Pyrazoles containing thiophene, thienopyrimidine and thienotriazolopyrimidine as COX-2 selective inhibitors: Design, synthesis, in vivo anti-inflammatory activity, docking and in silico chemo-informatic studies

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ABSTRACT

New thiophene and annulated thiophene pyrazole hybrids were synthesized and screened for their in vitro COX-1/COX-2 enzymatic inhibition and in vivo anti-inflammatory activities. All compounds were more COX-2 selective inhibitors than COX-1 with compound 13 exhibiting the highest COX-2 selectivity index. Compounds 3, 6a, 9 and 11 were the most promising in the acute anti-inflammatory assay while compounds 3, 5, 6a, 6c, 9, 10, 11 and 13 exerted promising anti-inflammatory activity in the sub-acute anti-inflammatory assay. Compounds 3, 6a, 6c, 9, 10 and 11 were evaluated for their ED50 values and were more potent than diclofenac sodium while compounds 6a, 6c and 9 were of greater potency than celecoxib with compound 6a being the most potent showing ED50 = 0.033 mmol/kg. These compounds were non-toxic and proved to be gastrointestinal safe compared to indomethacin, diclofenac sodium and celecoxib. Docking studies into COX-2 active site (PDB code 3LN1) revealed that compounds 3, 6a, 6c, 9, 10, 11 and 13 had binding modes and energies comparable to that of celecoxib. Compounds 3, 9, 10 and 11 complied with Lipinski's RO5 while compounds 6a and 6c showed one violation whereas compound 13 deviated by 2 violations. Compounds 6a, 6c and 13 showed 100% plasma protein binding (PPB) and showed no aqueous solubility while compounds 3, 10 and 11 demonstrated the best drug likeness model scores. Therefore, the thiophene analog 3 and the thienopyrimidine derivatives 10 and 11 are promising anti-inflammatory candidates that exert moderate selective COX-2 inhibition with acceptable physicochemical properties.

1. Introduction

Inflammation is the initial defense response of the body cells and tissues to various stimuli such as pathogens, infections, irritation, chemicals, mechanical or thermal injuries [1]. It is clinically manifested as pain, swelling, heat, redness and aches at the inflamed tissues [2]. These symptoms are due to the release of some inflammatory mediators including prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) [3]. Inflammatory diseases are commonly treated with non-steroidal anti-inflammatory drugs (NSAIDs) [4]. They exert their effect via the inhibition of cyclooxygenases (COXs) which catalyze the metabolism of arachidonic acid (AA) into PGs and TXs preventing their formation [4,5]. COXs were discovered to exist in three differently regulated isoforms COX-1, COX-2 and COX-3 [4,6]. COX-1 is a constitutive enzyme which is involved in the production of physiological PGs and TXs that govern the protection of gastric mucosa, normal kidney function and aggregation of platelets, respectively, thereby acting as a “house-keeper” enzyme [3,5,7]. On the other hand, COX-2 is induced at the sites of infection or inflammation as a response to the release of pro-inflammatory mediators and catalyzes the release of pathological PGs involved in the inflammatory process [1,3,7]. In addition, another variant of COX-1 known as COX-3 was discovered in the central nervous system [3,6]. It is to be noted that chronic use of non selective NSAIDs is accompanied by gastrointestinal (GI) complications such as ulceration and bleeding in addition to some renal malfunctions [3,6,8] and these adverse effects are attributed to the inhibition of COX-
Therefore, it was thought that the use of selective COX-2 inhibitors without interfering with COX-1 activity is a fruitful tool for treatment of inflammation with reduced risk of occurrence of side effects especially the GI problems [5–7]. The highly selective COX-2 inhibitors (coxibs) as valdecoxib (Bextra®) and rofecoxib (Vioxx®) that were approved for marketing due to their safe gastrointestinal use [3,5], caused an increased risk of significant cardiovascular (CV) problems upon their administration which led to their withdrawal from the market [1,3,11,12]. On the other hand, moderate selective COX-2 inhibitors like celecoxib (Celebrex®) represent safe anti-inflammatory drugs regarding their cardiovascular effects and are still marketed [12,13]. Unfortunately, the efficacy of celecoxib as an anti-inflammatory agent was not proved in all patients [14]. Thus, the design and synthesis of new COX inhibitors that show appropriate selectivity towards COX-2 inhibition with safe GI profile is a continuous need in order to overcome the CV and GI adverse effects of the currently available COX-2 inhibitors.

Pyrazoles and pyrazolines remain the scaffold of choice for the design of anti-inflammatory agents. Celecoxib (Celebrex®) I [4], dipyrone (dimethone*) II [4], compounds III [15] and IV [16] (Fig. 1) are pyrazole derivatives that exhibited anti-inflammatory activities. Recently, numerous COX-2 selective inhibitors containing pyrazole or pyrazoline moieties have been synthesized (V-VIII) [9,17,18] (Fig. 1). The presence of two aryl rings attached to a central pyrazole or pyrazoline moiety is a mutual feature of most COX-2 inhibitors [6]. The nature of substituents on these aryl groups impact the inhibitory potency and selectivity index of the produced COX-2 inhibitor. Phenyl sulfonylamino (PhSO2NH2) moiety is also a dominant pharmacophore in many selective COX-2 inhibitors with anti-inflammatory properties [3] (compounds V, VI). However, the pyrazole core was substituted with other different aryl moieties or attached to them through variable linkages. The trifluoromethylnaphthpyrazoles were known as selective COX-2 inhibitors (compound V [17]). However, in order to minimize the toxicity risk of fluorne, the trifluoromethyl functionality (CF3) was better replaced with an amino group (NH2) (compound VI) [9]. Moreover, pyrazolo[3,4-d]pyrimidin moieties (compounds VII, VIII) were proved to augment COX-2 selectivity via increase in the hydrophobic interactions with COX-2 rather than COX-1 active site [18]. Additionally, 3-aminopyrazole-4-carbonitriles, 3,5-dimethylpyrazoles as well as pyrazoles substituted with an ester or 4-chlorophenyl moieties are well known pharmacophoric scaffolds that improve the anti-inflammatory activity (Fig. 1). Incorporating the hydrazono functionality also showed enhanced COX-2 selectivity (compound VI) [9].

It is to be noted that substitution of the pyrazole core with thio-phenone moiety was also known to yield active anti-inflammatory agents (as in compounds IV [16]) or COX-2 selective inhibitors with anti-inflammatory activity (as in compound V [17]). In addition, tiaprofenic acid (Surgam®) IX and suprofen (Profenal®) X (Fig. 1) are NSAIDs possessing thiophene ring [19,20]. Moreover, CBS-1108 (XI) [3] and XII [21] are COX-2 selective inhibitors possessing the thiophene pharmacophore incorporating hydrazono functionality or substituted with 2-amino-3-cyano substituents. Furthermore, the tetrahydrobenzothienopyrimidine derivative XIII [22] showed increased COX-2 activity with high selectivity index while the thienopyrimidines XIV [23] revealed augmented anti-inflammatory activity. Additionally, the triazolothienopyrimidinones XV, XVI [22,24] revealed high COX-2 selective inhibition with high selectivity indices.

