

Synthesis of Naproxen Esters and Evaluation of their *In vivo* and *In silico* Analgesic and Anti-inflammatory Activities

Mansura Akter¹, Zobaer Al Mahmud¹, Mir Muhammad Nasir Uddin², Raju Das²
and S.M. Abdur Rahman¹

¹Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh

²Department of Pharmacy, University of Chittagong, Chittagong- 4331, Bangladesh

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ABSTRACT: Three consecutive alkyl esters methyl (1), ethyl (2) and isopropyl (3) esters were synthesized from naproxen by direct Fischer esterification reaction and were evaluated for their *in vivo* and *in silico* analgesic and anti-inflammatory activities. Methyl and ethyl ester showed potent peripheral analgesia with 82.09% and 82.59% writhing inhibition, respectively compared to that of naproxen (64.68% inhibition) at equivalent dose of 25 mg/kg bw. In anti-inflammatory study, all the three esters generated 96.75%, 91.54% and 90.65% inhibition of inflammation at 5th hour, similar to that obtained by naproxen (95.12% inhibition). Molecular docking study suggested that methyl ester had the highest binding energy towards COX-2 enzyme, while isopropyl ester possessed the lowest energy, and also exhibited the lower Vander Waals interaction that might affect COX inhibition. Moreover, all the compounds had satisfactory ADME profile. Again, methyl ester satisfied the safety issues, while other compounds might have cardio toxicity.

Key words: Naproxen, ester, analgesic, anti-inflammatory, COX-1, COX-2, ADME/Tox, molecular docking.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) consisting of arylacetic acids, arylpropionic acids, β -ketoenols, and diarylheterocycle chemical groups are the most useful drugs for treating osteoarthritis and several other inflammatory disorders.¹ Mechanism of NSAIDs in inhibiting inflammation and pain sensation are based on their ability to inhibit cyclooxygenase (COX), which is the key enzyme for the conversion of arachidonic acid to prostaglandins.² Several prostaglandins are biosynthesized by two isoforms of COX (COX-1 and COX-2) and are involved in inflammatory response via a number of G-protein coupled receptors.³ COX-1 is responsible for the synthesis of baseline levels of prostaglandins

and COX-2 produces prostaglandins through stimulation. In addition to acute inflammation, prostaglandins are also responsible for chronic inflammatory reactions such as autoimmune diseases, neurodegenerative disorders, vascular and metabolic diseases as well as cancer via amplification of cytokine response and tissue remodelling.⁴ As prostaglandins are responsible for several acute and chronic inflammation, regulation of their biosynthesis through inhibiting COX-1 and COX-2 might be beneficial for all inflammatory diseases.

Although NSAIDs are very useful drugs for the treatment of several inflammatory conditions, they possess several side effects such as gastric upset, irritation, ulceration, and renal, hepatic and cardiovascular toxicity.^{1,2,5,6} In order to minimize side effects, improve analgesic/anti-inflammatory activity, a number of NSAID derivatives were prepared and some of them were reported to have better bioavailability, less gastric irritation and improved

Correspondence to: S. M. Abdur Rahman
E-mail: <smarahman@du.ac.bd>

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anti-oxidant property etc.⁷⁻¹¹ Naproxen, a non-selective NSAID presents the least cardiovascular risk and provides the most effective relief for arthritic patients among all NSAIDs.¹² However, arthritic doses of naproxen are significantly toxic to the GI tract resulting from COX-1 inhibition.¹² Therefore, improvement of the potency and efficacy of naproxen via derivatization chemical modification is an important task. To increase the pharmacological activity, physicochemical properties as well as to reduce the potential toxicity, we synthesized several metal complexes of naproxen and other NSAIDs earlier¹³⁻¹⁸ and we found better pharmacological, toxicological and physicochemical properties of the synthesized derivatives in some instances.^{14,17,18} As ester prodrugs are found to be beneficial in terms of efficacy and safety in many cases,¹⁹ our continued interest in this topic directed us to synthesize some ester derivatives of naproxen to evaluate and compare their pharmacological activity and toxicity with parent drug. Herein, we disclose the synthesis of the naproxen esters along with their *in vivo* analgesic and anti-inflammatory efficacy as well as *in silico* binding mechanism against potential target protein for the above activity.^{2,20}

MATERIALS AND METHODS

Ethical statement. All experimental protocols were in compliance with the ICH guidelines and ethical approval was governed by the Ethical Review Committee (animal group) of the Faculty of Biological Sciences, University of Dhaka (approval number 2016/118).

