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Synthesis and biological evaluation of isoxazolo[4,5-*d*]pyridazin-4-(5*H*)-one analogues as potent anti-inflammatory agents

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ABSTRACT

In this study, eighteen new isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one derivatives possessing either a 1,3,4-thiadiazole or a 1,2,4-triazole-5-thione moiety were synthesized and tested for anti-inflammatory activity in vitro (COX-1/COX-2, 5-LOX) and in vivo (rat paw edema assay). Compounds **15**, **16**, **25**, **26** and **28–30** showed dual COX-2 (IC_{50}'s in the 2.1–10.9 μM range), and 5-LOX (IC_{50}'s in the 6.3–63.5 μM range) inhibitory activity. When administered orally to rats, dual COX-2/5-LOX inhibitors showed higher anti-inflammatory activity in vivo (30–45% reduction of the inflammatory response) than the reference drug ibuprofen (18%). Among dual COX-2/5-LOX inhibitors, the most potent compound (**28**) exhibited the best anti-inflammatory profile by inhibiting both COX-2 (IC_{50} = 2.1 μM) and 5-LOX (IC_{50} = 6.3 μM) enzymes. We investigated the binding interactions of compound **28** by an enzyme–ligand molecular modeling (docking) studies, which showed favorable binding interactions in both COX-2 and 5-LOX active sites. Furthermore, the dual acting COX-2/5-LOX compound **28** exhibited a superior gastrointestinal safety profile (ulcer index = 0.25) compared to the reference drug ibuprofen (UI = 7.0) when administered orally at the same molar dose. These observations suggest that isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one analogs represent a new scaffold to design potent, effective, and safe anti-inflammatory agents possessing dual COX-2/5-LOX inhibitory activity.

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1. Introduction

The major pharmacological mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs) is the enzymatic inhibition of cyclooxygenase (COX)-mediated production of pro-inflammatory prostaglandins (PGs) and thromboxanes (TXs). There are two types of COX enzymes, namely COX-1 and COX-2; COX-1 is generally regarded as a constitutive enzyme involved in the physiological production of PGs and provides maintenance functions such as cytoprotection in the stomach. In contrast, COX-2 has been regarded as an inducible enzyme expressed in inflammatory cells.[1] However, recent reports have challenged these 'traditional' roles for COX-1 and COX-2 enzymes, emphasizing the importance of re-evaluating their roles not only in the inflammatory process, but also in their mechanism-based toxicity.[2]

Epidemiological studies have shown a significant risk of gastrointestinal,[3–6] renal,[7] and hepatic[8] side-effects associated with the inhibition of COX-mediated PG synthesis.[9–13] In this regard, gastrointestinal erosions and bleeding are two of the most common toxic effects associated with the long-term administration of NSAIDs, even when administered at low prophylactic doses (i.e., aspirin 81 mg/day).[14] Furthermore, recent literature reports describe how the relatively high incidence of side-effects associated with the chronic use of NSAIDs has led some clinicians (and patients) to reduce their use.[15]

The development and clinical use of highly selective COX-2 inhibitors (coxibs) arrived with bold promises of improving gastrointestinal safety that have not been fully realized.[2,16] Even though these drugs produce less ulceration than conventional NSAIDs, coxibs still produce significant gastric and cardiovascular adverse events in susceptible individuals, especially if they are administered concomitantly with aspirin.[17,18] There is evidence in the literature to be concerned about the dose-dependent hypertensive effect of some NSAIDs, which is not easily predicted by their COX selectivity alone.[19] For this reason, Wallace et al. recently proposed that there is still a 'strong clinical need for effective anti-inflammatory drugs with improved safety profiles over existing NSAIDs'.[20] In this regard, dual inhibitors of COX/LOX enzymatic pathways constitute a rationale approach for the design of more effective anti-inflammatory agents with an improved safety profile, because of their ability to synergistically block both pathways of the arachidonic acid cascade.[21,22] This supposition is based on the premise that inhibiting only one of the COX/LOX pathways may shift the metabolism of

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arachidonic acid to the other uninhibited pathway, which may produce abnormally high concentrations of the corresponding COX-derived prostaglandins (if only the LOX pathway is inhibited) or the LOX-derived leukotrienes (if only the COX pathway is blocked).[23]

To improve the efficacy/safety profile of new NSAIDs, extensive structure–activity relationship (SAR) studies have been carried out using a wide variety of scaffolds exerting COX inhibitory profile, including (but not limited to) pyridazinones,[24–27] 1,3,4-thiadiazoles,[28,29] and 1,2,4-triazol-5-thione rings.[30] Michaux et al. reported the isoxazolo[4,5-*d*]pyridazinone ring template (compound **I**, Fig. 1) as a potent and selective COX inhibitor equipotent to rofecoxib[31]; Unsal-Tan et al. reported a similar isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (**II**) derivative as a suitable scaffold for the inhibition of COX enzymes,[26] and Varandas et al. showed that hybrid drugs formed by known anti-inflammatory agents with a 1,3,4-thiadiazole moiety resulted in COX-2 selective molecules.[28] Additionally, Navidpour et al. observed that compounds carrying a 1,2,4-triazol-5-thione group were more selective than celecoxib toward COX-2.[30]

Based on these observations, we were interested in evaluating the pharmacological properties of a new series of 7-benzyl-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one derivatives, bearing either a 1,3,4-thiadiazole (**13**–**21**) or a 1,2,4-triazol-5-thione (**22**–**30**) moiety (Fig. 2).

In this report we describe the chemical synthesis and biological evaluation of 7-benzyl-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one derivatives as anti-inflammatory agents. We also investigated the potential (unwanted) gastric side-effects exerted by an oral dose of these compounds, and finally, we conducted two molecular modeling (docking) experiments to support the observed in vitro and in vivo dual COX-2/5-LOX inhibitory profile exerted by these compounds. The results of our investigation provide evidence suggesting that 7-benzyl-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one derivatives represent a new scaffold to develop potent and safe anti-inflammatory agents.

2. Results and discussion

2.1. Chemistry

The synthesis of 7-benzyl-3-methyl-5-((5-alkylamino-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones **13–21** and 7-benzyl-5-((4-substituted-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones **22–30** is outlined in Scheme 1.

7-Benzyl-3-methylisoxazolo[4,5-*d*]pyridazin-5(4*H*)-ones (**1a–c**) were prepared as described in the literature.[32] Briefly, reaction of intermediates **1a–c** with ethyl bromoacetate in the presence of potassium carbonate in acetone yielded 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetates (**2a–c**), which were subsequently reacted with hydrazine hydrate under reflux to obtain a series of 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetohydrazides (**3a–c**). Ethanolic solutions

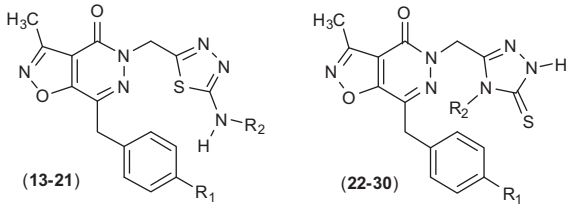


Figure 2. Chemical structure of 7-benzyl-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one derivatives, bearing a 1,3,4-thiadiazole (**13**–**21**) or a 1,2,4-triazol-5-thione moiety (**22**–**30**). R_1 and R_2 groups are described in Table 1.

of intermediates **3a–c** were refluxed with an appropriately substituted isothiocyanate (R_2NCS) to obtain 1-(2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-thiosemicarbazides (**4**–**12**).

