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RESEARCH PAPER

Computational Modeling, Docking, Synthesis, Characterization, and in vitro Cyclooxygenase Inhibitory Activity of Some Novel Non-Steroidal Anti-inflammatory Prodrugs

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A B S T R A C T:

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most prescribed analgesic and anti-inflammatory drugs. However, inhibition of cyclooxygenase1 and acidic groups such as carboxylic groups in most NSAIDs cause gastrointestinal (GI) side effects. Therefore, masking the acidic groups till it pass through the GI tract will decrease the direct GI side effects and because *N*-(2,6-dimethylphenyl)-acetamide **1** also has anti-inflammatory activity so the synthesized ester prodrugs might act as mutual prodrugs.

2-Chloro-*N*-(2,6-dimethylphenyl)-acetamide **1** was utilized to synthesize ester prodrug of various NSAIDs **2a-e**. The 2-Chloro-*N*-(2,6-dimethylphenyl)-acetamide **1** undergo substitution reaction at α position with various sodium carboxylate of NSAIDs **2a-e** in DMSO. The constitution of the newly synthesized ester prodrugs of NSAIDs **3a-e** had been confirmed depending on their IR, ^{1}H and ^{13}C-NMR spectral analysis. The synthesized ester prodrugs **3a-e** were screened for their *in vitro* inhibitory activities of COX-1 as well as COX-2 however, their COX inhibition activity increased compared with their starting **1** and **2a-e**.

Physicochemical properties and "Lipinski's rule of five" were assessed for compounds **3a-e**, and they all satisfied the rule. Furthermore molecular docking for compounds **3a-e** into COX-1 and COX-2 was done, in which they showed binding free energies ΔG_b in the range of (-8.9 to -9.8 kcal/mol) when docked into COX-1 and (-10.4 to -12.4 kcal/mol) into COX-2 enzymes.

KEY WORDS: NSAIDs; docking; prodrug; cyclooxygenase; acetamide.
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1. INTRODUCTION

The fact that non-steroidal anti-inflammatory drugs (NSAIDs) are considered to be among the most commonly prescribed medications is attributed to their broad range of medical indications, as they can be used as analgesic, anti-inflammatory, antirheumatic and antipyretic agents (Al-Turki *et al.*, 2017). Despite being commonly used; NSAIDs were shown to have extensive adverse effects, including gastrointestinal (GI) complications, hypertension, edema, renal disease, and cardiovascular risk. The most frequent among these are the gastrointestinal adverse effects such as dyspepsia, ulcers, bleeding and other associated GI complications (Pountos *et al.*, 2011). Hence, the need for designing new NSAIDs having less GI complications has become of a great importance. It has been approved that converting the acidic carboxylic groups into their corresponding amides and esters can effectively reduce the NSAIDs associated GI damage without changing their anti-inflammatory properties (Makhija, Somani and Chavan, 2013; Qasir, 2013; Ashraf *et al.*, 2016)(Ameen, 2020).

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One approach was to use amino acids such as L-glycine, L-histidine, and L-tryptophan for synthesizing new amide and ester derivatives by masking the acidic carboxylic group of some NSAIDs. The synthesized derivatives can also be considered as mutual prodrugs since these amino acids possess some anti-inflammatory properties as well (Meyers, Moonka and Davis, 1979). Other NSAID mutual prodrugs were synthesized by combining two anti-inflammatory and analgesic agents, for example acetylsalicylic acid and paracetamol were esterified to give Benorylate; a mutual prodrug with better gastric tolerability (Croft, Cuddigan and Sweetland, 1972). Prodrugs of flurbiprofen (Gairola *et al.*, 2005), diclofenac, and ketoprofen (Dhaneshwar *et al.*, 2009) were synthesized by esterification using phenylalanine, alanine, histidine, tryptophan, and glycine. They showed reduced ulcerogenic effect with improved analgesic and anti-inflammatory activity. Ibuprofen, naproxen, and mefenamic acid mutual prodrugs were synthesized as they were linked to chlorzoxazone through an ester linkage with the objective of ameliorating the GI adverse effects. After evaluating their anti-inflammatory activity, these prodrugs were comparable to the starting NSAIDs. They also showed an improved gastro-sparing profile. Docking study was also performed for predicting the binding free energy (ΔG_b) and configuration of the synthesized prodrugs. Ibuprofen and naproxen prodrugs exhibited the highest binding free energy (ΔG_b) scores when docked into COX-2 enzyme; as ibuprofen showed -11.69 while naproxen showed -12.65 kcal/mol (Abdel-Azeem *et al.*, 2009). Other mefenamic acid and ibuprofen ester prodrugs were designed and synthesized using 4-(4-substituted benzylideneamino) phenol Schiff base for condensation with the acidic NSAIDs. These ester prodrugs showed significant anti-inflammatory effect. Interestingly, when studying their GI toxicity profile they were all non-ulcerogenic. This result proves that modifying the acidic carboxylic group of NSAIDs reduces their ulcerogenicity while retaining their anti-inflammatory activity. Molecular docking study was performed for the synthesized prodrugs into both COX-1 and COX-2 enzymes and they exhibited higher binding free energies (ΔG_b) when docked into COX-2 than COX-1 (Hegazy and Ali, 2012).

The interest in synthesizing poly-functional molecules is increasing nowadays. Acetamides can be considered as a poly-functional molecules since their derivatives were found to possess various bioactive properties such as; anthelmintic (Sawant and Kawade, 2011), anticonvulsant (Kamiński *et al.*, 2011) (Jawed *et al.*, 2010), antioxidant (Ayhan-Kılcıgil *et al.*, 2012) (Autore *et al.*, 2010), anti-inflammatory (Jain *et al.*, 2013)(Autore *et al.*, 2010), anti-arthritic (Jain *et al.*, 2013), anti-bacterial (Kanagarajan and Gopalakrishnan, 2012) (Nayak *et al.*, 2014), antifungal (Bardiot *et al.*, 2015) (Kidwai *et al.*, 2012) (Kanagarajan, Thanusu and Gopalakrishnan, 2010), and anti-viral properties (Babkov *et al.*, 2015) (Kai *et al.*, 2001).