Chemistry. Naproxen was obtained from Advance Chemical Industries (ACI) Limited, Bangladesh and all solvents were purchased from Sigma Aldrich (Deisenhofen, Germany). ¹H-NMR spectra were recorded in CDCl₃ using Tetramethyl Silane (TMS) as the internal standard in Bruker GmbHD PX-400 MHz FT Spectrometer. Chemical shifts were recorded as δ (ppm). IR spectra were recorded with a Shimadzu IR Spectrometer on KBr disc. Shimadzu LC Solution system under isocratic condition at a flow rate of 1 mL/min of the mobile

phase (CH₃CN:H₂O: HOAc, 50:49:1) was used for HPLC analysis using Zorbax Eclipse XDB- C18 reversed phase column (150/4.6 mm, 5 μm) and detected at 254 nm. Chromatographic purification was done using silica gel (60 Å, 200–400 mesh) and the reaction was confirmed by TLC silica gel plate (Merck 60 F₂₅₄) using hexane-ethyl acetate solvent (9:1). Spots were visualized by short-wave UV light. Alkyl esters of naproxen were synthesized by slight modification of the reported methods.^{21,22}

Synthesis of esters. Naproxen (0.9212 gm, 0.004 moles) was added to respective alcohols in the presence of 1 ml of conc. H₂SO₄ and refluxed at 80°C for 2-4 hours with stirring. The reaction is presented in Figure 1. Dichloromethane (3x30 ml) and 1% NaOH were added to quench the reaction. Ethyl acetate was added and the organic layer was separated, washed with H₂O (3x30 ml), dried over anhydrous MgSO₄, filtered and evaporated to give respective ester residues which were purified by column chromatography (hexane: EtOAc 9:1) to give the reported esters as solid crystals.

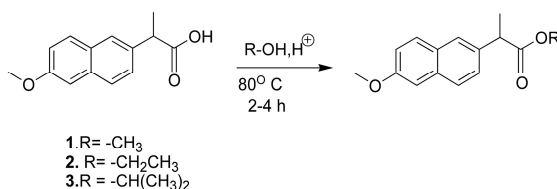


Figure 1. Synthesis of naproxen esters.

Methyl-2-(6-methoxy naphthyl) propionate (1)
 C₁₅H₁₆O₃, MW 244.28. Off white to brownish crystal. Yield: 0.773 g (84%). HPLC (R_t):11.39 min. IR (KBr, cm⁻¹): 2973, 1738, 1605, 1176.81; ¹H-NMR (400 MHz, CDCl₃, δ, ppm): 7.68 (dd, J = 8.4 Hz, 2H), 7.65 (s, 1H), 7.39 (dd, J = 2.0 Hz, 1H), 7.13 (dd, 1H), 7.10 (1H, bs), 3.88, (s, 3H), 3.66 (s, 3H), 1.58 (d, 3H);

Ethyl-2-(6-methoxy naphthyl) propionate (2)
 C₁₆H₁₇O₃, MW 258.29. White crystal. Yield: 0.792 g (86%). HPLC (R_t):15.54 min. IR (KBr, cm⁻¹): 2977, 1727, 1604, 1180. ¹H-NMR (400 MHz, CDCl₃, δ, ppm): 7.68 (d, J = 8.4, 3H), 7.40 (dd, J = 8.4 Hz, 1H),

7.13 (dd, $J = 8.8$ Hz, 2 H), 7.11(1H, bs), 4.12 (m, 2H), 3.90 (s, 3H), 3.83 (q, 1H, $J = 7.0$ Hz), 1.57 (d, $J = 7.0$ Hz, 3H), 1.20 (t, $J = 7.0$ Hz, 3H).

