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ORIGINAL PAPER

Synthesis, anti-inflammatory activities and docking studies of amide derivatives of meclofenamic acid

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Abstract NSAIDs constitute a heterogeneous class of pharmacological agents widely prescribed for the treatment of inflammation, pain and edema, as well as osteoarthritis, rheumatoid arthritis and musculoskeletal disorders. This class of drugs has proved efficacious on account of their analgesic, anti-pyretic and anti-inflammatory activities, but gastrointestinal toxicity exists as the biggest problem associated with their chronic use. Many attempts have been made to structurally modify conventional NSAIDs as selective COX-2 inhibitors based on the old and still prevalent common belief that selective inhibition of COX-2 would provide safer NSAIDs. The present work thus focused on the synthesis of amide derivatives of one of the conventional non-selective NSAID, meclofenamic acid utilizing the one pot procedure involving a selective agent, bis (2-oxo-3-oxazolidinyl) phosphonic chloride. The synthesized compounds were tested for their in vivo inflammatory activity using carrageenan rat paw edema assay, and were subsequently docked on COX-2 PDB code 4COX to have better insights into their mechanism of action. The amide derivative with *N*-4-methoxybenzyl moiety (TSN4) proved to have anti-inflammatory potential (72.8%) better than meclofenamic acid (56.75%). This compound also docked with the highest dock score among the synthesized compounds and was found to have both hydrogen bonding with Arg120 and Tyr355 and hydrophobic interactions with Val349, Leu352, Ser353, Tyr385, Trp387, Met522, Val523, Ala527 and Ser530. *N*-4-methoxybenzyl amide derivative (TSN4) followed by benzyl amide derivative (TSN1) of meclofenamic acid were identified as potential anti-inflammatory compounds in both in vivo and in silico studies.

Keywords NSAIDs · COX · Meclofenamic acid · Anti-inflammatory activity · Docking

Introduction

The rheumatic disorders are characteristic with high-frequency. Every seventh inhabitant on the Earth has some rheumatic disorders, in every third family there is a member with a rheumatic problem. More than 200 rheumatic diseases are known. About 300 million people in the world are only those suffering from arthritis and osteoporosis (Stoilov 2008). With over 30 million people taking daily a non-steroidal anti-inflammatory drug (NSAID), NSAIDs constitute one of the most used classes of drugs in modern medicine and are the mainstay in the treatment of inflammation, pain and edema, as well as osteoarthritis, rheumatoid arthritis and musculoskeletal disorders. NSAIDs exert their analgesic, anti-inflammatory and anti-pyretic effects through inhibition of cyclo-oxygenase (COX) enzyme, which is involved in the synthesis of prostaglandins responsible for fever, pain, sensitization and inflammation. Two isoforms of COX have been identified, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and is believed to generate prostaglandins (PGs) for normal physiological functions, while COX-2 is characterized by rapid induction through a variety of stimuli, including mitogens, hormones, cytokines, and growth factors (Narsinghani and Sharma 2014). The huge number of drugs in this class is placed in two major categories as—the non-selective COX inhibitors and selective

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COX inhibitors (Reddy and Roy 2013). NSAIDs inhibit both isoforms of COX; but with variations in affinities and selectivities. The non-selective inhibitors are the older, conventional, non-selective NSAIDs that inhibits both isoforms; COX-1 and COX-2 are preferential towards COX-1. There are no well-established structural reasons for preferential COX-1 inhibition in conventional NSAIDs. However, it is an established fact that inhibitors with a preference for an isoform are generally also "tight-binders" of that isoform. This tight-binding is time-dependent and involve two or three steps. In view of this concept of tight-binding, the conventional NSAIDs are sub-classified as (1) irreversible inhibitor, such as the time-dependent inhibitor Aspirin (2) slow reversible (tight-binding) inhibitors also termed as Class I inhibitors which include COX-1 selective inhibitors like indomethacin and flurbiprofen, which are competitive inhibitors with slow tight-binding kinetic profiles (3) time-independent Class II compounds, such as non-selective, competitive and reversible NSAID like ibuprofen (4) mixed-type class, Class III which are neither competitive, reversible nor classic time-dependent inhibitors but instead are classified as slow reversible inhibitors, e.g. meloxicam (Khan et al. 2015).

However, ulceration of the upper gastrointestinal (GI) tract is a well-known adverse effect of NSAIDs, which is related to inhibiting PG synthesis in tissues where PGs are responsible for physiological homeostasis and synthesis of these gastroprotective PGs is affected by inhibition of constitutive isoform of COX, i.e. COX-1. Selective inhibition of COX-2 is desirable to avoid gastric side-effects generally associated with inhibition of COX-1, which is responsible for gastric mucosal protection.

Many attempts have been made to convert older, conventional, non-selective NSAIDs into their corresponding ester or amide derivatives to confer COX-2 selectivity (Narsinghani and Sharma 2014). Indomethacin (Kalgutkar et al. 2000a, b, 2005); ketoprofen (Rajic et al. 2010; Zarghi and Ghodsi 2010); flurbiprofen (Halen et al. 2006); naproxen (Sadek et al. 2013), piroxicam (de Miranda et al. 2012); and lonazolac (Ismail et al. 2009) have been successfully converted into selective COX-2 inhibitors. However, the methodology utilized in NSAID modification does not follow a general scheme. In principle, the strategy consisted of introducing larger substituents to fit into the active site volume of COX-2 (Narsinghani and Sharma 2014).

Sir James Black, winner of the 1988 Nobel Prize in Physiology or Medicine, said that 'the most fruitful basis for the discovery of a new drug is to start with an old drug' (Raju 2000). Keeping in mind this statement from the Noble Laureate, the conventional non-selective NSAID meclofenamic acid was converted into its corresponding amide derivatives (Kalgutkar et al. 2002) with the objective of enhancing the potency while reducing the unwanted GI side-effects at the same time. The present work thus deals with the modification of carboxylate moiety of meclofenamic acid into its corresponding amide derivatives, evaluating their anti-inflammatory potential and performing docking of these synthesized derivatives on COX-2 enzyme.

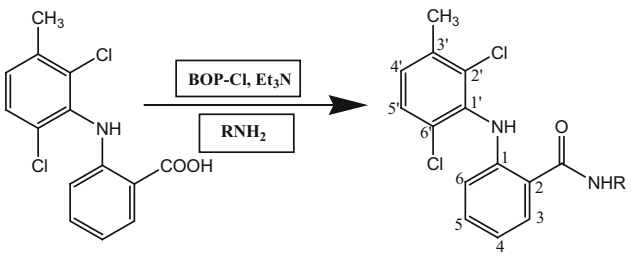
Experimental

Physical measurements

Melting point of all the compounds was determined by open capillary melting point apparatus and is uncorrected. Thin layer chromatography was performed using prepared precoated silica gel-G TLC plates. The IR spectra were recorded on FTIR, Shimadzu-8400S at School of Pharmacy, DAVV, Indore (MP). Mass spectra of compounds were obtained on JEOL-AccuTOF JMS-T100LC Mass Spectrometer having a DART (Direct Analysis in Real Time) source at Sophisticated Analytical Instrumentation Facility (SAIF), CDRI, Lucknow. ^{1}H-NMR and ^{13}C-NMR spectra of compounds were obtained on Bruker DRX-300 (300 MHz FT NMR) at SAIF, CDRI, Lucknow. Elemental analysis was also carried out using Elementar Vario EL III Carlo Erba 1108 at SAIF, CDRI, Lucknow.