In view of the mentioned reports, this study was aimed to design and synthesize new ester prodrugs of *N*-(2,6-dimethylphenyl)-acetamide **1** and NSAIDs **2a-e** in an attempt to reduce the gastric irritation of acidic NSAIDs. Using *N*-(2,6-dimethylphenyl)-acetamide **1** for masking the acidic group will form a mutual prodrug, since it also works as a mild anti-inflammatory agent (Autore *et al.*, 2010; Ayhan-Kilcigil *et al.*, 2012).

2.MATERIALS AND METHODS

This experimental study was designed for modelling of some NSAIDs prodrugs and docking them into the PDB models of COX-1 and COX-2 receptors to compare their binding affinity (ΔG_b) in order to decide which models should be synthesized. MarvinSketch 19.7 was used to draw the chemical structures of these models as 2D structures and convert them to 3D structures. SwissADME web tool was used to check the compliance of the designed compounds with "Lipinski's rule of five". For the preparation of COX-1 and COX-2 macromolecules (proteins); AutoDockTools 1.5.6 was used, while ligand preparation and docking were both performed using PyRx (Python Prescription 0.8). For molecular visualization; "The PyMOL Molecular Graphics System (Version 2.3.2 Schrödinger)" was used.

Importantly, the study was aimed to synthesize those prodrugs of NSAIDs which had higher docking score and to evaluate the ability of the synthesized compounds **3a-e** of inhibiting COX enzyme. All the synthetic procedures were done at Hawler Medical University, College of Pharmacy, Pharmaceutical Chemistry and Organic Chemistry

Lab, between 1st of June 2019 to 15th of August 2019. The NSAIDs **2a-e** were purchased from (Sigma Aldrich, UK and Apollo Healthcare Resources, Singapore) the 2-Chloro-*N*-(2,6-dimethylphenyl)-acetamide **1** purchased from (Apollo Healthcare Resources, Singapore). All the chemicals were used directly without being purified in advance. For the determination of the melting points a Gallenkamp electro-thermal apparatus was used through an open capillary method and the results were recorded without correction. Pre-coated TLC plates 60 F254 (Merck, Germany) were used to check the purity of the compounds, with a solvent system of toluene: acetone (2:1). For visualizing the developed chromatographic plates; UV (254 nm) was used. JASCO FTIR Spectrophotometer was used to record IR spectra, (at Hawler Medical University, College of Pharmacy, Pharmaceutical Chemistry Department). Bruker Ultra shield 400 MHz with internal TMS was used to measure ^{1}H and ^{13}C-NMR spectra with the chemical shifts represented as ppm (at University of Science and Technology/ Irbid- Jordan)**.** Finally, *in vitro* inhibitory properties (of COX-1 and COX-2 enzymes) for compounds **3a-e** and their starting **1** and **2a-e** were assessed at Central University of Lancashire, College of Pharmacy and Biomedical Science, Physiology and Biology Lab.

2.1Computational and docking study

Docking into both COX-1 and COX-2 enzymes was studied. X-ray Crystallographic Structures for these enzymes were retrieved from "Protein Data Bank" website (https://www.rcsb.org/); COX-1 bound with ibuprofen (PDB: 1EQG) and COX-2 bound with meclofenamic acid (PDB: 5IKQ). For identifying the main amino acids involved in the ligand-receptor interactions which form the binding site of a receptor; data from "PDBsum" website was used as a reference (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/) (Abdel-Aziz, Eltahir and Asiri, 2011).

Preparation of proteins

AutoDockTools 1.5.6 was used for the preparation of the protein structures. In this step the polar hydrogens were placed back to the protein. To avoid masking the enzyme surface with the water molecules which can affect the binding of the ligand; water molecules were removed. Ligands, cofactors, and all the molecules bound to the protein were also removed from the protein structure (Hegazy and Ali, 2012; Dhingra *et al.*, 2014).

Preparation of ligands

MarvinSketch 19.7 was used to draw the Structures of ligands **3a-e**. They were primarily sketched as 2D structures and then converted to the 3D format (pdb) using the same program. Then they were energetically minimized by using PyRx (Python Prescription 0.8) (Dallakyan and Olson, 2015).

Docking study

PyRx (Python Prescription 0.8) docking program was used to perform the docking of ligands **3a-e** into the previously prepared proteins: COX-1 (1EQG) and COX-2 (5IKQ) (Dallakyan and Olson, 2015). For the molecular visualization of the docked conformations (poses) and the receptor-ligand interactions; "The PyMOL Molecular Graphics System (Version 2.3.2 Schrödinger)" was used.

Prediction of ADME properties

Ligands **3a-e** were all tested for their compliance to "Lipinski's rule of five". Physicochemical descriptors of these compounds were computed to predict their ADME parameters, pharmacokinetic properties, and druglike nature. To perform all these calculations SwissADME web tool was used (Daina, Michielin and Zoete, 2017).

2.2Chemical Synthesis

Synthesis of the ester prodrugs of attempted NSAIDs with 2-Chloro-*N*-(2,6-dimethylphenyl)-acetamide

2-Chloro-*N*-(2,6-dimethylphenyl)-acetamide **1** (2.5 mmol) was added to a mixture of NSAIDs **2a-e** (2.5 mmol) and sodium bicarbonate ($NaHCO_3$) (2.5 mmol) in 20 ml of dimethyl sulfoxide (DMSO)**,** followed by stirring for 24 hours (Scheme 1). After the completion of the reaction, this mixture was cooled by adding it to an ice-water (100 ml), then the formed precipitate was collected through suction filtration and left to dry. After drying, the obtained solid residue was recrystallized using absolute ethanol (Hamad *et al.*, 2017)(hamadameen and Ameen, 2019). Table 4 shows the physical properties of these synthesized prodrugs **3a-e**.

2.3 Biological study

General procedure for the *in vitro* COX inhibitor screening

"Cayman's COX activity assay kit" was used for screening the COX inhibitory activity of the starting compounds **1** and **2a-e** as well as the synthesized compounds **3a-e**. This assay is a fluorescence-based technique in which ovine COX-1 and human recombinant COX-2 enzymes are used to screen for COX inhibiting properties (Nile *et al.*, 2016) (Aboraia *et al.*, 2017)(Salih *et al.*, 2020).

