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Synthesis, Docking Study & Anticancer Evaluation of Novel 3-Phenyl-2-(3-aryl-1-phenyl-1*H*-pyrazol-4-yl)thiazolidin-4-ones

Sumit Bansal [*1], Sharad Kumar [1], Anil Choudhary [2], Amit Tiwari [2], Vaibhav Aggarwal [3], Alex Joseph [1*]

[1] *Department of Pharmaceutical Chemistry, Manipal College of Pharmaceutical Sciences, Manipal-576104, India*

[2] *Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal-576104, India*

[3] *Department of Pharmacy, Jaypee University of Information Technology, Solan, Himachal Pradesh,India*

Address for Correspondance: bansalsumit50@gmail.com

ABSTRACT: On account of the reported anticancer activity of pyrazoles and thiazolidin-4-ones, we have designed and synthesized a series of novel 3-phenyl-2-(3-aryl-1-phenyl-1*H*-pyrazol-4-yl)thiazolidin-4-ones and investigated their COX-1 and COX-2 inhibition potential and also their *in vitro* cytotoxic activity by MTT assay. Amongst the tested compounds, the compound **3e** was found to be most potent COX-2 inhibitor with IC_{50} value of 4.4 µM and showed promising anticancer potential with IC_{50} values of 14.78 µg/ml and 12.45 µg/ml against HT-29 and COLO-205 cancer cells, respectively, compared to 5-FU as a standard drug. The newly synthesized compounds were characterized by melting point, IR, ^{1}H NMR, and MS analysis. Docking studies were performed on the active site of COX-1 and COX-2 enzymes.

KEYWORDS: Thiazolidin-4-ones; COX-2; Colorectal Cancer; Docking.

INTRODUCTION

Colorectal cancer (CRC) is the second most common cancer in women and the third in men worldwide and its chemoprevention currently is an area of intense investigation. Strategies for improving survival and reducing mortality from this disease are focused on prevention, early detection and improvement of current therapy [1]. Numerous studies have demonstrated the overexpression of cyclooxygenase-2 (COX-2) in solid malignancies including colon, breast, prostate, liver and lung cancer and the increased expression of COX-2 also seems to prevent cancer cells from undergoing apoptotic cell death [2,3,4]. Epidemiological, clinical, and preclinical investigations have provided convincing evidence that COX-2 inhibitors could induce apoptosis and act as chemopreventive agent [5,6].

Recent studies indicating the role of COX-2 inhibition as a tool to treat and prevent colorectal cancer still continues to attract researchers to develop clinically potent and safe COX-2 inhibitors [1]. Moreover, several studies demonstrated that selective COX-2 inhibitors (coxibs) such as celecoxib showed pro-apoptotic activity by suppressing the proliferation of various cells [7]. Various molecules having pyrazole nucleus have been synthesized and reported as selective COX-2 inhibitors [8]. Moreover, substituted 1,3-diarylpyrazoles are found to be interesting molecular scaffolds for designing selective COX-2 inhibitors and anticancer agents [9]. Thiazolidinone derivatives have been investigated for a range of pharmacologic indications such as anti-inflammatory [10], antiproliferative [11],antimicrobial [12], antitubercular [13], and anticonvulsant [14] activities but their anticancer effects have been less widely documented. Gududuru*et al.* [15], found that, thiazolidin-4-one moiety act as a biomimetic replacement for the phosphate group.

The present work is an extension of our ongoing efforts towards developing promising biologically active agents using

a hybrid pharmacophore approach [11,16]. Considering above observations, we decided to synthesize hybrids compounds by linking main structural unit of thiazolidin-4-one ring system with 1,3-diarylpyrazoles and investigated their COX inhibition potential and also *in vitro* anticancer effects on colorectal cancer cell lines.