Isopropyl-2-(6-methoxy naphthyl) propionate (3). $C_{17}H_{20}O_3$. MW 272.31. White crystal. Yield: 0.746 g (81%). HPLC (R_t): 21.74min. IR (KBr, cm^{-1}): 2972, 1729, 1604, 1638 1166.75. 1H -NMR (400 MHz, $CDCl_3$, δ , ppm): 7.69 (d, $J = 8.8$ Hz, 1H), 7.68 (d, $J = 8.4$ Hz, 1H), 7.66 (bs, 1H), 7.40 (dd, $J = 7.6$, 0.8 Hz, 1H), 7.12 (dd, 1H), 7.11 (bs, 1H), 5.00 (m, 1H), 3.90 (s, 3H), 3.78 (q, 1H), 1.55 (d, $J = 6.8$, 3H), 1.22 (d, $J = 6.4$ Hz, 3H), 1.11 (d, $J = 6.4$ Hz, 3H).

Experimental animals. Thirty Swiss albino mice of both sex (25-30 gm, aged 4-5 weeks) and thirty six Wister rat (100-150 gm) were purchased from the animal house of Jahangirnagar University and maintained with standard diet and husbandry conditions.²³ They were kept for 1 week before starting the experiment to minimize environment sensitivity.

Acetic acid (AA) - induced peripheral analgesic activity. In AA- induced writhing test,²⁴ the mice were subdivided into 5 groups (6 mice /group) that were treated intraperitoneally (i.p.) such as with 0.1 ml/10g bw AA and suspended saline of 0.25% w/v carboxy methyl cellulose (CMC) (control group), naproxen (reference group) and the test compounds **1-3** (equimolar dose of 25 mg/kg bw prepared in 0.25% w/v CMC suspended saline). Forty-five minutes later, 0.6% AA was administered. The total number of twisting, squirming movements or abdominal contractions (writhing) for each mouse was counted for the next 15 min, starting 5 min after the AA injection. Substance having analgesic activity reduces writhing count.

Carrageenan (CG)-induced rat's paw edema test. In CG-induced rat paw edema test,²⁵ the test animals were subdivided into 6 groups consisting of 6 rat/group that were received 0.25 % w/v CMC suspended saline for control, naproxen as reference and test samples at equimolar dose of 25 mg/kg bw by oral gavages 1 h prior to the CG injection (50 μ l 1% CG in CMC suspended saline) into the sub-

planter tissue of the right hind-paw pad. The paw volumes were measured by a plythesmometer (Model 7140, Ugo Basile, Italy) prior to and at 1, 2, 3 and 5 h after the CG injection.

Statistical analysis. The statistical analysis of the data was accomplished by using one way Analysis of Variance (ANOVA) followed by Dunnett's t test where $p < 0.05$ was considered as significant.

Molecular docking analysis. Three-dimensional crystal structure of COX-2 (Protein Data Bank, PDB id: 3NT1) was downloaded in pdb format from the protein data bank.²⁶ After that, structures were prepared and refined using the Protein Preparation Wizard of Schrödinger-Maestro v9.4. Then, bond orders and charges were assigned, all waters were deleted and hydrogens were added to the heavy atoms. Energy minimization was carried out setting maximum heavy atom root-mean-square-deviation (RMSD) to 0.30 Å using force field optimized potentials for liquid simulations (OPLS) version OPLS_2005. Compounds **1-3** were drawn by using chemdraw version 12. The 3D structures of the compounds were prepared by using Ligprep2.5 in Schrödinger Suite 2013 with an OPLS_2005 force field. Receptor grid was generated as described in literature.²⁷ The bounding box for docking experiment was set to 14 Å \times 14 Å \times 14 Å and flexible ligand docking was accomplished in Glide of Schrödinger-Maestro v9.4.^{28,29} docking was carried out as described in the literature. The best docked pose with the lowest Glide score value was recorded for each ligand.

