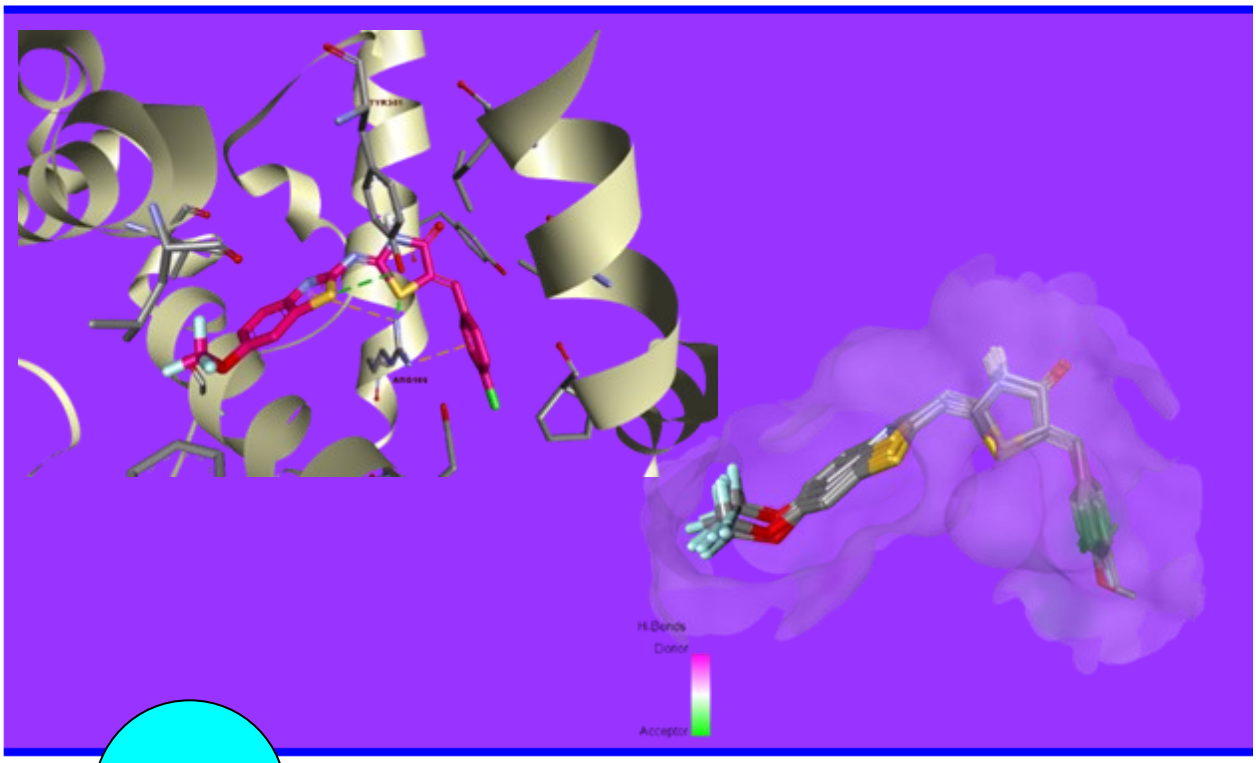

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Synthesis, molecular modeling, anti-cancer and COX-1/2 inhibitory activities of novel thiazolidinones containing benzothiazole core

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Abstract

In this study, new 1,3-thiazolidin-4-one derivatives containing arylmethylene groups in the 5-position were obtained from 6-(trifluoromethoxy)-1,3-benzothiazol-2-amine (riluzole). Synthesized compounds were characterized by spectral data and elemental analysis. *In vitro*, cytotoxic activities of the synthesized molecules were evaluated against the human lung cancer (A549) and human prostate cancer (PC-3) cell lines. Compounds were also tested on mouse embryonic fibroblast cells (NIH/3T3) to determine selectivity. Ten target compounds **3**-**12** were also screened for their COX-1 and COX-2 inhibitory activities. Of these compounds, **4** showed the highest COX-2 inhibition at 10 µM. Molecular docking calculations were performed to understand the binding interactions of compounds with COX-1 and COX-2 proteins. *In silico* studies of the tested compounds represented important binding modes that may be responsible for their anti-cancer activity via selective inhibition of the COX-2 enzyme. ADMET predictions were conducted to assess the drug-like properties of the novel compounds.

Introduction

Cancer is one of the most important health problems of the 21st century due to its increasing incidence and high mortality. According to the current data from the World Health Organization, approximately 18.1 million people were diagnosed with cancer in 2018, and 9.6 million people died due to cancer.

Compounds containing 5-arylmethylene-4-thiazolidinone and benzothiazole ring form the core structure of many synthetic compounds with their broad-spectrum biological activity, including anti-cancer effects. 5-arylmethylene-4-thiazolidionone derivatives have been reported in the literature as anti-inflammatory (Eleftheriou et al., 2012; Haroun et al., 2021; Omar et al., 2020; Ottanà et al., 2007, 2005), antimicrobial (Tatar et al., 2010; Vicini et al., 2006; Vicini et al., 2008) and anti-cancer (Havrylyuk et al., 2010; Ottanà et al., 2005; Zhou et al., 2008) effects (Figure 1). In recent years, promising results have been obtained in terms of anti-cancer effects in the literature. Anti-cancer effects of 5-arylmethylene-4-thiazolidinone derivatives which are responsible for many biological effects including COX-2 enzyme inhibitory effects have been promising for the treatment of cancer and inflammatory diseases (Apostolidis et al., 2013; Türe et al., 2021).

On the other hand, the benzothiazole ring is a heterocycle containing electron-rich sulfur and nitrogen atoms, and compounds bearing benzothiazole rings are

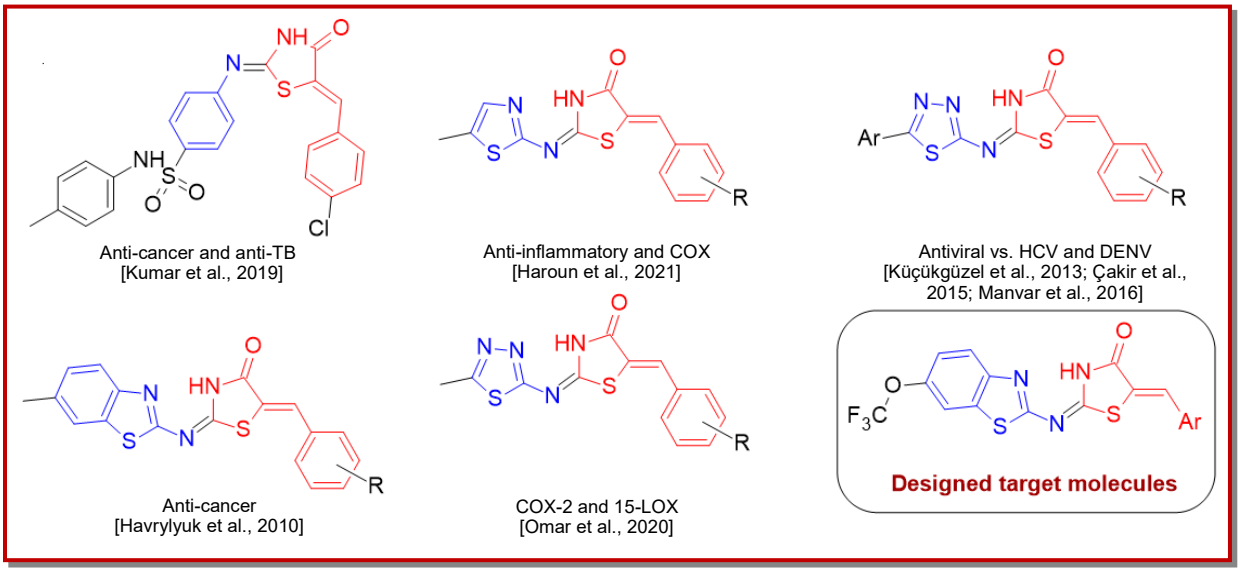


Figure 1: Structures of the reported 1,3-thiazolidine-4-ones and benzothiazole derivatives as biologically active agents and the newly designed benzothiazole-thiazolidinone conjugates

known to have many biological activities. There are numerous reports on the antimicrobial (Catalano et al., 2013; Saeed et al., 2010), anticonvulsant (Liu et al., 2016), anti-inflammatory (Shafi et al., 2012), anti-cancer (Akhtar et al., 2008) activities of benzothiazole derivatives. It has been reported that benzothiazole derivatives are effective on molecular targets such as fatty acid amide hydrolase (FAAH) (Wang et al., 2009), raf kinase (Raf-1) (Song et al., 2008) and BCL-2 (Zheng et al., 2007), especially in the discovery of new anti-cancer agents.

