamine, pyrrolidine, piperidine, 4-Me piperidine, morpholine) was added to the reaction mixture. The reaction flask was heated from room temperature to 50-60°C and reaction completion was controlled with the TLC method. The reaction mixture was cooled to room temperature, after observing that the starting material was finished (after 30 min-2 hours) and evaporated under vacuum. The obtained gel material was washed with diethyl ether to get solid material. All of the compounds **6a-d** and **7a-d** were purified with column chromatography (n-hexane: ethylacetate, 5:1).[30]

*N^1-(2-hydroxyphenyl)- N^2,N^2-diisopropyloxalamide **(6a)***



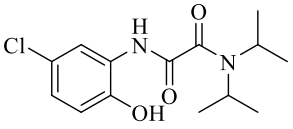
White solid, mp:204-205°C, Yield: 69%. ^{1}H NMR (400 MHz, d_6-DMSO) δ = 10.00 (s, 1H, -NH), 8.78 (bs, 1H, -OH), 8.18 (d, J=7.65 Hz, 1H, Ar-H), 6.90-6.79 (m, 2H, Ar-H), 6.77-6.69 (m, 1H, Ar-H), 3.33 (bs, 1H, -CH), 1.20 (bs, 12H, -CH_3). ^{13}C NMR (100 MHz, d_6-DMSO) δ= 163.2, 163.0, 146.5, 127.1, 123.7, 119.4, 118.8, 115.1, 46.5, 19.2. Anal. Calcd. for $C_{14}H_{20}N_2O_3$: C: 63.62; H: 7.63; N: 10.60; Found: C: 63.65; H: 7.61; N: 10.64.

*N^1-(2-hydroxy-4-methylphenyl)- N^2,N^2-diisopropyloxalamide **(6b)***



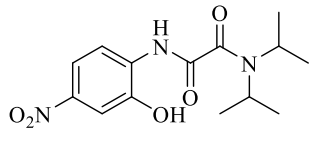
Gray solid, mp:206-209°C, Yield: 79%. ^{1}H NMR (400 MHz, d_6-DMSO) δ =9.92 (bs, 1H, -NH), 8.72 (bs, 1H, -OH), 8.03 (d, J=8.24 Hz, 1H, Ar-H), 6.66 (d, J=1.41 Hz, 1H, Ar-H), 6.55 (dd, J=1.41, J=8.24 Hz, 1H, Ar-H), 3.32 (h, J=6.47 Hz, 2H, -CH), 2.16 (s, 3H, Ar-CH_3), 1.19 (d, J=6.47 Hz, 12H, -CH_3). ^{13}C NMR (100 MHz, d_6-DMSO) δ= 163.2, 163.0, 146.4, 132.8, 124.7, 119.8, 118.7, 155.8, 46.5, 21.2, 19.2. Anal. Calcd. for $C_{15}H_{22}N_2O_3$: C: 64.73; H: 7.97; N: 10.06; Found: C: 64.70; H: 8.01; N: 10.03.

*N^1-(5-chloro-2-hydroxyphenyl)-N^2,N^2-diisopropyloxalamide **(6c)***



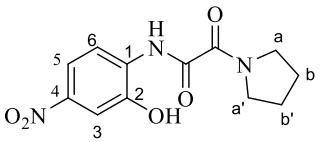
Dark yellow solid, mp:203-205°C decomp., Yield: 86%. ^{1}H NMR (400 MHz, d_6-DMSO) δ = 10.04 (s, 1H, -NH), 8.27 (d, J=2.38 Hz, 1H, Ar-H), 6.93-6.88 (m, 2H, Ar-H), 3.31 (h, J=6.49 Hz, 2H, -CH), 1.19 (d, J=6.49 Hz, 12H, -CH_3). ^{13}C NMR (100 MHz, d_6-DMSO) δ= 163.0, 162.7, 145.9, 128.1, 123.2, 122.4, 117.9, 116.1, 46.5, 19.2. Anal. Calcd. for $C_{14}H_{19}ClN_2O_3$: C: 56.28; H: 6.41; N: 9.38; Found: C: 56.31; H: 6.37; N: 9.42.