Prime MM-GBSA (Molecular Mechanics, The Generalized Born model and Solvent Accessibility) approach was used to determine ligand binding energies and ligand strain energies for a ligand and a single receptor.²⁷ The total free energy of binding is calculated as follows:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}})$$

Analysis of absorption, distribution, metabolism, excretion and toxicity (ADMET) property. ADMET properties determine drug-like activity of ligand molecules based on Lipinski's rule

of five. ADMET properties of naproxen and the synthesized compounds were analyzed using Schrodinger QikProp 3.2 module.³⁰ Several parameters related to physicochemical properties and pharmacokinetics such as molecular weight (MW), Hydrogen bond donor (HB donor) and acceptor (HB acceptor), Total solvent accessibility surface area (SASA), Predicted IC₅₀ value for blockage of HERG K + channel (QP log HERG), predicted aqueous solubility (QP log S), predicted partition coefficient (QP log P) and predicted human oral absorption were also measured.

RESULTS AND DISCUSSION

General synthesis of methyl-2-(6-methoxy naphthyl) propionate (1), ethyl-2-(6-methoxy naphthyl) propionate (2) and isopropyl-2-(6-methoxy naphthyl) propionate (3). The three esters namely methyl-2-(6-methoxy naphthyl) propionate (1), ethyl-2-(6-methoxy naphthyl) propionate (2) and isopropyl-2-(6-methoxy naphthyl) propionate (3) were synthesized from naproxen in very good yields (81%-86%). The isolated yield of the compound 3 was better (81%) than that of reported (72%) data²¹ and others were obtained in comparable yields.^{21,22} The compounds were identified and characterized by IR and NMR spectra where peaks for respective alkyl protons were observed cleanly. The esters were also confirmed by comparing the spectral data to that of the reported data and found to be identical.³¹ The retention time (R_t) of the esters in the HPLC chromatogram was increased gradually (11.39 min, 15.54 min, 21.74 min for compound 1, 2 and 3, respectively) upon increasing the alkyl side chain which indicated the masking of the polar acidic functional group of naproxen by hydrophobic alkyl moiety (Figure 2). All spectral data (NMR, IR), molecular formula, molecular weight (MW) and retention time are presented in the materials and method section.

In vivo peripheral analgesic activity. Compound 1 and 2 showed higher writhing inhibition compared to that of naproxen (82.09 % and 82.95 % inhibition respectively vs. 64.69% inhibition by

naproxen) at an equivalent dose of 25 mg/kg bw (Table 1), whereas compound 3 showed lower writhing inhibition (49.25% inhibition) than that of naproxen. The increased inhibition obtained by the compounds 1 and 2 might be due to increased absorption from GIT supported by some other reported synthesized esters.⁸

Table 1. Peripheral analgesic activity assessment via writhing inhibition by naproxen and its esters at equimolar dose of 25 mg/kg bw i.p.

Test compounds	Number of Writhing Mean \pm SEM (% inhibition)
Control/saline 0.1ml/10 g bw	20.1 \pm 0.620
Naproxen	7.1 \pm 1.83 ^{***} (64.68)
Compound 1	3.6 \pm 1.36 ^{***} (82.09)
Compound 2	3.5 \pm 2.32 ^{***} (82.59)
Compound 3	10.2 \pm 1.55 ^{***} (49.25)

Data are expressed as mean \pm standard error mean (SEM); Asterisk represents the statistically significant difference from the control which is calculated as p values (^{***} p < 0.001).

Anti-inflammatory activity test. At equimolar dose of 25 mg/kg bw, compound 1 showed highly significant inhibitory activities which was higher than that found by naproxen (72.97%, 76.79% & 96.75% inhibition of inflammation vs. 68.65%, 77.23% & 95.12% inhibition at 2nd, 3rd and 5th hours respectively). Besides, at 2nd & 3rd hours compounds 2 & 3 showed 40% & 56.25% and 47% & 70% inhibition, respectively which were lower than but statistically significant to that of naproxen (p < 0.05 & p < 0.01 at respective hours for both compounds). However, all the esters were found to be significantly comparable to that of naproxen (Table 2). Since it is assumed that prostaglandins are secreted in the third and fourth hours after CG injection,¹⁰ the result indicated that all naproxen esters still inhibited the arachidonic acid pathway effectively after 3rd and 5th hours. Among the esters, methyl ester which exhibited better analgesia than naproxen and others also showed highly significant anti-inflammation at equimolecular doses. Ethyl ester also showed higher analgesia & significant anti-inflammatory property

and in the case of isopropyl ester moderate analgesia with significant edema inhibition was also found. Previously, we also noticed that methyl esters showed lowest GI injury among the synthesized

esters and the parent drug.¹¹ Our results are consistent with the observation found from some aromatic esters and amide derivatives of naproxen and ibuprofen.³²