Synthesis of compounds

All the chemicals were of synthetic grade and were purchased from Aldrich and alfa-aesar. As depicted in Scheme 1, a reaction mixture containing meclofenamic acid (3.64 mmol) and BOP-Cl [bis(2-oxo-3-oxazolidinyl) phosphonic chloride)] (3.64 mmol), in 10 ml of anhydrous dichloromethane was treated with anhydrous triethylamine (7.28 mmol) and allowed to stir at room temperature for 5 min. The mixture was then treated with the appropriate amine (4 mmol) and stirred overnight at room temperature. Following dilution with dichloromethane (30 mL), the organic solution was washed with water (2 × 25 mL),



Scheme 1 Synthesis of title compounds. *BOP-Cl* bis(2-oxo-3-oxazolidinyl) phosphonic chloride), Et_3N Triethylamine, *RNH_2* Amine

dried ($MgSO_4$), filtered, and the solvent concentrated in vacuo. The crude amides were chromatographed [ethyl acetate: *n*-hexane (10:90)] to get the pure compounds.

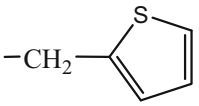
TSN1: R = -$CH_2C_6H_5$

TSN2: R = -C_6H_{11}

TSN3: R = -$CH_2(CH_2)_5CH_3$

TSN4: R = -$CH_2C_6H_4$(4-OCH_3)

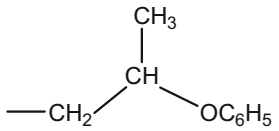
TSN5: R = -$CH_2C_6H_4$(4-Cl)

TSN6: R = 

TSN7: R = -$CH_2C_6H_4$(4-CF_3)

TSN8: R = -$CH_2C_6H_3$(3,4-diCl)

TSN9: R = -CH_2-C_6H_{11}

TSN10: R = 

N-Benzyl-2-(2,6-dichloro-3-methyl-phenylamino)-benzamide (TSN1)

Yield: 65%; $R_f = 0.4$; m.p. 105–110 °C; IR (KBr) ν (cm^{-1}): 1645 [C=O(amide)], 1455, 1607 (C=C aromatic), 3308 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 2.40 [s, 3H, CH_3], 4.59 [s, 2H, CH_2], 6.28–6.31 [d, 1H, ArH], 6.77–6.82 [t, 1H, ArH], 7.16–7.40 [m, 8H, ArH], 7.61–7.63 [dd, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.49 (3′-CH_3), 43.28 (CH_2-benzyl), 115.7 (C-6), 119.5 (C-2), 119.7 (C-4), 126.74 (C-5′), 127.54 (C-4′), 128.85 (C-2″ and 6″), 129.09 (C-4″), 129.11 (C-3″ and 5″), 129.26 (C-3), 131.38 (C-5), 131.89 (C-6′), 133.14 (C-3′), 134.35 (C-2′), 137.2 (C-1′), 138.1 (C-1), 146.79 (C-1″), 172.14 (CONH); MS (DART-MS) m/z: 385.1 $(M + H)^+$; Mol. formula: $C_{21}H_{18}Cl_2N_2O$; Exact mass: 384.08; Elemental analysis for $C_{21}H_{18}Cl_2N_2O$ (MW = 385.29) in wt% calc. C = 65.46, H = 4.71, N = 7.27, O = 4.15 and found to be C = 64.58, H = 4.50, N = 6.92, O = 4.52.

N-Cyclohexyl-2-(2,6-dichloro-3-methyl-phenylamino)-benzamide (TSN2)

Yield: 68%; $R_f = 0.46$; m.p. 120–125 °C; IR (KBr) ν (cm^{-1}): 1658 [C=O(amide)], 1452, 1602 (C=C aromatic), 3320 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 1.22–1.49 [m, 6H, H (C-3″,C-4″,C-5″)], 1.67–2.00 [m, 5H, H (C-1″, C-2″,C-6″)], 2.4 [s, 3H, CH_3], 6.27–6.29 [d, 1H, ArH], 6.77–6.82 [t, 1H, ArH], 7.15-7.21 [m, 2H, ArH], 7.34–7.36 [d, 1H, ArH], 7.54–7.57 [dd, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.52 (3′-CH_3), 23.1 (C-3″ and 5″), 27.5 (C-4″), 34.2 (C-2″ and 6″), 48.1 (C-1″), 115.62 (C-6), 119.37(C-2), 119.76 (C-4), 127.68 (C-5′), 129.01 (C-4′), 129.32 (C-3), 131.33 (C-5), 132.67 (C-6′), 134.2 (C-3′), 137.3 (C-2′), 137.87 (C-1′), 146.42 (C-1), 171.42 (CONH); MS (DART-MS) m/z: 377.13 $(M + H)^+$; Mol. formula: $C_{20}H_{22}Cl_2N_2O$; Exact mass: 376.11; Elemental analysis for $C_{20}H_{22}Cl_2N_2O$ (MW = 377.32) in wt% calc. C = 63.67, H = 5.88, N = 7.42, O = 4.24 and found to be C = 63.29, H = 5.95, N = 6.82, O = 4.76.

(2,6-Dichloro-3-methyl-phenylamino)-N-heptyl-benzamide (TSN3)

Yield: 70%; $R_f = 0.48$; m.p. 64–70 °C; IR (KBr) ν (cm^{-1}): 1650 [C=O(amide)], 1450, 1610 (C=C aromatic), 3330 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 0.86–0.91 [t, 3H, CH_3(C-7″)], 1.31–1.38 [m, 9H, CH_2 (alkyl side chain)],1.59–1.66 [m, 2H, CH_2 (alkyl side chain)], 2.39 [s, 3H, CH_3], 3.35–3.39 [t, 2H, CH_2 (C-1″)], 6.27–6.29 [d, 1H, ArH], 6.76–6.81 [m, 1H, ArH], 7.13–7.21 [m, 2H, ArH], 7.32–7.35 [d, 1H, ArH], 7.54–7.57 [dd, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 14.58 (C-7″), 20.78 (3′-CH_3), 23.81 (C-6″), 28.23 (C-3″), 30.31 (C-4″), 30.69 (C-2″), 33.14 (C-5″), 40.87 (C-1″), 115.53 (C-6), 119.33 (C-2), 119.97 (C-4), 127.6 (C-5′), 129.06 (C-4′), 129.18 (C-3), 131.27 (C-5), 132.78 (C-6′), 134.17 (C-3′), 137.25 (C-2′), 137.93 (C-1′), 146.26 (C-1), 171.95 (CONH); MS (DART-MS) m/z: 393.26 $(M + H)^+$; Mol. formula: $C_{21}H_{26}Cl_2N_2O$; Exact mass: 392.14; Elemental analysis for $C_{21}H_{26}Cl_2N_2O$ (MW = 393.35) in wt% calc. C = 64.12, H = 6.66, N = 7.12, O = 4.07 and found to be C = 64.83, H = 7.52, N = 6.99, O = 4.23.