For decades, studies on 2-imino-1,3-thiazolidin-4-one compounds have been ongoing in many research groups. In recent years, a significant increase has been observed in studies conducted with both 2-imino-1,3-thiazolidin-4-one compounds and compounds bearing benzothiazole rings. Many reports reveal that these compounds may be hepatitis C virus NS5B polymerase inhibitors (Çakir et al., 2015; Küçükgüzel et al., 2013) or dengue virus NS5 (RdRp) inhibitors (Manvar et al., 2016), or anti-cancer agents (Abdellatif et al., 2015; Geronikaki et al., 2008; Havrylyuk et al., 2010; Zhou et al., 2008).

The research described herein is an extension of ongoing efforts toward a search for novel anti-cancer agents bearing the thiazolidinone scaffold. Considering the above-mentioned literature on the biological effects of benzothiazole derivatives, combining the benzothiazole ring and the 5-arylmethylene-1,3-thiazolidin-4-one skeleton in one molecule was considered a promising approach for the development of new drug-like molecules.

In this current study, synthesis of 2-heteroarylimino-5-arylmethylene-1,3-thiazolidin-4-one derivatives starting from 6-(trifluoromethoxy)-1,3-benzothiazol-2-amine, their anti-cancer effects and COX-1/2 enzyme inhibitory properties are described. The potential interactions of the synthesized compounds with COX-1 and COX-2 target proteins were also investigated by *in silico* molecular modeling techniques.

Materials and Methods

Chemistry

All solvents and reagents were obtained from commercial sources and used without purification. All melting points (°C, uncorrected) were determined using Schmelzpunktbestimmer SMP II basic model melting point apparatus. In thin layer chromatography (TLC) studies, 0.2 mm silica gel F-254 (Merck) plates (20 x 20 cm) were used as adsorbent. Two different solvent systems S1 [petroleum ether: ethyl acetate (50:50 v/v)] and S2 [chloroform: acetone (65:35 v/v)] were used in the chromatographic control of the synthesized compounds. The solvent systems used were of a purity that could be used for chromatographic studies. Detection of the compounds was carried out at 254 nm and 366 nm UV lamps. Elemental analyses were obtained using Leco CHNS-932 and were consistent with the assigned structures. Infrared spectra were recorded on a Shimadzu FTIR 8400S and data are expressed in wavenumber υ (cm^{-1}). ^1H NMR and HMBC spectra were recorded on Bruker Avance 300 MHz for the chemical shifts expressed in δ (ppm) downfield from tetramethylsilane (TMS) using DMSO-d_6 as solvent. High-resolution mass spectra were acquired by using Jeol JMS700 instrument.

Synthesis of 2-chloro-N-[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]acetamide [**1**]

6-(Trifluoromethoxy)-1,3-benzothiazol-2-amine (10

mmol) was dissolved in dichloromethane. Triethylamine (0.012 mol) was added and stirred under reflux for 30 min. Chloroacetyl chloride (12 mmol) was then slowly added and the medium was refluxed for 7 hours. At the end of the reaction, the flask content was poured into ice, the precipitate was filtered, dried, and purified by crystallization from ethanol (Kulabaş et al., 2022).

Off-white powder. Yield, 44%. TLC %Rf, 68 (S1). M.p. 182°C. FTIR υ_{max} (cm^{-1}): 3179 (N-H); 3105, 3071 (C-H arom.); 3004, 2952 (C-H aliph.) 1739 (C=O); 1667 (-NH-bend.). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 4.49 (2H, s, -COC**H**$_2$Cl); 7.43-7.47 (1H, m, Ar**H**); 7.87 (1H, d, *J=9 Hz*, Ar**H**); 8.16 (1H, d, *J=2.1 Hz*, Ar**H**); 12.86 (1H, s, **NH**). HRMS (EI$^+$); (m/z, calcd./found): 309.9785/309.9781 $[M]^+$. Anal. calcd. for $C_{10}H_6ClF_3N_2O_2S$: C, 38.66; H, 1.95; N, 9.02; S, 10.32. Found: C, 38.64; H, 1.964; N, 9.06; S, 9.52.

Synthesis of 2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl] imino}-1,3-thiazolidin-4-one [**2**]

2-Chloro-*N*-[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl] acetamide [**1**] (0.004 mol) was dissolved in ethanol. Ammonium thiocyanate (0.006 mol) was added and boiled under reflux for 7 hours. Afterward, the reaction was terminated by pouring the flask content into ice. The resulting precipitate was filtered, dried, and purified by crystallization from ethanol (Kulabaş et al., 2017).

Gray shiny powder. Yield, 27%. TLC %Rf, 60 (S1); 72 (S2). M.p. 228°C. FTIR υ_{max} (cm^{-1}): 3140 (N-H); 3063, 2989 (C-H arom.); 2820 (C-H aliph.); 1726, 1699 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 4.09 (2H, s, -C**H**$_2$- thiazolidinone); 7.39-7.46 (1H, m, Ar**H**); 7.88 (1H, d, *J=9 Hz*, Ar**H**); 8.13 (1H, d, *J=1.2 Hz*, Ar**H**); 12.37 (1H, s, **NH**). HRMS (EI$^+$); (m/z, calcd./found): 332.9848/332.9849 $[M]^+$. Anal. calcd. for $C_{11}H_6F_3N_3O_2S_2.H_2O$: C, 37.61; H, 2.30; N, 11.96; S, 18.25. Found: C, 37.52; H, 2.178; N, 13.13; S, 17.81.

Synthesis of 5-(substituted aryl methylene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-ones [**3**-**12**]

2-{[6-(Trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**2**] (0.0011 mol) was dissolved in a methanolic solution of equimolar sodium methoxide. An appropriate aromatic/heteroaromatic aldehyde (0.0011 mol) was added to it. The mixture was heated in a water bath under reflux at 90°C for 4 hours. Then, the medium was cooled and neutralized with 10% acetic acid. The crude product was washed thoroughly with water, dried, and then purified by washing with boiling methanol (Çakir et al., 2015; Küçükgüzel et al., 2013).

5-(3-Fluorobenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**3**]

Yellow powder. Yield, 33%. TLC %Rf, 80 (S2). M.p. 368-370°C. FTIR υ_{max} (cm^{-1}): 3244 (N-H); 3196, 3011 (C-H arom.); 1643 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 7.19-7.59 (5H, m, Ar**H**); 7.38 (1H, d, *J=8.7 Hz*, Ar**H**); 7.50 (1H, s, =C**H**-Ar); 7.91 (1H, d, *J=1.5 Hz* Ar**H**). HRMS (EI$^+$); (m/z, calcd./found): 439.0067/439.0050 $[M]^+$. Anal. calcd. for $C_{18}H_9F_4N_3O_2S_2$.3 mol H_2O: C, 43.81; H, 3.06; N, 8.52; S, 13.00. Found: C, 43.69; H, 2.496; N, 8.521; S, 12.17.