Strongly convinced by the afore-mentioned studies and tempted by our findings in the field of anti-inflammatory agents [22,24–27], profound interest was developed to access various thiophene pyrazole analogs to be screened for their COX-2 selective inhibition and anti-inflammatory activities (Fig. 2). Our attention has been focused on 2-
aroylthiophene moiety that is a common pharmacophore in many NSAIDs as tiaprofenic acid (IX) [19] and suprofen (X) [20]. The fact that the electron donating amino group was incorporated in many promising compounds (as VI and VII), probing probably less toxicity and potential interaction with the receptor was not beyond our interest. Besides, the introduction of phenyl ring implying increased hydrophobicity and interaction with the receptor was also considered. Based on these data and inspired by the well-documented COX-2 selective inhibition and anti-inflammatory activity of the pyrazole ring, a basic scaffold consisting of 3-amino-4-phenyl-2-aroylthiophene was incorporated into variously substituted pyrazole rings either directly (structures A & B, Fig. 2) or through a hydrazinomethylene spacer (structure C, Fig. 2). Structures A & B contain the planar thiophene and pyrazole moieties separated by a flexible one atom spacer (C=O) while structure C contains the two aromatic rings separated by a flexible four atom spacer (O=C–NH–N=C) in order to study the effect of such hybridizations on COX-2 selective inhibition and anti-inflammatory activity. Substituents on the pyrazole ring include either electron donating groups (hydroxyl or methyl) or electron withdrawing groups (ester or phenyl either unsubstituted or substituted with various chions) in order to monitor the effect of such substitution on the activity.

Our interest has been also extended to construct two libraries including pyrazole and thieno[3,2-d]pyrimidine rings in order to develop new COX-2 selective inhibitors and anti-inflammatory agents. To achieve this interest, the two rings were incorporated into one structure either directly (structure D, Fig. 3) or through a three atoms spacer (NH–N=C) (structure E, Fig. 3). In addition, it was hypothesized that compounds containing both hydrophobic and hydrophilic components would be promising anti-inflammatory candidates due to variable interactions with receptor binding domain. Thus, variable substituents including amino, cyano, hydroxyl, methyl, ester, phenyl or p-chlorophenyl were introduced onto the thieno[3,2-d]pyrimidine pyrazole scaffold to study their impact on the anticipated COX-2 selective inhibition and anti-inflammatory activity.

Moreover, it was not beyond our scope to build up the thienotriazolopyrimidine scaffold from the thienopyrimidine ring system and to incorporate such tricyclic ring system directly into the traditional 1,3-diarylpyrazole scaffold (structure F, Fig. 3) to study the effect of increasing bulkiness, polarity and restricted rotation of the side chain on the COX-2 selective inhibition and anti-inflammatory activity.

The newly synthesized compounds were evaluated for their in vitro COX-1 and COX-2 enzymatic inhibition and their in vitro COX-2 selectivity indices were estimated. Moreover, all compounds were evaluated for their in vivo anti-inflammatory activities using the formalin-induced paw edema bioassay (acute and sub-acute inflammatory models) and using diclofenac sodium and celecoxib as reference standards. Furthermore, effective concentrations 50 (ED50), ulcerogenic activities and acute toxicities (ALD50) of the most active compounds in the in vivo anti-inflammatory assays were also evaluated and a detailed structure–activity relationship (SAR) study was discussed. Docking of the most active compounds in both in vitro and in vivo assays into the active site of COX-2 enzyme was performed to predict possible binding modes with the receptor. Computational determination of the in silico physicochemical properties of the most active compounds using variable tools was also within our attention.

2. Materials and methods

2.1. Chemistry

Synthesis of the intermediates and target compounds was
accomplished according to the steps illustrated in Schemes 1 and 2. The starting material methyl 3-amino-4-phenylthiophene-2-carboxylate 1 [28] was prepared by adding a cold sodium methoxide solution slowly to a mixture of ethyl thioglycolate and 2-cyano-2-phenylvinylbenzenesulfonate and heating the reaction mixture at 60°C for 2h following the conditions reported by Lisowski et al. [29]. Treating 1 with hydrazine hydrate 80% yielded 3-amino-4-phenylthiophene-2-carbodrazide 2 which was used as a precursor for preparation of compounds illustrated in Scheme 1. 1H NMR spectrum of compound 2 showed three D2O exchangeable singlets at 4.33, 6.19 and 7.56 ppm for hydrazino NH2, thiophene NH2 and hydrazino NH protons, respectively in addition to a multiplet integrated for six protons assigned for phenyl and thiophene-C5-Hs. Cyclization of 2 with diethyl ethoxymethylenemalonate was achieved to produce the expected product ethyl 1-(3-amino-4-phenylthiophene-2-carbonyl)-5-hydroxy-1H-pyrazole-4-carboxylate 3. IR spectrum of compound 3 showed two stretching absorption bands at 1693 and 1630 cm−1 corresponding to ester and ketonic carbonyl groups in addition to a broad band at 3483, 3436 and 3345 cm−1 for OH and NH2, respectively. 1H NMR spectrum of compound 3 revealed the expected triplet and quartet signals assigned for the ethyl moiety at 1.18 and 4.02 ppm, respectively in addition to a new singlet at 7.58 ppm for pyrazole-C3-H. The structure was confirmed by EI-MS that demonstrated the molecular ion peak at \( m/z = 357 \) in addition to the base peak at \( m/z = 294 \).

On the other hand, condensing 2 with acetylacetone in ethanol gave rise to the corresponding 3,5-dimethylpyrazole 5. 1H NMR spectrum of compound 5 revealed 2 upfield singlets each integrated for 3 protons at 2.24 and 2.51 ppm assigned for 2 methyl groups in addition to a singlet at 6.20 ppm assigned for pyrazole-C4-H. Moreover, reacting 2 with the appropriate pyrazole-4-carboxaldehyde furnished the corresponding carbohydrazides 6a-c. 1H NMR spectrum of compound 6a revealed the 4-chlorophenyl protons as two doublets while in the 1H NMR spectrum of compound 6c two of the 4-methylphenyl protons were included within the multiplet attributed for other aromatic protons and the other two resonated as a doublet. The spectrum of compound 6c also demonstrated an upfield singlet for methyl protons. EI-MS of compound 6c showed the molecular ion peak at \( m/z = 477 \) and the base peak at \( m/z = 365 \).

Furthermore, 4-chloro-7-phenylthieno[3,2-d]pyrimidine 7 [31] was prepared by heating under reflux a mixture of 7-phenylthieno[3,2-d]pyrimidine-4(3H)-one and phosphorus oxychloride for 2h and pouring the reaction mixture onto ice according to the conditions reported by Pédeboaco et al. [32]. Compound 7 was reacted with hydrazine hydrate 99% to give the hydrazinothienopyrimidine 8 which was used as a
starting material for the target compounds depicted in Scheme 2. 

1H NMR spectrum of compound 8 demonstrated two D2O exchangeable singlets for NH2 and NH protons in addition to two downfield singlets at 8.27 and 8.28 ppm for thienopyrimidine-C6 and C2-protons, respectively. Condensation of 8 with ethoxymethylenemalononitrile or ethyl ethoxymethylenecyanoacetate in absolute ethanol, in the presence of anhydrous potassium carbonate resulted in the formation of the corresponding 5-amino-1H-pyrazole-4-carbonitrile 9 and 5-amino-1H-pyrazole-4-carboxylate 10 derivatives, respectively. 1H NMR spectrum of compound 9 showed a broad downfield D2O exchangeable singlet for NH2 protons in addition to a three downfield singlets for thienopyrimidine-C6-H, pyrazole-C3-H and thienopyrimidine-C2-H, respectively. The same signals were detected in the 1H NMR spectrum of compound 10 in addition to a triplet and a quartet for the ethyl group. EI-MS of both compounds demonstrated the molecular ion peak as the base peak at m/z = 318 and 365, respectively. 

Analogously, upon reacting 8 with diethyl ethoxymethylene malonate in refluxing ethanol, the target 5-hydroxy-1H-pyrazolyl-4-carboxylate derivative 11 was obtained while reacting 8 with acetylacetone produced 3,5-dimethyl-1H-pyrazolyl analog 12. EI-MS of compound 11 demonstrated the molecular ion peak at m/z = 366 and the base peak at m/z = 320 while 1H NMR spectrum of compound 12 revealed 2 new upfield singlets at 2.31 and 2.78 ppm for the 2 methyl groups in addition to a new singlet at 6.31 ppm for pyrazole-C4-H. 