1. Preparation of initial activity wells: 10 µl of DMSO, 10 µl of enzyme (COX-1 or COX-2), 10 µl of ADHP (10-acetyl-3,7-dihyroxyphenoxazepine), 10 µl of heme, and 150 µl of assay buffer were mixed.
2. Preparation of background wells: 10 µl of DMSO, 10 µl of ADPH, 10 µl of heme, and 160 µl of assay buffer were mixed.
3. Preparation of Inhibitor wells: 10 µl of inhibitors*, 10 µl of enzyme (COX-1 or COX-2), 10 µl of ADPH, 10 µl of heme, and 150 µl of assay buffer were mixed.
4. For initiating the reactions; 10 µl of arachidonic acid solution were added to all the used wells.
5. Followed by two minutes incubation of the wells at room temperature.
6. An excitation wavelength of (530-540 nm) and an emission wavelength of (585-595 nm) were used for reading the plate.
7. For each sample the average fluorescence was calculated.
8. The next step was subtracting the fluorescence of the background wells from the fluorescence of the inhibitor and the initial activity wells.
9. To calculate the percentage of inhibition; the value of each inhibitor sample was subtracted from the value of initial activity sample, followed by dividing this result by the value of initial activity and multiplying it by %100.

* Solutions of 10, 100 and 1000 nm of each Inhibitor (compounds **1**, **2a-e** and **3a-e**) were prepared using DMSO as a solvent.

3.RESULTS

3.1 Computational study

Docking accuracy validation

For validating the molecular docking program which has been used; COX-1 enzyme bound with ibuprofen crystal structure (1EQG) was retrieved from "Protein Data Bank" website, then by re-docking ibuprofen into the binding site of the same enzyme, the best docking configuration was very close to the experimentally proven configuration showing a root-mean-square deviation (RMSD) of 0.45 Å (Fig. 1, a).

For COX-2 enzyme; meclofenamic acid was used as a reference (PDB code: 5IKQ). Meclofenamic acid was again docked into COX-2 enzyme binding site, and it gave a binding mode that seemed to be closely similar to the originally bound ligand, with an RMSD of 0.34 Å (Fig. 1, b).

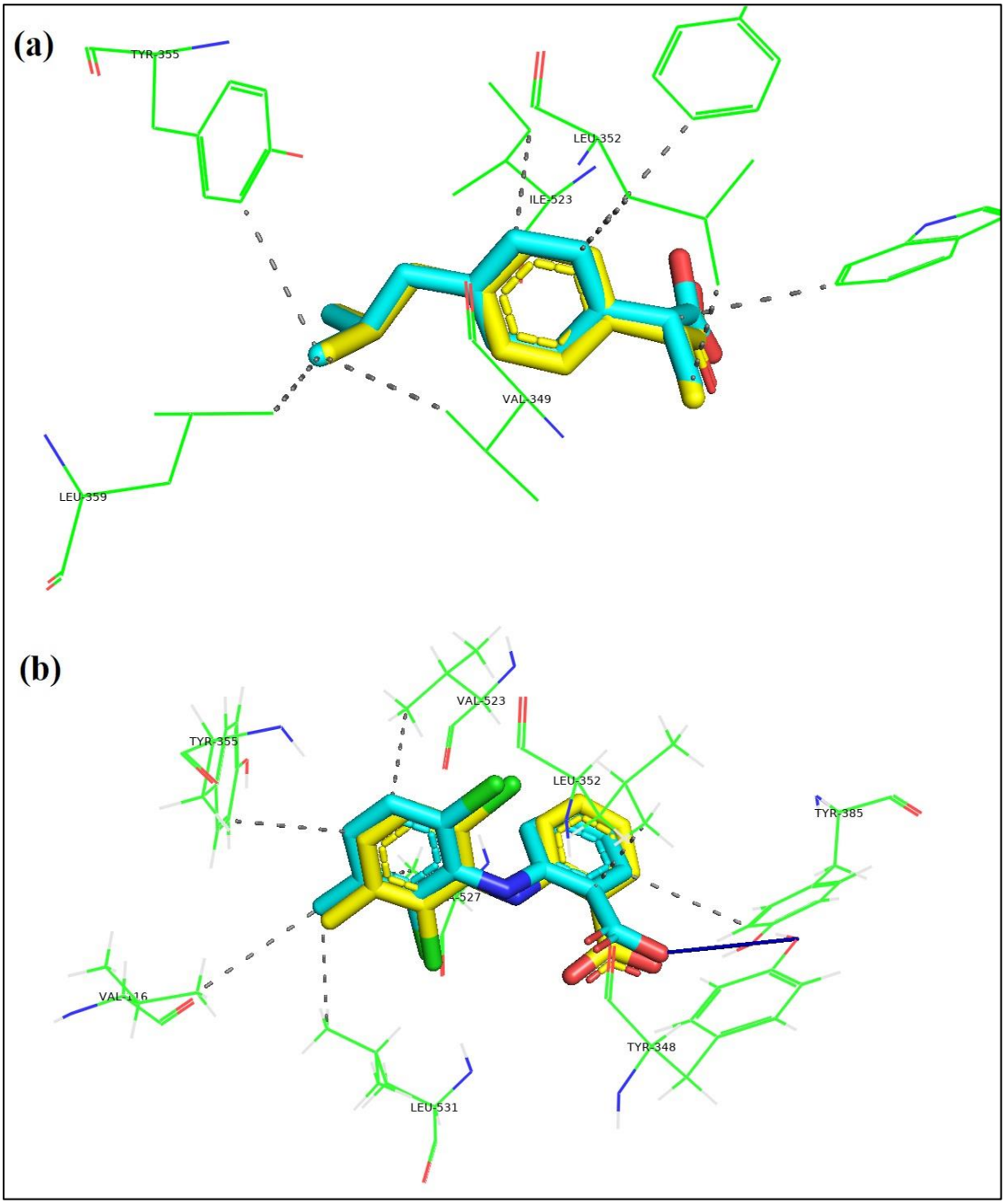


Figure 1. Validation of the docking accuracy. **(a)** Ibuprofen bound to COX-1. **(b)** Meclofenamic acid bound to COX-2. The native co-crystallized ibuprofen and Meclofenamic acid are shown in yellow, while the docked ligands are shown in cyan blue. All structures are colored by element. The hydrogen bonds are shown as dark blue lines. The docked ligands seem exactly superimposed on the native ones.

