Bioorganic & Medicinal Chemistry 17 (2009) 3665–3670

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Chlorzoxazone esters of some non-steroidal anti-inflammatory (NSAI) carboxylic acids as mutual prodrugs: Design, synthesis, pharmacological investigations and docking studies

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ARTICLE INFO

Article history:
Received 10 December 2008
Revised 23 March 2009
Accepted 29 March 2009
Available online 8 April 2009

Keywords:
Chlorzoxazone
Non-steroidal anti-inflammatory
Lipophilicity
Muscle relaxant
Ulcerogenicity
Docking

ABSTRACT

The discovery of the inducible isoform of cyclooxygenase enzyme (COX-2) spurred the search for anti-inflammatory agents devoid of the undesirable effects associated with classical NSAIDs. New chlorzoxazone ester prodrugs (**6**–**8**) of some acidic NSAIDs (**1**–**3**) were designed, synthesized and evaluated as mutual prodrugs with the aim of improving the therapeutic potency and retard the adverse effects of gastrointestinal origin. The structure of the synthesized mutual ester prodrugs (**6**–**8**) were confirmed by IR, ^{1}H NMR, mass spectroscopy (MS) and their purity was ascertained by TLC and elemental analyses. In vitro chemical stability revealed that the synthesized ester prodrugs (**6**–**8**) are chemically stable in hydrochloric acid buffer pH 1.2 as a non-enzymatic simulated gastric fluid (SGF) and in phosphate buffer pH 7.4 as non-enzymatic simulated intestinal fluid (SIF). In 80% human plasma, the mutual prodrugs were found to be susceptible to enzymatic hydrolysis at relatively faster rate ($t_{1/2} \approx 37$ and 34 min for prodrugs **6** and **7**, respectively). Mutual ester prodrugs (**6**–**8**) were evaluated for their anti-inflammatory and muscle relaxation activities. Scanning electromicrographs of the stomach showed that the ester prodrugs induced very little irritancy in the gastric mucosa of rats after oral administration for 4 days. In addition, docking of the mutual ester prodrugs (**6**–**8**) into COX-2 active site was conducted in order to predict the affinity and orientation of these prodrugs at the enzyme active site.

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1. Introduction

Limited utilization of a therapeutically significant drug in clinical practice may be attributed to various shortcomings like poor organoleptic properties, poor bioavailability, short duration of action, incomplete absorption or undesirable side effects.[1] The non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed drugs worldwide.[2] NSAIDs are widely used for indications extending from inflammation and pain to cardiovascular and genitourinary diseases.[3] Despite the intensive research that has been aimed at the development of NSAIDs, their clinical usefulness is still restricted by their gastrointestinal side effects like gastric irritation, ulceration, bleeding, and perforation and in some cases may develop into life threatening conditions.[4] GI mucosal injury produced by NSAIDs is generally believed to be caused by two different mechanisms.[5] The first mechanism involves a local action composed of a direct contact while the other has indirect effect on the GI mucosa. The direct contact effect can be attributed to a combination of a local irritation produced by acidic group of NSAIDs and local inhibition of prostaglandin synthesis in the GI tract. The indirect effect can be attributed to combination of an ion trapping mechanism of NSAIDs from the lumen into the mucosa. The second mechanism is based on a generalized systemic action occurring after absorption. Since the discovery of COX-2, a second subtype of cyclooxygenase, selective inhibitors or 'coxibs' were developed with the idea that this isoform was inducible at the site of inflammation. This new class of non steroidal anti-inflammatory agents was thought to be safer for ulcerations of the gastrointestinal mucosa observed with non selective COX-2 inhibitors. Nevertheless, at the end of September 2004, Merck & Co announced the voluntary withdrawal of rofecoxib worldwide because of an increased risk of cardiovascular events. This decision raised serious concerns about safety of selective COX-2 inhibitors which are actively marketed today, and the ones currently under development.[6,7] The GIT mucosal injury problems have been overcome by derivatization of carboxylic function of NSAIDs into ester and amide mutual prodrugs using amino acids like L-tryptophan, L-histidine, and L-glycine as carriers that have marked antiinflammatory activity of their own.[8] Other analgesic, antiinflammatory drugs like paracetamol and salicylamide have also been used as carriers to synthesize mutual prodrugs of NSAIDs. Benorylate is a mutual prodrug of aspirin and paracetamol, linked through ester linkage, which claims to have decreased