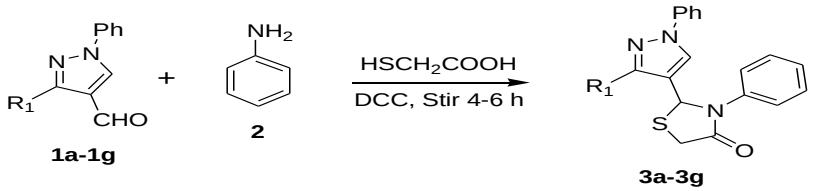
MATERIALS & METHODS

1. Chemistry

All the chemicals were purchased from Aldrich, Merck, and Spectrochem. The purity of all compounds was established by a single spot on the pre-coated silica gel plates (TLC silica gel F_{254}, Merck, Germany). The TLC solvent system used was hexane: ethylacetate (4:6). Iodine vapour was used as developing reagent. Infrared spectra were recorded on a Shimadzu FTIR-8310 (Shimadzu, Japan) using potassium bromide discs. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrophotometer (Bruker, USA). Chemical shifts are reported in parts per million (δ) units relative to an internal standard of tetramethylsilane. Coupling constants are given in Hz and the relative peak areas were in agreement with all assignments. Melting points were determined on a capillary melting point apparatus (Shital Scientific Industries, India) and are uncorrected.

2. Synthesis of 3-phenyl-2-(3-aryl-1-phenyl-1H-pyrazol-4-yl)thiazolidin-4-ones

A mixture of appropriate formylpyrazole (1.2 mmol) (**1a-1g**) and aniline (1.0 mmol) (**2**) were stirred in tetrahydrofuran under ice-cold condition for 5 min, followed by addition of the thioglycolic acid (2.0 mmol). After 5 min, dicyclohexylcarbodiimide (DCC, 1.2 mmol) was added to the reaction mixture at 0 °C and the reaction mixture was stirred for an additional 4-6 h at room temperature to complete the reaction [11]. Progress of the reaction was monitored by TLC using hexane: ethylacetate (4:6) as mobile phase. The precipitated dicyclohexylurea was filtered off; the filtrate was concentrated to dryness under reduced pressure. The extraction was done with water and ethylacetate. The organic layer was successively washed with 5% aqueous sodium hydrogen carbonate and sodium chloride. Finally organic layer was dried over anhydrous sodium sulphate. The crude solid obtained on evaporation of the solvent under reduced pressure was subjected for column chromatography using silica gel 100-200 mesh using hexane: ethyl acetate (5:5) as the solvent system and further recrystallized in ethanol to yield pure product.



1a: $R_1 = C_6H_5$
1b: $R_1 = 4\text{-}CH_3C_6H_4$
1c: $R_1 = 4\text{-}OCH_3C_6H_4$
1d: $R_1 = 4\text{-}FC_6H_4$
1e: $R_1 = 4\text{-}ClC_6H_4$
1f: $R_1 = 4\text{-}BrC_6H_4$
1g: $R_1 = 4\text{-}NO_2C_6H_4$

Scheme 1. Synthesis of novel 3-phenyl-2-(3-aryl-1-phenyl-1*H*-pyrazol-4-yl)thiazolidin-4-ones

a) 3-phenyl-2-(1,3-diphenyl-1H-pyrazol-4-yl)thiazolidin-4-one (3a): yield 74%, mp160-162°C. IR (KBR, cm^{-1}): 1633 (C=O of thiazolidinone ring), 1573 (C=C), 1401 (CN), 642 (CS). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.47 (s, 1H, =CH- of pyrazole ring), 7.33-7.67 (m, 15H, Ar-H), 5.91 (s, 1H, -CH- of thiazolidinone ring), 3.94-3.98 (d, *J* = 16 Hz, 1H, 5-H_a), 3.67-3.71 (d, *J* = 16 Hz, 1H, 5-H_b). ESI-MS (*m/z*): 398 ($C_{24}H_{19}N_3OS$, $[M+H]^+$).