Moreover, refluxing 8 with 3-(4-chlorophenyl)-1-phenyl-1H-pyrazole-4-carboxaldehyde yielded the corresponding methylenehydrazinylthieno[3,2-d]pyrimidine 13 which was reacted with bromine in glacial acetic acid at room temperature to give the unexpected product 8-bromo-3-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)-7-phenylthiophenylthio[2,3-e][1,2,4]triazolo[4,3-c]pyrimidine 14 where cyclization and bromination at C8 occurred. 1H NMR spectrum of compound 13 revealed 2 doublets for 4-chlorophenyl protons in addition to a downfield singlet for –CH besides a downfield D2O exchangeable singlet for NH. 1H NMR spectrum of 14 was characterized by the absence of the singlet characteristic for C8 proton and showed a singlet at 9.61 ppm for thienotriazolopyrimidine-C5-H. EI-MS of compound 14 showed [M+•+4] at m/z 586 corresponding to C28H16Br37ClN6S radical cation, [M+•+2] at m/z 584 corresponding to C28H16Br35ClN6S or C28H16Br37ClN6S radical cations and [M+•] at m/z 582 corresponding to C28H16Br35ClN6S radical cation.

2.2. Biological evaluation

2.2.1. In vitro COX-1 and COX-2 enzymatic inhibition assay [33]

All the newly synthesized compounds were evaluated for their in vitro inhibition of ovine COX-1 and COX-2 isoenzymes via measuring the peroxidase activity of both enzymes according to the instructions of using Cayman colorimetric COX (ovine) inhibitor screening assay kit. The concentration causing 50% enzyme inhibition (IC50, µM) for both enzymes in addition to the in vitro COX-2 selectivity indices (SI = IC50 COX-1/IC50 COX-2) of the test compounds were estimated and recorded in Table 1. Celecoxib and indomethacin were used as reference drugs as examples of selective and non selective COX-2 inhibitors, respectively.

Results revealed that all test compounds elicited weak inhibition of COX-1 isozyme (IC50 = 7.52–11.91 µM range) when compared to indomethacin (IC50 = 0.10 µM). Compounds 6c, 9, 10 and 13 were weak COX-1 inhibitors with potency close to that of celecoxib (IC50 = 10.11–11.91 µM range compared to IC50 = 14.70 for celecoxib) expecting that these compounds will have safe gastric profiles. 

On the other hand, the newly synthesized compounds showed variable inhibitory activities of COX-2 enzyme. Compound 13 exhibited
COX-2 selective inhibition activity nearly equal to that of celecoxib (IC$_{50}$ = 0.048 µM for 13 and 0.045 µM for celecoxib) while compounds 6c, 9 and 10 exhibited COX-2 selective inhibition activity (IC$_{50}$ = 0.059–0.071 µM range) comparable to that of celecoxib in the order of 10 > 9 > 6c. Compounds 4, 5, 6a, 11 and 14 demonstrated moderate COX-2 selective inhibition (IC$_{50}$ = 0.098–0.12 µM range) while compounds 3 and 12 showed weak activity with IC$_{50}$ values = 0.17 and 0.19 µM, respectively whereas compound 6b was the least selective COX-2 inhibitor with IC$_{50}$ = 0.23 µM.

Regarding in vitro COX-2 selectivity indices, all test compounds showed relatively higher selectivity towards COX-2 than COX-1 with selectivity indices ranging from 34.39 to 248.13 compared to selectivity indices of 326.67 and 1.25 for celecoxib and indomethacin, respectively. Compound 13 namely, 4-(2-((3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)methylene)hydrazinyl)-7-phenylthieno[3,2-d]pyrimidine elicited the highest COX-2 selectivity index (SI = 248.13) and was moderately selective COX-2 inhibitor with IC$_{50}$ = 0.23 µM.

It is well documented that high COX-2 selectivity causes thrombotic cardiovascular toxicity [3,12] whereas low COX-2 selectivity results in GI problems [5]. Therefore, the thiophene pyrazole derivative 6c and the thienopyrimidine pyrazole hybrids 9, 10 and 13 are moderately selective COX-2 inhibitors that could act as gastrointestinal safe anti-inflammatory agents.

**Table 1**

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>COX-1 IC$_{50}$ (µM)$^a$</th>
<th>COX-2 IC$_{50}$ (µM)$^a$</th>
<th>COX-2 SI$^b$ (COX-1/COX-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>7.52</td>
<td>0.17</td>
<td>44.24</td>
</tr>
<tr>
<td>4</td>
<td>8.11</td>
<td>0.11</td>
<td>73.73</td>
</tr>
<tr>
<td>5</td>
<td>8.76</td>
<td>0.12</td>
<td>73.00</td>
</tr>
<tr>
<td>6a</td>
<td>8.61</td>
<td>0.12</td>
<td>71.75</td>
</tr>
<tr>
<td>6b</td>
<td>7.91</td>
<td>0.23</td>
<td>34.39</td>
</tr>
<tr>
<td>6c</td>
<td>10.23</td>
<td>0.071</td>
<td>144.08</td>
</tr>
<tr>
<td>9</td>
<td>10.11</td>
<td>0.063</td>
<td>160.48</td>
</tr>
<tr>
<td>10</td>
<td>11.23</td>
<td>0.059</td>
<td>190.34</td>
</tr>
<tr>
<td>11</td>
<td>9.65</td>
<td>0.098</td>
<td>98.47</td>
</tr>
<tr>
<td>12</td>
<td>7.53</td>
<td>0.19</td>
<td>39.63</td>
</tr>
<tr>
<td>13</td>
<td>11.91</td>
<td>0.048</td>
<td>248.13</td>
</tr>
<tr>
<td>14</td>
<td>7.65</td>
<td>0.11</td>
<td>69.55</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>14.70</td>
<td>0.045</td>
<td>326.67</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.10</td>
<td>0.080</td>
<td>1.25</td>
</tr>
</tbody>
</table>

$^a$ Values are means of three determinations acquired using an ovine COX-1/COX-2 assay kit (catalog no.760111, Cayman Chemicals, MI, USA) and the deviation from the mean is < 10% of the mean value.

$^b$ In vitro COX-2 selectivity index SI (COX-1 IC$_{50}$/COX-2 IC$_{50}$).
2.2.2. In vivo anti-inflammatory activity

In order to verify results of the in vitro COX-1 and COX-2 enzymatic inhibition assay all the newly synthesized compounds were evaluated fore their in vivo anti-inflammatory activity using formalin-induced paw edema bioassay (acute inflammatory model) [30,34,35]. Diclofenac sodium and celecoxib were used as reference standards anti-inflammatory agents.

2.2.2.1. Formalin-induced paw edema bioassay (acute inflammatory model). The acute anti-inflammatory activities of all synthesized compounds were evaluated employing formalin-induced paw edema bioassay according to the reported method [30,34] using diclofenac sodium and celecoxib as reference drugs. The paw volume (mL) was measured at zero time and remeasured again 1h, 2h and 4h after administration of formalin. The edema was expressed as an increase in paw volume and the % of edema inhibition (EI%) was calculated. Results are presented in Table 2, Fig. 4 as the mean paw volume (mean in mL ± S.E.) and edema inhibition percentage (EI %).