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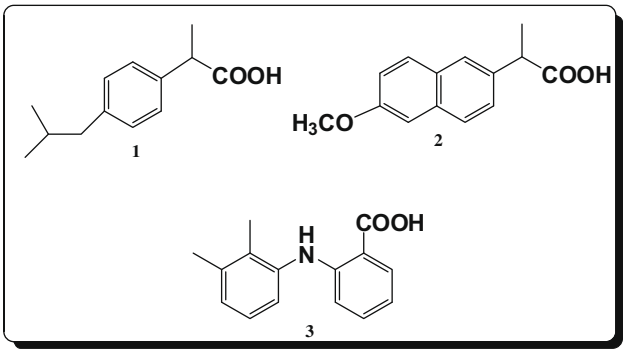
doi:10.1016/j.bmc.2009.03.065

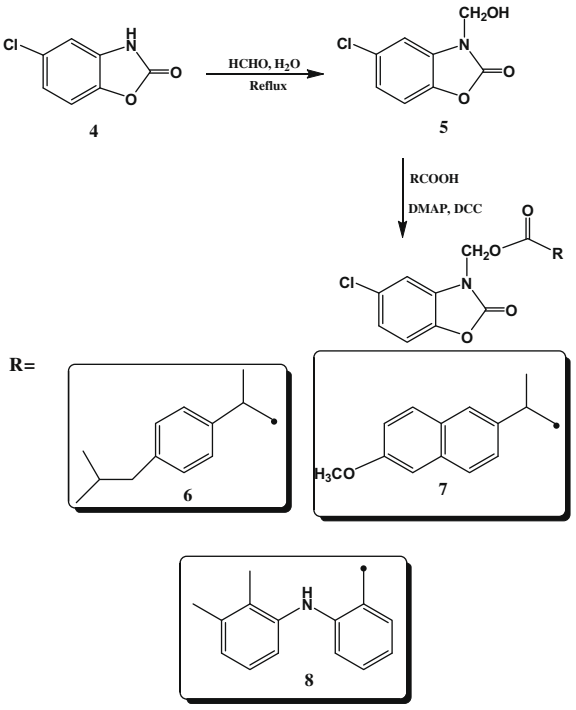
gastric irritancy with synergistic analgesic action.[9] Glycine methyl ester conjugate of ketoprofen,[10] histidine methyl ester conjugate of diclofenac,[11] and various conjugates of flurbiprofen with amino acid like L-tryptophan, L-histidine, phenylalanine and alanine as mutual prodrugs[12] were reported to have less ulcerogenicity with better anti-inflammatory and analgesic action than their parent drugs. Mutual prodrugs of ibuprofen with paracetamol and salicylamide have been reported with better lipophilicity and reduced gastric irritancy than the parent drug.[13] Naproxen propyphenazone mutual prodrugs were synthesized with an aim to improve therapeutic index through prevention of GI irritation and bleeding.[14] Chlorzoxazone [5-chloro-2(3*H*)-benzoxazolone] is an active muscle relaxant, while acetaminophen (*N*-acetyl-*p*-aminophenol) exhibits analgesic properties. Owing to their synergistic effects, these two drugs can be prescribed together.[15,16] Using this rationale, a mutual prodrug of chlorzoxazone and acetaminophen has been designed, and its synthesis and kinetics have been reported.[17] A mutual prodrug consists of two pharmacologically active agents coupled together so that each acts as a promoiety for the other agent and vice versa.[18] The active moiety selected may have the same biological action as that of the parent drug and thus might give synergistic action, or this moiety may have some additional biological action that is lacking in the parent drug, thus ensuring some additional benefit. The active moiety may also be a drug that might help to target the parent drug to a specific site or organ or cells or may improve site specificity of a drug. The drug may be used to overcome some side effects of the parent drugs as well.[18] Chlorzoxazone is a marketed as a single component muscle relaxant or in combination with some NSAIDs. On the basis of the mentioned reports, the present work reports on the design and synthesis of different mutual ester prodrugs of chlorzoxazone **4** and some NSAIDs (**1**–**3**) with the objective of minimizing gastrointestinal side effects of NSAIDs and improving pharmacokinetic properties of both chlorzoxazone **4** and NSAIDs (**1**–**3**) while maintaining the useful anti-inflammatory and skeletal muscle relaxation activities.



2. Results and discussion

2.1. Chemistry

Schematic representation of the reactions for the synthesis of the mutual ester prodrugs (**6**–**8**) is depicted in Scheme 1. The synthesis of the key intermediate, hydroxymethyl chlorzoxazone **5**, necessitate the reflux of chlorzoxazone **4** with formaldehyde solution (40%) according to the reported procedure.[19] Subsequent esterification of the resulting precursor **5** with the corresponding non-steroidal anti-inflammatory carboxylic acid drugs (**1**–**3**) afforded the mutual ester prodrugs (**6**–**8**) in good yields. The structure of the synthesized prodrugs (**6**–**8**) were confirmed by IR, ^{1}H NMR, mass spectroscopy (MS) and their purity was ascertained by TLC and elemental analyses.