b) 3-phenyl-2-(1-phenyl-3-p-tolyl-1H-pyrazol-4-yl)thiazolidin-4-one, (3b): yield 67%, mp169-170°C. IR (KBR, cm^{-1}): 1639 (C=O of thiazolidinone ring), 1576 (C=C), 1399 (CN), 639 (CS).^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.39 (s, 1H, =CH- of pyrazole ring), 7.15-7.64 (m, 15H, Ar-H), 5.82 (s, 1H, -CH- of thiazolidinone ring), 3.87-3.91 (d, *J* = 16 Hz, 1H, 5-H_a), 3.65-3.69 (d, *J* = 16 Hz, 1H, 5-H_b), 2.42 (s, 3H, -CH_3). ESI-MS (*m/z*): 412 ($C_{25}H_{21}N_3OS$, $[M+H]^+$).

c) 2-(3-(4-methoxyphenyl)-1-phenyl-1H-pyrazol-4-yl)-3-phenylthiazolidin-4-one (3c): yield 69%, mp 151-152°C. IR (KBR, cm^{-1}): 1630 (C=O of thiazolidinone ring), 1572 (C=C), 1395 (CN), 643 (CS). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.45 (s, 1H, =CH- of pyrazole ring), 6.94-7.69 (m, 15H, Ar-H), 5.89 (s, 1H, -CH- of thiazolidinone ring), 3.95-3.99 (d, *J* = 16 Hz, 1H, 5-H_a), 3.77-3.80 (d, *J* = 12 Hz, 1H, 5-H_b), 3.77 (s, 3H, -OCH_3). ESI-MS (m/z): 428 ($C_{25}H_{21}N_3O_2S$, $[M+H]^+$).

d) 2-(3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl)-3-phenylthiazolidin-4-one (3d): yield 65%, mp 175-176°C. IR (KBR, cm^{-1}): 1635 (C=O of thiazolidinone ring), 1573 (C=C), 1405 (CN), 649 (CS). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.47 (s, 1H, =CH- of pyrazole ring),

7.07-7.77 (m, 15H, Ar-H), 5.85 (s, 1H, -CH- of thiazolidinone ring), 3.93-3.97 (d, *J* = 16 Hz, 1H, 5-H_a), 3.65-3.68 (d, *J* = 12 Hz, 1H, 5-H_b).ESI-MS (*m/z*): 416 ($C_{24}H_{18}FN_3OS$, $[M+H]^+$).

e) 2-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)-3-phenylthiazolidin-4-one (3e): yield 70%, mp 183-184°C. IR (KBR, cm^{-1}): 1633 (C=O of thiazolidinone ring), 1569 (C=C), 1395 (CN), 635 (CS). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.55 (s, 1H, =CH- of pyrazole ring), 7.32-7.78 (m, 15H, Ar-H), 5.89 (s, 1H, -CH- of thiazolidinone ring), 3.85-3.89 (d, *J* = 16 Hz, 1H, 5-H_a), 3.62-3.65 (d, *J* = 12 Hz, 1H, 5-H_b). ESI-MS (*m/z*): 432 ($C_{24}H_{18}ClN_3OS$, $[M+H]^+$).

f) 2-(3-(4-bromophenyl)-1-phenyl-1H-pyrazol-4-yl)-3-phenylthiazolidin-4-one (3f): yield 62%, mp 201-201°C. IR (KBR, cm^{-1}): 1629 (C=O of thiazolidinone ring), 1571 (C=C), 1408 (CN), 648 (CS). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.38 (s, 1H, =CH- of pyrazole ring), 7.45-7.79 (m, 15H, Ar-H), 5.87 (s, 1H, -CH- of thiazolidinone ring), 3.89-3.93 (d, *J* = 16 Hz, 1H, 5-H_a), 3.61-3.65 (d, *J* = 16 Hz, 1H, 5-H_b). ESI-MS (*m/z*): 477 ($C_{24}H_{18}BrN_3OS$, $[M+H]^+$).

g) 2-(3-(4-nitrophenyl)-1-phenyl-1H-pyrazol-4-yl)-3-phenylthiazolidin-4-one (3g): yield 58%, mp 212-213 °C. IR (KBR, cm^{-1}): 1643 (C=O of thiazolidinone ring), 1572 (C=C), 1392 (CN), 639 (CS). ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 8.52 (s, 1H, =CH- of pyrazole ring), 7.31-7.79 (m, 15H, Ar-H), 5.89 (s, 1H, -CH- of thiazolidinone ring), 3.94-3.96 (d, *J* = 16 Hz, 1H, 5-H_a), 3.68-3.72 (d, *J* = 16 Hz, 1H, 5-H_b). ESI-MS (*m/z*): 445 ($C_{24}H_{18}N_4O_3S$, $[M+H]^+$).