5-(4-Chlorobenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**4**]

Yellow powder. Yield, 36%. TLC %Rf, 75 (S2). M.p. 287-289°C. FTIR υ_{max} (cm^{-1}): 3086 (N-H); 3026, 2972 (C-H arom.); 1699, 1688 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 7.50 (1H, d, *J=8.7 Hz*, Ar**H**); 7.65 (2H, d, *J=8.7 Hz*, Ar**H**); 7.72 (2H, d, *J=8.7 Hz*, Ar**H**); 7.79 (1H, s, =C**H**-Ar); 8.00 (1H, d, *J=8.4 Hz*, Ar**H**); 8.16 (1H, s, Ar**H**); 13.03 (1H, s, **NH**). ^{13}C NMR (150 MHz, DMSO-d_6/TMS) δ ppm: 115.44 (Ar**C**), 118.35 (**C**5, thiazolidinone), 120.47 (Ar**C**), 121.83 (=**C**H-Ar), 122.76, 124.69, 129.43 (Ar**C**), 131.83 (-O**C**F$_3$), 134.20, 135.00, 144.62, 149.67, 159.10 (Ar**C**), 166.75 (**C**2, thiazolidinone), 169.95 (**C**=O, thiazolidinone). HRMS (DART$^+$); (m/z, calcd./found): 455.9777/455.9853 $[M+H]^+$. Anal. calcd. for $C_{18}H_9ClF_3N_3O_2S_2$.3 mol H_2O: C, 46.51; H, 2.17; N, 9.04; S, 13.80. Found: C, 46.42; H, 2.054; N, 8.961; S, 12.60.

5-(4-Methoxybenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**5**]

Yellow powder. Yield, 6%. TLC %Rf, 73 (S2). M.p. 238-239°C. FTIR υ_{max} (cm^{-1}): 3036 (N-H); 3019 (C-H arom.); 2903, 2839 (C-H aliph.); 1713 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 3.83 (3H, s, -OC**H**$_3$); 7.11 (2H, d, *J=8.7 Hz*, Ar**H**); 7.35 (1H, d, *J=8.4 Hz*, Ar**H**); 7.59-7.62 (3H, m, =C**H**-Ar and Ar**H**); 7.79 (1H, d, *J=8.4 Hz*, Ar**H**); 7.98 (1H, s, Ar**H**). HRMS (EI$^+$); (m/z, calcd./found): 451.0267/451.0277 $[M]^+$. Anal. calcd. for $C_{19}H_{12}F_3N_3O_3S_2$. 3/2 mol H_2O: C, 47.80; H, 2.96; N, 8.80; S, 13.43. Found: C, 48.12; H, 2.965; N, 8.852; S, 12.52.

5-(4-Fluorobenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**6**]

Yellow powder. Yield, 24%. TLC %Rf, 70 (S2). M.p. 245-247°C. FTIR υ_{max} (cm^{-1}): 3119 (N-H); 3050, 2997 (C-H arom.); 1717 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 7.39-7.48 (3H, m, Ar**H**); 7.65-7.77 (3H, m, =C**H**-Ar and Ar**H**); 7.96 (1H, d, *J=9 Hz*, Ar**H**); 8.12 (1H, s, Ar**H**); 12.98 (1H, s, **NH**). HRMS (EI$^+$); (m/z, calcd./found): 439.0067/439.0059 $[M]^+$. Anal. calcd. for $C_{18}H_9F_4N_3O_2S_2$.1 H_2O: C, 47.26; H, 2.42; N, 9.19; S, 14.02. Found: C, 47.57; H, 2.418; N, 9.087; S, 12.65.

5-(2-Chlorobenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**7**]

Yellow powder. Yield, 59%. TLC %Rf, 55 (S2). M.p. 367° C. FTIR υ_{max} (cm^{-1}): 3090 (N-H); 3040 (C-H arom.); 1647 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 7.28 (1H, d, *J=8.7 Hz*, Ar**H**); 7.40 (1H, t, *J=7.5 Hz* and

J=7.8 Hz, Ar**H**); 7.53 (1H, t, *J=7.2 Hz* and *J=7.8 Hz*, Ar**H**); 7.59 (1H, d, *J=8.1 Hz*, Ar**H**); 7.67-7.73 (2H, m, Ar**H**); 7.74 (1H, s, =C**H**-Ar); 7.90 (1H, d, *J=1.5 Hz*, Ar**H**). HRMS (DART$^+$); (m/z, calcd./found): 455.9844/455.9852 $[M+H]^+$. Anal. calcd. for $C_{18}H_9ClF_3N_3O_2S_2$.1 H_2O: C, 45.62; H, 2.34; N, 8.87; S, 13.53. Found: C, 45.03; H, 1.811; N, 8.688; S, 11.55.

5-(2-Fluorobenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**8**]

Yellow powder. Yield, 62%. TLC %Rf, 48 (S2). M.p. 322-323°C. FTIR υ_{max} (cm^{-1}): 3295 (N-H); 3094, 3050 (C-H arom.); 1647 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 7.26-7.33 (2H, m, Ar**H**); 7.35-7.47 (2H, m, Ar**H**); 7.59 (1H, s, =C**H**-Ar); 7.63-7.70 (2H, m, Ar**H**); 7.90 (1H, d, *J=1.5 Hz* Ar**H**). HRMS (EI$^+$); (m/z, calcd./found): 439.0067/439.0071 $[M]^+$. Anal. calcd. for $C_{18}H_9F_4N_3O_2S_2$. 3 H_2O: C, 43.81; H, 3.06; N, 8.52; S, 13.00. Found: C, 43.86; H, 2.512; N, 8.485; S, 11.67.

5-(2-Methoxybenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**9**]

Yellow powder. Yield, 8%. TLC %Rf, 55 (S2). M.p. 240-242°C. FTIR υ_{max} (cm^{-1}): 3119 (N-H); 3046 (C-H arom.); 2995, 2791(C-H aliph.); 1721 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 3.89 (3H, s, -OC**H$_3$**); 7.08-7.12 (2H, m, Ar**H**); 7.28 (1H, d, *J=8.7 Hz*, Ar**H**); 7.38 (1H, t, *J=7.5 Hz*, Ar**H**); 7.55 (1H, d, *J=7.5 Hz*, Ar**H**); 7.68 (1H, d, *J=8.7 Hz*, Ar**H**); 7.80 (1H, s, =C**H**-Ar); 7.89 (1H, s, *Hz* Ar**H**). HRMS (DART$^+$); (m/z, calcd./found): 452.0345/ 452.0349 $[M+H]^+$. Anal. calcd. for $C_{19}H_{12}F_3N_3O_3S_2$.1 H_2O: C, 48.61; H, 3.01; N, 8.95; S, 13.66. Found: C, 48.06; H, 2.560; N, 8.769; S, 13.52.

5-(3-Methoxybenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**10**]

Yellow powder. Yield, 14%. TLC %Rf, 54 (S2). M.p. 225° C. FTIR υ_{max} (cm^{-1}): 3092 (N-H); 3196, 3011 (C-H arom.); 2938, 2915 (C-H aliph.); 1701 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 3.82 (3H, s, -OC**H$_3$**); 6.96 (1H, d, *J=7.8 Hz*, Ar**H**); 7.15-7.20 (2H, m, Ar**H**); 7.27 (1H, d, *J=9 Hz*, Ar**H**); 7.41 (1H, t, *J=7.8 Hz* and *J=8.1 Hz*, Ar**H**); 7.46 (1H, s, =C**H**-Ar); 7.68 (1H, d, *J=8.7 Hz*, Ar**H**); 7.89 (1H, s, *Hz* Ar**H**). HRMS (EI$^+$); (m/z, calcd./found): 451.0267/451.0286 $[M]^+$. Anal. calcd. for $C_{19}H_{12}F_3N_3O_3S_2$: C, 50.55; H, 2.68; N, 9.31; S, 14.21. Found: C, 50.26; H, 2.937; N, 9.223; S, 14.44.