Scheme 1. Pathway for the synthesis of the target ester prodrugs (**6**–**8**).

Lipophilicities of chlorzoxazone **4**, hydroxymethyl chlorzoxazone **5**, the synthesized prodrugs (**6**–**8**) and their parent non-steroidal anti-inflammatory drugs (**1**–**3**) were calculated using a PC-software package, which helps computation of the log*P* on the basis of the fragment method developed by Leo.[20] The results listed in Table 2 (Supplementary data) revealed that the lipophilicities of the prodrugs (**6**–**8**) increased vividly compared with the parent drugs (**1**–**3**). The lipophilicity increament of the synthesized mutual prodrugs (**6**–**8**) probably enhances their absorption and consequently their bioavailability.

2.2. Stability studies

The chemical and enzymatic hydrolysis of the synthesized ester prodrugs (**6**–**8**) of the selected NSAIDs were studied at 37 °C in aqueous buffer solutions of pH 1.2 and 7.4, which are considered as a non-enzymatic simulated gastric fluid (SGF) and non-enzymatic simulated intestinal fluid (SIF), respectively, as well as in 80% human plasma and the reactions were monitored by HPLC. The results indicate that the synthesized prodrugs (**6**–**8**) are sufficiently stable at pH 1.2 for about one day monitoring, so that no hydrolysis to the free acids might occur in the stomach. Similarly, the chemical stability of the ester prodrugs (**6**–**8**) at pH 7.4 for one day suggested that they are absorbed almost unchanged from the intestine. On the other hand, the enzymatic hydrolysis of the ester prodrugs (**6**–**8**) in human plasma demonstrates the susceptibility of these esters for plasma esterases. The results revealed that the rate of hydrolysis of ibuprofen and naproxen ester prodrugs **6** and **7** in human plasma are markedly accelerated, $t_{1/2}$ is 37 and 34 min, respectively, compared with those in aqueous buffers. The rate of hydrolysis of mepfenamic ester prodrug **8** was difficult to be determined owing to solubility problems in human plasma. The chemical stability of these orally designed ester prodrugs (**6**–**8**) at pH values simulating the gastric fluids and their liability to release the parent non-steroidal anti-inflammatory drugs (**1**–**3**) and the muscle relaxant chlorzoxazone **4** are essential requirements for improving the bioavailability.

2.3. Pharmacological evaluation

Non-steroidal anti-inflammatory drugs (**1**–**3**) and their corresponding mutual ester prodrugs (**6**–**8**) were evaluated for their in vivo systemic effect using carrageenan-induced paw edema bioassay in rats.[21] Table 3 shows the results (% inhibition) of the anti-inflammatory activity of the tested targets at different time intervals. Ibuprofen **1** and ibuprofen ester prodrug **6** showed maximum inhibition of inflammation ranging from 16.7% to 50%. On the other hand, naproxen **2**, mefenamic acid **3** and naproxen ester prodrug **7** were slightly active while the minimum inhibitory activity of inflammation was observed to mefenamic ester prodrug **8**. From the results obtained the mutual ester prodrugs (**6**–**8**) exhibited comparable anti-inflammatory activity to their parents carboxylic acid drugs (**1**–**3**). Also, muscle relaxant activity of the mutual ester prodrugs (**6**–**8**) was evaluated in comparison with muscle relaxant drug chloroxazone **4** using rat sciatic nerve tibialis muscle preparation.[22] The percentage inhibition of muscle contraction listed in Table 4 showed that all the tested targets (**6**–**8**) revealed muscle relaxation activity. Moreover, the ester prodrug **6** displayed muscle relaxation activity comparable to that of the reference drug chlorzoxazone **4**.

Gastrointestinal toxicity of ibuprofen-chlorzoxazone mutual prodrug ester **6** compared with the parent drug ibuprofen **1**, the most active anti-inflammatory drug tested, was examined under electron microscope.[23] Investigation of the stomach specimens of the treated experimental animals under scanning electron microscope afforded a highly precise method for examination of ulcerogenic potential of NSAIDs. Figure 1, represents scanning electromicrographs for stomach specimens of rats treated with chronic doses (150 mg/kg) of ibuprofen **1** (Fig. 1A), ibuprofen ester prodrug **6** (Fig. 1B), and the control group (Fig. 1C), which received only the vehicle. Ibuprofen **1** treated animals were characterized by complete damage of the mucous layer besides ulceration of submucosal cells compared with that of control. The ester prodrug of ibuprofen **6** treated animals showed damage of the mucosal layer but to mush less extent than that of ibuprofen **1**. From the above finding, ibuprofen ester mutual produrg **6** proved to have superior GI safety profile as compared to the reference drug ibuprofen **1**.