3. COX inhibition assay [17]

COX-1 and COX-2 inhibition activities of tested compounds were determined by using a COX Fluorescent Inhibitor Screening Assay Kit (catalogue no. 700100, Cayman Chemical, Ann Arbor, MI) consisting ovine COX-1 and human recombinant COX-2 enzymes. The minimum volume of DMSO was used to prepare stock solutions of tested compounds. A 10 µl of test solutions (0.01, 0.1, 1, 10, 50, and 100 µM) were added to 960 µl supplied buffer solution (0.1 M Tris-HCl pH 8.0 containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 µL) enzyme in the presence of 10 µl heme and 10 µl fluorometric substrate ADHP (10-acetyl-3,7-dihydroxyphenoxazine). These solutions were kept at 37 °C for 5 min, 10 µl of arachidonic acid solution (100 µM) was added and the COX reaction was stopped by the addition of 50 µl of 1 M HCl after 2 min. Fluorescence of resorufin, produced by the reaction between PGG2 and ADHP, were measured with an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The intensity of this fluorescence is proportional to the amount of resorufin, which is proportional to the amount of PGG2 present in the well during the incubation. Percentage inhibition was calculated by comparison with control value (no inhibitor). The IC_{50} values of tested compounds against COX-1 and COX-2 (µM) were calculated from the concentration inhibition response curve (triplicate determinations).

4. In vitro cytotoxic study (MTT assay) [18]

In vitro cytotoxicity of synthesized compounds was evaluated by MTT assay. In brief, exponentially growing human colon cancer cell lines (HT-29 and COLO-205)were plated in 96-wellplates (10^4 cells/well, in 100 µl of media) and incubated for 24 h to obtain 60-80% confluency. The test compounds were solubilized in 50 µl DMSO and were further diluted with media so as to get DMSO concentration less than 0.25%. The cells were then exposed to different concentration of test compounds (500, 250, 125, 62.5, 31.25, 15.62, 7.81 µg/ml) in the volume of 100 µl/well. The control wells received only cells containing media and 0.25% DMSO. 5-FU was used as positive control. Media was removed after 48 h, and 100 µl MTT reagent (1 mg/ml) was added to cell cultures, and then kept for incubation at 37 °C for 4 h. The viable cells developed formazan complex; was solubilized by addition of 100 µl DMSO. The plates were placed on micro-vibrator for 5 min. The absorbance was recorded on BIOTEK EL X800-MS microtiter plate reader at 540 nm and percentage cytotoxicity was calculated as (control-test/control) × 100.

5. Molecular docking studies [19]

3D Crystal structures of COX-1 and COX-2 were obtained from Protein Data Bank (PDB ID: 1Q4G and 1CX2, respectively). Ligands to be docked were prepared in PDB format using CS ChemDraw Ultra 8.0. Molecular docking study was carried out with AutoDock tools 1.5.4. Polar hydrogens were added to the target proteins and Gasteiger charges were allotted. Nonpolar hydrogens were merged and partial charges added to their parent carbon atoms. The grid boxes with dimensions 40 Å × 40 Å × 40 Å and center x = 22, y = 35, z = 210 for 1Q4G and dimensions 40 Å × 40 Å × 40 Å and center x = 25, y = 23, z = 19 for 1CX2, were defined as search space. The Lamarckian genetic algorithm (LGA) was used to find out conformations of lowest binding energy. Results of molecular docking studies were expressed as estimated free energy of binding in kcal/mol.