5-(3-Chlorobenzylidene)-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**11**]

Yellow powder. Yield, 6%. TLC %Rf, 68 (S2). M.p. 231-233°C. FTIR υ_{max} (cm^{-1}): 3337 (N-H); 3156, 3101 (C-H arom.); 1705 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 7.29 (1H, d, *J=8.7 Hz*, Ar**H**); 7.44 (1H, d, *J=8.7 Hz*, Ar**H**); 7.47 (1H, s, Ar**H**); 7.51-7.59 (2H, m, Ar**H**); 7.64 (1H, s, =C**H**-Ar); 7.69 (1H, d, *J=8.7 Hz*, Ar**H**); 7.90 (1H, s, Ar**H**). HRMS (EI$^+$); (m/z, calcd./found): 454.9771/454.9800 $[M]^+$. Anal. calcd. for $C_{18}H_9ClF_3N_3O_2S_2$: C, 47.43; H, 1.99; N, 9.22; S, 14.07. Found: C, 47.05; H, 2.389; N, 9.083; S, 14.07.

5-[(5-Ethylfuran-2-yl)methylidene]-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one [**12**]

Yellow powder. Yield, 6%. TLC %Rf, 63 (S2). M.p. 255° C. FTIR υ_{max} (cm^{-1}): 3127 (N-H); 3032, 2978 (C-H arom.); 2901, 2768 (C-H aliph.); 1715 (C=O). ^{1}H NMR (300 MHz, DMSO-d_6/TMS) δ ppm: 1.36 (3H, t, *J=7.5 Hz*, -CH_2C**H$_3$**); 2.84 (2H, q, *J=7.5 Hz*, -C**H$_2$**CH_3); 6.46 (1H, d, *J=3.3 Hz*, Ar**H**); 7.08 (1H, d, *J=3.3 Hz*, Ar**H**); 7.50 (1H, d, *J= 9.0 Hz*, Ar**H**); 7.55 (1H, s, =C**H**-Ar); 7.87 (1H, d, *J= 8.7 Hz*, Ar**H**); 8.15 (1H, s, Ar**H**); 12.77 (1H, s, **NH**). HRMS (EI$^+$); (m/z, calcd./found): 439.0267/439.0254 $[M]^+$. Anal. calcd. for $C_{18}H_{12}F_3N_3O_3S_2$.1 H_2O: C, 48.21; H, 2.92; N, 9.37; S, 14.30. Found: C, 48.68; H, 2.520; N, 9.347; S, 13.70.

Biological methods

Cell culture

Human lung cancer (A549), human prostate cancer (PC-3), and mouse embryonic fibroblast (NIH/3T3) cell lines were purchased from the American Type Culture Collection (USA). The cells were grown in Dulbecco's modified Eagle medium (DMEM) from Gibco, USA, which contains 10% fetal bovine serum and were kept in an incubator at 37°C and 5% CO_2. At 80–90% confluence, cell passage was carried out (Bülbül et al., 2022).

COX-1/2 inhibitory activity

The inhibitory potential of all synthesized compounds on COX-1 and COX-2 enzymes was evaluated using a COX inhibitor screening kit (Cayman Chemical, USA). The samples and control were dissolved in the DMSO and diluted with the reaction buffer to their final concentrations. DMSO served as a negative control for 100% initial activity. The inhibitor interference by adding the inhibitor to a boiled enzyme sample as a control was tested. The assay was conducted in duplicate and statistical analysis was carried out using GraphPad Prism 6.1 Software (Kulabaş et al., 2023).

Molecular docking studies

The structures of COX-1 (PDB code: 1Q4G, resolution: 2.0 Å) (Gupta et al., 2004) and COX-2 (PDB code: 3LN1, resolution: 2.4 Å) were obtained from the protein data bank (Wang et al., 2010). The target enzymes were prepared for docking studies using the AutoDock Tools program (Morris et al., 2009). Each enzyme was cleaned by removing the water molecules and co-crystallized inhibitors. The charge of the Fe atom in each enzyme was set to +2 manually. To validate the docking protocol, the co-crystallized inhibitors were docked into respective COX enzyme. Binding pockets for the COX-1 enzyme were detected using the CavityPlus web server (Xu et al., 2018; Yuan et al., 2020, 2013). The 3D structures of designed inhibitors were optimized with semi-empirical PM3 method via conformation search in

Box 1: MTT assay

Principle

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide] assay is a colorimetric method to evaluate the antiproliferative activity. It is based on the conversion of MTT to insoluble formazan crystals by mitochondrial enzymes. This reduction is directly related to the metabolic functionality and viability of the cells tested.

Requirements

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Human lung cancer cell line, A549 (ATCC, CCL-185); Human prostate cancer cell line, PC-3 (ATCC, CRL-1435); Microplate reader (BioTek, USA); Mouse embryonic fibroblast cell line, NIH/3T3 (ATCC, CRL-1658); sodium dodecyl sulfate buffer; Incubator; Synthesized compounds; 96-Well plate

Procedure

Step 1: In 96-well plates, the cells (1×10^4 cells/well) were seeded and incubated overnight.

Step 2: After removal of cell media, each culture well was washed with 200 µL of phosphate buffered saline (PBS) solution and the fresh medium was added. Then, the cells were treated with different concentrations (1-50 µM) of synthesised compounds 2-12 for 48 hours. At the end of the incubation, the cell medium was discarded and the wells were cleaned by adding of 200 µL of PBS. Then, PBS was discarded and 100 µL of the freshly prepared cell medium was distributed to all wells.

Step 3: MTT was added to each well at a final concentration of 0.5 mg/mL after the initial incubation time and incubated for an additional 4 hours.

Step 4: At the end of 4 hours, the cell media was discarded and 100 µL sodium dodecyl sulfate (SDS) was added to the wells and incubated at 37°C for 12 hours to dissolve the formazone crystals formed by MTT.

Step 5: Using a microplate reader, absorbances at 570 and 630 nm wavelengths were measured. All tests were repeated three times.

Calculation

The percentage of cell viability was calculated using the equation:

[mean OD of treated cells/mean OD of control cells] × 100

References

Bülbül et al., 2022

References (Video)

Bahuguna et al., 2017

Spartan 4 program and used for initial geometry in docking calculation (Stewart, 2007). The resultant docking files were analyzed to explain the mechanism of binding using BIOVIA Discovery Studio Visualizer program (https://discover.3ds.com/).

Calculation of molecular properties

Pharmacokinetic profiles, toxicity risks (mutagenicity, tumorigenicity, irritation, and reproductive effect), and physico-chemical properties (e.g. cLogP, TPSA, drug-score) of compounds were calculated by the methodology developed by OSIRIS Data Warrior software (http://www.openmolecules.org/datawarrior/) and ADME properties calculated using SwissADME (http://www.swissadme.ch/).

Results

Chemistry

Synthesis and characterization of compounds 1-12

In the present study, 10 original 5-arylmethylene-2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one derivatives were synthesized. In the first step, the corresponding chloroacetamide derivative **1** was prepared by reacting 6-(trifluoromethoxy)-1,3-benzothiazol-2-amine with chloroacetyl chloride in the presence of triethylamine. Compound **2**, identified as 2-{[6-(trifluoromethoxy)-1,3-benzothiazol-2-yl]imino}-1,3-thiazolidin-4-one was obtained by refluxing compound **1** with ammonium thiocyanate in an ethanolic medium. Finally, target compounds **3-12** were synthesized by Knoevenagel condensation of compound **2** with several aromatic aldehydes with the aid of sodium methoxide. All the newly synthesized compounds have been characterized by the use of IR, ^{1}H NMR, HMBC, and mass spectral data while their purities were proved with TLC and elemental analysis.