2.4. Docking studies

Docking studies of non-steroidal anti-inflammatory drugs (**1**–**3**) and their mutual prodrug esters with chlorzoxazone (**6**–**8**) were performed by MOE (Molecular Operating Enviroment) using murine COX-2 co-crystallized with SC-558 (PDB ID: 1CX2) as a template.[24] The recent determination of the three-dimensional co-crystal structure of murine COX-2 complexed with SC-558 has led to the development of a model for the topography of NSAIDs binding site in human COX-2. This might enable the prediction of the orientation and interaction of these drugs (**1**–**3**) and their ester prodrugs (**6**–**8**) into COX-2 active site.[25] We performed 100 docking iterations for each ligand and the top scoring configuration of each of the ligand-enzyme complexes was selected on energetic ground. The output of docking simulation are the scoring function which reflects the binding free energy dG in kcal/mol (S), value proportional to the sum of Gaussian $R_1R_2\exp(-0.5d^2)$, where R_1 and R_2 are the radii of atoms in Angstrom Å and d is the distance between the pair in Å (ASE) a linear combination of (S, ASE, E_{conf}) where E_{conf} is an estimated self-energy of the ligand in kcal/mol (E). Docking simulations revealed that the mutual ester prodrugs (**6**–**8**) inhibit COX-2 enzyme in a quiet similar manner to those of their parent non-steroidal anti-inflammatory drugs (**1**–**3**). These mutual ester prodrugs (**6**–**8**) docked on cyclooxygenase enzyme demonstrates $dG_{ester\ prodrug} \approx dG_{NSAIDs} \pm 1$ kcal/mol as shown in Table 5 (Supplementary data). Naproxen **2** and ibuprofen ester prodrug **6** showed the highest dG values of −12.65 and −11.69 kcal/mol, respectively. Figure 2 shows the orientation of these drug and ester prodrug (**2** and **6**, respectively) into COX-2 enzyme active site. The above mentioned observations may provide an explanation for the potent inhibitory activity of the ester prodrugs.

The introduction of mutual prodrug in human therapy has given successful results in overcoming undesirable properties like absorption, non-specificity, poor bioavailability and GIT toxicity. In vitro and in vivo evaluation of the synthesized mutual ester prodrugs (**6**–**8**) of chlorzoxazone **4** and some non-steroidal anti-inflammatory drugs (**1**–**3**) revealed adequately enhanced lipophilicity, chemical stability, reduced ulcerogenic liability compared

Table 3
Anti-inflammatory activity of non-steroidal anti-inflammatory drugs (**1**–**3**) and their target esters (**6**–**8**)

Compound no.	Anti-inflammatory activity % inhibition of edema (mean) ± SEM[a,*]					
	0.5 h	1 h	2 h	3 h	4 h	5 h
Control	0.00	0.00	0.00	0.00	0.00	0.00
1	16.7 ± 0.00	16.7 ± 0.00	45.8 ± 4.17	62.5 ± 4.17	50:0 ± 0.00	41.7 ± 4.81
2	9.4 ± 3013	16.3 ± 3.61	28.1 ± 3.13	46.9 ± 3.13	37.5 ± 0.00	18.8 ± 3.6
3	16.7 ± 1.36	18.3 ± 3.47	33.3 ± 4.1	41.7 ± 4.81	45.8 ± 4.17	33.3 ± 2.72
6	16.7 ± 0.00	16.7 ± 1.36	33.3 ± 0.00	45.8 ± 4.17	50:0 ± 0.00	45.8 ± 4.17
7	19.1 ± 0.95	19.6 ± 2.86	18.7 ± 2.35	33.3 ± 3.96	38.1 ± 4.17	23.8 ± 0.00
8	14.3 ± 1.1	14.8 ± 0.00	23.4 ± 3.21	30.5 ± 4.5	25.7 ± 3.3	20:0 ± 3.47

[a] SME denotes the standard error of the mean.
[*] All data are significantly different from control ($P < 0.01$).