*N^1-(2-hydroxy-4-nitrophenyl)- N^2,N^2—diisopropyloxalamide **(6d)***



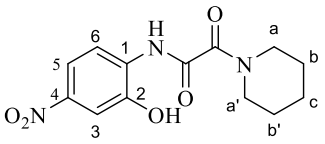
Orange solid, mp: 235-240°C decomp, Yield: 92%. ^{1}H NMR (400 MHz, d_6-DMSO) δ = 10.35 (bs, 1H, OH), 8.43 (d, J=8.89 Hz, 1H, Ar-H), 7.76-7.68 (m, 2H, Ar-H), 3.33 (h, J=6.49 Hz, 2H, -CH-), 1.20 (d, J=6.49 Hz, 12H, -CH_3).^{13}C NMR (100 MHz, d_6-DMSO) δ= 163.4, 162.3, 146.9, 142.5, 133.8, 117.2, 115.8, 109.2, 46.6, 19.2. Anal. Calcd. for $C_{14}H_{19}N_3O_5$: C: 54.36; H: 6.19; N: 13.58; Found: C: 54.37; H: 6.22; N: 13.59.

N-(2-hydroxy-4-nitrophenyl)-2-oxo-2-(pyrrolidin-1-yl)acetamide ***(7a)***



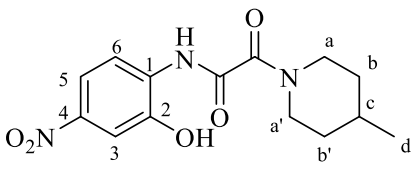
Dark orange solid, Mp: 178-179°C, Yield: 93%. ^{1}H NMR (400 MHz, d_6-DMSO) δ =8.24 (d, $J_{6,5}$=8.71 Hz, 1H, H-6), 7.32 (m, 1H, Ar-H), 7.26 (bs, 1H, Ar-H), 3.91 (t, $J_{a,b}$=6.73 Hz, 2H, H-a), 3.48 (t, $J_{a'b'}$=6.85 Hz, 2H, H-a′), 1.97-1.89 (m, 2H, H-b), 1.85-1.80 (m, 2H, H-b′). ^{13}C NMR (100 MHz, d_6-DMSO) δ=159.0, 158.6, 155.7, 144.0, 134.3, 116.1, 108.4, 108.3, 48.1, 47.4, 45.3, 26.2, 24.2, 23.0. Anal. Calcd. for $C_{12}H_{13}N_3O_5$: C: 51.61; H: 4.69; N: 15.05; Found: C: 51.63; H: 4.72; N: 15.09.

N-(2-hydroxy-4-nitrophenyl)-2-oxo-2-(piperidin-1-yl)acetamide ***(7b)***



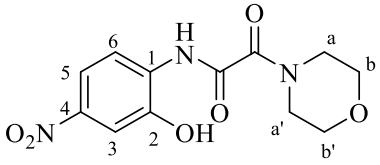
Light yellow solid, Mp: 190-191°C, Yield: 81%. ^{1}H NMR (400 MHz, d_6-DMSO) δ =10.32 (bs, 1H,-OH), 8.28 (d, $J_{6,5}$=8.95 Hz, 1H, H-6), 7.78 (dd, $J_{5,3}$=2.58, $J_{5,6}$=8.95 Hz, 1H, H-5), 7.72 (d, $J_{3,5}$=2.58 Hz, 1H, H-3), 3.54-3.49 (m, 4H, H-a,a′), 1.72-1.49 (m, 6H, H-b,b′,c). ^{13}C NMR (100 MHz, d_6-DMSO) δ=162.4, 161.4, 147.8, 143.4, 131.9, 120.4, 115.0, 109.3, 46.6, 42.1, 25.7, 24.8, 23.8. Anal. Calcd. for $C_{13}H_{15}N_3O_5$: C: 53.24; H: 5.16; N: 14.33; Found: C: 53.26; H: 5.18; N: 14.29.

N-(2-hydroxy-4-nitrophenyl)-2-(4-methylpiperidin-1-yl)-2-oxoacetamide ***(7c)***



Light yellow solid, Mp: 189-190°C, Yield: 89%. ^{1}H NMR (400 MHz, d_6-DMSO) δ =10.38 (bs, 1H, -OH), 8.50-8.24 (m, 1H, Ar-H), 7.82-7.69 (m, 2H, Ar-H), 4.32-3.91 (m, 1H, H-a), 3.29-3.26 (m, 1H, H-a), 3.14-3.08 (m, 1H, H-a′), 2.89-2.82 (m, 1H, H-a′), 1.77-1.58 (m, 4H, H-b,b′), 1.34-1.24 (m, 1H, H-c), 0.93 (d, $J_{d,c}$=6.52 Hz, 3H, H-d). ^{13}C NMR (100 MHz, d_6-DMSO) δ=163.2, 161.7, 146.1, 142.1, 133.4, 120.4, 116.9, 115.6, 114.9, 109.4, 108.8, 45.9, 43.2, 32.9, 30.3, 28.1, 21.5. Anal. Calcd. for $C_{14}H_{17}N_3O_5$: C: 54.72; H: 5.58; N: 13.67; Found: C: 54.75; H: 5.54; N: 13.71.