Docking results of compounds 3a-e

Ester prodrugs of NSAIDs with 2-chloro-N-(2,6-dimethylphenyl)-acetamide (**3a-e**) were docked into COX-1 and COX-2 crystal structures for comparing their binding affinities. The binding free energy (ΔG_b) measured in (kcal/mol) was used to compare the binding affinity. Table 1 and Table 2 show all the docking results for compounds **3a-e**. (Fig. 2) shows the best docked conformations of compounds **3a-e** bound to COX-1 (1EQG), while (Fig. 3) shows their best docked conformations bound to COX-2 (5IKQ).

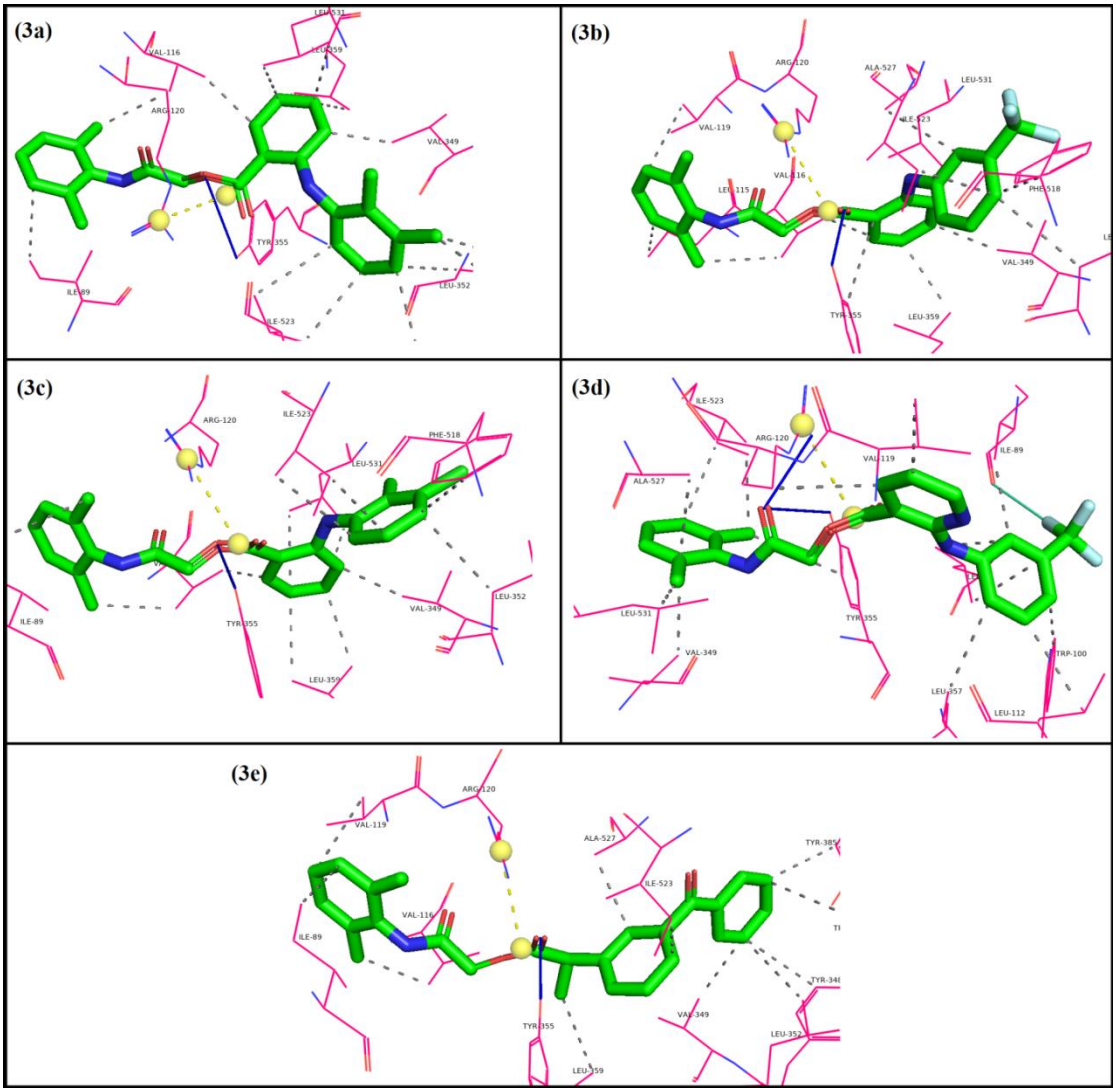


Figure 2. The best docking modes of compounds **3a-e** (colored in green) are shown in their binding site of COX-1 (1EQG). All structures are colored by element. The hydrogen bonds are shown as dark blue lines. The binding site of COX-1 is shown with labeled amino acids.