Chromatogram of Naproxen & Methyl, Ethyl and Isopropyl Ester of Naproxen

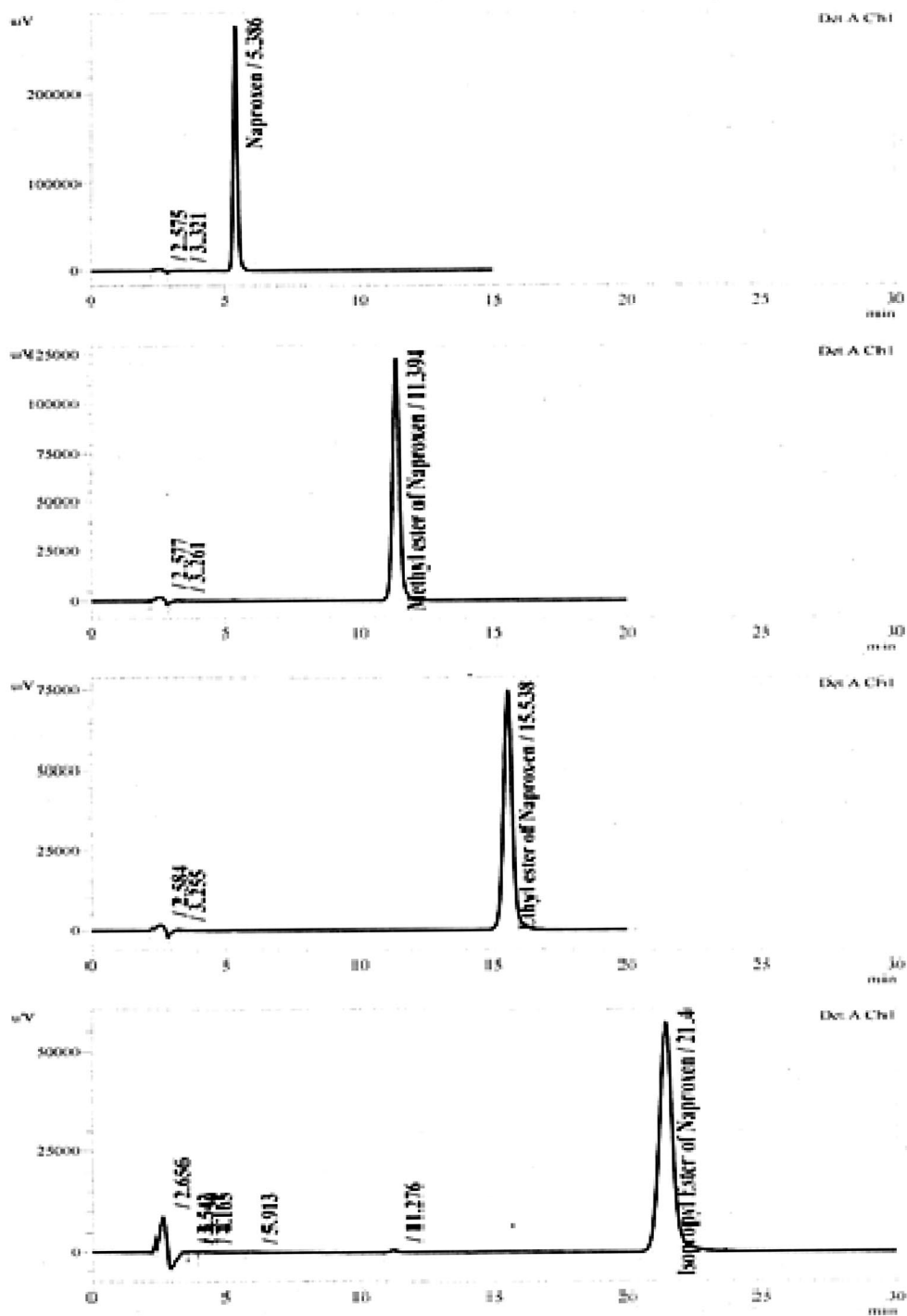


Figure 2. HPLC Chromatogram for naproxen and synthesized esters.