RESULTS & DISCUSSION

Chemistry

The synthetic strategies to obtain the target compounds are depicted in Scheme 1. The targeted compounds were synthesized by stirring 1,3-diarylpyrazole-4-carbaldehyde derivatives (**1a-1g**) with aniline (**2**) in the presence of thioglycolic acid and DCC in THF. All the final compounds were purified by column chromatography using hexane: ethyl acetate (5:5) as the solvent system and further recrystallized in ethanol to yield pure product. All the synthesized title compounds (**3a-3g**) were well characterized by melting point, IR, ^{1}H-NMR, and Mass spectroscopy.

IR spectra of all pyrazolyl substituted thiazolidin-4-ones showed C=O stretching in the range of 1629-1643 cm^{-1}. Other characteristic peaks C=C, C-N, and C-S appeared at 1569-

1576, 1392-1408, and 635-649 cm^{-1}, respectively. In ^{1}H-NMR spectra, formation of thiazolidinone ring was confirmed by the presence of two doublet peaks in the range of 3.61-3.99 ppm (J = 12-16 Hz) equivalent to two methylene protons in thiazolidinone nucleus. The chemical shift for =C-H- proton of pyrazole ring was observed as a singlet between 8.38-8.55 ppm while peak for -CH- of thiazolidinone ring was appeared as a singlet between 5.82-5.89 ppm. All aromatic protons were appeared as multiplet in the range of 6.94-7.79 ppm. All the final products showed molecular ion peak corresponding to their mass in ESI-MS spectra. Finally the elemental analysis results were within ±0.4% of the theoretical values assuring the purity of the final products for bioactivity studies.

Table 1 Data of the *in vitro* COX-1/COX-2 enzyme inhibition assay of the synthesized compounds

Compound	IC_{50} (µM)		Selectiviy Index
	COX-1	COX-2	
3a	>100	56.6	>1.77
3b	>100	6.2	>16.1
3c	>100	>100	>1
3d	>100	12.3	>8.1
3e	>100	4.4	>22.7
3f	>100	>100	>1
3g	>100	>100	>1
Celecoxib	>100	0.26	>384.6
Diclofenac	0.18	2.6	0.07

Table 2 IC_{50} of test compounds towards various cell lines by MTT assay method, after 48 h

Compound	IC_{50} µg/ml±SD[a]	
	HT-29	COLO-205
3a	35.78±1.2	32.88±0.9
3b	17.29±0.8	15.28±0.5
3c	39.85±1.6	38.56±1.5
3d	21.45±1.4	19.68±0.9
3e	14.78±0.6	12.45±0.5
3f	57.67±1.6	56.79±1.7
3g	48.77±1.3	41.90±1.5
5-FU	5.82±0.2	4.71±0.4

[a]Average of three determinations

In vitro cyclooxygenase (COX) inhibition assay

Synthesized 7 compounds and reference drugs celecoxib and diclofenac were evaluated for their inhibitory activities against COX-1 and COX-2 enzymes using an enzyme immunoassay (EIA) kit. The activities of the tested compounds were expressed as IC_{50} (concentration causing 50% enzyme inhibition) and selectivity index (SI values) was calculated by IC_{50} (COX-1)/ IC_{50} (COX-2) (Table 1).

The IC_{50} values of celecoxib on COX-1 and COX-2 were found to be >100 and 0.26 µM, respectively, indicating that celecoxib is a selective COX-2 inhibitor (SI = 384.6) while diclofenac showed non-selectivity towards COX-1 as well as COX-2. Among synthesized compounds, compound **3e** was found to be most potent COX-2 inhibitor with lowest IC_{50} i.e. 4.4µM. Halogen substitution was played significant role in COX-2 inhibition. Chloro substitution was found more favorable towards COX-2 inhibition over fluoro substitution.

However, bromo substitution resulted in complete loss of COX-2 selective inhibition. It was observed that compound possessing electron releasing methyl group (**3b**) resulted in improving the COX-2 inhibitory activity (IC_{50} = 6.2µM).