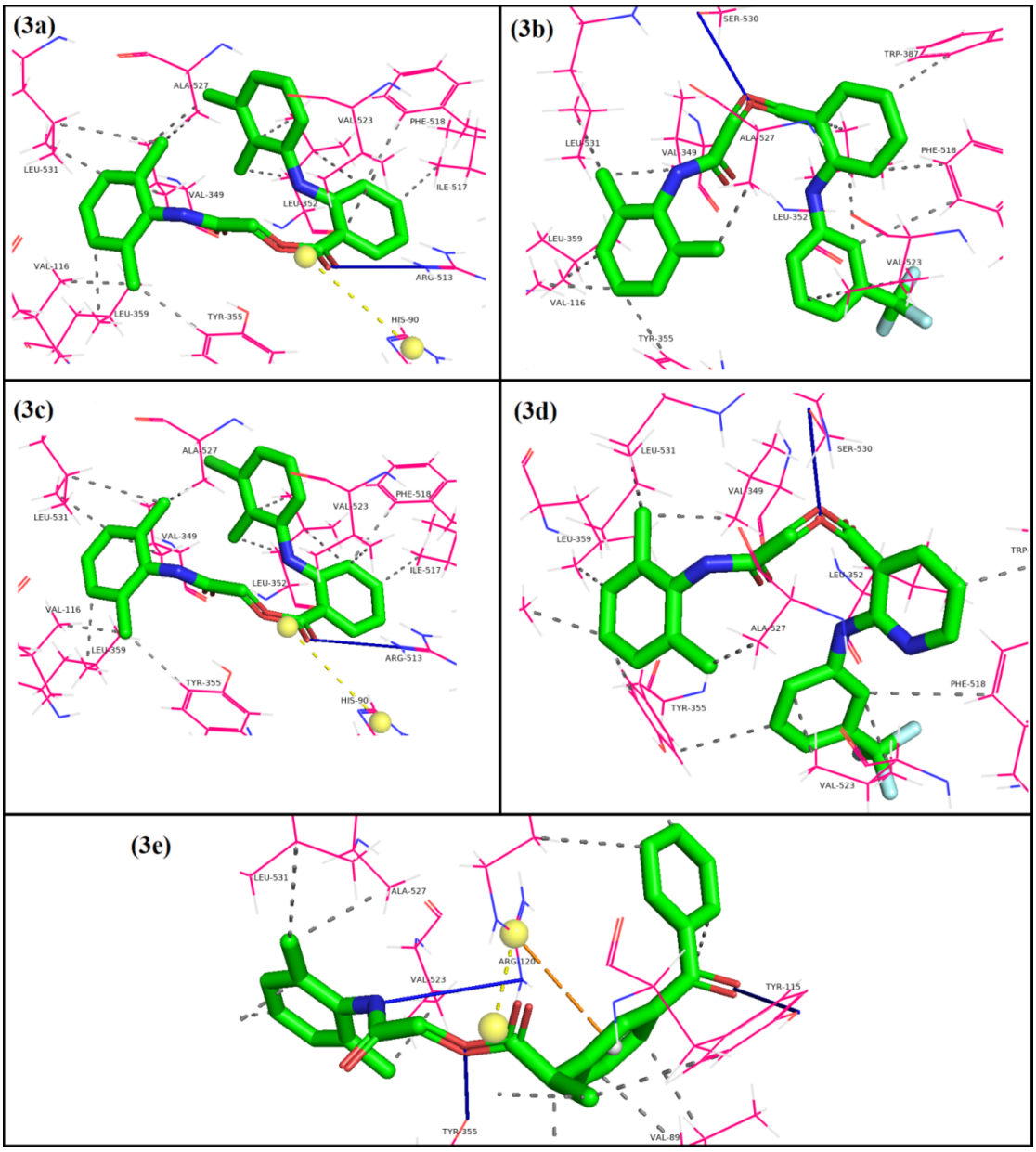


Figure 3. The best docking modes of compounds **3a-e** (colored in green) are shown in their binding site of COX-2 (5IKQ). All structures are colored by element. The hydrogen bonds are shown as dark blue lines. The binding site of COX-2 is shown with labeled amino acids.

ADME properties

SwissADME web tool was used to evaluate the physicochemical properties of compounds **3a-e**; these properties can affect their oral bioavailability, metabolism, and druggability. They were also checked for their "Lipinski's rule of five" compliance. All these results are shown in Table 3.

3.2Synthesis

The synthetic procedures that resulted in the generation of compounds **3a-e** are represented in Scheme 1. Characterization and purity of these compounds were monitored by measuring some of their physicochemical parameters such as their physical appearance, melting points, R*f* values, and percent yields which are shown in Table 4. Identification and characterization of these compounds was also done using spectral analysis such as FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy as shown in Table 5.

Table 1: Docking results of compounds 3a-e docked into COX-1 (1EQG)

Compound	ΔG_b (kcal/mol)	No. of HB	Residue	Amino Acid	Distance (A°)
3a	-9.1	1	355A	TYR	3.10
3b	-9.3	1	355A	TYR	1.82
3c	-9.1	1	355A	TYR	3.19
3d	-9.8	2	120A 355A	ARG TYR	2.18 3.03
3e	-8.9	1	355A	TYR	1.97

Table 2: Docking results of compounds 3a-e docked into COX-2 (5IKQ)

Compound	ΔG_b (kcal/mol)	No. of HB	Residue	Amino Acid	Distance (A°)
3a	-11	1	513A	ARG	2.86
3b	-10.4	1	530A	SER	2.54
3c	-11	1	513A	ARG	2.97
3d	-12.4	1	530A	SER	2.72
3e	-10.8	3	115A 120A 355A	TYR ARG TYR	3.22 3.85 2.11

Table 3: Calculation of various molecular properties of test compounds 3a-e

Compound	Mol. weight (MW) [a]	Rotatable bonds [b]	HB Donor [c]	HB Acceptors [d]	CLogP [e]	Lipinski violations [f]	TPSA [g]
3a	402.49	8	2	3	3.94	0	67.43
3b	442.43	9	2	6	3.92	0	67.43
3c	422.9	8	2	3	4.13	0	67.43
3d	443.42	9	2	7	3.29	0	80.32
3e	415.48	9	1	4	3.71	0	72.47

[a] (150 - 500 g/mol), [b] (≤ 9), [c] (≤ 5), [d] (≤ 10), [e] (< 5), [f] (≤ 1), and [g] (20 - 130 $Å^2$)

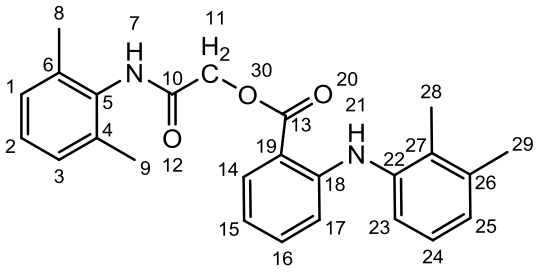
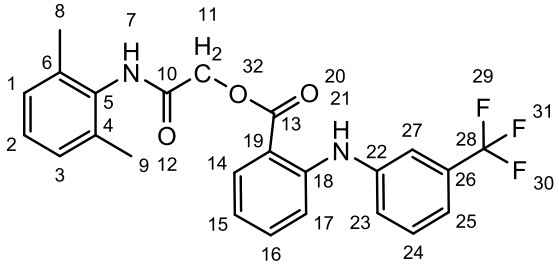
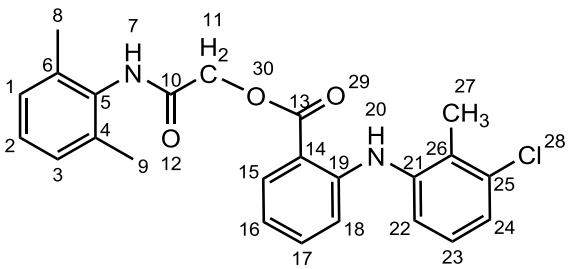
Table 4: Physical properties of synthesized compounds 3a-e

Compounds	Physical appearance	Yield (%)	Melting point °C	R*f* value *
3a	Colorless	67	217-219	0.82

	powder			
3b	Colorless powder	82	159-161	0.81
3c	Colorless powder	66	205-207	0.81
3d	Colorless powder	74	215-216	0.78
3e	Colorless powder	77	105-107	0.78

*R*f* value, toluene: acetone (2:1) used as eluent.