Table 4
Muscle relaxation activity of chlorzoxazone **4** and non-steroidal anti-inflammatory prodrugs (**6**–**8**)

Compound no	Muscle relaxation activity % inhibition of muscle contraction ± SEM[a]							
	10 min	20 min	30 min	45 min	60 min	75 min	90 min	120 min
4	18.5 ± 1.2[**]	25.88 ± 0.6[**]	33.43 ± 1.23[**]	35.26 ± 1.37[**]	33.45 ± 2.83[**]	21.65 ± 1.46[**]	15.25 ± 0.85[**]	0.00
6	2.5 ± 1.04	10.5 ± 0.68[**]	19.95 ± 1.96[**]	28.35 ± 5.07[**]	35.13 ± 2.33[**]	33.2 ± 1.72[**]	27.05 ± 1.68[**]	18.25 ± 1.3[**]
7	5.89 ± 0.49[**]	8.6 ± 0.66[**]	14.25 ± 0.63[**]	20.73 ± 0.93[**]	23.65 ± 3.89[**]	12.9 ± 1.45[**]	8.00 ± 0.51[**]	0.00
8	2.78 ± 1.00	5.59 ± 1.81[**]	10.78 ± 0.62[**]	16.55 ± 1.83[**]	19.48 ± 1.62[**]	9.65 ± 1.40[**]	4.58 ± 0.92[*]	0.00

[a] SEM denotes the standard error of the mean.
[*] Data are significantly different from control ($P < 0.05$).
[**] All data are significantly different from control ($P < 0.01$).

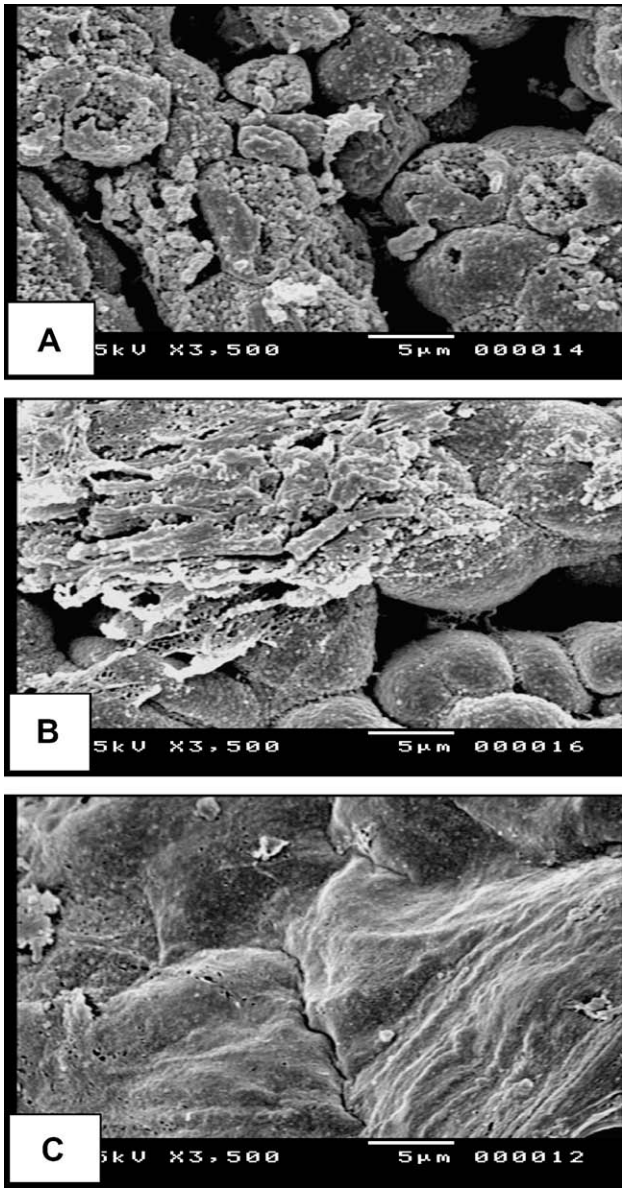


Figure 1. Scanning electromicrographs of rat stomach specimen after chronic doses (4 days) of: (A) ibuoprofen; (B) chlorzoxazone ester with ibuoprofen; (C) control.