N-(2-hydroxy-4-nitrophenyl)-2-morpholino-2-oxoacetamide ***(7d)***



Yellow solid, Mp: 226-227°C, Yield: 92%. ^{1}H NMR (400 MHz, d_6-DMSO) δ =11.31 (bs, 1H, -NH), 10.33 (bs, 1H, -OH), 8.30 (d, $J_{6,5}$=8.95 Hz, 1H, H-6), 7.79 (dd, $J_{5,3}$=2.59, $J_{5,6}$=8.95 Hz, 1H, H-5), 7.73 (d, $J_{3,5}$=2.59 Hz, 1H, H-3), 3.69-3.62 (m, 6H, H-b,b′,a), 3.58-3.51 (m, 2H, H-a′). ^{13}C NMR (100 MHz, d_6-DMSO) δ=161.5, 161.4, 147.6, 143.4, 131.8, 120.2, 115.1, 109.3, 66.0, 65.6, 46.2, 41.9. Anal. Calcd. for $C_{12}H_{13}N_3O_6$: C: 48.82; H: 4.44; N: 14.23; Found: C: 48.86; H: 4.41; N: 14.25.

2.3. Biological Evaluation

2.3.1. Cell Culture

In this study, MCF-7 (human breast adenocarcinoma cell line), MDA-MB-231 (human breast cancer cell line), and WI-38 (human lung normal cell line) cells were purchased from American Type Culture Collection (ATCC). The medium used in our study was DMEM containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. MCF-7, MDA-MB-231, and WI-38 cells were cultured in DMEM in an incubator with 5% CO_2 at 37 °C.[31]

2.3.2. Cytotoxicity Tests

Cytotoxicity effects of the compounds on MCF-7, MDA-MB-231, and WI-38 cells were determined by XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)5[(phenylamino)carbonyl] 2H

tetrazolium hydroxide) assay 24 h after treatment. The cell viability was determined according to the intensity of the orange color observed at the end of the incubation period, which was measured in a microplate reader in the reference range of 475 nm. All absorbance was compared to control samples (cells without any test compound) which represented 100% viability.[31]

2.3.3. Antibody Staining

In this study, Anti-COX1/Cyclooxygenase 1 antibody (Cat No: ab244261) Anti-COX2/Cyclooxygenase 2 antibody (Cat No: ab15191), and Anti-beta Actin-antibody (Cat No: ab8227) were used as primary antibodies. Western Breeze Kit (Invitrogen, Chromogenic Immunodetection System, Rabbit Primary Antibody Cat: WB7105) was used for antibody staining. GEN-BOX imager CFX was used as the imaging device. The results obtained were analyzed with the Image J program.

2.3.4. Protein Isolation

1 mL of 1X PBS was added to the culture cell and washed using a centrifuge for 3 min at 2500 rpm. After PBS was removed, 1 ml ProtinExTotal Protein Extraction Solution (GeneAll, Cat No: 701-001) was added to the cell pellet and pipetting was performed. 15 minutes at +4°C at 16000 rpm. centrifugation was done. The supernatant was placed in a clean tube. The study was carried out on ice. After PBS was removed, 1 ml ProtinExTotal Protein Extraction Solution (GeneAll, Cat No: 701-001) was added to the cell pellet and pipetting was performed. It was centrifuged at 16000 rpm for 15 minutes at +4°C. The supernatant was placed in a clean tube. The study was carried out on ice. Protein amount measurement was performed using the Qubit® Protein Assay Kits (Thermo Fisher Scientific, Cat No: Q33211) with Qubit®3.0 Fluorometer device (Thermo Fisher Scientific, Cat No: Q33216).