The acid-catalyzed cyclization of thiosemicarbazides **4**–**12** using concentrated sulfuric acid, afforded the corresponding 7-benzyl-3-methyl-5-((5-alkylamino-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (**13–21**), whereas the base-catalyzed cyclization of thiosemicarbazides **4**–**12** using sodium bicarbonate resulted in the formation of the corresponding 7-benzyl-5-((4-substituted-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (**22–30**). Both acid- and base-catalyzed procedures were based on the work published by Onkol et al.[33]

2.2. Determination of physicochemical properties

Considering that the COX-1 active site consists of a long narrow channel about 8 × 25 Å (total volume 316 Å^2), and the COX-2 binding site is about 25% larger (394 Å^3),[34] bulkier molecules normally fit better into the bigger active site of COX-2, and consequently, larger compounds generally show a higher selectivity for this enzyme.[35] This is a well established concept reported in the literature which has been employed in the development of potent and selective COX-2 inhibitors by several groups.[36–38] Consequently, we determined the molecular length (Å), surface (Å^2), and volume (Å^3) of experimental drugs to assess if they would possess the minimum essential structural requirements to enter the COX binding site. In this regard, after a standard energy minimization procedure using Alchemy 2000 (Version 2.0, 1997, Tripos Inc.), we determined these parameters for compounds **13–30** and the results are summarized in Table 1.The majority of test compounds (except **13** and **22**) showed a molecular volume higher than that required to fit into the active site of COX-1, which suggested that they would probably show a weak binding affinity for this enzyme. However, the calculated molecular volumes (309–390 Å^3) for compounds **13–30** suggested that these molecules would be able to access the long narrow channel of the COX-2 enzyme active site (394 Å^3), which in turn, would likely produce a higher binding

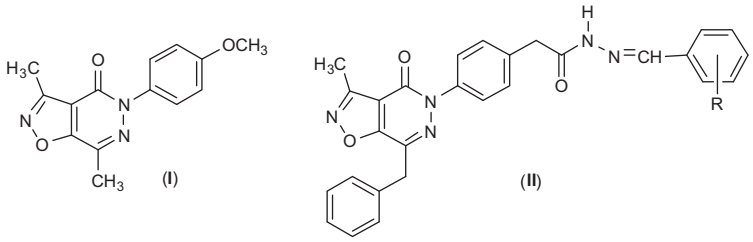
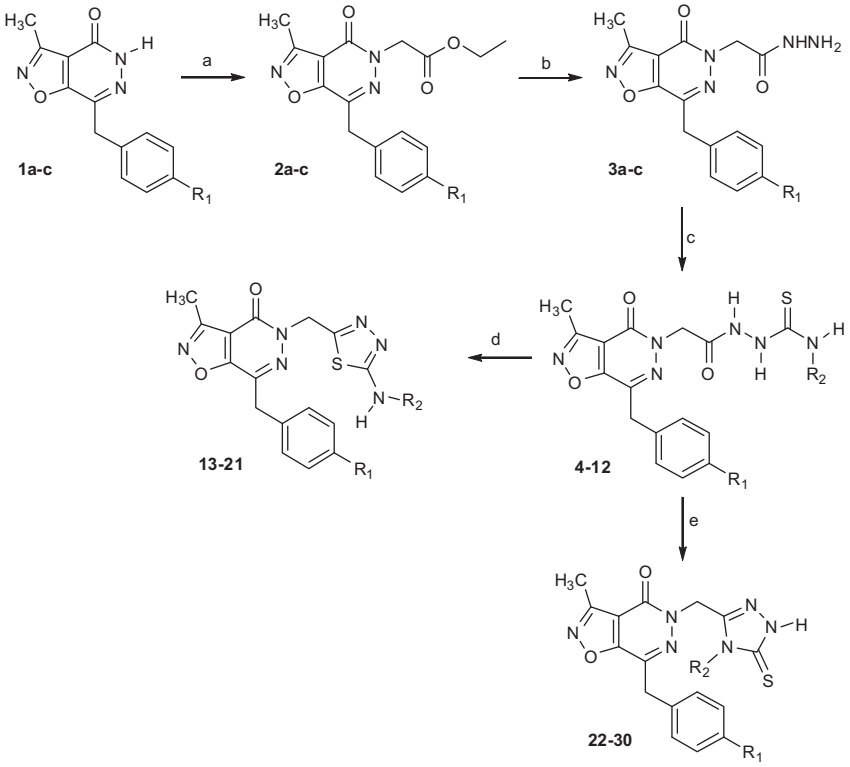


Figure 1. Chemical structure of reported isoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones SPB04674 (**I**), and (**II**).



Scheme 1. Synthesis of target compounds **13**–**30**. Reagents and conditions: (a) K_2CO_3, acetone, reflux 4 h, then $EtOCOCH_2Br$ and reflux 2 h; (b) NH_2NH_2, EtOH, reflux 1 h; (c) R_2NCS, EtOH, reflux 4 h; (d) H_2SO_4, H_2O, 25 °C,1 h; (e) $NaHCO_3$, EtOH, 25 °C, 1 h.

Table 1
Prediction of physicochemical parameters for compounds **13**–**30**: molecular volume (expressed in cubic Angstroms, Å^3); Length (expressed in Angstroms, Å); negative logarithm of the distribution coefficient of each compound between *n*-octanol/water (theoretical value, expressed as Log*P*)

Compd	R_1[a]	R_2[a]	Volume[b]	Length[c]	Log*P*[d]
13	H	CH_3	309.6	11.0, 13.2	0.67 ± 1.0
14	H	C_2H_5	325.8	11.8, 14.3	1.20 ± 1.0
15	H	C_6H_5	364.1	11.8, 15.2	1.68 ± 1.1
16	NO_2	CH_3	335.7	13.0, 13.2	0.40 ± 1.0
17	NO_2	C_2H_5	341.5	12.3, 14.1	0.93 ± 1.0
18	NO_2	C_6H_5	388.6	13.1, 16.2	1.41 ± 1.1
19	OCH_3	CH_3	334.7	13.2, 13.6	0.59 ± 1.0
20	OCH_3	C_2H_5	351.0	13.7, 14.1	1.12 ± 1.0
21	OCH_3	C_6H_5	389.1	13.4, 14.7	1.59 ± 1.1
22	H	CH_3	309.2	10.7, 11.5	−0.68 ± 1.0
23	H	C_2H_5	325.9	11.6, 11.9	−0.15 ± 1.0
24	H	C_6H_5	363.6	11.8, 12.7	1.07 ± 1.0
25	NO_2	CH_3	335.3	10.6, 13.9	−0.95 ± 1.1
26	NO_2	C_2H_5	351.6	11.4, 14.0	−0.42 ± 1.1
27	NO_2	C_6H_5	390.4	11.2, 11.5	0.80 ± 1.1
28	OCH_3	CH_3	334.1	11.4, 13.0	−0.76 ± 1.1
29	OCH_3	C_2H_5	350.3	11.7, 13.9	−0.23 ± 1.1
30	OCH_3	C_6H_5	388.3	13.2, 15.6	0.99 ± 1.1

[a] See Scheme 1.
[b] Calculated using Alchemy 2000 (Version 2.0, 1997, Tripos Inc.) after energy minimization.
[c] From the energy-minimized structures, different lengths were calculated by measuring the linear distance between atoms farthest apart from each other.
[d] Calculated using ACD/ChemSketch (Version 12.01 freeware, 2010, ACD Labs Inc.).

affinity compared to COX-1. Therefore, it was expected that compounds **13**–**30** would possess a selective COX-2 inhibitory profile.

In addition to molecular volumes, we also determined molecular lengths (Å) for all test compounds. Considering the three dimensional structure of pyridazin-4(5*H*)-ones (**13–30**), we determined two different linear distances between the two atoms farthest apart from each other in the corresponding energy-minimized structures (Table 1). Based on these parameters, we observed that all test compounds (10.6–16.2 Å long) could theoretically fit into the active site of COX (25 Å long). These preliminary parameters, along with the corresponding molecular volumes, suggested that pyridazin-4(5*H*)-ones had appropriate physicochemical parameters to access the COX channel. Nevertheless, we recognized that even though pyridazin-4(5*H*)-ones possessed proper volumes and lengths, they may or may not interact favorably with essential residues within the binding site of either enzyme (this would be better assessed by specific molecular modeling—docking—studies, see discussion below).

2.3. Inhibition of cyclooxygenase enzymes

The target compounds **13–30** were evaluated for their ability to inhibit COX-2 (human recombinant) and COX-1 (ovine) enzymatic activity using a fluorescent inhibitor screening assay kit (Cayman Chemicals, Cat. No. 700100).The potency (IC_{50} values) of test compounds was determined and compared to that of the reference molecules DuP-697 (selective COX-2 inhibitor) and SC-560 (selective COX-1 inhibitor); these results are summarized in Table 2.