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>Volume of edema (mL) (mean ± S.E.)</th>
<th>EI %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>1 h</td>
</tr>
<tr>
<td>Control</td>
<td>0.30 ± 0.01</td>
<td>0.53 ± 0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.32 ± 0.01</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.33 ± 0.02</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.33 ± 0.02</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>6a</td>
<td>0.32 ± 0.01</td>
<td>0.43 ± 0.02</td>
</tr>
<tr>
<td>6b</td>
<td>0.25 ± 0.01</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>6c</td>
<td>0.34 ± 0.01</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>9</td>
<td>0.39 ± 0.01</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>10</td>
<td>0.33 ± 0.02</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>11</td>
<td>0.34 ± 0.01</td>
<td>0.47 ± 0.02</td>
</tr>
<tr>
<td>12</td>
<td>0.31 ± 0.01</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>13</td>
<td>0.33 ± 0.01</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>14</td>
<td>0.31 ± 0.02</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>0.30 ± 0.01</td>
<td>0.43 ± 0.01</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>0.33 ± 0.01</td>
<td>0.40 ± 0.02</td>
</tr>
</tbody>
</table>

a: Dose levels, test compounds and diclofenac sodium (20mg/kg body weight, p.o).
b: Values are expressed as mean ± S.E. (n = 5).
c: Significantly different compared to corresponding control, p ≤ 0.05.

2.2.2.2. Formalin-induced paw edema bioassay (sub-acute inflammatory model). Sub-acute anti-inflammatory activities of all test compounds were conducted on the rats of the first experiment according to the reported method [34,35]. The changes in the volume of paw were measured at day 1, day 2, day 3, day 4, day 5 and day 8. Results are presented in Table 3, Fig. 5 as the mean paw volume (mL, mean ± S.E.) and edema inhibition percentage (EI %).

The obtained data revealed that test compounds showed a wide range of EI% in the range of 30% to 72% after the three time intervals used. After 1h, compounds 3, 6a and 9 exhibited greater anti-inflammatory activity than diclofenac sodium with activity in the order 9 > 6a > 3 (EI% = 67%, 64% and 61%, respectively compared to 58% for diclofenac sodium). Meanwhile, compounds 5 and 11 were as effective as diclofenac sodium. However, all compounds demonstrated lower activity than celecoxib except for compounds 9 and 6a that showed slight comparable activity to celecoxib (EI% = 67% and 64%, respectively compared to EI% = 68% for celecoxib). After 2h, compounds 6a and 9 were found to be superior to the two references (EI% = 68% and 70%, respectively compared to EI% = 56% for diclofenac sodium and EI% = 60% for celecoxib). Compound 11 was more potent than diclofenac sodium while eliciting comparable activity to that of celecoxib whereas compound 3 was equipotent to diclofenac sodium with less potency than celecoxib. Taking the 4h test as a criterion for comparison revealed that compound 9 was more potent than both references with EI % = 72% while compound 6a displayed similar potency to that of celecoxib and greater activity than diclofenac sodium with EI % = 64%. Similarly, compounds 3 and 11 elicited enhanced activity than diclofenac sodium whereas compound 6c was as effective as the latter. Other compounds displayed variable activities.

2.2.2.3. Determination of effective dose 50 (ED50) [30]. Compounds 3, 6a, 6c, 9, 10 and 11 that showed promising in vivo anti-inflammatory activities were further tested at 5, 10, 20, 40, and 50 mg/kg body weight and their ED50 values were determined by measuring the inhibition of the edema volume 2h after formalin injection. Results
**Table 3**

*In vivo* anti-inflammatory activities for test compounds, diclofenac sodium and celecoxib in formalin-induced rat paw edema bioassay (sub-acute inflammatory model) represented as volume of edema (mL) and edema inhibition percentage (EI %).

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>Volume of edema (mL) (mean ± S.E.)</th>
<th>EI %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Day 1 Day 8 Day 1 Day 8</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.30 ± 0.01 0.63 ± 0.02 0.85 ± 0.02</td>
<td>0 0</td>
</tr>
<tr>
<td>3</td>
<td>0.32 ± 0.01 0.50 ± 0.02 0.61 ± 0.01</td>
<td>45 47</td>
</tr>
<tr>
<td>4</td>
<td>0.33 ± 0.02 0.55 ± 0.01 0.73 ± 0.04</td>
<td>33 26</td>
</tr>
<tr>
<td>5</td>
<td>0.33 ± 0.02 0.49 ± 0.02 0.61 ± 0.02</td>
<td>51 49</td>
</tr>
<tr>
<td>6a</td>
<td>0.32 ± 0.01 0.47 ± 0.02 0.60 ± 0.02</td>
<td>54 49</td>
</tr>
<tr>
<td>6b</td>
<td>0.25 ± 0.01 0.46 ± 0.02 0.55 ± 0.02</td>
<td>36 45</td>
</tr>
<tr>
<td>6c</td>
<td>0.34 ± 0.01 0.57 ± 0.03 0.60 ± 0.03</td>
<td>30 52</td>
</tr>
<tr>
<td>9</td>
<td>0.39 ± 0.01 0.56 ± 0.02 0.62 ± 0.04</td>
<td>48 58</td>
</tr>
<tr>
<td>10</td>
<td>0.33 ± 0.02 0.52 ± 0.02 0.60 ± 0.03</td>
<td>42 51</td>
</tr>
<tr>
<td>11</td>
<td>0.34 ± 0.01 0.51 ± 0.01 0.60 ± 0.03</td>
<td>48 52</td>
</tr>
<tr>
<td>12</td>
<td>0.31 ± 0.01 0.55 ± 0.03 0.64 ± 0.02</td>
<td>27 39</td>
</tr>
<tr>
<td>13</td>
<td>0.33 ± 0.01 0.51 ± 0.02 0.63 ± 0.02</td>
<td>48 45</td>
</tr>
<tr>
<td>14</td>
<td>0.31 ± 0.02 0.56 ± 0.01 0.66 ± 0.02</td>
<td>24 35</td>
</tr>
<tr>
<td>Diclofenac sodium</td>
<td>0.30 ± 0.01 0.49 ± 0.01 0.55 ± 0.03</td>
<td>42 54</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>0.33 ± 0.01 0.49 ± 0.01 0.54 ± 0.02</td>
<td>51 61</td>
</tr>
</tbody>
</table>

a Dose levels, test compounds and diclofenac sodium (20 mg/kg body weight, po).

b Values are expressed as mean ± S.E. (n = 5).

c Significantly different compared to corresponding control, p ≤ 0.05.

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**Fig. 5.** *In vivo* anti-inflammatory activity (EI %) of the most active compounds of the formalin-induced rat paw edema bioassay (sub-acute inflammatory model).

**Fig. 6.** Effective dose 50 (ED$_{50}$) (mmol/kg) for diclofenac sodium. On the other hand, compounds 6a, 6c and 9 showed superior potency than celecoxib while compound 11 was slightly equipotent to celecoxib. Other compounds were less potent than celecoxib. The potency was in the order 6a > 6c > 9 > 11 > 3 > 10 (Fig. 6) with compound 6a being the most potent showing ED$_{50}$ = 0.033 mmol/kg.

2.2.2.4. Determination of ulcerogenic activity. Compounds 3, 6a, 6c, 9, 10 and 11 that exhibited pronounced in vivo anti-inflammatory profiles were further evaluated for their ulcerogenic potential in rats according to the reported method [36] using diclofenac sodium, indomethacin and celecoxib as reference standards. Results are presented in **Table 4** as % ulceration. Gross observation of the isolated rat stomachs showed a normal stomach texture for compounds 6c, 9 and 11 with no observable hyperemia indicating a superior GI safety profile in the population of the test animals. On the other hand, compounds 3, 6a and 10 showed weak ulcerative effect (10%) compared to both celecoxib and diclofenac sodium (no ulceration). All test compounds were less ulcerogenic than indomethacin that showed 100% ulceration.