In vitro cytotoxic studies (MTT assay)

All the synthesized compounds were screened for their *in vitro* growth inhibitory activities against two human cultured cell lines HT-29 and COLO-205 at 100 µg/ml by MTT assay method and the results are expressed as IC_{50} ± SD. Among synthesized compounds, **3b** and **3e** showed maximum percentage cytotoxicity at 100 µg/ml. Further cytotoxicity studies of compound **3b** against HT-29 and COLO-205 cell lines exhibited IC_{50} values at 17.29 and 15.28 µg/ml, respectively, while compound **3e** exhibited IC_{50}; 14.78 and 12.45 µg/ml, respectively. The IC_{50} values of reference drug 5-FU against HT-29 and COLO-205 cells were found to be 5.82 and 4.71 µg/ml respectively (Table 2).

Molecular docking analysis

Designed ligands were docked into the binding sites of COX-1 and COX-2 proteins. Outcomes of molecular docking studies pointed out that all the docked ligands showed higher binding affinity towards COX-2 than COX-1. Series of compounds docked toward COX-2 exhibited estimated free energy of binding ranging between –8.95 and–9.36 kcal/mol, while compounds that were docked into the binding site of COX-1 showed estimated free energy of between –4.32 and–5.90 kcal/mol. Based on the estimated free energy of binding of docked compounds, it can be inferred that docked compounds were more selective towards COX-2 protein than COX-1. Among the ligands docked into the binding site of COX-2, compound **3e** showed lowest estimated free energy of binding of –9.36 kcal/mol, while estimated free energy of binding of marketed drug diclofenac was found to be –9.23 kcal/mol. This difference in estimated free energy of binding between **3e** and diclofenac in terms of COX-2 binding affinity indicates superior binding potential of **3e** towards COX-2 than its counterpart diclofenac.

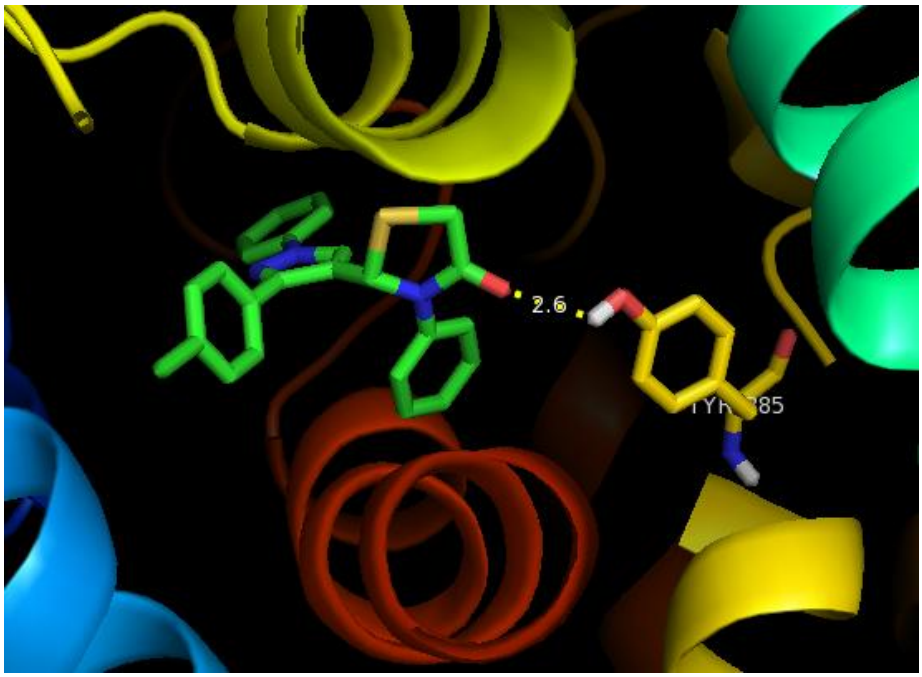


Fig. 1. Stereoview of the docked complex formed by docking of 3e into the active site of COX-2. The amino acid Tyr-385 involved in hydrogen bonding with 3e, is highlighted.