2.3.5. Protein Electrophoresis

For denaturing protein samples, distilled water was added to the protein sample (50 µg) with 4X NuPAGE LDS SampleBuffer (ThermoFisherScientific, Cat No: NP0004) (5 µl), 10X NuPAGE Sample Reducing Agent (ThermoFisherScientific, Cat No: B0004) (2 µl). The prepared mixture was incubated at 70 ° C for 10 minutes and then taken on ice for 2 minutes. Buffercore vertical gel system was placed in the tank (XCellSureLock, Invitrogen). 4-12% Bis-Trisgradient gel (Invitrogen, NuPAGE 4-12% Bis-Tris Gel) was removed from the plastic packaging, the white tape on the gel cassette was removed and the comb was carefully removed without breaking the gel wells. Loading of Samples in Gel and SDS-PAGE Electrophoresis, Buffercore vertical gel system tank (XCellSureLock, Invitrogen), and 4-12% Bis-Trisgradient gel (Invitrogen, NuPAGE 4-12% Bis-Tris Gel) were used. 20X MES RunningBuffer (ThermoFisher, B0002) was diluted to 1X MES buffer with water. 5 µl marker (NZY Color Protein Marker II) was loaded from the wells located in the gel cassette to the foremost and trailing. 20 µl protein sample was loaded from the protein sample prepared for the remaining readers. IBLOT GEL TRANSFER System and Iblot transfer stack Nitrocellulose (NC) (Invitrogen, IB23001) kit was used in the blotting section.

2.4. Molecular Docking Studies

The calculations of the geometrical parameters were performed using the Gaussian 09W program package and B3LYP (Becke's Three Parameter Hybrid Functional using the LYP Correlation Functional) approach in conjunction with the 6-311G(d,p) basis set. The resulting optimization output files are saved as pdb extension files. The protein macromolecules were obtained from the protein data bank complexed with a selective inhibitor, celecoxib (as a co-ligand for both COX-1 and COX-2). Docking processes started with the removal of unwanted solvents, water, and co-ligands in crystal structures. By determining the amino acids with which the crystalline co-ligand interacts, an active site area with a volume of 50x50x50 A° was created in a cubic box. For COX-1 (PDB ID: 3KK6), X: -32.191, Y: 41.786, Z: -4.226, and for COX-2 (PDB ID: 3LN1), X: 26.318, Y: -21.114, Z: -19.903

coordinate centers are determined. Then, after hydrogen addition and charge assignment to the crystal structures, the preparations of the compounds to be tested were completed with Discovery Studio 2020 software.

In the molecular docking studies, the cluster RMSD (Root-Mean-Square Deviation) value was calculated for the validation of the targeted active site of both COX-1 and COX-2. For the docking studies to be valid, this value is required to be in the range of 0-2 A°. In the validation of molecular docking of COX-1; celecoxib co-ligand with PDB ID: 3HNG code was obtained from the protein data bank. The re-docking was done in the grid box created for the target region. It was determined that the cluster RMSD value of the ligand molecule, which was tested with 10 different conformations, was 0.16 A°. For COX-2 validation, celecoxib co-ligand with PDB ID: 2XIR code was obtained from the protein data bank and the re-docking was done in the grid box created for the target region. The cluster RMSD value of the ligand molecule, which was tested with 10 different conformations, was determined as 0.20 A°.

Compounds **6a-d** and **7a-d** were docked under validated docking conditions using Autodock 4.2 software. Docking processes were set as 50 repetitive tests, 10 different conformations, and target molecule-crystal structure binding energies, ligand efficiency, and theoretical inhibition concentrations were obtained according to the obtained outputs. The interactions of COX-1 and COX-2 with the compounds 6a-d and 7a-d were analyzed using the Discovery Studio Client 4.1 program.

3. Results and Discussion

3.1. Chemistry

In this study, the targeted oxalamide derivative compounds were obtained in high yield by reacting 2-aminophenol derivatives with diethyl oxalate and then with various secondary amines. The structures of the synthesized compounds are presented in Fig. 4. The structures of the compounds were characterized by elemental analysis and NMR, and ^{1}H-NMR and ^{13}C-NMR spectra of all compounds are presented in the Supp Info file.