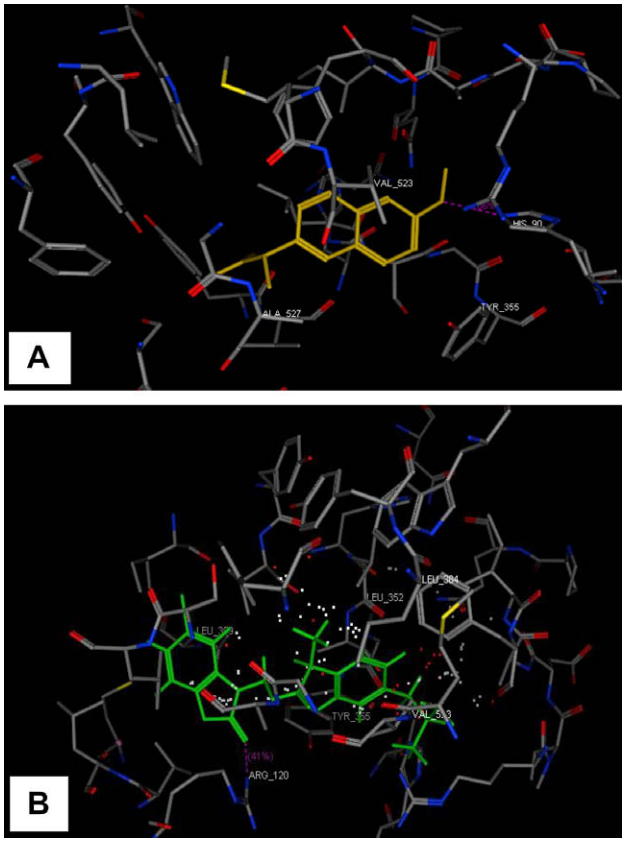


Figure 2. Docking of (A) naproxen **2** (yellow stick) and (B) ibuprofen ester prodrug **6** (green stick) in the active site of COX-2 enzyme. Hydrogen atoms of the amino acid residues have been removed to improve clarity.

with the corresponding parent drugs. The minimized side effects obtained in the prodrug might be due to inhibition of direct contact of carboxyl group of the drug to the gastric mucosa, which is mainly responsible for the damage. It is also due to negligible dissolution as well as hydrolysis in acidic buffer (pH 1.2). On the basis of the results, it is concluded that prodrug approach can be successfully applied in attaining the goal of minimized gastrointestinal toxicity without loss of desired anti-inflammatory and analgesic activities of the drug. As indicated, mutual prodrug approach offers a very fruitful area of research and an efficient tool for improving the clinical and therapeutic effectiveness of a drug that is suffering from some undesirable properties hindering its clinical usefulness otherwise.

3. Experimental

Melting points were determined on an electrothermal melting point apparatus (Stuart scientific, England) and are uncorrected. Thin layer chromatography was carried out on pre-coated Silica gel 60 F254 plates (0.25 mm thickness, Merck, Darmastat, Germany) and spots were detected under UV light. Silica gel 60 (60–230 mesh, Merck, Darmastat, Germany) was used for column chromatography. IR spectra were recorded as KBr discs on a shimadzu IR 400-91527 (Shimadzu, Kyoto, Japan) at the Faculty of Pharmacy, Assiut University, Assiut, Egypt. ^{1}H NMR spectra were measured on Varian EM-360L, NMR Spectrophotometer (60 MHz) (Varian, USA) at the Faculty of Pharmacy, Assiut University, Assiut, Egypt. Chemical shifts were given in δ ppm relative to tetramethylsilane (TMS). Electron impact (EI) Mass Spectra were measured with JEOL spectrophotometer (JEOL, Tokyo, Japan) at an ionization voltage of 70 eV at the central laboratory, Assiut University, Assiut, Egypt and at the Microanalytical center, Cairo University, Cairo, Egypt. Elemental microanalyses were performed at the Microanalytical Center, Faculty of Science, Assiut University, Assiut, Egypt. All chemicals used were of analytical grade.

HPLC analysis was carried out a Knauer HPLC system, consisting of a pump (KNAUER WellChrom Mini-Star K-500 HPLC pump, Germany), a variable wavelength UV detector (KNAUER), a Shimadzu C-R 6A chromatopac recording integrator, and a 20 µL injection loop (KNAUER). The column used was a reversed phase SUPELCO Discovery C18 (250 × 4.6 mm; 5 µm particle) connected with a cartridge guard column. The chromatographic conditions for each of the studied compounds are summarized in Table 2 (Supplementary data). Quantification of the eluted compounds was obtained from the area under the peak measurements in relation to those of standards chromatographed under the same conditions.

The anti-inflammatory and muscle relaxation activities were performed at the Department of Pharmacology, Faculty of

Medicine, Assiut University, Assiut, Egypt. The ulcerogenic potential was detected with a JEOL, JSM-5400LV Scanning Electron Microscope (Electron Microscope Unit, Assiut University, Assiut, Egypt).