(2,6-Dichloro-3-methyl-phenylamino)-N-(4-methoxybenzyl)-benzamide (TSN4)

Yield: 72%; $R_f = 0.35$; m.p. 100–110 °C; IR (KBr) ν (cm^{-1}): 1646 [C=O(amide)], 1485, 1610 (C=C aromatic),

3315 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 2.4 [s, 3H, CH_3], 3.78 [s, 3H, OCH_3], 4.52–4.58 [d, 2H, CH_2], 6.27–6.30 [d, 1H, ArH], 6.7-6.81[t, 1H, ArH], 6.88–6.91 [d, 2H, ArH], 7.17–7.23 [m, 2H, ArH], 7.30–7.37 [m, 3H, ArH], 7.58–7.61 [dd, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.65 (3′-CH_3), 44.82 (CH_2-benzyl), 54.21 (OCH_3), 114.24 (C-3″ and 5″), 115.21 (C-6), 119.12 (C-2), 119.67 (C-4), 126.65 (C-5′), 129.07 (C-4′), 129.23 (C-2″ and 6″), 129.32 (C-3), 131.23 (C-5), 131.88 (C-6′), 133.10 (C-3′), 134.25 (C-2′), 137.12 (C-1′), 138.21 (C-1″), 146.34 (C-1), 158.15 (C-4″), 172.02 (CONH); MS (DART-MS) m/z: 415.15 $(M + H)^+$; Mol. formula: $C_{22}H_{20}Cl_2N_2O_2$; Exact mass: 414.09; Elemental analysis for $C_{22}H_{20}Cl_2N_2O_2$ (MW = 415.31) in wt% calc. C = 63.62, H = 5.16, N = 6.75, O = 7.7 and found to be C = 63.37, H = 5.61, N = 6.19, O = 7.85.

N-(4-chlorobenzyl)-(2,6-dichloro-3-methyl-phenylamino)-benzamide (TSN5)

Yield: 67%; $R_f = 0.4$; m.p. 98–112 °C; IR (KBr) ν (cm^{-1}): 1652 [C=O(amide)],1465, 1605 (C=C aromatic), 3325 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 2.40 [s, 3H, CH_3], 4.56 [s, 2H, CH_2], 6.28–6.30 [d, 1H, ArH], 6.77–6.82 [t, 1H, ArH], 7.17-7.23 [m, 2H, ArH], 7.32–7.39 [m,5H, ArH], 7.61–7.63 [dd, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.47 (3′-CH_3), 44.12 (CH_2-benzyl), 115.12 (C-6), 119.02 (C-2), 119.34 (C-4), 125.14 (C-5′), 126.22 (C-4′), 129.1 (C-3″ and 5″), 129.23 (C-2″ and 6″), 129.42 (C-3), 131.62 (C-5), 131.75 (C-6′), 133.23 (C-4″), 134.65 (C-3′), 136.51 (C-2′), 137.12 (C-1′), 138.15 (C-1″), 146.57 (C-1), 171.23 (CONH); MS (DART-MS) m/z: 419.16 $(M + H)^+$; Mol. formula: $C_{21}H_{17}C_{l3}N_2O$; Exact mass: 418.04; Elemental analysis for $C_{21}H_{17}C_{l3}N_2O$ (MW = 419.73) in wt% calc. C = 60.09, H = 4.85, N = 6.67, O = 3.81 and found to be C = 59.26, H = 5.08, N = 6.12, O = 3.92.

2-(2,6-dichloro-3-methyl-phenylamino)-*N*-thiophen-2-yl-methyl-benzamide (TSN6)

Yield: 75%; $R_f = 0.33$; m.p. 128–132 °C; IR (KBr) ν (cm^{-1}): 1648 [C=O(amide)], 3083 (C-H str. in thiophene), 1470, 1600 (C=C aromatic), 3310 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 2.4 [s, 3H, CH_3], 4.74 [s, 2H, CH_2], 6.27–6.30 [d, 1H, ArH], 6.76–6.81 [t, 1H, ArH], 6.94–6.97 [m, 1H, ArH], 7.06-7.07 [m, 1H, ArH], 7.17–7.23 [m, 2H, ArH], 7.28–7.30 [dd, 1H, ArH], 7.34–7.37 [d, 1H, ArH], 7.56–7.59 [dd, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.34 (3′-CH_3), 42.62 (CH_2-thiophenyl), 115.12 (C-6), 119.12 (C-2), 119.55 (C-4), 123.23 (C-3″), 125.72 (C-5″), 126.45 (C-4″), 127.48 (C-5′), 129.05 (C-4′), 129.36 (C-3), 131.45 (C-5), 132.78 (C-6′), 134.32 (C-3′), 137.15 (C-2′), 137.54 (C-1′), 141.28 (C-1), 146.56 (C-1″), 171.16 (CONH); MS (DART-MS) m/z: 391.09 $(M + H)^+$; Mol. formula: $C_{19}H_{16}Cl_2N_2OS$; Exact mass: 390.04; Elemental analysis for $C_{19}H_{16}Cl_2N_2OS$ (MW = 391.31) in wt% calc. C = 58.32, H = 4.08, N = 7.16, O = 4.09, S = 8.19 and found to be C = 58.14, H = 4.94, N = 6.54, O = 4.25, S = 5.86.

2-(2,6-Dichloro-3-methyl-phenylamino)-*N*-4-trifluoromethyl-benzyl)-benzamide (TSN7)

Yield: 77%; $R_f = 0.4$; m.p. 130–144 °C; IR (KBr) ν (cm^{-1}): 1635 [C=O(amide)], 1495, 1607 (C=C aromatic), 3315 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 2.4 [s, 3H, CH_3], 4.67 [s, 2H, CH_2], 6.28–6.31 [d, 1H, ArH] 6.79–6.83 [t, 1H, ArH], 7.17–7.24 [m, 4H, ArH], 7.34–7.37 [d,1H, ArH], 7.56–7.67[m, 4H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.75 (3′-CH_3), 43.86 (CH_2-benzyl), 115.55 (C-6), 119 (C-2), 119.3 (C-4), 124.42 (CF_3), 125.08 (C-3″ and 5″), 126.54 (C-5′), 127.58 (C-2″ and 6″), 129.06 (C-4′), 129.19 (C-4″), 129.31 (C-3), 131.59 (C-5), 133.23 (C-6′), 134.49 (C-3′), 137.16 (C-2′), 138.01 (C-1′), 145.19 (C-1), 146.79 (C-1″), 172.05 (CONH); MS (DART-MS) m/z: 453.14 $(M + H)^+$; Mol. formula: $C_{22}H_{17}Cl_2F_3N_2O$; Exact mass: 452.07; Elemental analysis for $C_{22}H_{17}Cl_2F_3N_2O$ (MW = 453.28) in wt% calc. C = 58.29, H = 3.78, N = 6.18, O = 3.53 and found to be C = 57.46, H = 4.75, N = 6.01, O = 3.67.