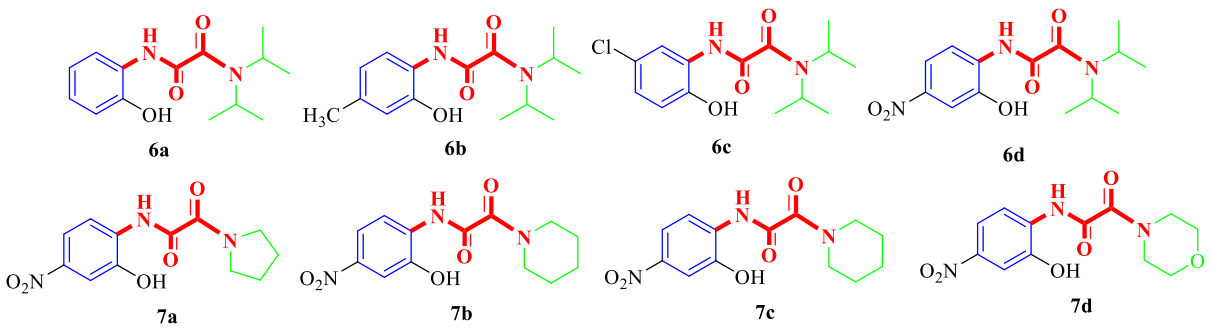


Figure 4. Structures of synthesized compounds

3.2. Anti-proliferative Activity

The novel target compounds were evaluated *in vitro* for their anti-proliferative activity against MCF-7 (human breast adenocarcinoma *cell line)*, MDA-MB-231 (human breast cancer *cell line)*, and WI-38 (human lung normal cell line) cell lines. The results were expressed in terms of IC_{50} values (the concentration that resulted in a 50% inhibition) and selectivity indexes (SI, IC_{50} for normal cell line/IC_{50} for cancerous cell line) where the well-known anticancer agents 5-Fluorouracil (5-FU) was used as a positive control.

From the results of anti-proliferative activity, it can be deduced that the compounds have a higher IC_{50} value in healthy cells and a lower IC_{50} value in breast cancer cell lines. This indicates that the compounds show an anti-proliferative effect with higher selectivity for the cancer cell line. For compounds **6a-d**, the substitution of the benzene ring seems to cause a significant change in

antiproliferative activity, and among this series, compound **6c** (containing -Cl substituent) with a lower IC_{50} value has a toxic effect both on the healthy and cancer cell lines. In addition, compounds **6a-d** appear to have a lower selectivity index for cancer cell lines compared to 5-FU, but are more selective for the MCF-7 cell line against the MDA-MB-231 cell line. However, compound **6d** (containing -NO_2 substituent) has higher selectivity for both MCF-7 (SI: 1.41) and MDA-MB-231 (SI: 1.17) cancer cell lines.

Like compound **6d**, similar IC_{50} values were obtained for nitro-substituted compounds **7a-d**. Compound **7d** appears to have the lowest IC_{50} value among all compounds. Also, it was obvious that the compound **7d** was the most potent candidate against both MCF-7 and MDA-MB-231 cell lines with IC_{50} = 4.72 µM and IC_{50} = 6.37 µM, respectively. In addition, the selectivity index of compound **7d** against both MCF-7 (2.09) and MDA-MB-231 (1.55) cancer cell lines is comparable to the positive control 5-FU (2.44 and 1.54 respectively). As a result of compounds other than **7d** in the MCF-7 cell line, compounds **6b**, **6c**, **6d,** and **7b** appear to have higher antiproliferative effects in MCF-7 cells. In addition, **6c** and **7b** compounds appear to have higher antiproliferative effects in MDA-MB-231 cells (Table 1).

Table 1. IC_{50} and SI values of compounds **6a-d** and **7a-d** against different breast cancer cells after 24 h.

	IC_{50} µM			**Selectivite Index (SI)**	
Compounds	**MCF-7**	**MDA-MB-231**	**WI-38**	**MCF-7**	**MDA-MB-231**
6a	8.33	13.44	13.81	1.65	1.02
6b	7.01	32.23	11.59	1.65	0.36
6c	5.60	7.07	7.60	1.35	1.07
6d	7.05	8.45	9.94	1.41	1.17
7a	9.12	9.46	12.37	1.35	1.31
7b	7.47	7.70	8.22	0.75	1.06
7c	8.43	8.92	9.49	1.12	1.06
7d	**4.72**	**6.37**	**9.90**	**2.09**	**1.55**
5-FU	3.95	6.26	9.64	2.44	1.54

*****IC_{50} for normal cell line/IC_{50} for cancerous cell line

*3.3. Western Blotting for COX1/2 Treated with **6a-d** and **7a-d***

Cyclooxygenase enzymes are potential therapeutic targets frequently studied in cancer research because of the important role they play in cancer development. Accordingly, the effects of non-steroidal anti-inflammatory drugs (NSAIDs) selective or non-selective for COX enzymes in cancer prevention or treatment of cancer is a frequently researched topic. However, the mechanism of the drugs used as COX inhibitors in the prevention or treatment of cancer is not fully known and serious studies are being carried out on this subject.[32] Because the antiproliferative effect of COX inhibitors despite the lack of expression of COX enzymes in some cancer cell lines reveals that COX inhibitors show antiproliferation in another mechanism.[33] Therefore, precise determination of the expression levels of COX proteins in cancerous cells is critical. With this interest, the protein levels of COX-1 and COX-2 in the MCF-7 cell line of the compounds synthesized in this study were tested by Western Blot analysis.