In the FTIR spectra of compounds **2-12**, the N-H stretching bands of the thiazolidinone ring had been observed as weak bands in the range of 3036-3337 cm^{-1}, while the C=O stretchings were detected at 1643-1761 cm^{-1} as strong bands. When the ^{1}H NMR data of the target compounds **2-12** were evaluated, the NH protons of the 4-thiazolidinone ring were detected as broad singlets in the range of 12.37-13.03 ppm. The NH proton of the ring could not be detected in the spectra of compounds **3**, **5**, and **7-11**. It was considered that the NH proton was exchanged by D_2O during the analysis of these compounds and therefore could not be observed. In the ^{1}H-NMR spectrum of compound **2**, while the singlet appeared at 4.09 ppm corresponding to the active methylene –CH_2– group, this proton disappeared for the ^{1}H NMR spectrum of compounds **3-12**.

The ^{1}H NMR spectra of compounds **3-12** also exhibited a methine proton (Ar–CH=C<), which was deshielded by the neighboring C=O moiety and appeared at d 7.46-7.80 ppm field. Furthermore, the additional four aromatic protons belonging to benzylidene structures were detected between 6.46-7.77 ppm for compounds **3-12**.

In the mass spectra of compounds **1**-**12** recorded by EI and DART techniques, molecular ion peaks were detected for all compounds. The difference between the calculated and found values was less than 0.05 for each compound, confirming the structures of the compounds.

HMBC analysis was applied to elucidate the carbon skeleton of compound **4**, one of the arylmethylidene derivatives, and thus the structure was determined using ^{13}C-^{1}H interactions at two, three, or more bond distances (See Supplementary data). When the 107-128 ppm range of the HMBC spectrum was examined, the chemical shift value of the carbons of the benzothiazole ring (C11, C14, and C15), the carbons of the thiazolidin-4-one ring (C8), and the C1 and C7 carbon of the benzene ring were determined. The C14 carbon of the benzothiazole ring was detected at 115.44 ppm, with two symmetrical contours with the H14 proton attached to it. In the 129-154 ppm range of the spectrum, the chemical shift values of the carbon atoms of the 1,4 disubstituted benzene ring (C2-6) and the carbon atoms of the benzothiazole ring (C10, C12, C13, and C18) were observed and were identified as a result of their coupling with the surrounding hydrogen atoms. The peak observed at 131.83 ppm was attributed to the C18 carbon of the benzothiazole ring due to its contour with the H12 proton around 7.5 ppm, whereas the peak at 144.62 ppm was attributed to the C10 carbon due to its interactions with H14, H11, and H12 hydrogens. The interactions of the C4 carbon with the H2, H3, H5, and H6 protons on the 1.4 disubstituted benzene ring confirmed that this carbon resonated at 135.00 ppm. The C17 carbon atom of the thiazolidin-4-one ring was detected at 166.75 ppm due to its interaction with the methine proton.

Biological studies

Anti-cancer activity

The antiproliferative effects of the synthesized compounds were first investigated on A549 (lung cancer) and PC-3 (prostate cancer) cell lines using the MTT technique. NIH3T3 cells were used to determine the selectivity of the measured cytotoxic effect values. In general, the addition of a benzylidene group to the structure of compound **2** did not create a strong dramatic anti-cancer effect, but it is noteworthy that the most effective derivatives were found with the 4-substitution of benzylidene groups (Table I). Among the screened benzothiazole-thiazolidinone conjugates, compound **5** was determined as the most effective derivative with IC_{50} values of 22.8 µM against A549 cells and 43.4 µM against PC-3 cell lines. The selectivity index of this molecule was determined as 2.5 for A549 cells and 1.5 for PC-3 cells.

Inhibition of COX-1 and COX-2 enzymes

The results obtained in the COX-1/2 inhibition assays of the compounds are given in Table II. Celecoxib was used as a positive control in the present study. Accoring to the enzyme assay results, target molecules **2**, **4**, **5**, and **12** showed more than 70% inhibition against COX-2 at 1 µM. Compound **4** had higher activity compared to celecoxib, while the remaining molecules showed similar or lower activity. Another advantage of the active compounds was that they showed 6% or less inhibition of COX-1 enzyme even at 50 µM concentration and were more selective inhibitors than celecoxib. The binding energies calculated in the molecular docking study reflected the results of *in vitro* inhibition. While high scores were obtained with all arylmethylene derivatives (**3**-**12**), it was observed that none of the target molecules could interact with the COX-1 binding pocket. Compound **5**, which had the highest anti-cancer activity, remarkably had a selective inhibition of the COX-2 enzyme. Among the target molecules, the highest COX-2 inhibitory effect was observed in the derivatives carrying chlorine or methoxy at the 4-position, while the substitution shift to other positions slightly decreased the activity. It was understood with compound **12**

Table I

***In vitro* antiproliferative effects of compounds 3-12 against A549 and PC-3 cancer cell lines, and cytotoxic effect on NIH3T3 cell lines**

		IC_{50} (µM)		
Compound	Ar	A549 cells	PC-3 cells	NIH3T3 cells
2	-	157.53	154.93	129.49
3	3-fluorophenyl	174.22	96.60	227.19
4	4-chlorophenyl	40.70	73.14	28.45
5	4-methoxyphenyl	22.84	43.42	56.73
6	4-fluorophenyl	35.05	38.46	32.47
7	2-chlorophenyl	297.27	112.63	79.13
8	2-fluorophenyl	146.74	94.62	70.03
9	2-methoxyphenyl	113.87	73.56	81.13
10	3-methoxyphenyl	172.30	152.00	73.92
11	3- chlorophenyl	168.95	156.70	92.58
12	5-ethylfuran-2-yl	126.07	120.07	83.25

Table II

COX-1 and COX-2 enzyme inhibition and the binding energy in the cyclooxygenase active site of synthesized compounds

Compound	% Inhibition		Docking score ΔG (kcal/mol)			
			COX-1			COX-2
	COX-1 (50 µM)	COX-2 (1 µM)	Pocket A (active site)	Pocket B	Gate Pocket of A	
2	n.a.[a]	75.8	n.d.[b]	n.d.	-7.8	-8.5
3	n.a.	60.0	n.d.	-9.8	n.d.	-10.8
4	1.7	88.1	n.d.	-9.0	n.d.	-10.4
5	1.0	77.8	n.d.	-9.1	n.d.	-10.7
6	n.a.	63.2	n.d.	-9.2	n.d.	-10.7
7	6.0	65.3	n.d.	-9.7	n.d.	-9.9
8	6.0	65.6	n.d.	-8.1	n.d.	-10.1
9	5.3	64.8	n.d.	-8.9	n.d.	-10.5
10	n.a.	59.5	n.d.	-8.5	n.d.	-10.9
11	5.5	65.8	n.d.	-9.3	n.d.	-10.8
12	6.0	70.0	n.d.	-8.8	n.d.	-9.8
Celecoxib (1 µM)	27.9	78.7	-10.5	-	-	-12.0

[a]Not active, [b]Not detected

that the presence of furylmethylene group instead of benzylidene groups also preserved the inhibitory effect. Compounds **4** and **6**, which were found to have anti-cancer activity, also showed inhibition of COX-2 but were ineffective against COX-1 at 50 µM. However, it could be said that there was a complete correlation between COX-2 inhibitory properties and anti-cancer activities for all synthesized compounds.