N-(3,4-dichlorobenzyl)-2-(2,6-dichloro-3-methyl-phenylamino)-benzamide (TSN8)

Yield: 70%; $R_f = 0.4$; m.p. 144–148 °C; IR (KBr) ν (cm^{-1}): 1640 [C=O(amide)], 1469, 1613 (C=C aromatic), 3331 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 2.4 [s, 3H, CH_3], 4.55–4.57 [s, 2H, CH_2], 6.28–6.31 [d, 1H, ArH], 6.78–6.83 [t,1H, ArH], 7.17–7.24 [m, 2H, ArH], 7.31–7.37 [m, 2H,ArH], 7.47–7.54 [m, 2H, ArH], 7.62–7.65 [dd, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.23 (3′-CH_3), 44.65 (CH_2-benzyl), 115.09 (C-6), 119.12 (C-2), 119.54 (C-4), 126.36 (C-5′), 127.52 (C-4′), 128.15 (C-6″), 128.89 (C-2″), 129.06 (C-3), 129.18 (C-5″), 130.65 (C-4″), 131.45 (C-5), 133.48 (C-3″), 134.78 (C-6′), 135.42 (C-3′), 136.78 (C-2′), 137.28 (C-1′), 138.36 (C-1), 146.68 (C-1″), 171.18 (CONH); MS (DART-MS) m/z: 453.07 $(M + H)^+$; Mol. formula: $C_{21}H_{16}Cl_4N_2O$; Exact mass: 452; Elemental analysis for $C_{21}H_{16}Cl_4N_2O$ (MW = 454.18) in wt% calc. C = 55.53, H = 3.55, N = 6.17, O = 3.52 and found to be C = 55.8, H = 3.90, N = 6.01, O = 3.8.

N-Cyclohexylmethyl-2-(2,6-dichloro-3-methyl-phenylamino)-benzamide (TSN9)

Yield: 75%; $R_f = 0.5$; m.p. 134–142 °C; IR (KBr) ν (cm^{-1}): 1655 [C=O(amide)], 1450, 1604 (C=C aromatic), 3318 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ: 1.155–1.366 [m, 6H, H(C-3″, C-4″, C-5″)], 1.595–1.855 [m, 5H, H(C-1″,C-2″,C-6″)], 2.4 [s, 3H, CH_3], 3.21-3.23 [s, 2H, CH_2], 6.27–6.29 [d, 1H, ArH], 6.77–6.82 [t, 1H, ArH], 7.16–7.21 [m, 3H, ArH], 7.34–7.36 [d, 1H, ArH], 7.54–7.57 [d, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.22 (3′-CH_3), 23.7 (C-3″ and 5″), 26.8 (C-4″), 34.25 (C-2″ and 6″), 42.75 (C-1″), 47.91 (CH_2-cyclohexyl), 115.23 (C-6), 119.29 (C-2), 119.64 (C-4), 127.85 (C-5′), 129.12 (C-4′), 129.35 (C-3), 131.09 (C-5), 132.45 (C-6′), 134.21 (C-3′), 137.44 (C-2′), 137.97 (C-1′), 146.12 (C-1), 171.45 (CONH); MS (DART-MS) m/z: 391.24 $(M + H)^+$; Mol. formula: $C_{21}H_{24}Cl_2N_2O$; Exact mass: 390.13; Elemental analysis for $C_{21}H_{24}Cl_2N_2O$ (MW = 391.33) in wt% calc. C = 64.45, H = 6.18, N = 7.16, O = 4.09 and found to be C = 64.4, H = 8.05, N = 6.96, O = 4.3.

(±)-2-(2,6-dichloro-3-methyl-phenylamino)-*N*-(2-phenoxy-propyl)-benzamide (TSN10)

Yield: 70%; $R_f = 0.43$; m.p. 110–118 °C; IR (KBr) ν (cm^{-1}): 1660 [C=O(amide)], 1469, 1604 (C=C aromatic), 3316 (N–H); ^{1}H-NMR (300 MHz, CD_3OD) δ:1.26-1.36 [t, 3H, CH_3 of propyl], 2.4 [s, 3H, CH_3], 3.58–3.61 [s, 2H, CH_2 of propyl], 4.67–4.73 [m, 1H, O–CH of propyl], 6.26–6.28 [d, 1H, ArH], 6.73–6.78 [m, 1H, ArH], 6.86–6.91 [t,1H, ArH], 6.97–7.00 [d, 2H, ArH], 7.16–7.26 [m, 4H, ArH], 7.34–7.36 [d, 1H, ArH], 7.46–7.49 (dd, 1H, ArH]; ^{13}C-NMR (300 MHz, CD_3OD) δ: 20.46 (3′-CH_3), 21.12 (CH_3 of propyl), 47.92 (CH_2 of propyl), 74.92 (O–CH of propyl), 114. 6 (C-2″ and 6″), 115.21 (C-6), 119.42 (C-2), 119.65 (C-4), 121.5 (C-4″), 127.57 (C-5′), 129.08 (C-4′), 129.17 (C-3), 129.33 (C-3″ and 5″), 131.24 (C-5), 133.23 (C-6′), 134.46 (C-3′), 137.18 (C-2′), 138.49 (C-1′), 146.62 (C-1), 158.12 (C-1″), 171.12 (CONH); MS (DART-MS) m/z: 429.16 $(M + H)^+$; Mol. formula: $C_{23}H_{22}Cl_2N_2O_2$; Exact mass: 428.11; Elemental analysis for $C_{23}H_{22}Cl_2N_2O_2$ (MW = 429.34) in wt% calc. C = 63.62, H = 4.85, N = 6.75, O = 7.7 and found to be C = 64.21, H = 5.85, N = 6.15, O = 7.89.

Anti-inflammatory activity

Carrageenan rat paw edema assay

The anti-inflammatory activity was performed using carrageenan rat paw edema assay as per the guidelines and rules laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Male or female Sprague–Dawley rats with a body weight between 100 and 150 g were used. To insure uniform hydration, the rats received 5 ml of water by stomach tube (controls) or the test drug dissolved or suspended in the same volume. Thirty minutes later, the rats were challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hind paw. The paw volume was measured plethysmographically (LE7500 Digital Plethysmometer, Panlab Harvard Apparatus) immediately after injection, again 1, 2, 3 h after challenge and compared with the initial paw volume of each rat for determining the edema volume (Winter et al. 1962). The difference between the initial and subsequent paw volume reading gave the actual edema volume. The percent inhibition of inflammation/protection was calculated using the formula:

$$\%\text{inhibition} = \frac{(V_t - V_0)_{\text{control}} - (V_t - V_0)_{\text{treated}} \times 100}{(V_t - V_0)_{\text{control}}}$$

where V_t represents paw volume at given time 't'; V_0: paw volume before giving any treatment.

Statistical analysis

Anti-inflammatory activity was expressed as mean paw volume ± standard error of the mean (SEM) and mean ± SEM percentage inhibition of edema in treated animals in comparison with the control group. The statistical significance of differences between control and treated groups was determined by ANOVA, followed by Dunnett's test. A value of $p < 0.05$ was considered to be statistically significant.

Docking of synthesized compounds on COX-2 enzyme

Docking studies were performed using Surflex Dock module of SYBYL X 2.1.1 (Tripos Inc, St. Louis, MO, USA). The title compounds were docked on COX-2 PDB ID 4COX (COX-2 in complex with indomethacin); resolution 2.9 Å. This COX-2 enzyme is a dimer and it is reported that COX-2 is functionally active as a monomer, and thus docking was carried out on chain A of the enzyme. The protomol was generated by extracting the ligands, removing all water molecules and other co-factors. In addition to this, polar hydrogen atoms and AMBER7FF99 charges were added. Generation of protomol was carried out by keeping the threshold and bloat values as 0.5 and 1, respectively. The synthesized compounds and the standard NSAIDs tested for in vivo anti-

inflammatory activities in present study were subjected to docking on this validated protomol generated from COX-2 PDB 4COX. Finally, the docked ligands were ranked on the basis of various scoring functions, primarily single consensus score (C-Score) reporting the output of total score (Raghavendra et al. 2012).