Table 2. Anti-inflammatory activity assessment via Paw edema inhibition at equimolar dose of 25 mg/kg bw p.o.

Test compounds	Swelling in thickness (mm) \pm SEM (% paw edema inhibition)			
	1 st hour	2 nd hour	3 rd hour	5 th hour
Control	0.295 \pm 0.069	0.4625 \pm 0.086	0.56 \pm 0.082	0.615 \pm 0.101
Naproxen	0.233 \pm 0.052 (21.02)	0.145 \pm 0.009* (68.65)	0.127 \pm 0.054** (77.23)	0.03 \pm 0.021*** (95.12)
Compound 1	0.220 \pm 0.025 (20.34)	0.125 \pm 0.074* (72.97)	0.13 \pm 0.055** (76.79)	0.02 \pm 0.022*** (96.75)
Compound 2	0.315 \pm 0.093 (18.64)	0.2775 \pm 0.092* (40)	0.245 \pm 0.077** (56.25)	0.052 \pm 0.074*** (91.54)
Compound 3	0.315 \pm 0.051 (16.27)	0.245 \pm 0.047* (47.03)	0.167 \pm 0.051** (70.03)	0.0575 \pm 0.020*** (90.65)

Data are expressed as mean \pm standard error mean (SEM); Asterisk represents the statistically significant difference from the control which is calculated as p values (*p < 0.05, **p < 0.01, ***p < 0.001).

Molecular docking analysis. After observing the significant analgesic and anti-inflammatory activity of the naproxen esters *in vivo*, we performed molecular simulation study to understand their binding modes particularly in COX-2 enzyme. In this part of analysis, we have considered these compounds as analogues of naproxen, not its prodrug. It is well known that COX-2 is one of major enzymes responsible for analgesic and anti-inflammatory activity. Regarding this, we first redocked ligand (naproxen) structure into the active site of protein by glide with standard precision mode, and on the basis of the highest minimum docking scores obtained, pose (Table 3) had been selected to visualize the ligand interactions in the active site of protein and we found the similar binding confirmation of experimental naproxen. After that, we docked other three esters in the same active sites and the best scored pose files had been subjected to further analysis. In post docking analysis, all three esters of naproxen had been found in participating hydrogen bonding with arginine (ARG) ARG¹²⁰, where, compound 1 and compound 2 were also involved in additional hydrogen bonding with tyrosine (TYR) TYR³⁵⁵ (Figure 3). As shown in Figure 3, the binding modes of all these esters were same as reference ligand (naproxen) and with the other members of the 2-arylpropionic acid family of NSAIDs. Here, the *p*-methoxy group of methyl and

ethyl esters were also oriented toward the top of the COX-2 active site, and also formed van der Waals (vdW) interactions with tryptophan (TRP) TRP³⁸⁷ and TRP³⁸⁵. In contrast, the orientation of isopropyl ester was slightly different from other esters. According to the report by Duggan et al, it appeared that the vdW interaction with TRP³⁸⁷ is unique to this inhibitor.³³ In our result, we estimated per residue's contribution to the ligand bindings, where we found that the vdW energy for methyl, ethyl and isopropyl ester compounds for TRP³⁸⁷ were -1.320 kcal/mol, 0.059 kcal/mol, 0.377 kcal/mol with corresponding distance from ligand were 2.409 Å, 2.200Å, 2.174Å, respectively. In the case of naproxen, the vdW energy was -1.579 kcal/mol with distance 2.433 Å. Moreover, we used MM-GBSA approach to calculate the binding energy of these moieties with COX-2 enzyme by Prime module of Schrodinger. The Prime MM-GBSA is one of the appropriate methods to predict the binding energy of protein ligand complex, which is to be correlated to some extent with biological data.³⁴ Here, more the negative value, stronger the binding will be, and it was observed that methyl ester showed the highest binding energy ($\Delta G_{\text{bind}} = -66.33$ kcal/mol), while isopropyl ester possessed the lowest value ($\Delta G_{\text{bind}} = -33.12$ kcal/mol). Additionally, naproxen showed slightly lower binding energy ($\Delta G_{\text{bind}} = -63.94$ kcal/mol), than that of methyl ester. Henceforth, from this molecular

modeling it can be predicted that the substitution by bulk group like isopropyl in the carboxylic position of naproxen may alter the orientation of the compound such as that decrease the distance

from TRP³⁸⁷ which subsequently may affect the vdW interaction and thus may reduce the COX-2 inhibitory activity.