2.2.2.5. Determination of acute toxicity. The most active compounds (3, 6a, 6c, 9, 10 and 11) were further evaluated for their approximate acute lethal dose (ALD$_{50}$) using a literature method [37]. Results are presented in **Table 4** indicating that all test compounds proved to be non-toxic and were well tolerated by the experimental animals. Test compounds showed a high safety margin when screened at graded doses 100–400 mg/kg, po, where their ALD$_{50}$ values were found to be > 400 mg/kg.

2.2.2.6. Structure-Activity Relationship (SAR) study of the in vivo anti-inflammatory activities results:

- A collective interpretation of the in vivo anti-inflammatory activity of the test compounds in the pre-mentioned screens Tables 2 and 3 revealed that the thiophene pyrazole hybrids 3, 6a and 6c in addition to the thienopyrimidine pyrazole derivatives 9, 10 and 11 showed pronounced activity in the formalin-induced paw edema screen (both the acute and sub-acute models). These facts would suggest that such type of compounds might be effective in managing chronic as well as acute inflammatory conditions.

- Inspection of the structures of the test compounds revealed that within the tested thiophene pyrazole hybrids, compound 3 containing 5-hydroxy-2-pyrazole-4-carboxylate moiety attached to the thiophene ring through a carbonyl group was more active than diclofenac sodium especially in the acute inflammatory model while being less potent than celecoxib in both models.

- Replacement of the 5-hydroxy-2-pyrazole-4-carboxylate moiety by dimethylpyrazole ring system in compound 5 decreased the in vivo acute anti-inflammatory activity while its sub-acute anti-
inflammatory activity was increased especially on day 1 of the sub-
acute anti-inflammatory assay.

- Introduction of diarylpyrazole functionality separated from the
aminothiophene ring by 4 atoms spacer produced variable activities.
Compound 6a (R = H) was found to be more active or of compar-
able activity to both reference standards especially in the acute in-
nflammatory model and on day 1 of the sub-acute anti-inflammatory
assay. In addition, compound 6c (R = CH$_3$) was equipotent to di-
clofenac sodium after 4 h of formalin injection while compound 6b
(R = Cl) exhibited the least in vivo anti-inflammatory activity within
this series.

- Among the five tested thienopyrimidine derivatives, compound 9
containing aminopyrazolecarbonitrile moiety proved to be the most
active in the in vivo acute and sub-acute inflammatory models.

- Replacement of the carbonitrile moiety with carboxylate function-
ality in compound 10 decreased the in vivo anti-inflammatory ac-
tivity in both models.

- Replacement of the amino group in compound 10 with hydroxyl
group (compound 11) increased the in vivo anti-inflammatory ac-
tivity in both models. However, the introduction of either di-
methylpyrazole (compound 12) or diarylpyrazole functionality
(compound 13) decreased the in vivo anti-inflammatory activity
especially in the acute model.

- Thienotriazolopyrimidine derivative 14 was less active than its
thienopyrimidine precursor 13 in both models.

2.2.2.7. Statistical analysis. Data obtained are presented as
means ± S.E. of the mean. The concentration-dependent effects of
various drugs in vitro were evaluated statistically by the randomized
block design analysis of variance (ANOVA) followed by Student-
Newman-Keuls Multiple Comparison Test. The difference in results
was considered significant when p < 0.05.

2.2.3. In silico studies

2.2.3.1. Docking studies. Further, to determine the plausible modes of
binding of the most active compounds in both in vitro and in vivo assays
with COX-2 enzyme, molecular docking studies of compounds 3, 6a,
6c, 9, 10, 11 and 13 were performed using Molecular Operating
Environment (MOE) version 2014.09 [38]. Crystal structure of COX-2
complexed with celecoxib (PDB code 3LN1) [39] was retrieved from the
Protein Data Bank. Docking scores, amino acid residues forming
hydrophobic or hydrogen bonding interactions and their lengths were
summarized in Table 5.

Regarding COX-2 enzyme, Celecoxib sulfonyl moiety (SO$_2$) and 4-
tolyl ring made one H-bonding interaction with His 200 amino acid and
one arene–arene interaction with His 374 amino acid in a distance of
3.60 and 3.97 Å, respectively with an energy score equals to-5.05 kcal/
Mol.

Compounds that were subjected to molecular docking fitted well to
COX-2 active site inside the pocket and showed good binding energy
scores ranged from −5.17 to − 8.60 kcal/Mol. Docking results re-
vealed that compounds 3, 6a, 10, 11 and 13 showed arene–arene in-
teraction with His 374 amino acid while compounds 6c and 9 exhibited
arene-cation interaction with the same amino acid residue indicating
that all test compounds shared pretty similar binding mode with cel-
ecoxib. Compound 6c showed H-bonding interaction with His 200 amino
acid similar to that of celecoxib while compounds 10 and 13
demonstrated H-bonding interactions with Thr 198 amino acid.
Compounds 6a and 13 showed arene-cation interaction with His 200
amino acid while compounds 3, 6c, 11 and 13 revealed additional
arene-cation interaction with His 372 amino acid. On the other hand,
the docking poses of compounds 6c, 9, 10 and 13 that elicited the highest in
vivo COX-2 selectivity indices, appeared to access more re-
sidues in the binding site of COX-2 enzyme compared to the co-crys-
tallized ligand. Compound 6c revealed a third arene-cation interaction
with Lys 197 while compound 10 exhibited a second arene-cation
interaction with Trp 373 amino acid. Furthermore, both compounds 9
and 13 showed two additional arene-cation interactions with Gin 189
and His 193 amino acids for compound 9 and with Gin 189 amino acid
for compound 13. Therefore, docking studies results proved that all test
compounds showed high binding affinity to COX-2 enzyme in addition
to promising binding patterns comparable to that of celecoxib.
The proposed binding modes and 2D interactions of celecoxib and com-
 pounds 3, 6a, 6c, 9, 10, 11 and 13 with amino acid residues in COX-2
receptor active site (PDB code 3LN1) were presented in Figs. 7A and 7B
while their proposed 3D interactions were demonstrated in Figs. 8A and
8B.

2.2.3.2. In silico Chemo-informatic properties evaluation. Bioavailability
is a factor that must be considered in clinical applications. It is affected
by the physicochemical properties of compounds including molecular
weight, fat-water partition coefficient and others [40]. In an attempt
to explain the different behavior of the newly synthesized ligands in the in
vitro and in vivo assays, the most active ligands in both screenings
namely, compounds 3, 6a, 6c, 9, 10, 11 and 13 were analyzed
computationally to predict the best ligand on the basis of Lipinski’s
rule of five (ROS), chemical and bio-molecular properties. In this study,
Molinspiration tool [41] is used to predict the drug-likeness properties
of the most active ligands and their violations from Lipinski’s ROS
[42–44]. Moreover, the % absorption (% ABS) of compounds 3, 6a, 6c,
9, 10, 11 and 13 were calculated [44]. The solubility (Mol log S in mg/
L) and the drug likeness model score of the most active compounds
were predicted using Molsoft software [45,46]. Furthermore, the
predicted human intestinal absorption (HIA), blood–brain barrier
penetration (BBB) and plasma protein binding (PPB) were evaluated
by Pre ADMET calculator [47] to evaluate the overall pharmacokinetic
profile of the compounds. The predicted chemo-informatic properties
are mentioned in Table 6.