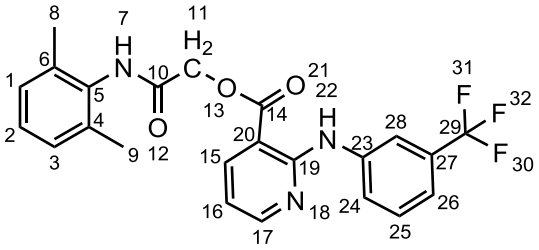
Table 5: Spectroscopic data of the synthesized compounds (3a-e)

Compounds	Spectroscopic data
3a 	**IR in cm⁻¹:** 3319, 3244 (NH str. amine and amide), 1689 (C=O ester), 1660 (C=O amide), 1222 (C-O ester). **¹HNMR** (400 MHz, DMSO- d_6): δ 1.24(s, 3H, $_{29}CH_3$), 1.31(s, 6H, $_8CH_3$, $_9CH_3$), 1.44 (s, 3H, $_{28}CH_3$), 4.13 (s, 2H, $_{11}CH_2$), ((6.68, d, 1H, *J*=8Hz), (6.75, t, 1H), (7.08,s, 4H), (7.13,s,2H),(7.36, t, 1H), (8.04, d, 1H, *J*=4Hz) 10Ar-H), 9.12(s, 1H, Ar-NH), 9.57(s, 1H, Ar-NHCO). **¹³CNMR** (101 MHz, DMSO-d_6): δ C_{29}-14.09, C_8,C_9- 18.46, , C_{28}-20.69, C_{11}-63.41, Ar (C) -110.69-149.16, C_{10}-166.00, C_{13}-167.98.
3b 	**IR in cm⁻¹:** 3323, 3201 (NH str. amine and amide), 1685 (C=O ester), 1658 (C=O amide), 1228 (C-O ester). **¹HNMR** (400 MHz, DMSO- d_6): δ 2.17 (s, 6H, $_8CH_3$, $_9CH_3$), 5.01 (s, 2H, $_{11}CH_2$), ((9.98, t, 1H) (7.08, s, 3H) (7.34, d, 2H, *J*=8Hz)(7.50, m, 4H) (8.06, d, 1H, *J*=8Hz) 11Ar-H), 9.29 (s, 1H, Ar-NH), 9.60 (s, 1H, Ar-NHCO). **¹³CNMR** (101 MHz, DMSO-d_6): δ C_8,C_9- 17.80, C_{11}-62.76, C_{28}- 114.16, Ar (C) -115.45-144.76, C_{10}-165.22, C_{13}-166.58.
3c 	**IR in cm⁻¹:** 3313, 3246 (NH str. amine and amide), 1691 (C=O ester), 1666 (C=O amide), 1222 (C-O ester). **¹HNMR** (400 MHz, DMSO- d_6): δ 2.18 (s, 6H, $_8CH_3$, $_9CH_3$), 2.26 (s, 3H, $_{27}CH_3$), 5.00 (s, 2H, $_{11}CH_2$), ((6.84, t, 2H) 7.09, s, 3H) (7.25, m, 3H)(7.43, t, 1H) (8.06, d, 1H, *J*=8Hz) 10Ar-H), 9.20

(s, 1H, Ar-NH), 9.59 (s, 1H, Ar-NHCO).

^{13}CNMR (101 MHz, DMSO-d_6): δ C_{27}-14.46, C_8,C_9-17.80, C_{11}-62.84, Ar (C) -111.16-147.25, C_{10}-165.25, C_{11}-167.17.

3d

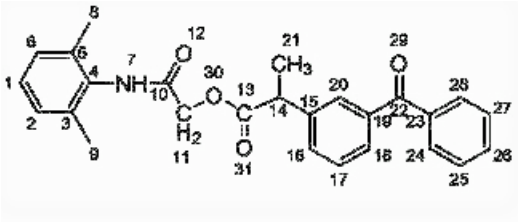


IR in cm^{-1}: 3265 (NH str. amine and amide overlaped), 1697 (C=O ester), 1668 (C=O amide), 1116 (C-O ester).

^{1}HNMR (400 MHz, DMSO- d_6): δ 2.18 (s, 6H, $_8CH_3$, $_9CH_3$, 5.07 (s, 2H, $_{11}CH_2$), ((7.02, d, 4H, *J*=20Hz) (7.35 ,s, 1H)(7.55, s, 1H) (7.89, s, 1H) (8.29, s, 1H) (8.43, d, 2H, *J*=28Hz) 10Ar-H), 9.63 (s, 1H, Ar-NH), 10.15 (s, 1H, Ar-NHCO).

^{13}CNMR (101 MHz, DMSO-d_6): C_8,C_9-17.89, C_{11}-63.30, C_{29}-107.85, Ar (C) -114.81-154.45, C_{10}-165.10, C_{14}-166.11.

3e



IR in cm^{-1}: 3346 (NH str. amide), 1747 (C=O str. ester), 1697 (C=O str. amide), 1116 (C-O str. ester).

^{1}HNMR (400 MHz, DMSO): δ 1.49 (d, 3 H, $_{2\ 1}$ CH_3), 2.12 (s, 6 H, $_8$ CH_3 , $_9$ CH_3), 4.13 (q, 1 H, $_{1\ 4}$ CH), 4.73 (dd, 2 H, $_{1\ 1}$ CH_2), (7.08 (s, 3 H), 7.56 (t, 3 H), 7.64 (m, 6 H), 12 Ar-H), 9.40 (s, 1 H, Ar-NHCO).