Results and discussion

Chemistry

The synthesis of the title compounds was carried out by converting the carboxylate moiety of meclofenamic acid to amide functionality utilizing the one pot procedure involving a selective agent, bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP-Cl) serving as the carboxylic acid activator (Diago-Meseguer and Palomo-Coll 1980). The desired derivatives were prepared by treating the mixture of acid and BOP-Cl in anhydrous dichloromethane with appropriate amine in the presence of BOP-Cl and triethylamine. The reaction was monitored by performing TLC using ethyl acetate: *n*-hexane as the mobile phase (2:8). This one pot procedure afforded the desired compounds in 60–75% yield. The pure compounds obtained after column chromatography were characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR and mass spectral analysis. The C=O str. in amides was observed between 1635–1660 cm^{-1} and N–H str. was seen between 3308 and 3331 cm^{-1}. ^{1}H-NMR spectra depicted methyl signal at 2.4 δ and aromatic protons between 6.27 and 7.67. In ^{13}C-NMR spectra, methyl signal was seen at 20 δ while the carbonyl carbon was observed at 171 δ. Mass spectra of all the synthesized compounds depict the presence of $[M + H]^{+}$ along with isotopic peaks for chlorine. In addition to these spectral data, elemental analysis was also in good agreement with the reported values.

Anti-inflammatory activity

All the synthesized compounds were evaluated for their anti-inflammatory activity by carrageenan rat paw edema assay at the dose of 14 mg/kg along with the standard drugs meclofenamic acid and indomethacin (Fig. 1). Two derivatives, TSN4 and TSN1 were found to display high potency in protection of carrageenan induced inflammation at 1, 2 and 3 h. of inflammation ($p < 0.001$). Compound TSN4 was the most active compound of the series with percentage inhibition of 72.8% as compared to meclofenamic acid (56.75% inhibition) and comparable to indomethacin (75.15%). It was followed by TSN1 (70.35%), TSN3 (66.55%) and TSN10 (63.75%). TSN7 (54.28%) showed comparable percentage inhibition of edema to meclofenamate (56.75%), while TSN8 (15.56%) was observed to be least active among the series of synthesized compounds.

Analysis of structure–activity relationship of synthesized compounds revealed that the electron-releasing group substituted on the para position of benzyl group attached to amide nitrogen of TSN4 resulted in most significant anti-inflammatory activity ($p < 0.001$) when compared to the control group. The second most promising among the synthesized compounds was TSN1 with *N*-benzyl group linked to the amide functionality ($p < 0.001$). Substitution of electron withdrawing groups resulted in decreased anti-inflammatory activity when compared to TSN4 and TSN1, as evident from comparable percentage inhibition of TSN8 with 4-trifluoromethyl group with meclofenamate and loss of activity in TSN5 and TSN8 with 4-chloro and 3,4-dichloro groups. In addition to this, substitution of alkyl

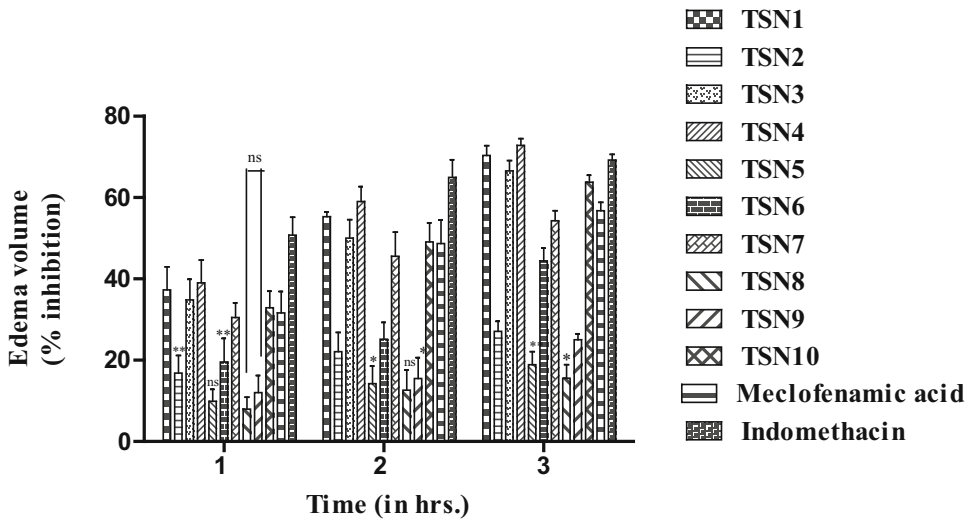


Fig. 1 Acute anti-inflammatory effect of tested amide derivatives (TSN1-TSN10) on carrageenan induced rat paw edema, in comparison with standard. Data shown are means ($n = 6$) ± SEM, *$p < 0.05$, **$p < 0.01$, all unmarked ***$p < 0.001$, ns: $p > 0.05$ when compared with control

chain in the form of TSN3 (heptyl amide) also resulted in potent anti-inflammatory activity when compared to meclofenamic acid.

Furthermore, on substituting 2-phenoxy propyl group to amide moiety (TSN10), significant reduction in edema was observed with respect to control group, but the reduction was lower than TSN4, TSN1 and TSN3. Heterocyclic ring in the form of thiophene moiety (TSN6) was also tried and this compound displayed moderate anti-inflammatory activity (44.4%) in comparison to meclofenamate and indomethacin. When saturated cyclic ring (cyclohexyl) was attached directly to amide nitrogen, percentage inhibition of inflammation decreased and upon inserting a methylene group in between cyclohexyl and amide nitrogen (TSN9), there was a fall in activity in comparison to TSN2.

Results of biological evaluation highlighted the significant anti-inflammatory activity of compounds TSN4, TSN1 followed by TSN3 and TSN10 predominantly at second and third hour of inflammation (Fig. 1), i.e. in the second phase of carrageenan induced inflammation, which is sensitive to drugs like indomethacin (Vinegar et al. 1969).

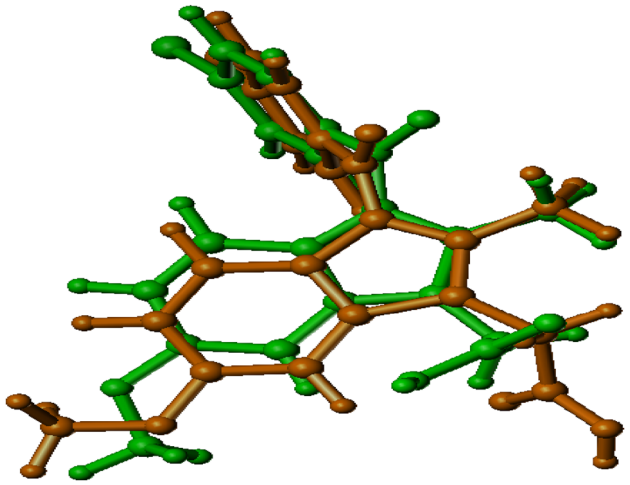


Fig. 2 Comparison of redocking results of ligand (indomethacin) to X-ray crystallographic mode of binding (model *coloured green* represent docked ligand while model *coloured orange* is experimentally verified binding pose) (colour figure online)

Molecular docking

To gain insights into the anti-inflammatory activities of synthesized compounds and correlate the in vivo results to the structure of title compounds, molecular docking was carried out on COX-2 PDB ID 4COX. The docking methodology adopted was validated by redocking the extracted ligand (indomethacin) and overlapping the docked and co-crystallized ligands. The two ligands overlapped in the same position (Fig. 2).