Docking studies were carried out at the Department of Pharmaceutical Medicinal Chemistry, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

3.1. Chemical synthesis

3.1.1. Synthesis of 5-chloro-3-(hydroxymethyl)benzo[d]oxazol-2(3H)-one (5)[19]

A suspension of chlorzoxazone **4** (1.7 g, 0.01 mol) and 5 mL formaldehyde solution (40%) in water (100 mL) was refluxed for three hours. The hot solution was left to cool where crystals are formed. The separated crystalline product was filtered and dried to afford hydroxymethyl chloroxazone **5**. Physical, IR, and ^{1}H NMR data are listed in Table 1 (Supplementary data).

3.1.2. General method for the synthesis of the target derivatives (6–8)

To an ice-cold solution of the appropriate non-steroidal anti-inflammatory drug (3 mmol) in 30 ml dichloromehane, hydroxymethyl chlorzoxazone **5** (3 mmol), dimethylaminopyridine (DMAP, 20 mg), and dicyclohexylcarbodiimde (DCC, 3.3 mmol) were added. The reaction mixture was stirred at 4 °C for 1 h and kept overnight at room temperature. The precipitated formed was separated by filtration and the filtrate was washed with cooled 0.05 N HCl followed by saturated solution of $NaHCO_3$ and finally with brine, dried over anhydrous Na_2SO4, and evaporated under reduced pressure. The residue was purified with column chromatography using a mobile phase system of hexane and ethyl acetate in ratio (9:1) for esters (**6 and 7**), and ratio (20:1) for ester (**8**). Physical, IR, and ^{1}H NMR data are listed in Table 1 (Supplementary data).

3.2. Calculation of log*P* values

The log*P* values of the NSAIDs (**1**–**3**), chloroxazone **4**, hydroxymethyl chlorxazone **5** and the target derivatives (**6**–**8**) were computed with a routine method called calculated log*P* (*C*log*P*) contained in a PC-software package (Mac log*P* 2.0, BioByte Corp., CA, USA). A representation of the molecular structure where hydrogens are omitted, or suppressed (SMILES notation), is entered into the program, which computes the log*P* based on the fragment method developed by Leo.[20] Results are given in Table 2 (Supplementary data).

3.3. In vitro experiments

3.3.1. Chemical hydrolysis

The hydrolysis of the synthesized derivatives (**6**–**8**) was studied in hydrochloric acid buffer (pH 1.2) and phosphate buffer (pH 7.4), containing 1% tween 20. The ionic strength (μ) for each buffer was maintained 0.2 by adding a calculated amount of potassium chloride. The reactions were initiated by adding 200 μL of stock methanolic or acetonitrile solution of the target derivative (3 mg/mL) to 9.8 mL of preheated buffer solutions in screw-capped test tubes. The solutions were kept in a water bath at constant temperature (37 °C), at appropriate time intervals, 20 μL were withdrawn and analyzed by HPLC for the residual target derivative.

3.3.2. Enzymatic hydrolysis

The reactions were initiated by adding 50 μL of stock solution of the target derivatives (**6**–**8**) in DMSO (4 mg/mL) to 1.95 mL of pre-incubated 80% human plasma with isotonic phosphate buffer (pH 7.4) and incubated at 37 °C. At appropriate time intervals 200 μL of the plasma reaction were withdrawn and deproteinized by mixing with 300 μL of acetonitrile. After centrifugation for 10 min at 104 rpm, 20 μL of the clear supernatant was analyzed by HPLC.

3.4. Biological evaluations

3.4.1. Anti-inflammatory activity

The anti-inflammatory activity of the synthesized derivatives (**6**–**8**) in comparison with their parent NSAI drugs (**1**–**3**) was evaluated against carrageenan-induced rat paw edema.[21] Adult male Wister rats weighing (100–130 g) were divided into seven groups, four animals each. Target compounds were suspended in aqueous solution of carboxymethyl cellulose (CMC, 0.5% w/v) and administered orally at a dose level of 150, 40, 20 mg/kg of NSAI drugs (**1**–**3**), respectively and their equivalent doses of the target derivatives (**6**–**8**). Control animals were similarly treated with aqueous solution of carboxymethyl cellulose (CMC, 0.5% w/v). After 30 min 0.1 mL of freshly prepared 1% carrageenan solution in normal saline was injected into the subplantar region of the right hind paw of each rat. The thickness of paw edema was determined by means of skin caliper at different time intervals (1/2, 1, 2, 3, 4, and 5 h) after administration of the test compounds. The difference between the thicknesses of two paws (right and left) was taken as a measure of edema. The percent inhibition of edema was calculated as follows:

$$\%\ \text{Edema inhibition} = \frac{(V_R - V_L)\ \text{control} - (V_R - V_L)\ \text{treated} \times 100}{(V_R - V_L)\ \text{control}}$$

Where V_R represents the mean of the right paw displacement volume, and V_L represents the mean of the left paw displacement volume. The results of the anti-inflammatory evaluation are listed in Table 3.