Molecular docking studies

Computer-assisted molecular docking techniques have been utilized to estimate the possible inhibitory activities and mechanism of binding to COX enzymes of the synthesized compounds. The interactions of these compounds with the active site of both COX enzymes were investigated to support enzyme inhibition studies, and celecoxib was used as a reference ligand. Binding energy was obtained from the docking studies of compounds **2**-**12** by using AutoDock Vina software (http://autodock.scripps.edu). While the binding energies of all synthesized compounds show high affinity to COX-2 active site, not detected bind to the the active site pocket of COX-1 as compatible with the *in vitro* tests in Table II. When the docked conformations of compounds **2**-**12** in COX-1 in more detail were analyzed, was noticed that bind to different pockets of COX-1 with better affinities, instead of its active site. Using an online tool, CavityPlus web server, four different binding sites A, B, C, and D were identified for COX-1 protein structure (PDB code: 1Q4G) (Figure 2). Compounds **3**-**12** were found to interact with pocket B at various binding energies while not interacting with the active site identified as pocket A. Similarly, compound **2** also interacted with the gate pocket of A. These findings support the fact that the synthesized compounds have less than 6% inhibition at 50 µM dose. The docking scores are given in Table II.

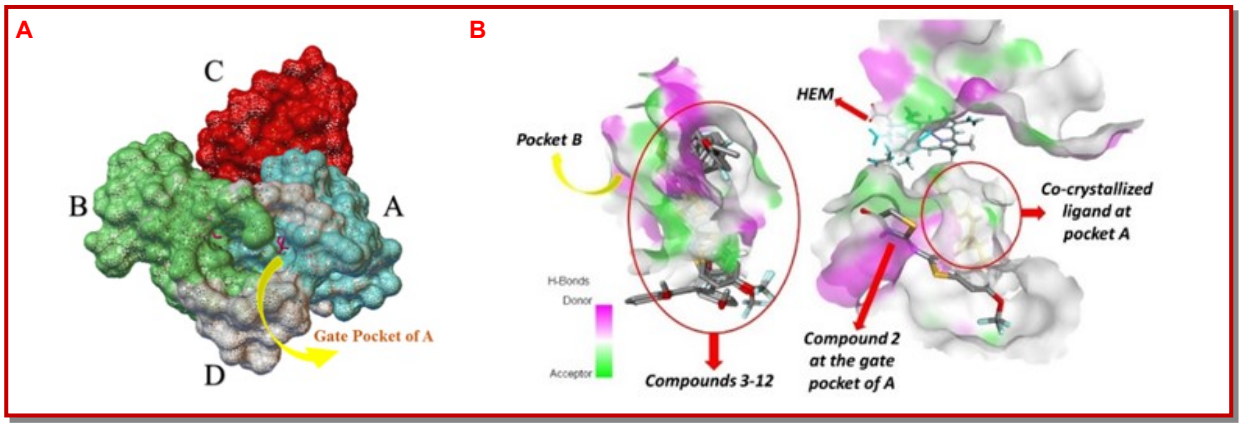


Figure 2: The predicted druggable binding sites of COX-1 according to CavityPlus web server. Pocket A is the cyclooxygenase active site and pocket C is peroxidase binding site (red colored 'A'). Interactions of alternative bindings of compounds **2**-**12** with the binding sites of COX-1 enzyme (red colored 'B')

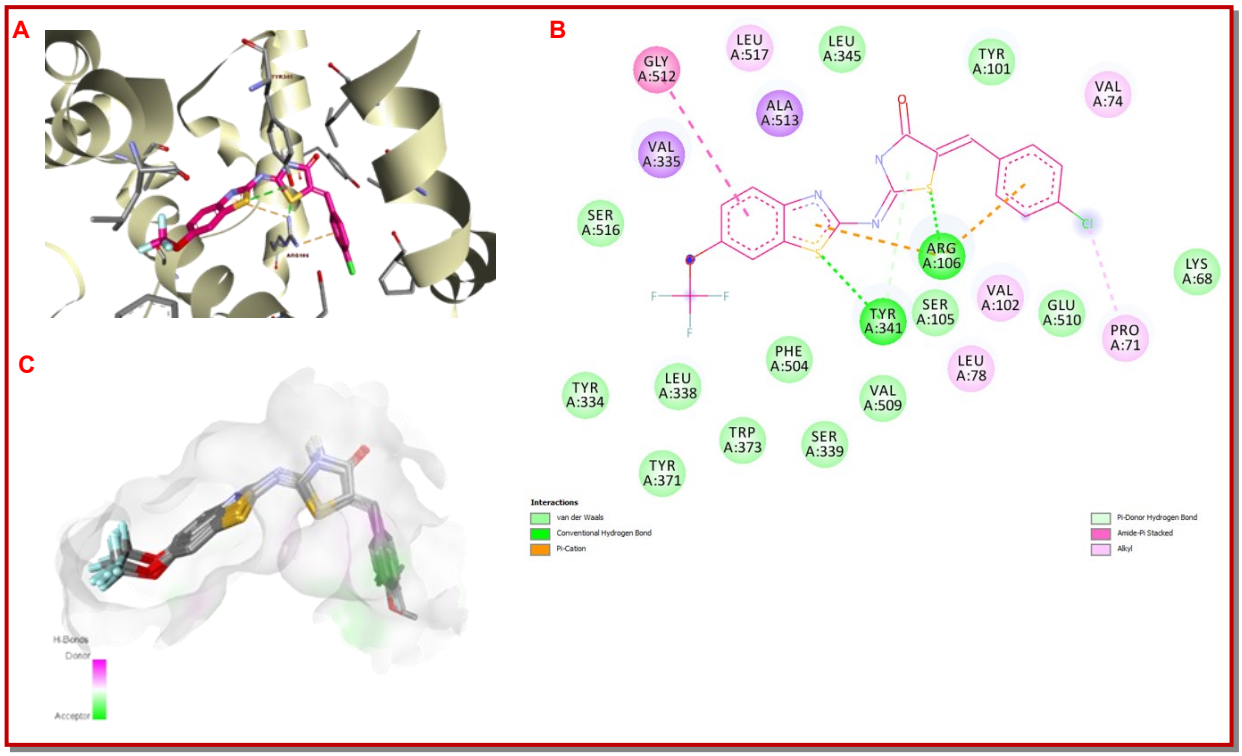


Figure 3: 3D diagram of binding orientation of compound **4** in cyclooxygenase active site of COX-2 (A). 2D diagram of the interaction of the compound 4 with active site of COX-2 enzyme (B). The binding pose of compounds **3-12** at active site of COX-2 enzyme (C)

As the most effective and selective COX-2 inhibitor, the binding orientation and interactions of compound **4** at the COX-2 active site are shown in Figures 3A and 3B. When the interactions of compound **4** were examined, a hydrogen bond between the sulfur atom of the benzothiazole ring and the side chain of Tyr341 was detected. Moreover, two pi-cation interactions were observed between Arg106 and both benzothiazole and arylmethylene aromatic rings. The pi-stacked interaction of Gly512 with benzothiazole ring is thought to promote the inhibitory effect. When the interactions of the synthesized 5-arylmethylene-2-imino-1,3-thiazolidin-4-one derivatives with COX-2 active site were examined, all compounds exhibited similar binding poses with a range of -9.8 via -10.9 kcal/mol binding energy (Figure 3C).

In silico ADMET studies

Oral bioavailability plays an important role in the development of bioactive molecules such as therapeutic agents. For this reason, a computational study was carried out for the prediction of ADME properties by determining the lipophilicity of the molecules, topological polar surface area (TPSA), absorbance (%ABS), simple molecular descriptors using the Lipinski's rule of five (Table III). The calculated number of hydrogen bond donors was 1 for all of the compounds, whilst the number of hydrogen bond acceptors varied from 5 to 6. These new compounds, which can show absorption in the range of 64.06-81.59%, have a log P value of less than 5, except compounds **4**, **7**, and **11**. Investigation of Lipinski parameters of the synthesized compounds showed that all heterocyclic derivatives of riluzole, compounds **2**-**12**, might be considered drug-like candidates for novel COX-2 inhibitors, as they obeyed the rule of five without violating more than one of them.