Lipinski’s ROS refers to four physicochemical properties of a mo-
lecule to become a drug, i.e. molecular weight (MW) should be < 500,
logP < 5, H-bond donors < 5 and H-bond acceptor < 10. Compounds that comply or show only one violation from Lipinski’s ROS
were known to exhibit good oral bioavailability and were suitable for
oral administration [42,43]. Results revealed that compounds 3, 9, 10
and 11 comply with Lipinski’s ROS with no violations while compounds
6a and 6c show only 1 violation whereas compound 13 demonstrated 2
violations from Lipinski’s ROS. This can explain the difference between
the in vivo and in vitro screenings results of these compounds. Test
compounds were administered orally during the in vivo anti-in-
nflammatory assays so oral bioavailability will reflect on the in vivo re-
sults. Therefore, compounds 3, 6a, 6c, 9, 10 and 11, complying with
ROS, exhibited variable in vivo anti-inflammatory activities. On the
other hand, compound 13, the most active compound in the in vitro
COX-1 and COX-2 enzymatic inhibition assay, did not show remarkable
in vivo anti-inflammatory activity as it showed 2 violations from Li-
piniski’s ROS, i.e. has certain bioavailability problems.

Another factor that affect drug bioavailability is the topological
polar surface area (TPSA) of the compound where passively absorbed
compounds with a TPSA > 140 Å$^2$ are thought to have low oral bio-
availability [48]. All compounds showed acceptable TPSA results in the
range of 68.00–107.45 Å$^2$. Moreover, all compounds were predicted to
display good % absorption in the range of 71.93–85.54%. Furthermore,
all compounds could be well absorbed across the intestinal epithelium
(HIA > 91%) while they were predicted to have weak blood brain
barrier penetration (BBB: 0.06–0.22). In addition, compounds 3, 9, 10
and 11 showed high affinity to plasma protein (PPB: 88–96%) while
compounds 6a, 6c and 13 showed 100% PPB. This parameter may also
account for their diminished in vivo anti-inflammatory activities com-
pared with the promising in vitro results. In addition, compounds 6a, 6c
and 13 were insoluble in water (mol log S = 0) while other compounds
displayed good water solubility (0.19–0.56). Predicted drug likeness
model scores revealed that the best score was for compound 11

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Table 5
The docking scores and proposed binding interactions of compounds 3, 6a, 6c, 9, 10, 11 and 13 inside COX-2 active site.

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>Docking scores (Kcal/mol)</th>
<th>Hydrogen bonding</th>
<th>Other interactions</th>
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<tr>
<td></td>
<td></td>
<td>Residues involved (distance Å)</td>
<td>Functional group</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>−5.0500</td>
<td>His 200 (3.60)</td>
<td>SO₂⁻O</td>
</tr>
<tr>
<td>3</td>
<td>−5.8330</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6a</td>
<td>−6.3438</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6c</td>
<td>−8.6037</td>
<td>His 200 (3.34)</td>
<td>Pyrazole-N₂</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>9</td>
<td>−5.8014</td>
<td>−</td>
<td>−</td>
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<td></td>
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<td>−</td>
<td>−</td>
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<td></td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>10</td>
<td>−5.1697</td>
<td>Thr 198 (2.97)</td>
<td>Carbonyl-O</td>
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<tr>
<td>11</td>
<td>−5.9462</td>
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<td>13</td>
<td>−8.2479</td>
<td>Thr198 (2.94)</td>
<td>N1-thienopyrimidine ring</td>
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</tbody>
</table>

Fig. 7A. The proposed 2D interactions of celecoxib and compounds 3, 6a and 6c with amino acids residues in COX-2 receptor active site (PDB code 3LN1).
Fig. 7B. The proposed 2D interactions of compounds 9, 10, 11 and 13 with amino acids residues in COX-2 receptor active site (PDB code 3LN1).

Fig. 8A. The proposed 3D interactions of celecoxib and compounds 3, 6a and 6c with amino acids residues in COX-2 receptor active site (PDB code 3LN1).
followed by compounds 10 and 3 (Drug likeness model score = 0.78, 0.67 and 0.54, respectively) indicating that these compounds displayed acceptable drug-likeness parameters and are suitable anti-inflammatory candidates that can be orally administered.

3. Conclusion

Design and synthesis of new thiophene - pyrazole hybrids were achieved. All compounds were found to be more COX-2 selective inhibitors than COX-1 with compound 13 exhibiting activity nearly equal to that of celecoxib and compounds 6c, 9 and 10 eliciting comparable activity to that of celecoxib in the order of 10 > 9 > 6c. Selectivity indices of compounds 6c, 9, 10 and 13 revealed that they are moderately selective COX-2 inhibitors. Acute in vivo anti-inflammatory assays showed that compounds 3, 6a, 9 and 11 were the most active. Compounds 5 and 6a were the most active on day 1 of the sub-acute anti-inflammatory assay compared to celecoxib while compounds 3, 5, 6a, 6c, 9, 10, 11 and 13 elicited promising activity superior or comparable to that of diclofenac sodium on day 1 of the sub-acute anti-inflammatory assay compared to celecoxib. Other compounds showed binding modes analogous to that of celecoxib.

Table 6

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>Molinspiration</th>
<th>Pre ADMET</th>
<th>Molsoft</th>
</tr>
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<tr>
<td>LogP</td>
<td>MW</td>
<td>n-ON</td>
<td>n-OHNH</td>
</tr>
<tr>
<td>3</td>
<td>2.93</td>
<td>357.39</td>
<td>7</td>
</tr>
<tr>
<td>6a</td>
<td>5.46</td>
<td>463.57</td>
<td>6</td>
</tr>
<tr>
<td>6c</td>
<td>5.91</td>
<td>477.59</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>2.58</td>
<td>318.37</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>3.03</td>
<td>365.42</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>3.33</td>
<td>366.40</td>
<td>7</td>
</tr>
<tr>
<td>13</td>
<td>6.44</td>
<td>507.02</td>
<td>6</td>
</tr>
</tbody>
</table>

Fig. 8B. The proposed 3D interactions of compounds 9, 10, 11 and 13 with amino acids residues in COX-2 receptor active site (PDB code 3LN1).
directly attached or separated with one atom spacer from a pyrazole moiety substituted at the 4-position with an ester functionality is a promising scaffold for the design of novel biologically active anti-inflammatory ligands having moderate COX-2 selectivity and hence safe gastrointestinal and cardiovascular profiles. Therefore, the thiophene analogs 3 and the thiophene-pyrazine derivatives 10 and 11 are considered promising non-toxic, gastrointestinal safe anti-inflammatory candidates with moderate COX-2 selectivity, good oral bioavailability and physicochemical properties.

4. Experimental

4.1. Chemistry

All reagents and solvents were purchased from commercial suppliers and were dried and purified when necessary by standard techniques. Melting points were determined in open glass capillaries on a Stuart capillary melting point apparatus (Stuart scientific Stone, Staffordshire, UK) and were considered promising non-toxic, gastrointestinal safe anti-inflammatory candidates with moderate COX-2 selectivity, good oral bioavailability and physicochemical properties.

4.1.1. 3-Amino-4-phenylthiophene-2-carboxylic acid (2)

A mixture of 1 (0.47 g, 2 mmol) and hydrazine hydrate 80% (0.2 mL) in ethanol (5 mL) was heated under reflux for 5 h. The reaction mixture was cooled to room temperature and the obtained precipitate was filtered, washed with ethanol, dried and crystallized from ethanol. White crystals (0.38 g, 83%); mp 132–134 °C; IR (KBr, cm⁻¹): 3483, 3436, 3345 (OH, NH₂), 1639 (C=O, C=O-Ar); ¹H NMR (300 MHz, δ ppm): 4.33 (s, 2H, NHNH₂, D₂O exchangeable), 6.19 (s, 2H, thiophene NH₂, D₂O exchangeable); ¹³C NMR (75 MHz, δ ppm): 74.5, 100.9, 128.5, 131.6, 141.0, 154.3, 164.7; EI-MS m/z (%): 175 (M⁺) (23), 157 (M⁺-29) (100); Anal. Calcd for C₇H₈N₂O₆S (238.4): C, 46.70; H, 3.82; N, 14.28. Found: C, 46.47; H, 3.79; N, 14.24.