In general, 7-benzyl-3-methyl-5-((5-alkylamino-1,3,4-thiadiazol-2-yl) methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (**13–21**), showed a modest cyclooxygenase inhibition in vitro (COX-1 IC_{50}'s = 19–99.1 μM; COX-2 IC_{50}'s = 2.1–39.6 μM) compared to reference compounds SC-560 (IC_{50} = 8.5 nM) and DuP-697 (IC_{50} = 41.6 nM). The most potent COX inhibitors in this series were compounds **19** (COX-1 IC_{50} = 19.0 μM; COX-2 IC_{50} = 14.7 μM) and **16** (COX-1 IC_{50} = 67.0 μM; COX-2 IC_{50} = 6.0 μM).

Structure–activity relationship studies showed that the nature of substituents (R_2) on the 1,3,4-thiadiazol-2-yl ring was not a major determinant on COX-1 inhibition, whereas substitution at the

Table 2
In vitro COX-1 and COX-2 enzyme inhibition for compounds **13**–**30**

Compd	COX-1 IC_{50} (μM)[a]	COX-2 IC_{50} (μM)[a]	SI[b]
13	99.1	21.3	4.6
14	>100	>100	n.d.[c]
15	>100	10.9	>10
16	67.0	6.0	11.1
17	46.7	39.6	1.1
18	77.4	>100	n.d.[c]
19	19.0	14.7	1.3
20	>100	>100	n.d.[c]
21	>100	>100	n.d.[c]
22	6.4	5.5	1.1
23	0.9	6.1	0.1
24	>100	>100	n.d.[c]
25	47.7	4.6	10.3
26	>100	9.2	>10
27	>100	>100	n.d.[c]
28	26.5	2.1	12.6
29	20.5	2.2	9.15
30	>100	10.8	>10
SC-560	0.0085	n.d.[c]	—
DuP-697	n.d.[c]	0.04	—

[a] The in vitro test compound concentration required to produce 50% inhibition of enzymatic activity. The result (IC_{50}, μM) is the mean of three determinations acquired using the COX Fluorescent Inhibitor Screening Assay Kit (Catalog No. 700100, Cayman Chemicals Inc., Ann Arbor, MI, U.S.A.) and the deviation from the mean is <10% of the mean value.
[b] In vitro COX-2 selectivity index (COX-1 IC_{50}/COX-2 IC_{50}).
[c] Not determined.

Table 3
In vitro 5-LOX enzyme inhibition, in vivo anti-inflammatory and ulcerogenic activity for compounds **15**, **16**, **25**, **26**, **28**, **30**

Compd	5-LOX IC_{50} (μM)[a]	AI[b]	Ulcer index[c]
13	79.2	n.d.	n.d.
14	72.8	n.d.	n.d.
15	63.5	40.3 ± 6.2	0.50 ± 0.2
16	41.2	30.2 ± 3.5	0.75 ± 0.3
17	52.8	n.d.	n.d.
18	58.9	n.d.	n.d.
19	48.7	n.d.	n.d.
20	39.5	n.d.	n.d.
21	46.2	n.d.	n.d.
22	34.7	n.d.	n.d.
23	40.3	n.d.	n.d.
24	45.1	n.d.	n.d.
25	13.5	35.8 ± 3.5	0.25 ± 0.2
26	14.8	38.0 ± 4.8	0
27	16.5	n.d.	n.d.
28	6.3	45.0 ± 2.8	0.25 ± 0.2
29	8.6	n.d.	n.d.
30	7.7	45.0 ± 3.9	0
Caffeic acid	4.0	—	—
Zileuton	1.1[d]	—	—
Ibuprofen	—	18.5 ± 3.0	7.00 ± 0.8

[a] The in vitro test compound concentration required to produce 50% inhibition of potato 5-LOX (Cayman Chemicals Inc. Catalog No. 60401). The result (IC_{50}, μM) is the mean of two determinations acquired using a LOX assay kit (Catalog No. 760700, Cayman Chemicals Inc., Ann Arbor, MI, U.S.A.) and the deviation from the mean is <10% of the mean value.
[b] Inhibitory activity in a carrageenan-induced rat paw edema assay. The results are expressed as the % inhibition of inflammation at 3 h following an oral dose (0.14 mmol) of the corresponding test compounds equivalent to 30 mg/kg of ibuprofen. Results are expressed as the mean ± SEM ($n = 4$).
[c] The average overall length (in mm) of individual ulcers in each stomach ± SEM, $n = 4$, 6 h after oral administration of the test compounds (0.14 mmol equivalent to 30 mg/kg of ibuprofen).
[d] Ref. value.43

7-benzyl ring (R_1) modulated the potency of compounds in this series; molecules possessing an electronegative nitro group (**16–18**) were more active than unsubstituted compounds (R_1 = H, **13–15**), and molecules having an electron donating methoxy group (**20**, **21**) were the least potent COX-1 inhibitors, with the notable exception of compound **19**, which was the most potent in this group.

A similar analysis conducted on 7-benzyl-5-((4-substituted-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (**22–30**) revealed that inhibition of COX-1 and COX-2 was modulated mostly by the size of substituents (R_2) located on the 1,2,4-triazol-3-yl ring, since compounds possessing small alkyl groups (**22**, **25**, **28** R_2 = methyl; **23**, **26**, **27** R_2 = ethyl) showed increased potency on both COX-1 (IC_{50}'s in the 0.9–47.7 μM range) and COX-2 (IC_{50} = 2.1–9.2 μM) enzymes, compared to analogs possessing a larger phenyl group (**24**, **27**, **30**) at the same position, which did not inhibit cyclooxygenase activity at the highest test compound concentration (100 μM). The only exception was compound **30**, which displayed a relatively strong COX-2 enzyme inhibition (IC_{50} = 10.8 μM). In this regard, we also observed that compounds **22–30** showed an increased selectivity toward COX-2 (selectivity indices = 0.15–12.65) compared to analogs possessing a 5-substituted-1,3,4-thiadiazol-2-yl moiety (**13–21**). Compounds **26** and **30** were selective COX-2 inhibitors (SI >10), but their potency (COX-2 IC_{50} = 9.2 and 10.8 μM) was not nearly as good as that of the corresponding reference compound DuP-697 (IC_{50} = 41.6 nM). The most potent COX-2 inhibitor (**28**, IC_{50} = 2.1 μM) was about 52 fold-less potent than the reference drug DuP-697 (IC_{50} = 0.04 μM).

2.4. Anti-inflammatory activity

Based on the inhibitory potencies obtained in vitro (COX-2 selectivity), we screened a group of compounds (**15**, **16**, **25**, **26**, **28**, and **30**) for in vivo anti-inflammatory activity using the traditional carageenan-induced rat paw edema assay[39] (Table 3). We observed that these compounds were significantly more active (30.2–45.0% decrease in the inflammatory response) than the reference drug ibuprofen (18.5% decrease) on a molar basis (0.14 mmol/kg, equivalent to 30 mg/kg of ibuprofen), exerting a 1.6–2.4 fold-increase in anti-inflammatory activity. These results are in agreement with a previous report by Amir et al. describing the in vivo anti-inflammatory profile of 2,5-disubstituted 1,3,4-oxadiazoles and 1,2,4-triazoles as 'arylacetic acid derivatives' with significant analgesic and anti-inflammatory activity.[40]

There are several plausible explanations for the increased anti-inflammatory profile exerted by these compounds (especially **28** and **30**) compared to ibuprofen. One of them is the modest increase in their selectivity towards COX-2 enzyme (COX-1/COX-2 selectivity index = 10 or higher). However, we recognize the limitations of correlating in vitro potency and selectivity with in vivo results, because the incubation of test compounds in the presence of purified enzymes does not account for all the pharmacokinetic and pharmacodynamic factors evaluated in the animal model. Another possible explanation may be that compounds **15**, **16**, **25**, **26**, **28**, and **30** are absorbed at a higher extent compared to ibuprofen, which contains an ionizable COOH group; however, without a proper pharmacokinetic study we cannot conclude this is the case. In this regard, one of the main factors controlling the passive diffusion of molecules across cell membranes is their lipophilic character, and therefore, we determined the theoretical Log*P* values for compounds **13–30** (Table 1). However, the result of these calculations showed a lower lipophilic character for the test compounds (Log*P* values in the −0.95–1.68 range), compared to the reference drug ibuprofen (Log*P* = 3.72 ± 0.23). Based on these results, we could not correlate theoretical Log*P* values to the observed in vivo anti-inflammatory activity; for example, compounds **15** (Log*P* = 1.68) and **25** (Log*P* = −0.95) showed similar in vivo anti-inflammatory potencies (40.3% and 35.8% decrease in inflammatory response

respectively). This wide range of Log*P* values suggested that the in vivo anti-inflammatory response exerted by 7-benzyl-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one derivatives is (probably) not modulated by their lipophilic character.