^{13}CNMR (101 MHz, DMSO): C_8 ,C_9 -17.50, $C_{2\ 1}$ -18.16, $C_{1\ 4}$ -43.57, $C_{1\ 1}$ -62.38, Ar (C) -126.22-140.42, $C_{1\ 0}$ -164.75, $C_{1\ 3}$ -172.87, $C_{2\ 2}$ -195.12.

3.3COX inhibition activity

Screening for the COX inhibition activity of compounds **1**, **2a-e** and **3a-e** was done using Cayman's assay kit. Results of COX-1 inhibition screening are shown in Table 6, while results of COX-2 inhibition screening are shown in Table 7.

Table 6: *In vitro* ovine COX I assay results

Compounds	COX I % inhibition					
	F	10 nM	F	100 nM	F	1000 nM
1	13884±363	14	17452±378	17	1751±279	18
2a	24004±274	37	23118±251	39	22841±218	40
3a	29187±666	23	27534±445	27	26993±234	29
2b	27149±612	28	26601±184	30	232621±265	39
3b	33823±567	11	25695±408	33	23080±53	40
2c	31612±404	17	27505±499	28	24247±160	37
3c	28413±258	26	27191±112	29	24935±325	35
2d	29818±625	22	28602±407	25	25839±169	32
3d	33622±444	12	25693±213	33	22824±160	41

2e	33312±240	13	29051±234	24	25244±116	34
3e	32252±105	16	30295±501	21	26852±79	30

F= Mean±SD of the initial fluorescence activity. nM= Nano-molar concentrations of the synthesized prodrugs **3a-e** and their precursor **2a-e**.

Table 7: *In vitro* human recombinant COX-2 assay results

Compounds	COX II % inhibition					
	F	10 nM	F	100 nM	F	1000 nM
1	7255±247	10	12354±392	13	10124±582	16
2a	29428±353	22	24895±417	35	22225±235	41
3a	29551±583	23	25401±324	33	22323±209	42
2b	30511±442	22	26895±897	32	24279±531	39
3b	29506±597	23	25489±572	33	22863±528	40
2c	27556±386	26	27293±664	28	23987±266	35
3c	28099±21	27	26725±208	29	24729±292	37
2d	27047±437	32	26625±461	33	23780±408	40
3d	24931±459	37	22437±697	44	21063±680	47
2e	29167±444	23	25670±347	31	21229±81	35
3e	28158±63	26	26725±208	32	24557±287	44

F= Mean±SD of the initial fluorescence activity. nM= Nano-molar concentrations of the synthesized prodrugs **3a-e** and their precursor **2a-e**.

4.DISCUSSION

In this study; new NSAIDs ester prodrugs were designed and synthesized by modifying some acidic NSAIDs with the main goals of decreasing side effects, increasing tolerability, and increasing efficacy. As mentioned in many literatures, masking the carboxylic acid group of an NSAID, gives less gastrotoxic prodrugs, with comparable or higher anti-inflammatory activity (Kalgutkar *et al.*, 2000; Le Borgne *et al.*, 2000).

Before using the docking program, its reliability should be investigated. Validating the docking program showed excellent results since the obtained RMSD values from redocking ibuprofen and meclofenamic acid into their binding sites of proteins 1EQG and 5IKQ respectively were both ≤ 2, which according to literatures (Dhingra *et al.*, 2014)(Hegazy and Ali, 2012); if the RMSD value is within this range it means that the used scoring method is accurate and very reliable. The smaller RMSD values we get, the more confident we are in the applied docking program, because this means that the used program could reproduce the same binding mode (pose) of the native ligand, and as close as possible to the binding mode found experimentally. We can clearly see in (Fig. 1) how ibuprofen and meclofenamic acid ligands' theoretically predicted conformations looked almost identical with the experimentally found ligand conformations of proteins 1EQG and 5IKQ respectively.

Docking of compounds **3a-e**, which were both the most potent and the most selective ones, showed that they were able to bind at the conventional binding site of COX-1 and COX-2 receptors as described in many literatures (Limongelli *et al.*, 2010)(Zarghi and Arfaei, 2011).

All the docked compounds (**3a-e**) showed higher binding affinities when docked into COX-2 than COX-1 (Table 1 and Table 2). These results indicate that these compounds (**3a-e**) are more likely to be selective COX-2 inhibitors which might be due to becoming bulkier after modifying their chemical structures and this has made them fit more easily in the bigger active site of COX-2 than COX-1 (Aboraia *et al.*, 2017).

Comparing the docking results; compound **3d** showed the highest binding affinity as it had the highest binding free energy (ΔG_b: -12.4 kcal/mol) when it was docked into COX-2 (5IKQ) binding site (Table 2), and exhibited one hydrogen bond with Ser530 (Fig. 3). This predicts compound **3d** to have the highest ant-inflammatory activity among the evaluated compounds.

Computerized ADME predicting tool was used to determine certain parameters that can affect the bioavailability, cell permeability, and metabolism of the test compounds (**3a-e**). Their druglike nature was tested by measuring their compliance with "Lipinski's rule of five". This rule works as a guideline to assess the druglikeness of a certain molecule and mainly to predict its oral bioavailability profile (Lipinski *et al.*, 2012). It states that for a molecule to have good oral bioavailability; its molecular weight should not be more than 500 (g/mol), number of H-bond donors not exceeding five, number of H-bond acceptors not more than ten, and (CLogP) value should not be greater than five. If a molecule violates more than one of the mentioned rules, then it's more probably going to have bad oral bioavailability (Barret, 2018).