The newly synthesized compounds (TSN1-TSN10) docked and occupied the same binding site as that of celecoxib, indomethacin and meclofenamic acid (Fig. 3). Figure 3 depicts the overlapped view of ball and stick models of TSN1, TSN4 and standards.

Indomethacin was shown to have hydrogen bond interactions between its oxygen of benzoyl moiety and hydroxyl oxygen of Ser530 (2.02 Å), and second, oxygen atom of acidic group and hydrogen atom of Arg120 (2 Å). Celecoxib displayed two hydrogen bond interactions, viz. one between oxygen of sulfonamide group and hydrogen atom of Ser530 (2.73 Å) and the other between fluorine atom of trifluoromethyl group and hydrogen atom of Arg120 (2.45 Å). The nitrogen atom of amide group of TSN4 formed

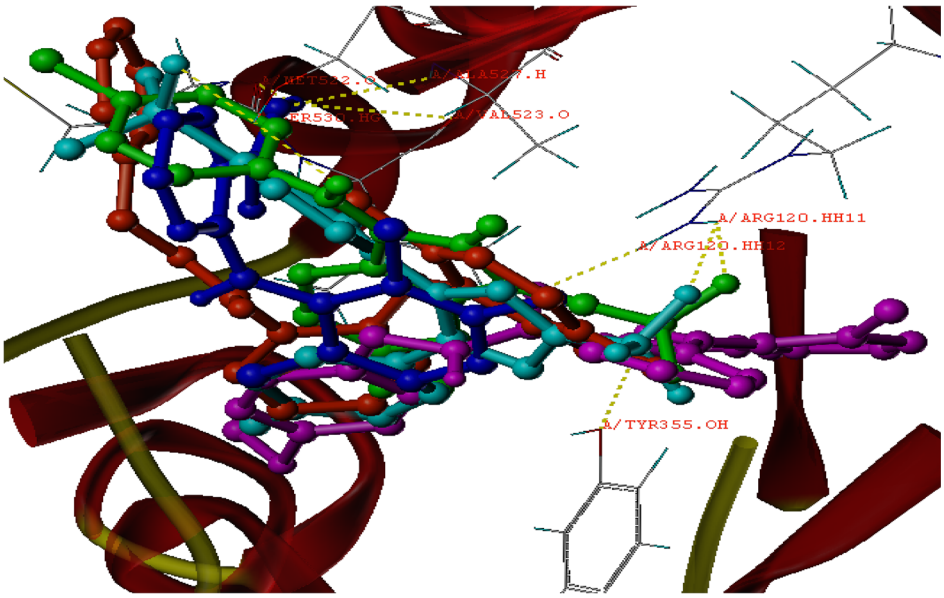


Fig. 3 Overlap of ball and stick models of docked compounds: TSN1 (*red–orange colour*), TSN4 (*magenta colour*), Celecoxib (*cyan colour*), Indomethacin (*green colour*) and meclofenamic acid (*blue colour*) displaying hydrogen bond interactions (*yellow coloured dotted lines*) with amino acids in the active site of COX-2, PDB Code 4COX (COX-2 co-crystallized with indomethacin) (colour figure online)

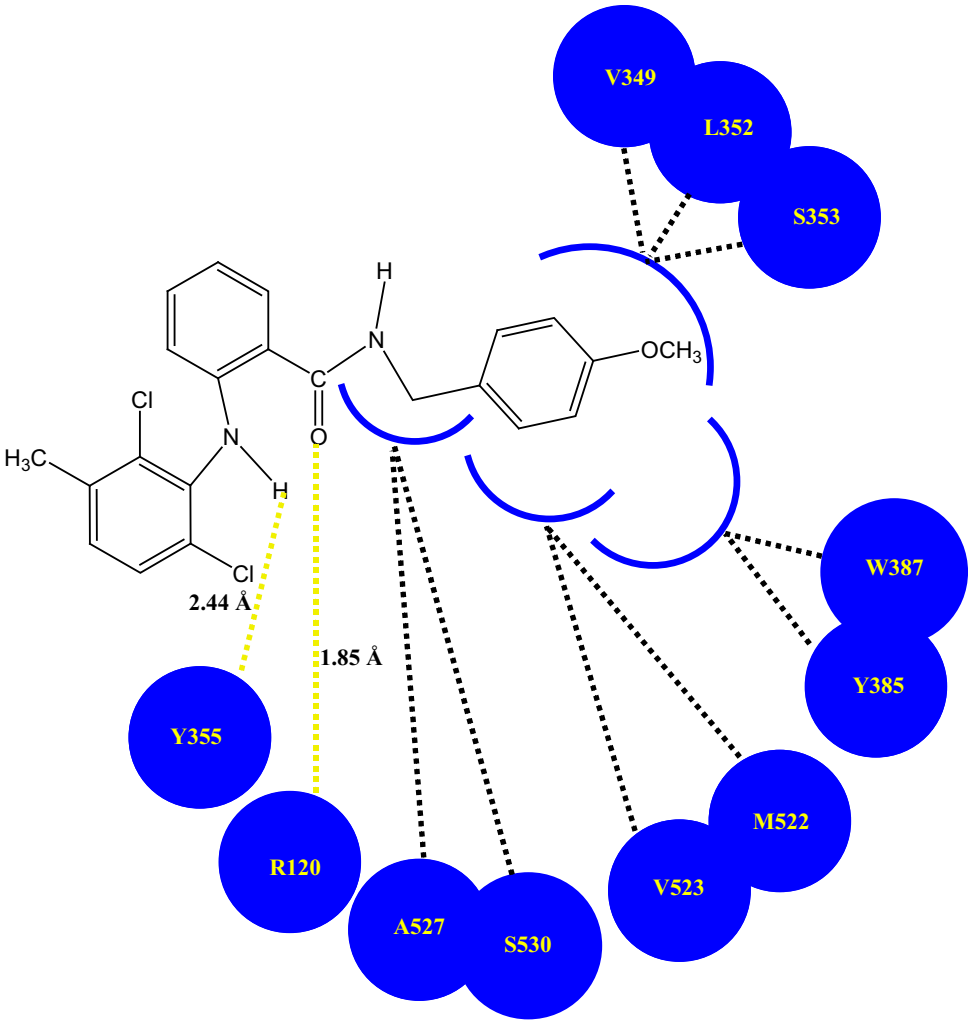


Fig. 4 Binding map of top scoring compound (TSN4) on COX-2 (4COX); *yellow coloured dotted lines* indicate hydrogen bonding interactions along with the distances, *black dotted lines* indicate hydrophobic interactions (colour figure online)

hydrogen bond (Fig. 4) with hydroxyl oxygen of Tyr355 (2.44 Å) while the carbonyl oxygen of amide group of this compound was involved in hydrogen bonding with hydrogen of Arg120 (1.85 Å). Meclofenamic acid displayed three hydrogen bond interactions with Met522 (1.85 Å), Val523 (2.61 Å) and Ala527 (2.5 Å).

Research findings have reported that interaction between Arg120 and the carboxylate moiety of arachidonic acid is the fundamental requirement for COX reaction to happen (Bhattacharyya et al. 1996; Malkowski et al. 2000). The most active compound (TSN4) is also displaying hydrogen bond interaction with Arg120. Another important residue known to bind carboxylate of arachidonate through phenolic hydroxyl is Tyr355, which plays a structural role in COX inhibition by various NSAIDs (Rowlinson et al. 2003). Our most potent compound (TSN4) is also involved in hydrogen bonding with phenolic hydroxyl of Tyr355 (Fig. 4).