A third possible explanation for the improved in vivo anti-inflammatory profile observed for the test compounds, is the inhibition of other enzymes involved in the inflammatory process. One of these enzymes is 5-lipoxygenase (5-LOX), which mediates the production of leukotrienes (LTs), species implicated not only in inflammation but also in fever, arthritis, and bronchospasm.[21,22]

2.5. Inhibition of 5-lipoxygenase (5-LOX)

It has been suggested that inhibition of only one of the COX/LOX pathways could shift the metabolism of arachidonic acid towards the other pathway, thereby inducing potential side-effects.[23] Therefore, to support the hypothesis of dual COX/5-LOX inhibition by the test compounds, we evaluated the in vitro inhibitory profile of drugs **15**, **16**, **25**, **26**, **28**, and **30** (Table 3). We observed that the 5-LOX inhibitory potency of the test compounds was moderate to relatively high (IC_{50}'s in the 6.3–79.2 μM range), especially for compounds **28** (IC_{50} = 6.3 μM) and **30** (IC_{50} = 7.7 μM) which were the most active, showing an inhibitory potency similar to that of the reference compound caffeic acid (IC_{50} = 4.0 μM). However, these inhibitory potencies represent a 5 to 7-fold decrease in potency compared to Zileuton (Zyflo®), an orally active inhibitor of 5-LOX.

The dual inhibition of COX and LOX enzymatic pathways has been extensively reported in the literature, and constitutes a rationale method for the design of more effective anti-inflammatory agents, because of their ability to synergistically block both pathways of the AA cascade. In this regard, considering that the reported mechanisms of enzymatic inhibition of 5-LOX involve either chelation of the iron atom present in the active site (hydroxamic acids and *N*-hydroxyureas),[41] or direct inhibition by reversible binding interactions (classical enzyme inhibition),[42] we can assume that 7-benzyl-3-methyl-5-((5-alkylamino-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (**15**, **16**) and 7-benzyl-5-((4-substituted-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (**25**, **26**, **28**, and **30**) inhibited 5-LOX by classical enzyme (reversible binding) interactions. At this point we do not have evidence to support a possible chelation of iron as the potential mechanism of LOX inhibition. Nevertheless, these compounds represent a new scaffold to design relatively potent and selective dual COX/5-LOX inhibitors.

2.6. Molecular modeling

To support the in vitro and in vivo anti-inflammatory profile exerted by isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (compound **28**), we also carried out enzyme–ligand molecular modeling (docking) studies, which showed that the test compounds exerted favorable interactions with both COX-2 (Fig. 3) and 5-LOX (Fig. 4).

The pyridazin-4(5*H*)-one ring was oriented in the central region of the COX-2 active site and the fused isoxazole ring was within the vicinity of COX-2 secondary pocket, surrounded by His90, Ser353, Tyr355, Arg513 and Val523. The isoxazole nitrogen atom formed a hydrogen bond with N*H* of His90 (distance = 2.30 Å) and the oxygen atom of isoxazole was about 3.55 Å away from O*H* of Tyr355, which suggests favorable binding interactions allowing this ring to enter the mouth of the COX-2 active site. The substituted 1,2,4-triazole-5-thione ring was oriented toward the apex of the COX-2 active site in a region comprised of Phe381, Leu384, Tyr385, Trp387, Met522, Gly526 and Ala527. The C=*S* moiety underwent nonpolar interactions with the side chain of Met522 (distance <5 Å) and was about 4.28 Å away from O*H* of Tyr385,

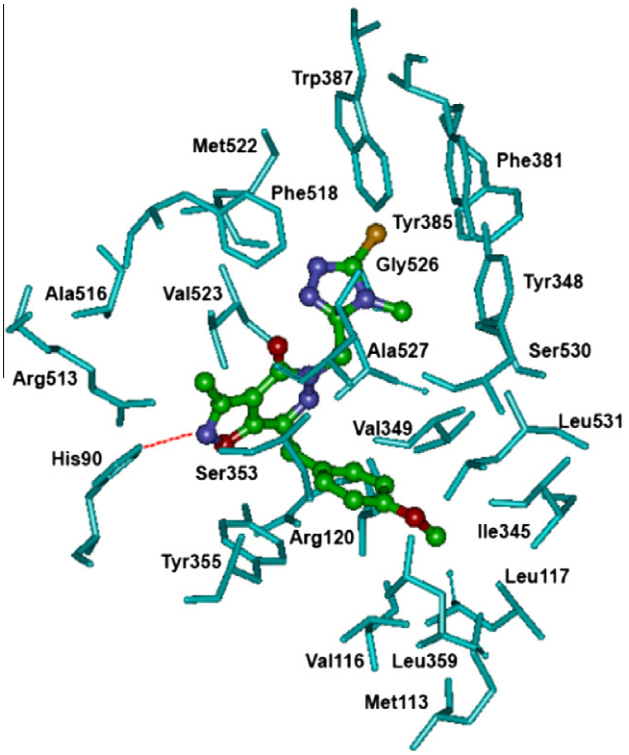


Figure 3. The orientation of **28** (ball-and-stick) in the active site of mammalian COX-2. Red dotted line represents hydrogen bonding. Hydrogen atoms are not shown for clarity.

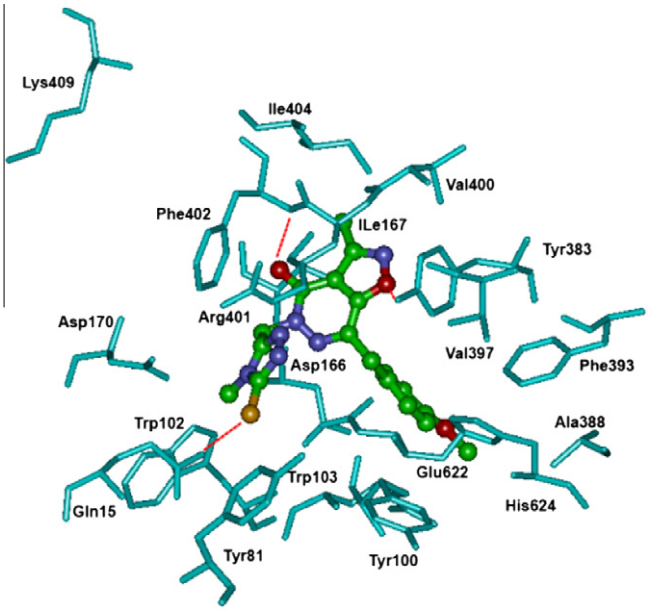


Figure 4. The orientation of **28** (ball-and-stick) in the active site of mammalian 5-LOX. Red dotted line represents hydrogen bonding. Hydrogen atoms are not shown for clarity.

whereas the N-CH_3 group was oriented toward Tyr348, Ala527 and Ser530. The *para*-methoxy-benzyl substituent of isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one was oriented in a hydrophobic region comprised of Met113, Leu117, Ile345, Leu359, and Leu531 closer to the mouth of the COX-2 active site (distance <5 Å). This orientation differs from that reported by Unsal-Tan et al.,[23] who reported the benzyl ring, and not the 1,2,4-triazole-5-thione ring, to be the

group orienting into the cavity occupied by Phe381, Leu384, Tyr385, Trp387, Phe513 and Ser530.