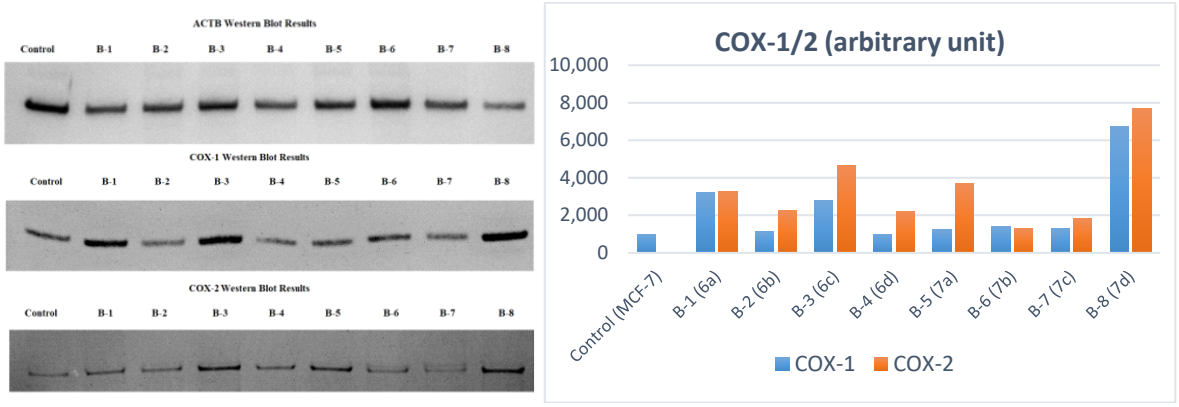


Figure 5. The expression of the COXs enzymes with compounds **6a-d** and **7a-d**

This study's *in vitro* enzyme assay kit consisted of ovine COX-1 and human COX-2. MCF-7 control group was used for both cyclooxygenase enzymes. The results show that among the synthesized molecules, **7d** molecule increased the expression of COX-1 and COX-2 enzymes more. Compound **7d** increased the expression of COX-1 approximately 2.3-fold than the other more effective compound **6a** and the expression of COX-2 approximately 1.5-fold than the other more effective compound **6c**. In the effect of the other compounds, it is observed that **6a** and **6c** compounds increase COX-1 expression, and **6a**, **6c** and **7a** compounds increase COX-2 expression (Figure 5).

3.4. Docking Analysis

Crystal structures with celecoxib as an inhibitor ligand in the active site of COX-1 and COX-2 enzymes obtained from the protein data bank were selected. The active site was determined by simulating the complex binding modes of this inhibitor, and docking studies were done under validated conditions. The docking scores of the compounds in the active site of COX-1 and COX-2 enzymes are presented in Table 2.

Table 2. The docking scores of the compounds **6a-d** and **7a-d** against human COX-1/2 enzymes

	COX-1 (PDB ID: 3KK6)			**COX-2 (PDB ID: 3LN1)**		
Compounds	**Binding Energy (kcal/mol)**	**Ligand Efficiency**	**Inhibitory Conc. (µM)**	**Binding Energy (kcal/mol)**	**Ligand Efficiency**	**Inhibitory Conc. (µM)**
6a	-6.80	-0.34	10.37	-7.24	-0.38	4.94
6b	-6.74	-0.34	11.39	-7.14	-0.36	5.85
6c	-6.76	-0.32	11.14	-7.49	-0.37	3.22
6d	-6.73	-0.31	11.60	-7.19	-0.33	5.37
7a	-6.72	-0.34	11.92	-7.38	-0.37	3.88
7b	-6.73	-0.32	11.64	-6.76	-0.32	11.06
7c	-6.65	-0.30	13.27	-6.85	-0.31	9.50
7d	**-6.93**	**-0.36**	**8.36**	**-7.73**	**-0.37**	**2.16**
Celecoxib	-10.97	-0.42	0.0093	-11.28	-0.42	0.0054

According to the results presented in Table 2, compound **7d** appears to have the highest binding affinity for both COX-1 and COX-2, -6.93 and -7.73 kcal/mol, respectively. It can also be said that the compounds are more targeted for the COX-2 active site. However, not all compounds appeared to have as high a binding affinity for both enzymes as celecoxib. However, the compounds can be said to be moderately COX inhibitors. The amino acid residues in the active site of the COX-1/2 enzymes and the H-bond and other non-covalent interactions of the compounds were examined and presented in Table 2.