While analysing the hydrophobic interactions of docked compounds (Fig. 5), it was seen that amino acids, viz. Val349, Leu352, Ser353, Tyr385, Trp387, Met522, Val523 and Ala527 were enclosing TSN4, TSN1, celecoxib, indomethacin and meclofenamic acid.

The amide moiety and the *N*-4-methoxybenzyl and *N*-benzyl groups of TSN4 (Figs. 4, 6a) and TSN1 (Fig. 6b), respectively, are seen in close vicinity to Val349, Leu352 and Ser353 similar to indomethacin. Indomethacin is known to place its indoly-2-methyl group at alpha position that inserts itself in lipophilic pocket above Val349 to form a strong inhibitor-enzyme complex (Kurumbail et al. 1996; Prusakiewicz et al. 2004) as depicted in Fig. 6c. A variety of COX inhibitors are known to have hydrophobic interactions with these amino acids (Fig. 6d). Interactions of title compounds with these residues are in good agreement with biological activity.

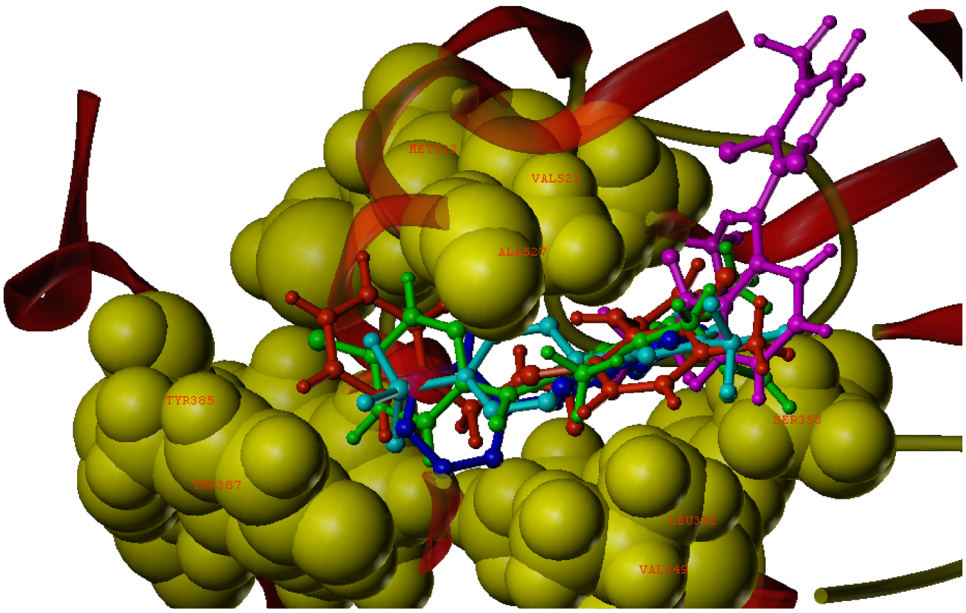


Fig. 5 Hydrophobic amino acids (*yellow coloured space fill*) enclosing ball and stick models of docked compounds: TSN1 (*red–orange colour*), TSN4 (*magenta colour*), Celecoxib (*cyan colour*), Indomethacin (*green colour*) and meclofenamic acid (*blue colour*) displaying hydrogen bond interactions with amino acids in the active site of COX-2, PDB Code 4COX (COX-2 co-crystallized with indomethacin) (colour figure online)

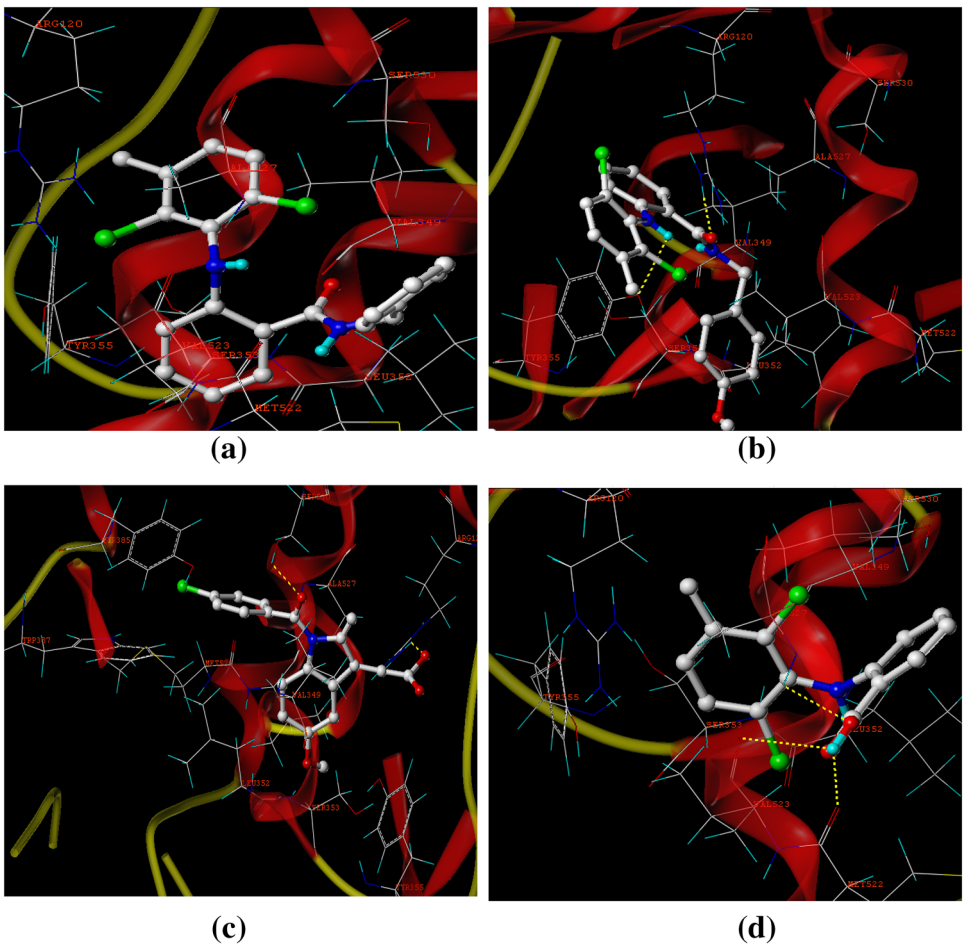


Fig. 6 Binding poses of ball and stick models of **a** TSN1, **b** TSN4, **c** indomethacin and **d** meclofenamic acid complexed with COX-2 enzyme showing hydrogen bonding (*yellow dotted lines*) and hydrophobic interactions (colour figure online)