We also investigated the binding interactions of compound **28** (5-LOX IC_{50} = 6.3 μM), which was the most potent LOX inhibitor in vitro (Fig. 4). This study showed that the fused isoxazolopyridazin-4(5*H*)-one ring present in compound **28** was at the center of the active site, and the pyridazin-4-one C=*O* group was oriented toward the entrance of 5-LOX active site (Lys409) and formed a hydrogen bond with the N*H* group of Phe402 (distance = 2.52 Å). The fused isoxazole ring was oriented in a region comprised of Ile167, Tyr383, Phe402 and Arg401. The isoxazole oxygen formed a hydrogen bond with O*H* of Tyr383 (distance = 2.39 Å), whereas the methyl substituent underwent non-polar interactions with Val400, Ile404 and Ala405 (distance <5 Å). The substituted 1,2,4-triazole-5-thione ring was oriented toward a polar pocket comprised of Gln15, Tyr81, Trp102, Asp170 and Glu622 (distance <5 Å). Interestingly, the C=*S* of the triazole ring also formed a hydrogen bond with the NH_2 group of Gln15 (distance = 2.44 Å), and additionally, the triazole nitrogens underwent electrostatic interactions with the polar side chain of Arg401 (distance ~3 Å). The *para*-methoxy-benzyl substituent of isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one was oriented toward the catalytic site of 5-LOX in a region comprised of Tyr383, Phe393, Val397 and His624 (distance <5 Å). These studies indicate that compound **28** (molecular volume of 334.1 $Å^3$) fits well within the large boot-shaped active site of 5-LOX (volume = 663 $Å^3$).[51] The results obtained in these docking studies suggest that compound **28** is able to form several binding interactions, which allow it to inhibit the catalytic activity of 5-LOX, establishing a correlation with its relatively high in vitro inhibitory potency.

2.7. Ulcer index assay (UI)

Gastrointestinal erosions and ulcers are two of the most common side-effects associated with the chronic administration of NSAIDs in subpopulations of patients classified at high risk. Therefore, we were interested in determining if isoxazolo[4,5-*d*]pyridazin-5(4*H*)-ones produce toxicity when administered orally at their anti-inflammatory dose. To evaluate the potential (unwanted) ulcerogenic side-effects of dual COX/5-LOX inhibitors **15**, **16**, **25**, **26**, **28**, and **30**, we conducted a toxicity assay by administering the corresponding test compounds (0.14 mmol/kg), and the reference compound ibuprofen (0.14 mmol/kg, equivalent to 30 mg/kg) po to rats. All drugs were suspended in 1% methylcellulose solution.

Interestingly, the test compounds showed negligible toxicity (UI = 0–0.75) compared to the reference drug ibuprofen (UI = 7.0, Table 3). These results showed that the test compounds were significantly less ulcerogenic (p <0.001) than the reference drug ibuprofen when administered orally at equimolar doses, suggesting that the 3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one ring possessing either a 1,3,4-thiadiazole (**15**, **16**) or a 1,2,4-triazole (**25**, **26**, **28**, and **30**) moiety at *N*-5 have an improved efficacy/safety profile compared to a classical NSAID such as ibuprofen.

A plausible explanation for the lack of acute gastric toxicity exerted by the test compounds is the lack of acidic carboxylic acid groups in isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one derivatives. These results showed that in spite of exerting a relatively high COX inhibitory profile in vitro and a potent anti-inflammatory activity in vivo, the test compounds did not exert significant gastric toxicity, and the inhibition of COX activity seems to be unrelated to the extent of gastric toxicity. This observation is in agreement with several reports described previously. For example, Ligumsky et al. demonstrated that intragastrically- and parenterally-administered aspirin exerted the same degree of gastric prostaglandin biosynthesis inhibition, but only the former induced significant gastric injuries to the stomachs of rats.[44] Darling et al. found that NSAIDs can induce gastric injury in transgenic mice deficient in either COX-1 or COX-2, suggesting that simple inhibition of gastric COX-1 activity cannot provide a suitable explanation for the NSAID-induced gastric pathogenesis.[45] Orally administered NSAIDs associated with phosphatidylcholine (PC) inhibit gastric PGE_2 to the same extent as NSAIDs administered alone; however, PC-NSAIDs do not produce significant GI toxicity.[46]

It has been proposed that NSAIDs possessing carboxylic acid groups produce epithelial damage at sites of contact. The re-ionization of the uncharged, lipid-soluble drug within the gastric cells can lead to osmotic swelling and lysis of the cells, and this process can uncouple mitochondrial respiration leading to cell death.[47] The theoretical Log*P* values calculated for isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one derivatives (Log*P* values in the −0.9–1.6 range) suggests low lipophilicity for test compounds, especially for 7-benzyl-3-methyl-5-((5-(substituted amino)-1,3,4-thiadiazol-2-yl)-methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (**13**–**21**). However, despite their low lipophilic character, compounds **15**, **16**, **25**, **26**, **28**, and **30** showed an improved anti-inflammatory activity compared to the reference drug ibuprofen (calculated Log*P* value = 3.72 ± 0.23), but were devoid of gastric significant gastric toxicity at a 0.14 mmol dose (equivalent to 30 mg/kg of ibuprofen).

3. Conclusion

We propose the 3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one ring with a central 4-substituted benzyl ring at C-7, and a 1,3,4-thiadiazole (13–21) or 1,2,4-triazole (22–30) moiety at *N*-5 as a new scaffold for dual COX/5-LOX inhibition. Structure–activity relationship studies showed that the nature of substituents (R_2) on the 1,3,4-thiadiazol-2-yl ring was not a major determinant on COX inhibition, whereas substitution at the 7-benzyl ring (R_1) modulated their potency in vitro. The addition of a 5-alkylamino-1,3,4-thiadiazol-2-yl, or a 5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl moiety to the central 3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one ring provides a new class of anti-inflammatory agents with negligible ulcerogenicity in vivo.

4. Experimental

4.1. Chemistry

Melting points were determined with a Thomas–Hoover capillary melting point apparatus (Philadelphia, PA, U.S.A.) and are uncorrected. IR spectra (KBr) were recorded on a Jasco FTIR420 Fourier spectrometer. The ^{1}H NMR spectra (DMSO-d_6) were recorded on a Varian Mercury 400 FT NMR spectrometer using TMS as internal reference (Chemical shift represented in δ ppm). The ESI-MS spectra were determined using a micromass ZQ-4000 single quadruple mass spectrometer. Elemental analyses (C, H, N) were performed on a Leco CHNS 932 analyzer (University of Hacettepe). 7-benzyl-3-methylisoxazolo[4,5-*d*]pyridazin-5(4*H*)-ones (**1a–c**),[32] ethyl 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetate (**2a**),[26] and 7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetohydrazide (**3a**)[26] were synthesized following previously reported methods.

4.1.1. General procedure for the preparation of ethyl 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetates (2b–c)

Anhydrous potassium carbonate (0.075 mol) was added to a solution of 0.05 mol of **1a–c** in acetone (100 mL). This reaction mixture was stirred under reflux for 4 h before adding a solution of ethyl bromoacetate (0.05 mol) in acetone (25 mL) dropwise.

After 2 h of continuous reflux, the mixture was poured into a beaker containing about 250 mL of ice. The solid was filtered out and purified by recrystallization from ethanol/water.

4.1.1.1. Ethyl 2-(7-(4-nitrobenzyl)-3-methyl-4-oxoisoxazolo-[4,5-*d*]pyridazin-5(4*H*)-yl)acetate (2b). 60% yield; mp 128–129 °C. IR: 1733 (C=O, ester), 1689 (C=O, pyridazinone).^{1}H NMR (DMSO-d_6); δ 1.16 (3H, t, –CH_2–CH_3, *J*: 6.8 Hz), 2.52 (3H, s, –CH_3), 4.13 (2H, q, –CH_2–CH_3, *J*: 6.8 Hz), 4.41 (2H, s, –CH_2–C_6H_5), 4.93 (2H, s, –CH_2COO–), 7.57 (2H, d, phenyl-H_2 and H_6, *J*: 8.8 Hz), 8.17 (2H, d, phenyl-H_3 and H_5, *J*: 8.8 Hz). ESI-MS (*m*/*z*); 395 $[M+Na]^+$ (100%), 373 $[M+H]^+$. Anal. calcd for $C_{17}H_{16}N_4O_6$: C, 54.84; H, 4.33; N, 15.05. Found: C, 54.78; H, 4.28; N, 15.04.