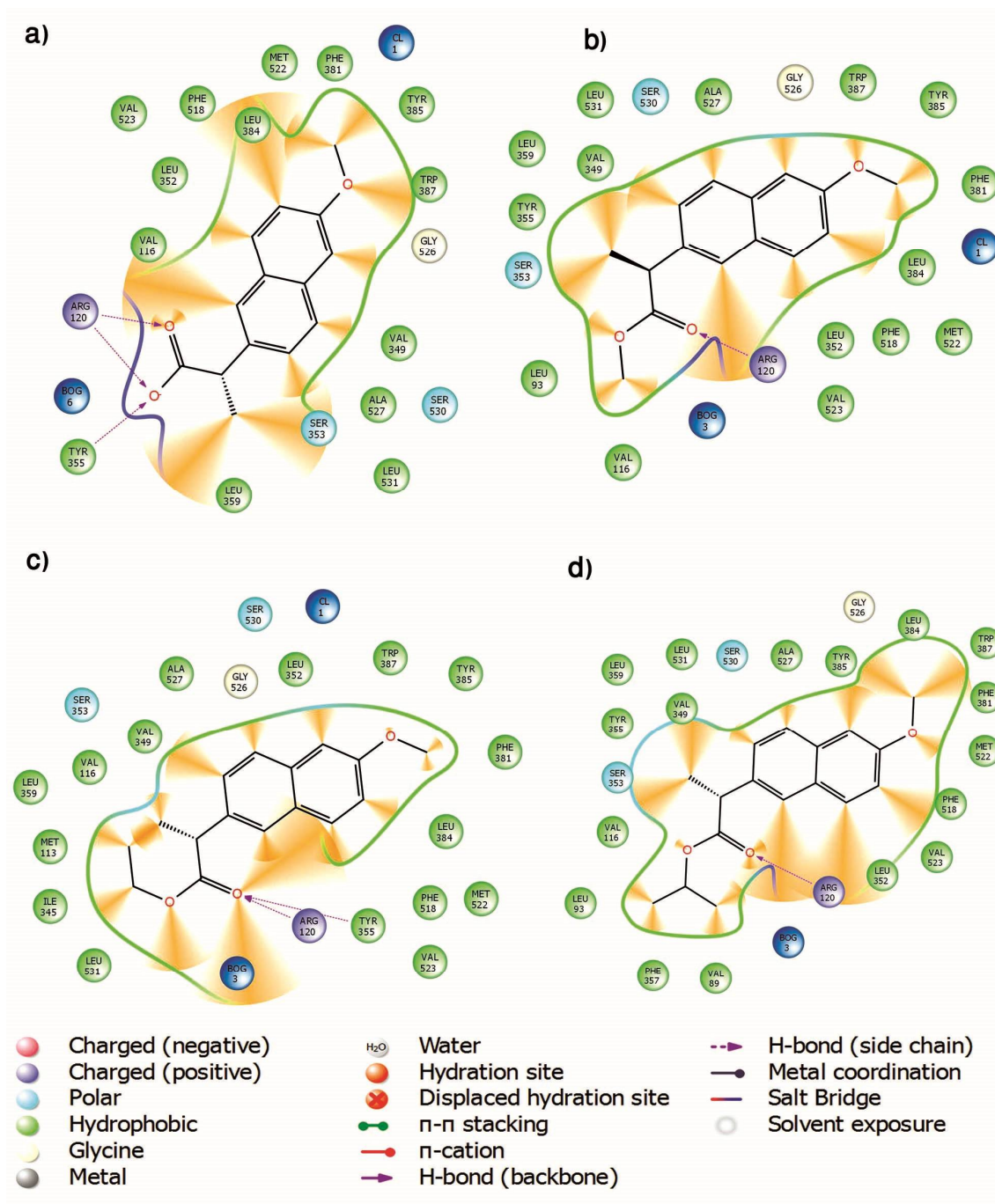


Figure 3. Binding patterns of synthesized naproxen esters in COX-2 active site. Here, a) Naproxen, b) Compound 1, c) Compound 2 and d) Compound 3.