4.1.2. Ethyl 1-(3-amino-4-phenylthiophene-2-carbonyl)-5-hydroxy-1H-pyrazole-4-carboxylate (3)

A mixture of 2 (0.47 g, 2 mmol), diethyl ethoxymethylenemalonate (0.43 g, 0.4 mL, 2 mmol) and anhydrous potassium carbonate (0.28 g, 2 mmol) in absolute ethanol (5 mL) was heated under reflux for 6 h. After being cooled to room temperature, the produced solid was filtered, suspended in water, neutralized with 10% hydrochloric acid. The obtained precipitate was filtered, washed with water, dried and crystallized from ethanol. White crystals (0.58 g, 71%); mp 292–294 °C; IR (KBr, cm⁻¹): 3443 (OH, NH₂), 1657 (C=O, C=O-Ar), 1542 (C=O, C=O-Ar), 1280, 1111 (ω, and γ, C–O–C); ¹H NMR (300 MHz, δ ppm): 1.18 (t, J = 7.2 Hz, 2H, CH₂=CH₂), 6.82 (s, 2H, NH₂, D₂O exchangeable), 7.36-7.51 (m, 5H, Ar-H), 7.53 (s, 1H, thiophene-C⁵-H), 7.58 (s, 1H, pyrazole-C⁵-H); ¹³C NMR (75 MHz, δ ppm): 15.3, 56.5, 90.1, 101.2, 127.8, 128.4, 129.4, 131.6, 134.5, 144.0, 155.2, 163.6, 164.5, 165.7; EI-MS m/z (%): 357 (M⁺) (23), 294 (100); Anal. Calcd for C₁₇H₁₄N₄O₆S (375.38): C, 57.13; H, 4.23; N, 11.76; S, 8.97. Found: C, 57.39; H, 4.27; N, 11.89; S, 9.03.

4.1.3. Ethyl 3-(2-(3-amino-4-phenylthiophene-2-carbonyl)hydrazinyl)-2-cyanoprop-2-enoate (4)

A mixture of 2 (0.47 g, 2 mmol), ethyl ethoxymethylenecyaanoacetate (0.34 g, 2 mmol) and anhydrous potassium carbonate (0.28 g, 2 mmol) in absolute ethanol (5 mL) was heated under reflux for 6 h. After being cooled to room temperature, the solid product was filtered, washed with water, dried and crystallized from dioxane. White crystals (0.51 g, 71%); mp 292–294 °C; IR (KBr, cm⁻¹): 3443 (OH, NH₂), 1657 (C=O, C=O-Ar), 1542 (C=O, C=O-Ar), 1280, 1111 (ω, and γ, C–O–C); ¹H NMR (300 MHz, δ ppm): 1.15 (t, J = 7.2 Hz, 2H, CH₂=CH₂), 7.97-8.0 (m, 2H, phenyl-C²,6-H), 8.25 (s, 2H, NH₂, D₂O exchangeable), 8.34 (s, 1H, thiophene-C⁵-H), 8.37 (s, 1H, =CH); ¹³C NMR (75 MHz, δ ppm): 15.4, 58.2, 121.6, 124.7, 128.1, 128.4, 128.9, 131.0, 131.4, 136.6, 147.8, 153.2, 155.8, 165.8, 169.1; HRMS calculated [M+H⁺] for C₁₇H₁₇N₄O₃S: 357.102, found: 357.099; Anal. Calcd for C₁₇H₁₇N₄O₃S (356.40): C, 57.29; H, 4.52; N, 15.72; S, 9.00. Found: C, 57.54; H, 4.49; N, 15.98; S, 9.12.

4.1.4. 1-(3-Amino-4-phenylthiophen-2-yl)carbonyl-3,5-dimethyl-1H-pyrazole (5)

Acetylacetone (0.3 g, 0.31 mL, 3 mmol) was added to a solution of 2 (0.47 g, 2 mmol) in absolute ethanol (5 mL) and the reaction mixture was heated under reflux for 10 h. The reaction mixture was concentrated and left to cool to room temperature. The obtained precipitate was filtered, dried and crystallized from ethanol. White crystals (0.33 g, 55%); mp 140–142 °C; IR (KBr, cm⁻¹): 3456, 3393 (NH₂), 1639 (C=O), 1590, 1536 (C=N, C=O-Ar); ¹H NMR (300 MHz, δ ppm): 2.24 (s, 3H, CH₃), 2.51 (s, 3H, CH₃), 6.20 (s, 1H, pyrazole-C⁵-H), 7.13 (s, 2H, NH₂, D₂O exchangeable), 7.40–7.49 (m, 5H, Ar-H), 7.80 (s, 1H, thiophene-C⁵-H); ¹³C NMR (100 MHz, δ ppm): 14.0, 14.7, 99.6, 111.0, 128.1, 128.5, 129.1, 131.6, 134.7, 134.9, 150.4, 157.3, 163.1; Anal. Calcd for C₁₇H₁₇N₂O₃S (397.37): C, 64.62; H, 5.08; N, 14.13; S, 9.08. Found: C, 64.73; H, 5.12; N, 14.28; S, 10.86.

4.1.5. General procedure for the synthesis of 3-amino-4-phenyl-N'-(1-phenyl-3-(4-substituted phenyl)-1H-pyrazol-4-yl)(methylenetriphenylcarbodiimide (6a-c)

To a suspension of 2 (0.47 g, 2 mmol) in ethanol (5 mL), the appropriate pyrazole-4-carboxaldehyde (2 mmol) was added. The reaction mixture was heated under reflux for 12 h then allowed to cool to room temperature. The obtained precipitate was filtered, washed with ethanol, dried and crystallized from dioxane/ethanol.

4.1.5.1. 3-Amino-4-phenyl-N'-(1,3-diphenyl-1H-pyrazol-4-yl)(methylene) triphenylcarbodiimide (6a)

Pale yellow crystals (0.73 g, 78%); mp 245–247 °C; IR (KBr, cm⁻¹): 3454, 3429, 3232 (NH, NH₂), 1627 (C=O), 1591, 1504 (C=N, C=O-Ar); ¹H NMR (400 MHz, δ ppm): 6.71 (br s, 2H, NH₂, D₂O exchangeable), 7.37-7.60 (m, 12H, 11Ar-H, thiophene-C⁵-H), 7.64 (s, 1H, pyrazole-C⁵-H), 7.72 (d, J = 7.04 Hz, 2H, pyrazole
4.1.7. 5-Amino-1-(7-phenylthieno[3,2-d]pyrimidin-4-yl)-1H-pyrazole-4-carboxylate (10)