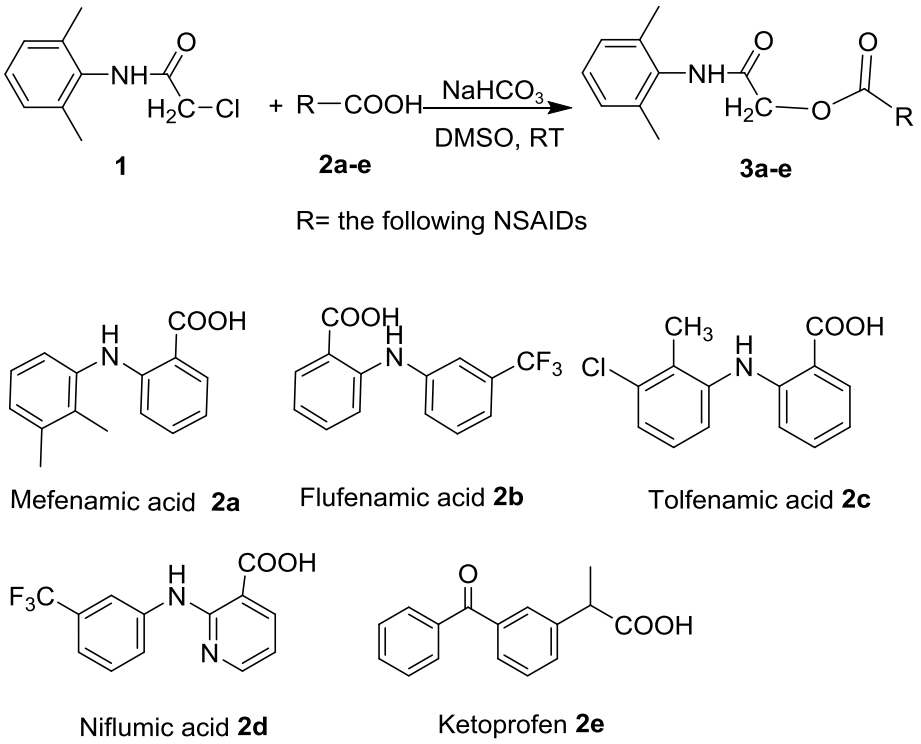
Another predicted parameter was TPSA (topological polar surface area). TPSA measures the polarity of a molecule, more specifically its hydrogen bond forming ability, which is also associated with the bioavailability profile (Ertl, Rohde and Selzer, 2000). In order to have good oral bioavailability from passive absorption; the molecule's TPSA should be between (20 and 130 $Å^2$). ADME prediction results of compounds **3a-e** fall within the ideal ranges (Table 3).

The reaction of the synthesis of compounds **3a-e** is demonstrated in Scheme 1, it is an esterification by S_N2 reaction, which is a nucleophilic substitution reaction (Bruice, 2007); in this study it involves the substitution of the halide group (leaving group) of a primary alkyl halide with a carboxylate anion (nucleophile), as a result an ester is formed (Hamad *et al.*, 2017)(Hamlin, Swart and Bickelhaupt, 2018).

The main aim was to mask the carboxylic groups of the NSAIDs molecules to be modified (**2a-e**), through esterifying them using *N*-(2,6-dimethylphenyl)-acetamide **1**. At first the carboxylic groups of NSAIDs (**2a-e**) were converted to their sodium carboxylate salts by mixing them with sodium bicarbonate using dimethyl sulfoxide (DMSO) as a solvent. These sodium carboxylate salts provide the carboxylate anion (nucleophile). After the addition of 2-Chloro-*N*-(2,6-dimethylphenyl)-acetamide **1;** the carboxylate anion , prepared previously, substitutes the chloride group at the alpha position**.** The negatively charged oxygen of the carboxylate anion attacks the alpha carbon; at that point a transition-state is formed. This transition-state ends up by forming the alpha carbon-nucleophile bond and breaking the alpha carbon-chloride bond instantaneously producing the target compounds (**3a-e**). The target compounds (**3a-e**) were formed in high yields (66-82%).

The progress of the reaction was monitored using thin layer chromatography technique. FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy were used for authenticating the final products chemical structures and to determine their purity. IR spectra of the synthesized compounds showed expected values. Correspondingly, peaks for ^{1}H and ^{13}C-NMR appeared at expected δ values (Table 5). The observed IR, ^{1}H-NMR, and ^{13}C-NMR values showed a good agreement with the ones reported in the literature (Silverstein, Webster and Kiemle, 2005)(Smith and March, 2006) which accordingly confirm the structures of the synthesized compounds.

For evaluating the ability of these synthesized compounds (**3a-e**) as well as the starting compounds **1** and **2a-e** for inhibiting COX-1 and COX-2 enzymes, they were screened using "Cayman's COX activity assay kit". Results of their COX-1 and COX-2 inhibitory activity are shown in Table 6 and Table 7 respectively. Compounds **3a-e** showed COX-1 inhibition activity which was comparable with the starting compounds (**1** and **2a-e**), while their COX-2 inhibition activity was superior to that of the starting compounds. Importantly, compound **3d** showed maximum *in vitro* COX-2 inhibition (37-47%) due to its better ability to fit with COX-2 enzyme. These results moreover confirm the theoretical predictions which were obtained from the molecular docking study as compound **3d** showed the highest binding free energy ΔG_b (-12.4 kcal/mol) among the designed compounds when it was docked into COX-2 (5IKQ) binding site.



Scheme 1: synthesis of prodrugs of NSAIDS 3a-e

5.CONCLUSIONS

An efficient and simple approach as well as an easy work-up had been applied for the synthesis of prodrugs of NSAIDs **3a-e** which was helpful in masking the irritating carboxylate moiety, to increase *in vitro* COX inhibitory activity i.e. anti-inflammatory activity of the parent NSAIDs **2a-e**. The approach and the reaction condition were mild to afford in suitable time the desired products with good yields. A simple recrystallization technique had been applied to separate conveniently the prepared products **3a-e** from the reaction mixture in high purity. Docking study was performed for the designed compounds **3a-e** showing binding free energies ΔG_b of (-8.9 to -9.8 kcal/mol) when docked into COX-1 and (-10.4 to -12.4 kcal/mol) into COX-2 enzymes. Compound **3d** showed the highest binding free energies ΔG_b (-9.8 and -12.4 kcal/mol) when it was docked into COX-1 and COX-2 binding sites, respectively. The synthesized prodrugs **3a-e** were screened for their COX inhibition activity which revealed that they possess greater inhibitory activity than the parent NSAIDs **2a-e**. Compound **3d** exhibited the highest COX-2 inhibition (37-47%) and this further supports the docking result.

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