Table 3. Docking results and binding energy of selected hits in kcal/mol.

Test compounds	COX-2					
	Docking score	Glide gscore	Glide emodel	Glide energy	ΔG_{bind}	ΔG_{bind} (NS)
S- Naproxen	-9.11	-9.11	-29.02	-60.27	-63.94	-64.765
Compound 1	-8.39	-8.39	-32.17	-49.17	-66.33	-71.138
Compound 2	-8.65	-8.65	-36.75	-45.69	-51.04	-66.463
Compound 3	-9.04	-9.04	-32.64	-24.01	-33.12	-53.084

Table 4. ADMET and drug likeness properties of the selected ligands by QikProp.

Name	MW ^b	HB donor ^c	HB acceptor ^d	SASA ^e	QP log HERG ^f	QP log S ^g	QP log Po/w ^h	% Human Oral Absorption ⁱ
Compound 1	244.290	0	2.750	519.962	-4.962	-4.118	4.677	100
Compound 2	258.316	0	2.750	557.671	-5.249	-4.694	3.682	100
Compound 3	272.343	0	2.750	578.682	-5.260	-5.092	4.328	100

^b acceptable range: <500, ^c acceptable range: ≤ 5 , ^d (acceptable range: ≤ 10), ^e acceptable range: 300–1000 using a probe with a 1.4 radius, ^f Predicted IC₅₀ concern: below -5. ^g acceptable range: -6.5–0.5 in mol/dm⁻³. ^h (acceptable range: -2–6.5). ⁱ (<25% is poor and >80% is high).

ADMET analysis. The ADMET properties of the synthesized molecules had been explored out by using QikProp module as stated above. The ADMET properties of naproxen and the compounds **1**, **2** and **3** are shown in Table 4. All the predicted properties of the synthesized compounds satisfied the Lipinski's rule of five to be considered as drug like potential. Here, we found that all the compounds had satisfactory ADMET properties, however, compound **2** and compound **3** may produce cardiotoxicity, because of having high concerning value of hERG K⁺ channel which indicates the potential of a compound for cardiac toxicity. From a previous *in vivo* toxicity study we also found that all the esters showed negligible microscopic as well as macroscopic GI injury and bleeding where methyl ester was found to be the least toxic.¹¹

CONCLUSION

In summary, we have successfully synthesized and characterized three esters of naproxen in a single experiment. The synthesized esters retained significant anti-inflammatory and analgesic activities of naproxen; and in some cases (methyl and ethyl ester) the analgesic activity was increased greatly.

Their anti-inflammatory action was also significant and sometimes better than that reported. The synthesized compounds were compared with redocked naproxen-COX-2 complex using docking score, glide emodel, glide score, hydrogen bond and hydrophobic interactions, and binding free energy. Compounds **1** & **2** had better binding energy compared to naproxen bound COX-2 complex. However, all the esters had better glide emodel and comparable docking score, glide score and glide energy. Besides, hydrogen bond and hydrophobic interactions were analyzed for the compounds that have exhibited favorable interactions with the active site residues. In particular their binding mode on COX-2 enzyme, the molecular docking analysis showed that vdW interaction with TRP³⁸⁷ as well as the binding energy were reduced with increase of alkyl side chain on the compounds i.e. the bulky group and that may affect COX inhibition accordingly. We found that among the compounds, methyl ester was the most potent candidate with better analgesic, anti-inflammatory and COX-2 binding energy with least toxicity satisfying all the ADMET properties *in silico*.

Authors' contributions. MA performed synthesis & chemical analysis, participated in biological studies and drafted these parts in the manuscript, ZAM participated in carrying out the experiment of analgesic, anti-inflammatory studies and performed statistical analysis, MMNU participated in performing anti-inflammatory study and molecular docking analysis, RD performed molecular docking and SMAR designed the studies, supervised and coordinated the research work and critically revised the manuscript.

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