Swissadme server was also used for boiled-egg plot denoted (Figure 4). In the graphic yellow area was defined as well penetrated within the brain with good intestinal absorption. The white area was defined as intestinal absorption, while the gray area indicated poor intestinal absorption. None of the synthesized compounds, including compound **2**, exhibited the potential to cross the blood-brain barrier, whereas only compound **2** was predicted to be passively absorbed by the gastrointestinal tract. According to the estimated toxicity profiles of the compounds, only compound **12** showed mutagenic effects, while both compound **7** and compound **12** showed tumorigenic profiles. None of the compounds showed irritant properties besides the low reproductive effect detected for compound **8**.

Discussion

In this study, 10 new benzothiazole-thiazolidone conjugate compounds were synthesized based on riluzole, a 2-aminobenzothiazole derivative. In the reactions that

Table III

Predicted ADME, Lipinski parameters and molecular properties of the synthesized compounds 2-12

Compound	M.W. (g/mol)	cLogP	H-Acceptors	H-Donors	%ABS	TPSA	Drug-likeness
2	333.314	2.72	5	1	68.5936	117.12	-6.4621
3	439.412	4.71	5	1	68.5936	117.12	-5.7999
4	455.867	5.44	5	1	68.5936	117.12	-5.1221
5	451.448	4.76	6	1	65.4093	126.35	-5.1177
6	439.412	4.93	5	1	68.5936	117.12	-5.1839
7	455.867	5.44	5	1	68.5936	117.12	-5.1221
8	439.412	4.93	5	1	68.5936	117.12	-5.1839
9	451.448	4.76	6	1	65.4093	126.35	-5.1177
10	451.448	4.76	6	1	65.4093	126.35	-5.1177
11	455.867	5.44	5	1	68.5936	117.12	-5.1221
12	439.437	4.83	6	1	64.0603	130.26	-3.8862
Riluzole	234.201	2.88	3	1	82.6489	76.38	-8.5405

%ABS: Percentage of absorption - calculated according to the equation %ABS = 109-0.345 x TPSA (Zhao et al., 2002); TPSA: Topological polar surface area

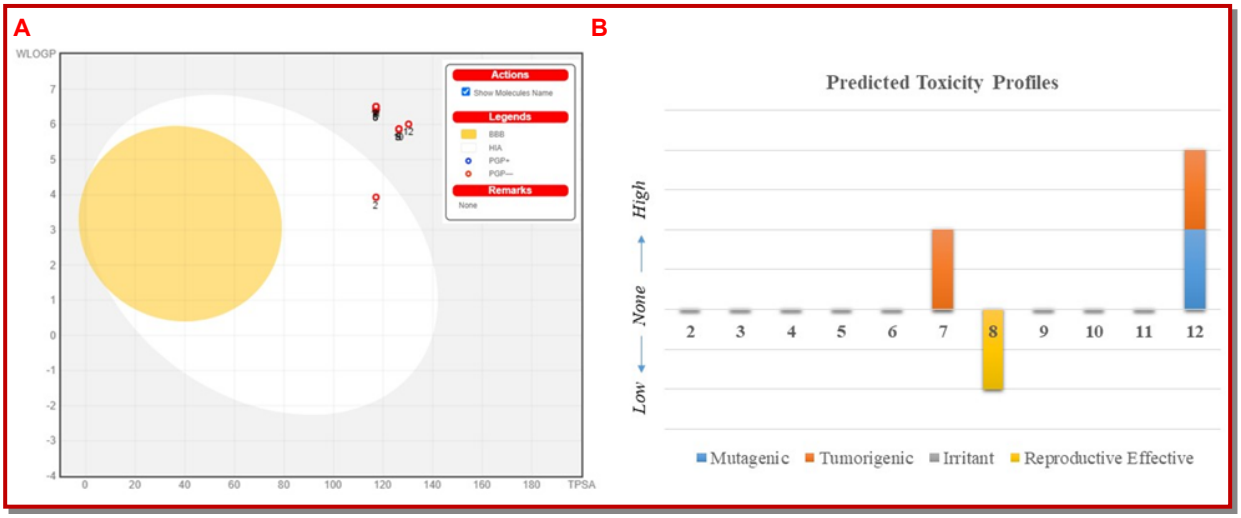
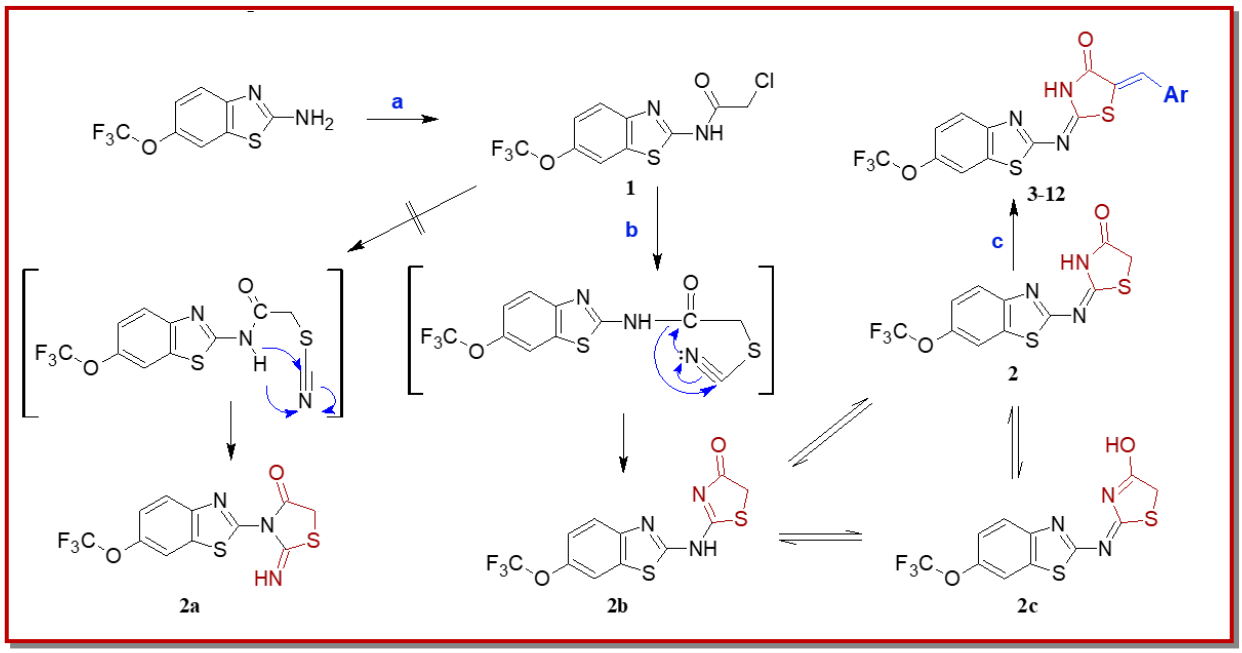