A mixture of 8 (0.48 g, 2 mmol), ethyl ethoxymethylenecycloacetate (0.34 g, 2 mmol) and anhydrous potassium carbonate (0.28 g, 2 mmol) in absolute ethanol (5 mL) was heated under reflux for 6 h. After being cooled to room temperature, the produced solid was separated, washed with water, dried and crystallized from ethanol. White crystals (0.53 g, 74%); mp 179–181 °C; IR (KBr, cm⁻¹): 3445, 3332 (NH₂), 1686 (C=O ester), 1617 (C=N), 1556, 1483 (C=Ar), 1301, 1129 (ν₉ and ν₁O–C); 1H NMR (300 MHz, δ ppm): 1.31 (t, J = 7.2 Hz, 3H, CH₃CH₂), 4.27 (q, J = 7.2 Hz, 2H, CH₂CH₂), 7.41–7.54 (m, 3H, phenyl-C₃,C₄,C₅-H), 7.99 (br s, 2H, NH₂, D₂O exchangeable), 8.03 (s, 1H, thiopyrimidine-C₆-H), 8.04–8.08 (m, 2H, phenyl-C₃,C₄-H), 8.75 (s, 1H, pyrazole-C₇-H), 9.10 (s, 1H, thiopyrimidine-C₇); ¹³C NMR (75 MHz, δ ppm): 19.0, 56.4, 94.9, 118.9, 128.4, 125.0, 129.0, 133.3, 134.7, 141.2, 151.0, 150.7, 150.7, 157.0, 160.0; Anal. Calc: C₂₀H₁₅N₄O₃S (365.44): C, 59.07; H, 4.12; N, 14.67; S, 6.82.
1.1.2. 8-Bromo-3-(3-(4-chlorophenyl)-1-phenyl-1H-pyrazol-4-yl)-7-phenylthieno[2,3-e][1,2,4]triazolo[4,3-c]pyrimidine (14)

A mixture of 13 (0.51 g, 1 mmol) and anhydrous sodium acetate (0.25 g, 3 mmol) in glacial acetic acid (3 mL) was treated with bromine (0.32 g, 0.1 mL, 2 mmol) and the reaction mixture was stirred at room temperature for an overnight then poured onto ice/cold water. The obtained precipitate was filtered, washed with water, dried and crystallized from dioxane. White crystals (0.56 g, 95%) mp: 254–256°C; IR (KBr, cm⁻¹): 1630(C=O), 1575, 1499(C=N), 1301, 130.4, 130.5, 131.5, 133.9, 135.6, 139.5, 142.2, 143.9, 153.1, 157.0; Anal. Caled for C₈₃H₆₃BrClN₆S: C, 57.60; H, 2.76; N, 14.39. Found: C, 57.89; H, 2.79; N, 14.62.

δ (ppm): 85.3, 103.8, 119.3, 119.7, 128.5, 128.6, 128.8, 128.9, 129.0, 129.9, 130.1, 130.2, 130.5, 130.7, 131.2, 133.9, 135.1, 141.5, 147.1, 151.0, 154.4, 157.0, 161-MS m/z (%): 586 (M++ 4) (21), 584 (M++ 2) (68), 582 (M++) (48), 63 (100); Anal. Caled for C₈₃H₆₃BrClN₆S (583.89): C, 57.60; H, 2.76; N, 14.39. Found: C, 57.89; H, 2.79; N, 14.62.

4.2. Biological activity

4.2.1. In vitro COX-1 and COX-2 enzymatic inhibition assay [33]

The inhibitory COX activity of the newly synthesized compounds in addition to celecoxib and indomethacin were assayed according to the instructions of the use of Cayman colorimetric COX (ovine) inhibitor screening assay kit (Catalog No. 760111, Cayman chemicals, MI, USA).

4.2.2. In vivo anti-inflammatory activity

Male albino rats weighing 180–200 g were used to study the anti-inflammatory, ulcerogenic liability and acute toxicity. Animals (five per cage) were housed under controlled laboratory conditions and were allowed standard chow and water ad libitum. All experimental procedures involving animals were performed in strict accordance with The Institutional Animal Care and Use Committee ethical standards. Experimental protocol was approved by Institutional Animal Care and Use Committee, Faculty of Medicine, Alexandria University, Alexandria, Egypt (AlexU-IACUC code: 0634).

4.2.2.1. Formalin-induced paw edema bioassay (acute inflammatory model).

The acute anti-inflammatory activity for the newly synthesized compounds and the reference drugs diclofenac sodium and celecoxib were evaluated using formalin-induced paw edema bioassay and the measurement of paw volume (mL) was done at zero time and after 1 h, 2 h and 4 h of administration of formalin according to the reported method [30,34]. The edema was expressed as an increase in the paw volume and the percentage of edema inhibition (EI%) was obtained as follows:

\[ EI\% = \left( V_t - V_0 \right) / V_0 \times 100 \]

where \( V_t \) = volume of edema at specific time interval and \( V_0 \) = volume of edema at zero time interval.

4.2.2.2. Formalin-induced paw edema bioassay (sub-acute inflammatory model).

Sub-acute anti-inflammatory activities of all test compounds were conducted on the rats of the first experiment and the changes in the volume of paw were measured plethysmographically at day 1 and day 8 according to the reported method [34,35].

4.2.2.3. Determination of effective dose 50 (ED₅₀) [30]. Compounds 3, 6a, 6c, 9, 10 and 11 that showed promising in vivo anti-inflammatory activities and the reference diclofenac sodium and celecoxib were further tested at 5, 10, 20, 40, and 50 mg/kg body weight and the ED₅₀ was determined by measuring the inhibition of the edema volume 2 h after formalin injection according to the reported method [30].

4.2.2.4. Determination of ulcerogenic activity. Compounds 3, 6a, 6c, 9, 10 and 11 that exhibited pronounced in vivo anti-inflammatory profiles were further evaluated for their ulcerogenic potential in male albino rats according to the reported method using diclofenac sodium, indomethacin and celecoxib as reference standards [36]. Test compounds were given at 0 and 12 h for three successive days at a dose of 300 mg/kg per day. Animals were sacrificed and their stomachs were inspected for any evidence of hyperemia, hemorrhage, definite hemorrhagic erosion or ulcer.

4.2.2.5. Determination of acute toxicity. The most active compounds (3, 6a, 6c, 9, 10 and 11) were further evaluated for their approximate acute lethal dose (ALD₅₀) using a literature method [30,37]. The compounds were given orally in graded doses of 100–400 mg/kg body weight, screened for their acute lethal doses (ALD₅₀) and mortalities were recorded at each dose level after 24 h.

4.2.3. In silico studies

4.2.3.1. Docking studies. Docking studies were performed using Molecular Operating Environment (MOE) version 2014.09 [38] running on an Intel Core i5PC running Windows 10 as operating system. Crystal structure of COX-2 complexed with celecoxib (PDB code 3LN1) [39] was retrieved from the Protein Data Bank. 3D structures for compounds 3, 6a, 6c, 9, 10, 11 and 13 were constructed using the Builder module of MOE and optimized by energy minimization using MMFF94X forcefield. All docking calculations were carried out using the MOE Dock module. The parameters used were alpha triangle as placement methodology and London dG as scoring function with forcefield refinement. Ligand interactions were generated using the Ligand Interactions module in MOE. The docking protocol was validated by docking celecoxib in the active site of 3LN1, where the docked celecoxib showed 0.4 Å Root Mean Square Distance (RMSD) from the co-crystallized celecoxib.

4.2.3.2. Chemo-informatic properties evaluation. ADME properties of compounds 3, 6a, 6c, 9, 10, 11 and 13 were obtained computationally through Molinspiration tool [41] (http://www.molinspiration.com/cgi-bin/properties) where numbers of hydrogen donors (n-OHNH) and acceptors (n-ON), c logP and total polar surface area (TPSA) in addition to violations from Lipinski’s RO5 were calculated. Moreover, % absorption (% ABS) was calculated from the following equation: % ABS = 109 − 0.345 TPSA [44]. The aqueous solubility (Mol log S in mg/L) and the drug likeness model score of the most active compounds were predicted using Molsoft software (http://molsoft.com/mprop/) [43,45]. Furthermore, the predicted human intestinal absorption (HIA), blood–brain barrier penetration (BBB) and plasma protein binding (PPB) (ADME properties) were evaluated by Pre ADMET calculator [46] to evaluate the overall pharmacokinetic profiles of the compounds.

Conflicts of interest

The authors have no conflict of interest to declare.

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