4.1.1.2. Ethyl 2-(7-(4-methoxybenzyl)-3-methyl-4-oxoisox-azolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetate (2c). 93% yield; mp 102–104 °C. IR; 1742 (C=O, ester), 1689 (C=O, pyridazinone).^{1}H NMR (DMSO-d_6); δ 1.20 (3H, t, –CH_2–CH_3, *J*: 6.8 Hz), 2.51 (3H, s, –CH_3), 3.68 (3H, s, –OCH_3), 4.13–4.15 (4H, m, –CH_2–CH_3 and –CH_2–C_6H_5), 4.94 (2H, s, –CH_2COO–), 6.85 (2H, d, phenyl-H_3 and H_5, *J*: 8.4 Hz), 7.19(2H, d, phenyl-H_2 and H_6, *J*: 8.4 Hz). ESI-MS (*m*/*z*); 380 $[M+Na]^+$ (100%), 358 $[M+H]^+$. Anal. calcd for $C_{18}H_{19}N_3O_5$: C, 60.50; H, 5.36; N, 11.76. Found: C, 60.39; H, 5.28; N, 11.75.

4.1.2. General procedure for the preparation of 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetohydrazides (3b–c)

A solution of hydrazine hydrate (0.2 mol) and the corresponding ethyl 2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetate (**2a–c**, 0.01 mol) in absolute ethanol (50 mL) was refluxed for 1 h with vigorous stirring. The solvent was evaporated under vacuum and the residue was purified by recrystallization from ethanol.

4.1.2.1. 2-(7-(4-Nitrobenzyl)-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetohydrazide (3b). 87% yield; mp 206–208 °C. IR; 3321 (N–H), 1698 (C=O, pyridazinone), 1655 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6); δ 2.54 (3H, s, –CH_3), 4.29 (2H, br s, –NH_2), 4.41 (2H, s, –CH_2–C_6H_5), 4.73 (2H, s, –CH_2–CONH–), 7.61 (2H, d, phenyl-H_2 and H_6, *J*: 8.4 Hz), 8.20 (2H, d, phenyl-H_3 and H_5, *J*: 8.4 Hz), 9.25 (1H, br s, –NH–). ESI-MS (*m*/*z*); 381 $[M+Na]^+$ (100%), 359 $[M+H]^+$. Anal. calcd for $C_{15}H_{14}N_6O_5$: C, 50.28; H, 3.94; N, 23.45. Found: C, 50.42; H, 3.78; N, 23.25.

4.1.2.2. 2-(7-(4-Methoxybenzyl)-3-methyl-4-oxoisoxazolo-[4,5-*d*]pyridazin-5(4*H*)-yl)acetohydrazide (3c). 88% yield; mp 190–192 °C. IR; 3282 (N–H), 1694 (C=O, pyridazinone), 1656 (C=O, hydrazide). ^{1}H NMR (DMSO-d_6); δ 2.50 (3H, s, –CH_3), 3.68 (3H, s, –OCH_3), 4.11 (2H, s, –CH_2–C_6H_5), 4.26 (2H, br s, –NH_2), 4.71 (2H, s, –CH_2–CONH–), 6.85 (2H, d, phenyl-H_3 and H_5, *J*: 8.4 Hz), 7.20(2H, d, phenyl-H_2 and H_6, *J*: 8.4 Hz), 9.24 (1H, br s, –NH–). ESI-MS (*m*/*z*); 366 $[M+Na]^+$ (100%), 345 $[M+H]^+$. Anal. calcd for $C_{16}H_{17}N_5O_4$: C, 55.97; H, 4.99; N, 20.40. Found: C, 55.67; H, 5.02; N, 20.05.

4.1.3. General procedure for the preparation of 1-(2-(7-benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-substituted thiosemicarbazides (4–12)

An equimolar amount of the corresponding isothiocyanate was added to a solution of hydrazide (**3a–c**) in ethanol (50 mL); this reaction mixture was refluxed for 4 h and then allowed to reach room temperature. The precipitated solid was filtered out, washed with water, and purified by recrystallization from ethyl acetate.

4.1.3.1. 1-(2-(7-Benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-methylthiosemicarbazide (4). Obtained as a white solid (66% yield) by reacting **3a** with methyl isothiocyanate; mp 169–171 °C. IR; 3288, 3163 (N–H), 1693 (C=O, pyridazinone and hydrazide), 1205 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.53 (3H, s, –CH_3), 2.88 (3H, d, –NH–CH_3, *J*: 4.4 Hz), 4.22 (2H, s, –CH_2–C_6H_5), 4.91 (2H, s, –CH_2–CONH–), 7.23–7.34 (5H, m, –CH_2–C_6H_5), 7.94 (1H, q, –N*H*–CH_3, *J*: 4.4 Hz), 9.38 (1H, br s, –NH–N*H*–CS–), 10.15 (1H, br s, –CO–N*H*–NH–). ESI-MS (*m*/*z*); 409 $[M+Na]^+$ (100%), 387 $[M+H]^+$. Anal. calcd for $C_{17}H_{18}N_6O_3S$: C, 52.84; H, 4.70; N, 21.75; S, 8.30. Found: C, 52.44; H, 4.59; N, 21.41; S, 8.21.

4.1.3.2. 1-(2-(7-Benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-ethylthiosemicarbazide (5). Obtained as a white solid (87% yield) by reacting **3a** with ethyl isothiocyanate; mp 189–191 °C. IR; 3356, 3262 (N–H), 1684 (C=O, pyridazinone), 1670 (C=O, hydrazide), 1204 (C=S). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 1.11 (3H, t, –$CH_2$$CH_3$, *J*: 6.8 Hz), 2.54 (3H, s, –CH_3), 3.46–3.49 (2H, m, –$CH_2$$CH_3$), 4.24 (2H, s, –$CH_2$–$C_6H_5$), 4.91 (2H, s, –$CH_2$–CONH–), 7.23–7.37 (5H, m, –CH_2–C_6H_5), 7.87 (1H, t, –N*H*–CH_2CH_3, *J*: 5.2 Hz), 9.38 (1H, br, –NH–N*H*–CS–), 10.19 (1H, br, –CO–N*H*–NH–). ESI-MS (*m*/*z*); 423 $[M+Na]^+$ (100%), 401 $[M+H]^+$. Anal. calcd for $C_{18}H_{20}N_6O_3S$: C, 53.99; H, 5.03; N, 20.99; S, 8.01. Found: C, 53.76; H, 4.73; N, 20.74; S, 7.78.

4.1.3.3. 1-(2-(7-Benzyl-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-phenylthiosemicarbazide (6). Obtained as a white solid (70% yield) by reacting **3a** with phenyl isothiocyanate; mp 186–188 °C. IR; 3496, 3173 (N–H), 1714 (C=O, pyridazinone), 1659 (C=O, hydrazide), 1205 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.52 (3H, s, –CH_3), 4.24 (2H, s, –CH_2–C_6H_5), 4.97 (2H, s, –CH_2–CONH–), 7.17–7.52 (10H, m, –CH_2–C_6H_5 and –NH–C_6H_5), 9.53 (1H, br s, –N*H*–C_6H_5), 9.83 (1H, br s, –NH–N*H*–CS–), 10.47 (1H, br s, –CO–N*H* –NH–). ESI-MS (*m*/*z*); 471 $[M+Na]^+$ (100%), 449 $[M+H]^+$. Anal. calcd for $C_{22}H_{20}N_6O_3S$: C, 58.92; H, 4.49; N, 18.74; S, 7.15. Found: C, 58.41; H, 4.48; N, 18.84; S, 6.95.