Figure 4: Graphical distribution of compounds according to the boiled-egg predictive model (A), and their toxicity profiles (B)

took place in three steps, first compound **1** was obtained by chloroacetylation, and then compound **2** was obtained by the thiazolidone ring closure. The proposed mechanism of cyclocondensation of compound **1** to compound **2** and its hypothetically tautomeric forms (**2**, **2a-c**) are shown in Scheme 1. The absence of a band that can be attributed to the OH group in the FTIR spectrum, the detection of the NH group of the lactam structure around 3140 cm^{-1}, the detection of the C=O group in the range of 1726-1699 cm^{-1} confirmed that the ring is in the thiazolidinone structure (tautomeric forms **2**, **2a** or **2b**) (Vicini et al., 2008; Havrylyuk et al., 2010). The g-lactam structure of **2** was confirmed based on the ^1H NMR spectrum which exhibited an NH proton appeared at 12.37 ppm as the previously reported NH resonances appeared around 12-13 ppm. These findings showed that this proton corresponds to a lactam proton of tautomeric form **2** but not to an imine proton of tautomeric form **2a** which is expected to appear at a much higher field at around 9.0 ppm (Abdellatif et al., 2016; Kulabaş et al., 2017; Türe et al., 2021). In previous studies, it has been reported that these two tautomers cannot be isolated separately experimentally, since the minimum energy change was detected between imino (**2**) ⇋ amino (**2b**) tautomers, one of the tautomeric forms of the 1,3-thiazolidinone ring (Nowaczyk et al., 2014). The presence of two strong carbonyl bands (1726 cm^{-1} and 1699 cm^{-1}) in the FTIR spectrum of compound **2** can be explained by the presence of two tautomeric forms which 2-amino-4-thiazolinone (**2b**) and the 2-imino-4-thiazolidinone (**2**) structure. Although the presence of both 2-amino-4-thiazolinone (**2b**) and the 2-imino-4-thiazolidinone tautometic forms (**2**) in solid form is detected in the FTIR spectrum, it is observed that only the 2-imino-4-thiazolidinone form (**2**) is in solution. Additionally, the singlet appeared at 4.09 ppm corresponds to the active methylene $-CH_2-$ group, supporting the cyclization of the 4-thiazolidinone ring (Küçükgüzel



Scheme 1: General procedure for the synthesis of compounds **3-12**. Reagents and conditions: a) Cl-CO-CH_2-Cl, TEA, DCM, reflux; b) NH_4SCN, EtOH, reflux; c) Ar-CH=O, NaOMe, MeOH, reflux. **3** (Ar: 3-fluorophenyl); **4** (Ar: 4-chlorophenyl); **5** (Ar: 4-methoxyphenyl); **6** (Ar: 4-fluorophenyl); **7** (Ar: 2-chlorophenyl); **8** (Ar: 2-fluorophenyl); **9** (Ar: 2-methoxyphenyl); **10** (Ar: 3-methoxyphenyl); **11** (Ar: 3-chlorophenyl); **12** (Ar: 5-ethylfuran-2-yl)

et al., 2002; Bekhit et al., 2008).

Target compounds **3-12** were obtained from the Knoevenagel reaction of compound **2** with several aldehydes. Although it has been reported in many studies that this reaction occurs in the presence of sodium acetate/acetic acid (Jung et al., 2011; Rashid et al., 2014), the sodium methoxide/methanol medium was preferred as reported in previous studies (Küçükgüzel et al., 2013; Çakır et al., 2015; Türe et al., 2021). The findings indicating that the target compounds **3-12** were obtained are the detection of an additional four aromatic protons belonging to benzylidene structures and methine proton (=CH-Ar) between 6.46-7.77 ppm and 7.46-7.80 ppm, respectively. Moreover, -CH_2- protons which belong to the thiazolidinone ring of com-pound **2**, at 4.09 ppm were not observed in the spectrum of compounds **3-12**. When the *E* and *Z* geo-metric isomers of the C=C exocyclic double bond in the 5th position of benzylidene derivatives were examined, the =CH-Ar proton of compounds **3-12** was detected between 7.46-7.80 ppm. In many previously reported studies, the =CH-Ar proton detected in the range of 6.90-7.97 ppm was reported as the *Z* isomer, while the related proton for *E* isomer was reported to be detected in the higher energy area (about 6 ppm) (Havrylyuk et al., 2010; Mushtaque et al., 2012; Ottanà et al., 2005; Vicini et al., 2008, 2006). This finding confirms that the target compounds **3-12** prefer the (*Z*)-isomer due to the -C=C- bond.

Anti-cancer and anti-inflammatory effects of hybrid compounds containing thiazole, thiadiazole, and benzothiazole rings and 4-thiazolidinone rings in the same structure have been reported in the literature (Havrylyuk et al., 2010). Therefore, the cytotoxic effects and COX-1/2 enzyme inhibition potential of the synthesized compounds were examined on A549 and PC-3 cells. Among compounds **2-12** whose anti-cancer effects were examined by the MTT technique, only compounds **4-6** showed higher activity on A549 and PC -3 cells compared to the others. However, when the effects of these compounds on cancer cells were compared to their effects on NIH3T3 cells, which are healthy, it was observed that their selectivity indexes were low. Therefore, further mechanistic studies on these compounds were not deemed necessary.

Looking at the structure-activity relationships, it was understood that the benzylidene group at the 5-position of the 1,3-thiazolidin-4-one ring represented by compound **2** was necessary for activity, but no effect was observed with 2- or 3-substitution in the benzylidene group. The highest cytotoxic effect was achieved in the presence of halogen or methoxy at the 4-position of the benzylidene group. There is widespread knowledge that the COX-2 enzyme has a crucial role in various solid tumors. Therefore, this enzyme is targeted by medicinal chemists in anti-cancer drug development studies (Şenkardeş et al., 2020).

In the inhibition studies conducted on COX-1 and COX-2 enzymes, it was noteworthy that compounds **4-6**, which had the highest antiproliferative activity, also

selectively inhibited the COX-2 enzyme at high rates. Compound **5** had a higher effect than celecoxib used as a reference, with an inhibition value of 88.1% at 1 µM concentration. Although there was no exact correlation between the COX-1 and COX-2 enzyme inhibitions of the studied compounds and their docking scores, it was observed that the compounds showing high binding scores mostly selectively inhibited the COX-2 enzyme. A modeling study focusing on compound **4**, which is the most effective and selective COX-2 inhibitor, showed that binding of both benzothiazole and thiazolidone rings with sulfur atoms as hydrogen bond acceptors was effective in binding the inhibitors to the COX-2 active site. In addition, benzothiazole and benzylidene rings were bound to the COX-2 enzyme by pi-cation interaction.

Lipinski's rule of five states that most drug-like molecules with good membrane permeability should have log P ≤5, formula weight ≤500, number of hydrogen bond acceptors (nON) ≤10 and number of hydrogen bond donors (nOHNH) ≤5. Violation of more than one rule indicates problems with bioavailability. Polar surface area, together with lipophilicity, is an important property of a molecule in transport across biological membranes. Too high TPSA values give rise to poor bioavailability and absorption of a drug (Lipinski et al., 1997; Veber et al., 2002). An increase in hydrogen bond acceptors resulted in a slight decrease in the calculated %ABS for compounds **5**, **9**, **10**, and **12**.

There were no direct correlations observed between simple molecular properties such as log P and anticancer or COX-1/2 inhibitory activity. Nevertheless, it was notable that compound **4**, the most active COX-2 inhibitor, had the highest Log P value among the series.

Conclusion

Ten novel benzothiazole-thiazolidinone hybrids **3**-**12**, were synthesized and characterized in detail by the use of IR, ^{1}H NMR, HMBC, and MS spectral techniques. Compounds **4**-**6** containing 4-substitution at arylmethylene moiety exhibited the highest cytotoxic activity towards A549 and PC-3 cell lines, whereas all synthesized compounds inhibited COX-2 enzyme selectively at 1 µM. Molecular docking studies reveal the binding interactions and justify selective inhibition of COX-2 enzyme by compounds **3**-**12**.

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Ethical Issue

Cell lines derived from expansion of primary cell cultures *in vitro* are not relevant material, as all of the original cells will have divided and so the cell line has been created outside of the human body. The storage and use of cell lines created from primary human tissue, for research purposes, does not require an ethical approval.

Conflict of Interest

Authors declare no conflict of interest

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