3.4.2. Muscle relaxant activity

The muscle relaxant activity of hydroxymethyl chlorzoxazone **5**, and the target esters (**6**–**8**) in comparison with chlorzoxazone **4** was evaluated using rat sciatic nerve tibialis muscle preparation.[22] Male adult Wister rats (100–130 g), were divided into four groups, four rats each, were anesthetized by intraperitoneal injection with 50 mg/kg urethane. The anterior tibialis muscle was carefully dissected and exposed so that the blood and nerve supply were intact. The sciatic nerve which supplies the muscle was exposed in the gluteal region. The distal tendon of the muscle was tied to silk threads which were attached to a T_2 isotonic transducer and an amplifier of a two-channel oscillograph MD_2 (Bioscience, Kent, UK). The hind limb was tidily fixed in a horizontal position and the muscle was adjusted to resting length. The nerve was stimulated using an electronic square wave physiograph stimulator with pulses of 2 ms duration, 5 V intensity, and 5 S^{-1} frequencies. After the preparation becomes stable, the height of the tibialis muscle contractions was determined before and after injection, intraperitoneally at a dose of 50 mg/kg chlorzoxazone **4** or equivalent dose of the target ester prodrugs (**6**–**8**), at different time intervals of any of the tested compounds under investigation into the animal. The percentage change in the height of contraction was determined as indicator of muscle relaxation activity. The results of the muscle relaxation evaluation are listed in Table 4.

3.4.3. Ulcerogenicity studies[23]

Male adult Wister rats (150–200 g) were divided into three groups, three animals each. Rats were starved but had free access to water for 12 h prior to the administration of the drug. The first group was administered a daily oral dose, (150 mg/kg) as a 1 mL suspension of Ibuprofen **1** in 0.5% solution of carboxymethyl

cellulose (CMC), for four successive days. In a similar manner, the second group received equivalent doses of the corresponding ester **6**. The third group was administered equivalent amount of vehicle and considered as the control group. Food was withdrawn from all groups until 24 hr after the last dose. The rats were then sacrificed; so that the stomach could be removed, opened along the greater curvature and cleaned gently by dipping with saline. Randomly selected specimens were then taken and prepared for scanning in an electron microscope. Specimens were fixed by soaking in glutaraldehyde solution (5% in cacodylate buffer; pH 7.2) for 24 h followed three washings each for 20 min with cacodylate buffer. The specimens were then treated with osmium tetraoxide (1% solution) for 2 h and washed with cacodylate buffer as shown above. The specimens were subjected to dehydration by treatment for 30 min with each of 30%, 50% and 70% ethanolic solution followed by 90% ethanol for 1 h and finally in absolute ethanol for two days. After discharge of the alcohol the specimens were soaked in amyl acetate solution for two days, dried under reduced pressure, mounted on holders and coated for scanning in electron microscopy.

3.5. Docking studies

Docking studies were performed using 'Molecular Operating Environment (MOE) version 2005.06, Chemical Computing Group Inc., 1010 Sherbrooke Street West, Suite 910, Montreal, H3A 2R7, Canada. The program operated under 'Windows XP' operating system installed on an Intel Pentium IV PC with a 2.8 MHz processor and 512 RAM. Non-steroidal anti-inflammatory drugs (**1**–**3**) and their corresponding ester prodrugs (**6**–**8**) were built using the builder interface of the MOE program and subjected to energy minimization tool using the included MOPAC 7.0. The produced model was subjected to Systematic Conformational Search where all items were set as default with RMS gradient of 0.01 kcal/mol and RMS distance of 0.1 A°.

The X-ray crystallographic structure of cyclooxygenase-2 (murine COX-2) complexed with SC-558 (PDB ID: 1CX2) was obtained from the Protein Data Bank.[26] The enzyme was prepared for docking studies where: (i) the ligand molecule with any existing solvent molecules were removed from the enzyme active site. (ii) Hydrogen atoms were added to the structure with their standard geometry. (iii) MOE Alpha Site Finder was used for the active sites search in the enzyme structure and dummy atoms were created from the obtained alpha spheres. The obtained ligand–enzyme complex model was then used in calculating the energy parameters using MMFF94x force field energy calculation and predicting the ligand–enzyme interactions at the active site.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.03.065.

References and notes

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