4.1.3.4. 1-(2-(7-(4-Nitrobenzyl)-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-methylthiosemicarbazide (7). Obtained as a white solid (63% yield) by reacting **3b** with methyl isothiocyanate, mp 187–189 °C. IR; 3476, 3143 (N–H), 1680 (C=O, pyridazinone and hydrazide), 1209 (C=S). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.55 (3H, s, –CH_3), 2.89 (3H, d, –NH–CH_3, *J*: 4.4 Hz), 4.23 (2H, s, –CH_2–C_6H_5), 4.90 (2H, s, –CH_2–CONH–), 7.61(2H, d, phenyl-H_2 and H_6, *J*: 8.8 Hz), 7.94 (1H, q, –N*H*–CH_3, *J*: 4.4 Hz), 8.20 (2H, d, phenyl-H_3 and H_5, *J*: 8.8 Hz), 9.40 (1H, br s, –NH–N*H*-CS–), 10.15 (1H, br s, –CO–N*H*–NH–). ESI-MS (*m*/*z*); 454 $[M+Na]^+$ (100%), 432 $[M+H]^+$. Anal. calcd for $C_{17}H_{17}N_7O_5S$: C, 47.33; H, 3.97; N, 22.73; S, 7.43. Found: C, 47.40; H, 4.12; N, 22.51; S, 6.96.

4.1.3.5. 1-(2-(7-(4-Nitrobenzyl)-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-ethylthiosemicarbazide (8). Obtained as a white solid (89% yield) by reacting **3b** with ethyl isothiocyanate; mp 203–205 °C. IR; 3256 (N–H), 1681 (C=O, pyridazinone and hydrazide), 1208 (C=S). ^{1}H NMR1H NMR (DMSO-d_6); δ 1.08 (3H, t, –$CH_2$$CH_3$, *J*: 6.8 Hz), 2.53 (3H, s, –CH_3), 3.43–3.46 (2H, m, –$CH_2$$CH_3$), 4.41 (2H, s, –$CH_2$–$C_6H_5$), 4.86 (2H, s, –$CH_2$–CONH–), 7.59 (2H, d, phenyl-H_2 and H_6, *J*: 8.8 Hz), 7.82 (1H, t, –N*H*–CH_2CH_3, *J*: 5.2 Hz), 8.18 (2H, d, phenyl-H_3 and H_5, *J*: 8.8 Hz), 9.33 (1H, br s, –NH–N*H*–CS–), 10.14 (1H, br s, –CO–N*H*–NH–). ESI-MS (*m*/*z*); 468 $[M+Na]^+$ (100%), 446 $[M+H]^+$. Anal. calcd for $C_{18}H_{19}N_7O_5S$: C, 48.53; H, 4.30; N, 22.01; S, 7.20. Found: C, 48.45; H, 4.46; N, 21.91; S, 6.94.

4.1.3.6. 1-(2-(7-(4-Nitrobenzyl)-3-methyl-4-oxoisoxazolo[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-phenylthiosemicarbazide (9). Obtained

as a white solid (90% yield) by reacting **3b** with phenyl isothiocyanate; mp 195–197 °C. IR; 3324 (N–H), 1668 (C=O, pyridazinone and hydrazide), 1208 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.51 (3H, s, –CH_3), 4.41 (2H, s, –CH_2–C_6H_5), 4.93 (2H, s, –CH_2–CONH–), 7.14–8.18 (9H, m, –CH_2–C_6H_4 and –NH–C_6H_5), 9.47 (1H, br, –N*H*–C_6H_5), 9.75 (1H, br s, –NH–N*H*–CS–), 10.40 (1H, br, –CO–N*H*–NH–). ESI-MS (*m*/*z*); 516 [M+Na]$^+$ (100%), 494 [M+H]$^+$, 327, 299, 102. Anal. calcd for $C_{22}H_{19}N_7O_5S$: C, 53.54; H, 3.88; N, 19.87; S, 6.50. Found: C, 53.39; H, 3.65; N, 19.66; S, 6.33.

4.1.3.7. 1-(2-(7-(4-Methoxybenzyl)-3-methyl-4-oxoisoxazolo-[4, 5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-methylthiosemicarbazide (10). Obtained as a white solid (83% yield) by reacting **3c** with methyl isothiocyanate, mp 186–188 °C. IR; 3305, 3195 (N–H), 1685 (C=O, pyridazinone and hydrazide), 1209 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.51 (3H, s, –CH_3), 2.88 (3H, d, –NH–CH_3, *J*: 4.4 Hz), 3.68 (3H, s, –OCH_3), 4.13 (2H, s, –CH_2–C_6H_5), 4.88 (2H, s, –CH_2–CONH–), 6.86 (2H, d, phenyl-H_3 and H_5, *J*: 8.4 Hz), 7.20 (2H, d, phenyl-H_2 and H_6, *J*: 8.4 Hz), 7.94 (1H, q, –N*H*–CH_3, *J*: 4.4 Hz), 9.39 (1H, br s, –NH–N*H*–CS–), 10.15 (1H, br s, –CO–N*H* –NH–). ESI-MS (*m*/*z*); 439 [M+Na]$^+$ (100%), 417 [M+H]$^+$. Anal. calcd for $C_{18}H_{20}N_6O_4S$: C, 51.91; H, 4.84; N, 20.18; S, 7.70. Found: C, 51.69; H, 4.98; N, 19.97; S, 7.69.

4.1.3.8. 1-(2-(7-(4-Methoxybenzyl)-3-methyl-4-oxoisoxazolo-[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-ethylthiosemicarbazide (11). Obtained as a white solid (83% yield) by reacting **3c** with ethyl isothiocyanate; mp 193–194 °C. IR; 3364, 3161 (N–H), 1675 (C=O, pyridazinone and hydrazide), 1202 (C=S). ^{1}H NMR (DMSO-d_6); δ 1.08 (3H, t, –CH_2CH_3, *J*: 6.8 Hz), 2.51 (3H, s, –CH_3), 3.43–3.45 (2H, m, –CH_2CH_3), 3.69 (3H, s, –OCH_3), 4.14 (2H, s, –CH_2–C_6H_5), 4.87 (2H, s, –CH_2–CONH–), 6.86 (2H, d, phenyl-H_3 and H_5, *J*: 8.8 Hz), 7.20 (2H, d, phenyl-H_2 and H_6, *J*: 8.8 Hz), 7.82 (1H, t, –N*H*–CH_2CH_3, *J*: 5.6 Hz), 9.33 (1H, br, –NH–N*H*–CS–), 10.15 (1H, br, –CO–N*H*–NH–). ESI-MS (*m*/*z*); 453 [M+Na]$^+$ (100%), 431 [M+H]$^+$. Anal. calcd for $C_{19}H_{22}N_6O_4S$: C, 53.01; H, 5.15; N, 19.52; S, 7.45. Found: C, 53.36; H, 4.91; N, 19.84; S, 7.18.

4.1.3.9. 1-(2-(7-(4-Methoxybenzyl)-3-methyl-4-oxoisoxazolo-[4,5-*d*]pyridazin-5(4*H*)-yl)acetyl)-4-phenylthiosemicarbazide (12). Obtained as a white solid (83% yield) by reacting **3c** with phenyl isothiocyanate; mp 184–185 °C. IR; 3335, 3217 (N–H), 1673 (C=O, pyridazinone and hydrazide), 1204 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.51 (3H, s, –CH_3), 3.70 (3H, s, –OCH_3), 4.16 (2H, s, –CH_2–C_6H_5), 4.95 (2H, s, –CH_2–CONH–), 6.85–7.51 (9H, m, –CH_2–C_6H_4 and –NH–C_6H_5), 9.51 (1H, br s, –N*H*–C_6H_5), 9.80 (1H, br s, –NH–N*H*–CS–), 10.45 (1H, br, –CO–N*H*–NH–). ESI-MS (*m*/*z*); 501 [M+Na]$^+$ (100%), 479 [M+H]$^+$. Anal. calcd for $C_{23}H_{22}N_6O_4S$: C, 57.73; H, 4.63; N, 17.56; S, 6.70. Found: C, 57.69; H, 4.72; N, 17.41; S, 6.47.