Table 1 Surflex-Dock GeomX score of synthesized compounds and standards

Compd	C-Score	Crash Score	Polar Score	G Score	PMF Score	D Score	Chem Score
Indomethacin[a]	8.33	−1.61	1.08	−240	−67.7	−172	−37.2
TSN4	6.43	−3.49	1.07	−292	−63.6	−171	−45.5
TSN1	5.86	−4.22	0.00	−303	−77.9	−161	−40.9
Celecoxib[b]	5.35	−0.69	0.00	−189	−60.0	−461	−33.4
TSN3	5.28	−3.66	0.00	−327	−45.7	−172	−42.3
TSN10	5.09	−3.54	0.04	−301	−87.6	−179	−48.6
TSN7	4.93	−3.74	1.16	−265	−99.4	−156	−41.4
TSN6	4.33	−4.55	1.22	−292	−57.2	−170	−45.5
TSN2	4.31	−5.98	0.05	−320	−40.2	−170	−42.1
Meclofenamic acid	4.31	−1.69	0.97	−197	−53.7	−114	−35.0
TSN9	4.29	−4.58	2.59	−263	−62.1	−147	−39.8
TSN5	4.23	−6.24	0.61	−333	−56.8	−186	−46.9
TSN8	2.69	−6.31	1.05	−319	−57.9	−193	−50.3

[a] Extracted co-crystallized ligand (4COX)

[b] Extracted co-crystallized ligand (3LN1)

Table 2 Summary of results of docking analysis and in vivo anti-inflammatory activity

Compd.	Dock Score	% inhibition (3 h)	Interacting amino acids (HBD Å)	HB:HA	Residues involved in hydrophobic interactions
TSN1	5.86	70.35	–	–	Val349, Leu352, Ser353, Tyr355, Tyr385, Trp387, Met522, Val523, Ala527, Ser530
TSN2	4.31	27.08	Tyr355 (2.32 Å)	Tyr355 (OH):O::Lig(H):NH (anthranilic acid)	Val349, Leu352, Ser353, Tyr385, Trp387, Met522, Val523, Ala527
TSN3	5.28	66.55	–	–	Val349, Leu352, Ser353, Tyr355, Leu384, Trp387, Met522, Val523, Ala527, Ser530
TSN4	6.43	72.80	Arg120 (1.85 Å) Tyr355 (2.44 Å)	Arg120(NH_2):H::Lig(O):CONH Tyr355 (OH):O::Lig(H):NH (anthranilic acid)	Val349, Leu352, Ser353, Tyr385, Trp387, Met522, Val523, Ala527, Ser530
TSN5	4.23	18.91	–	–	Val349, Leu352, Ser353, Tyr355, Tyr385, Trp387, Met522, Val523, Ala527
TSN6	4.33	44.4	Arg120 (1.9 Å)	Arg120 (NH_2):H::Lig(O):CONH	Val349, Leu352, Ser353, Tyr355, Tyr385, Trp387, Met522, Val523, Ala527, Ser530
TSN7	4.93	54.28	Arg120 (1.82Å) Tyr355 (2.43 Å) Ser530 (2.32 Å)	Arg120 (NH_2):H::Lig(O):CONH Tyr355 (OH):O::Lig(H):NH (anthranilic acid) Ser530(CH_2OH):H::Lig(F):CF_3	Val349, Leu352, Ser353, Tyr355, Tyr385, Trp387, Met522, Val523, Ala527
TSN8	2.69	15.56	Arg120 (1.94Å)	Arg120 (NH_2):H::Lig(O):CONH	Val349, Leu352, Ser353, Tyr355, Tyr385, Trp387, Val523, Ala527
TSN9	4.29	25.02	Arg120 (1.82 and 2.31 Å) Tyr355 (2.53 Å)	Arg120 (NH_2):HH12 and HH22::Lig(O):CONH Tyr355 (OH):O::Lig(H):NH (anthranilic acid)	Val349, Leu352, Ser353, Trp387, Met522,Val523, Ala527
TSN10	5.09	63.75	Arg120 (3 Å)	Arg120 (NH_2):H::Lig(O):2-phenoxy-propyl	Val349, Leu352, Ser353, Tyr355, Tyr385, Trp387, Met522, Val523, Ala527, Ser530

Table 2 continued

Compd.	Dock Score	% inhibition (3 h)	Interacting amino acids (HBD Å)	HB:HA	Residues involved in hydrophobic interactions
Meclofenamic acid	4.31	56.75	Met522 (1.85 Å) Val523 (2.61 Å) Ala527 (2.5 Å)	Met522 (COOH) O::Lig(H):COOH Val523 (COOH):O::Lig(H):COOH Ala527 (NH_2) H::Lig(O):O attached to acidic proton	Val349, Leu352, Ser353, Tyr355, Tyr385, Trp387, Ser530
Indomethacin[a]	8.33	75.15	Arg120 (2 Å) Ser530 (2.02 Å)	Arg120 (NH_2):H::Lig(O):COOH Ser530 (CH_2OH):H::Lig(O):C=O (benzoyl)	Val349, Leu352, Ser353, Tyr355, Tyr385, Trp387, Met522, Val523, Ala527

[a] Extracted co-crystallized ligand (4COX) used as reference structure to validate the docking method

Table 1 summarizes the results obtained from docking in the form of various scores. TSN6, TSN2, TSN9, TSN5 and TSN8 were having crash scores (the degree of inappropriate penetration into the protein) >-4.5 kcal/mol, suggesting inappropriate penetration into the binding site of COX-2 enzyme. Compounds TSN3, TSN10 and TSN7 were having better C-Scores as is evident from their good anti-inflammatory activities comparable to meclofenamate.

G-score values indicating hydrogen bonding, complex (ligand–protein), and internal (ligand–ligand) energies were better for TSN1 and TSN4 than celecoxib, indomethacin, meclofenamic acid and other synthesized compounds. TSN1 was preferred over TSN4 on the basis of Helmholtz free energies of interactions for protein–ligand atom pairs (Potential of Mean Force, PMF Scores). In addition to this, TSN7 and TSN10 also displayed significant PMF Scores. D-Score values indicating charge and van der Waals interactions between the protein and the ligand were better for celecoxib but TSN4 and TSN1 were having better D-Scores over meclofenamic acid. Chemscore points for hydrogen bonding, lipophilic contact, rotational entropy, along with an intercept term (Chem Score) places TSN4 above TSN1 and the standard NSAIDs. Polar Score represents the contribution of hydrogen bonding to the total score. The tabulated scoring functions are in favour of indomethacin, followed by TSN4 and TSN1, celecoxib, TSN3, TSN10, TSN7, and finally meclofenamic acid.

Molecular docking results from this present study suggest that all the newly synthesized compounds are displaying contact whether through hydrogen bonding (Arg120, Tyr355) or hydrophobic interactions (Val349, Leu352, Ser353, Met522, Val523 and Ala527) with the residues of COX-2 enzyme (Table 2), thus accounting for the high potency of TSN4 and TSN1, followed by TSN3, TSN10 and TSN7.

Conclusions

To summarize and conclude the present findings, reported herein is the synthesis of amide derivatives of meclofenamic acid, their in vivo biological evaluation and subsequent docking on COX-2 enzyme. The in vivo studies of screened compounds revealed the promising anti-inflammatory activity of compound TSN4 with *N*-(4-methoxybenzyl) group followed by TSN1 with *N*-benzyl group. The results obtained from docking analysis are in good correlation with anti-inflammatory activity of title compounds. Further insights into the structures of docked complexes revealed hydrogen bond and hydrophobic interactions with the crucial residues (Arg120, Val329, Leu352, Ser353, Tyr355, Tyr385, Trp387, Val523, Ala527 and Ser530), reported for COX-2 inhibition. Both the in silico and in vivo studies have placed compound TSN4 on the top followed by TSN1. These two compounds thus can be further explored to improve their anti-inflammatory activity and safety profile.

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