Table 3. The docking interactions of the compounds B1-8 with human COX-1/2

	COX-1 (PDB ID: 3KK6)		COX-2 (PDB ID: 3LN1)	
Compounds	**H-bonding**	**Other non-covalent interactions**	**H-bonding**	**Other non-covalent interactions**
6a	Met522, Ala527	Tyr355, Ile523, Gly526	Tyr341	Trp373, Arg499, Phe504, Ala513
6b	Ile523	Val116, Leu352, Val349, Tyr355, Leu359, Leu531, Trp387, Gly526, Ala527	Tyr341	His75, Arg499, Ala502, Val509, Ala513
6c	Ser530	Val116, Leu352, Val349, Tyr355, Leu384, Trp384, Met522, Ala527	Tyr341	Val335, Leu338, Ser339, Arg499, Ala502, Ile503, Phe504, Val509
6d	Arg120, Ser530	Val116, Leu352, Trp387, Phe518	Tyr341, Ile503, Phe504	Val335, Val509, Ala513, Leu517
7a	Arg120, Ser530	Tyr385, Trp387, Leu531, Ala527	Tyr341, Arg499	Tyr371, Val509
7b	Arg120, Ser530	Val349, Trp387, Ala527, Leu531	Arg106	Val335, Tyr371, Trp373, Ala513
7c	Arg120	Leu352, Tyr385, Ile523, Ala527	Tyr371, Met508	Val102, Tyr341, Trp373, Ala513
7d	Arg120, Ser530	Tyr385, Trp387, Met522, Leu531, Gly526, Ala527	Arg499, Leu338	His75, Tyr371, Trp373, Ala502, Val509, Gly512
Celecoxib	Ser516, Ile517, Phe518	Leu352, Val349, Tyr355, Leu359, Leu384, Trp387, Gly526, Ala527	His75, Gln178, Arg499, Phe504	Val335, Tyr341, Leu345, Trp373, Val509, Gly512

According to Table 3, the control ligand celecoxib made three different hydrogen bonds in the active site of COX-1 and interacted non-covalently with the residues Leu352, Val349, Tyr355, Leu359, Leu384, Trp387, Gly526, Ala527. On the other hand, the most potential compound **7d**, interacted non-covalently with Trp387, Trp387, Gly526, and Ala527, which shared with celecoxib, by making two different hydrogen bonds at the active site. Furthermore, control ligand celecoxib H-bonded with His75, Gln178, Arg499 and Phe504 interacted non-covalently with Val335, Tyr341, Leu345, Trp373, Val509 and Gly512 at the COX-2 active site. Similarly, compound **7d** interacted with His75, Trp373, Arg499, Val509 and Gly512 in common with celecoxib. The binding types and interactions of the compound **7d** in the active site of COX enzymes were examined in 2D and 3D and the results are presented in Figures 6 and 7.

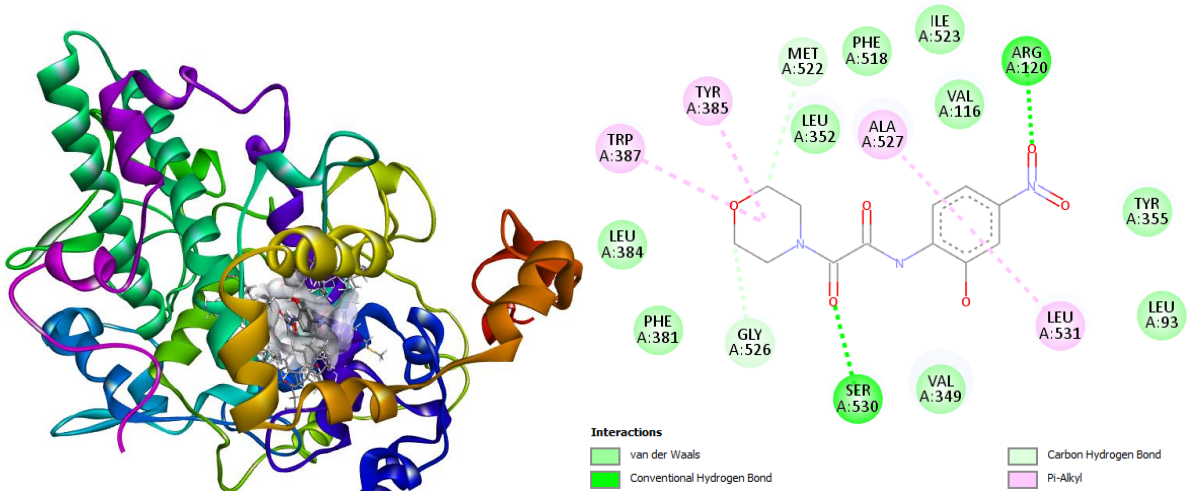


Figure 6. 3D and 2D ligand-protein interactions of COX-1 active site with compound **7d**