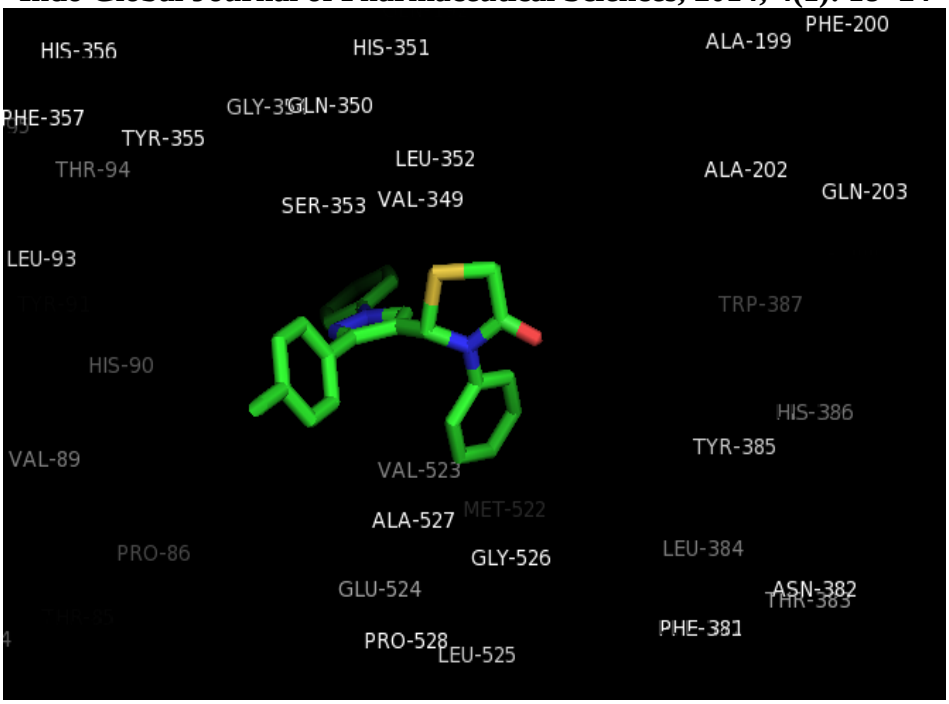


Fig. 2. Molecular docking of 3e in the binding site of COX-2. The amino acid residuesof COX-2 participated in hydrogen, hydrophobic and van der Waal interactions with 3e, are highlighted.

Stereoview of docked structure of **3e** within the binding site of COX-2 protein shows that carbonyl or 4-one function of thiazolidinone moiety was essential for COX-2 inhibitory potency. This carbonyl oxygen of thiazolidin-4-one moiety of **3e** was hydrogen bonded to hydroxyl hydrogen of Tyr-385 (C=O…H−O, 2.6 Å (Fig. 1)). Besides this hydrogen bonding observed between **3e** and COX-2, phenyl ring of thiazolidinone moiety displayed strong hydrophobic interactions with Met-522 and Val-523 residues of the COX-2 (Fig. 2). Phenyl ring of thiazolidin-4-one further showed weak van der Waals and hydrophobic interactions with Leu-384, Leu-525, and Pro-528 residues of the target protein. Phenyl substituted pyrazole scaffold of **3e** interacted with Val-349 and Leu-352 amino acids by means of hydrophobic contacts, while 4-chlorophenyl ring of pyrazole moiety exhibited hydrophobic and van der Waals interactions with Val-89 and Leu-93 residues of the COX-2 (Fig. 2).

CONCLUSION

This study has demonstrated that 3-phenyl-2-(3-aryl-1-phenyl-1H-pyrazol-4-yl)thiazolidin-4-ones bearing a variety of substituents in the aromatic part showed COX-2 Inhibitory potential with potent anticancer activities against colon cancer cell lines. Excellent correlation existed between docking score and COX inhibitory potency of screened drugs. Among synthesized compounds, **3e** showed highest COX-2 inhibition with potent anticancer activity. Docking studies revealed higher binding affinity of all compounds (**3a-3g**) towards COX-2 than COX-1 and concluded that carbonyl oxygen are crucial for COX-2 inhibition. However, COX-2 inhibitory potential and selectivity index towards COX-2 of all compounds are not well as standard drug celecoxib. There is a need to explore this hybrid pharmacophore to optimize COX-2 inhibition in future.

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