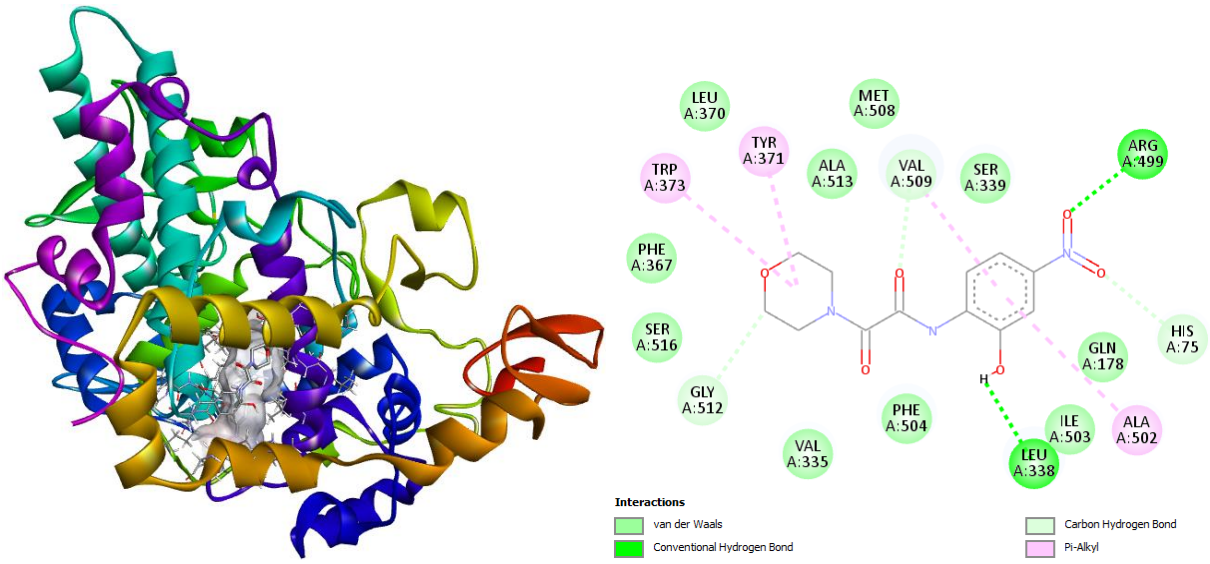


Figure 7. 3D and 2D ligand-protein interactions of COX-2 active site with compound **7d**

4. Conclusion

In recent studies, it has been observed that immunomodulatory drugs have *in vitro* antiproliferative activity in various cancer cell lines, especially breast cancer. The fact that thalidomide is an effective COX inhibitor in addition to its anticancer activity provides strong evidence for the relationship between anticancer effect and inflammation. This study found that oxalamide derivative compounds designed based on thalidomide molecule showed an antiproliferative effect comparable to the 5-FU control compound in breast cancer cell lines. The highest potent compound **7d** was found to have an antiproliferative effect on both MCF-7 and MDA-MB-231 breast cancer cell lines, with IC_{50} = 4.72 µM and IC_{50} = 6.37 µM, respectively. In addition, the expression of COX-1 and COX-2 in the MCF-7 cell line of the synthesized compounds was investigated by Western blot analysis to investigate the mechanism of death in cancerous cells, whether it was through the COX-dependent antiproliferative effect. It was observed that the synthesized compounds, especially the **7d** compound, considerably increased the COX-1/2 expression. In silico molecular docking studies indeed showed that the **7d** compound had a higher COX-1/2 enzyme inhibition potential. It can be said that the oxygen atoms in the NO_2 and morpholine groups in the **7d** compound are more active due to the hydrogen bonding for the active site of the COX enzymes. With the information obtained, it is planned to develop potential anticancer and COX inhibitor drug candidates by investigating the design and activity of molecules that provide stronger and selective COX inhibition in the future.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/organic-communications

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