4.1.4. General procedure for the preparation of 7-benzyl-3-methyl-5-((5-(substituted amino)-1,3,4-thiadiazol-2-yl)-methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (13–21)

Concentrated sulfuric acid (1 mL) was added dropwise to the corresponding thiosemicarbazide (**4**–**12**, 1 mmol), stirring at room temperature for 1 hour. The reaction mixture was poured into a beaker containing ice (50 mL), and the precipitated solid was filtered out, washed with water, and purified by recrystallization.

4.1.4.1. 7-Benzyl-3-methyl-5-((5-(methylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (13). Obtained as a white solid (63% yield, from compound **4**); crystallized from acetone/water; mp 152–154 °C. IR; 3230 (N–H), 1686 (C=O). ^{1}H NMR (DMSO-d_6); δ 2.54 (3H, s, –CH_3), 2.84 (3H, d, –NH–CH_3, *J*: 4.4 Hz), 4.22 (2H, s, –CH_2–C_6H_5), 5.50 (2H, s, –N–CH_2–), 7.24–7.33 (5H, m, –CH_2–C_6H_5), 7.69 (1H, q, –N*H*–CH_3, *J*: 4.4 Hz). ESI-MS (*m*/*z*); 391 [M+Na]$^+$ (100%), 369 [M+H]$^+$. Anal. calcd for $C_{17}H_{16}N_6O_2S$: C, 55.42; H, 4.38; N, 22.81; S, 8.70. Found: C, 55.67; H, 4.37; N, 22.28; S, 8.68.

4.1.4.2. 7-Benzyl-3-methyl-5-((5-(ethylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (14). Obtained as a white solid (72% yield from **5**); crystallized from acetone/water; mp 180–182 °C.IR; 3384 (N–H), 1683 (C=O). ^{1}H NMR (DMSO-d_6); δ 1.15 (3H, t, –CH_2CH_3, *J*: 6.8 Hz), 2.54 (3H, s, –CH_3), 3.23–3.27 (2H, m, –CH_2CH_3), 4.24 (2H, s, –CH_2–C_6H_5), 5.49 (2H, s, –N–CH_2–), 7.24–7.33 (5H, m, –CH_2–C_6H_5), 7.75 (1H, t, –N*H*–CH_2CH_3, *J*: 5.6 Hz). ESI-MS (*m*/*z*); 405 [M+Na]$^+$ (100%), 383 [M+H]$^+$, 325, 233, 102. Anal. calcd for $C_{18}H_{18}N_6O_2S$: C, 56.53; H, 4.74; N, 21.97; S, 8.38. Found: C, 56.65; H, 4.64; N, 21.59; S, 8.32.

4.1.4.3. 7-Benzyl-3-methyl-5-((5-(phenylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (15). Obtained as a white solid (83% yield from **6**); crystallized from acetone; mp 207–210 °C (dec). IR; 3255 (N–H), 1686 (C=O). ^{1}H NMR (DMSO-d_6); δ 2.55 (3H, s, –CH_3), 4.25 (2H, s, –CH_2–C_6H_5), 5.60 (2H, s, –N–CH_2–), 6.99–7.59 (10H, m, –CH_2–C_6H_5 and –NH–C_6H_5), 10.40 (1H, s, N*H*–C_6H_5). ESI-MS (*m*/*z*); 453 [M+Na]$^+$(100%), 431 [M+H]$^+$. Anal. calcd for $C_{22}H_{18}N_6O_2S$: C, 61.38; H, 4.21; N, 19.52; S, 7.45. Found: C, 61.29; H, 4.03; N, 19.41; S, 7.55.

4.1.4.4. 7-(4-Nitrobenzyl)-3-methyl-5-((5-(methylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (16). Obtained as a white solid (75% yield from **7**); crystallized from ethanol; mp186–188 °C. IR; 3216 (N–H), 1682 (C=O). ^{1}H NMR (DMSO-d_6); δ 2.53 (3H, s, –CH_3), 2.81 (3H, d, –NH–CH_3, *J*: 4.8 Hz), 4.40 (2H, s, –CH_2–C_6H_5), 5.44 (2H, s, –N–CH_2–), 7.58(2H, d, phenyl-H_2 and H_6, *J*: 8.8 Hz), 7.65 (1H, q, –N*H*–CH, *J*: 4.8 Hz), 8.16 (2H, d, phenyl-H_3 and H_5, *J*: 8.8 Hz). ESI-MS (*m*/*z*); 436 [M+Na]$^+$ (100%), 414 [M+H]$^+$, 325, 233, 149, 102. Anal. Calcd for $C_{17}H_{15}N_7O_4S$: C, 49.39; H, 3.66; N, 23.72; S, 7.76. Found: C, 49.18; H, 3.69; N, 23.19; S, 7.71.

4.1.4.5. 7-(4-Nitrobenzyl)-3-methyl-5-((5-(ethylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (17). Obtained as a white solid from (65% yield from **8**); crystallized from ethanol; mp 183–185 °C. IR; 3187 (N–H), 1686 (C=O). ^{1}H NMR (DMSO-d_6); δ 1.12 (3H, t, –CH_2CH_3), 2.53 (3H, s, –CH_3), 3.21 (2H, q, –CH_2CH_3, *J*: 6.8 Hz), 4.40 (2H, s, –CH_2–C_6H_5), 5.44 (2H, s, –N–CH_2–), 7.58 (2H, d, phenyl-H_2 and H_6, *J*: 8.8 Hz), 7.71 (1H, t, –N*H*–CH_2CH_3, *J*: 5.6 Hz), 8.16 (2H, d, phenyl-H_3 and H_5, *J*: 8.8 Hz). ESI-MS (*m*/*z*); 450 [M+Na]$^+$ (100%), 428 [M+H]$^+$. Anal. Calcd for $C_{18}H_{17}N_7O_4S$: C, 50.58; H, 4.01; N, 22.94; S, 7.50. Found: C, 50.30; H, 4.05; N, 22.63; S, 7.60.

4.1.4.6. 7-(4-Nitrobenzyl)-3-methyl-5-((5-(phenylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (18). Obtained as a white solid (90% yield from **9**); crystallized from ethyl acetate/acetone; mp 234–236 °C dec. IR; 3198 (N–H), 1698 (C=O). ^{1}H NMR (DMSO-d_6); $\delta\delta$ 2.53 (3H, s, –CH_3), 4.41 (2H, s, –CH_2–C_6H_5), 5.55 (2H, s, –N–CH_2–), 6.96–8.16 (9H, m, –CH_2–C_6H_4 and –NH–C_6H_5), 10.33 (1H, s, N*H*–C_6H_5). ESI-MS (*m*/*z*); 498 [M+Na]$^+$(100%), 476 [M+H]$^+$. Anal. Calcd for $C_{22}H_{17}N_7O_4S$: C, 55.57; H, 3.60; N, 20.62; S, 6.74. Found: C, 55.66; H, 3.78; N, 20.48; S, 6.81.

4.1.4.7. 7-(4-Methoxybenzyl)-3-methyl-5-((5-(methylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (19). Obtained as a white solid (40% yield from **10**); crystallized from acetone/water; mp 186–188 °C. IR; 3220 (N–H), 1688 (C=O). ^{1}H NMR (DMSO-d_6, 400); δ 2.51 (3H, s, –CH_3), 2.81 (3H, d, –NH–CH_3, *J*: 4.8 Hz), 3.68 (3H, s, –OCH_3), 4.13 (2H, s, –CH_2–C_6H_5), 5.45 (2H, s, –N–CH_2–), 6.85 (2H, d, phenyl-H_3 and H_5, *J*: 8.8 Hz), 7.20 (2H, d, phenyl-H_2 and H_6, *J*: 8.8 Hz), 7.65 (1H, q, –N*H*–CH_3, *J*: 4.8 Hz). ESI-MS (*m*/*z*); 421 [M+Na]$^+$ (100%), 399 [M+H]$^+$. Anal. Calcd for $C_{18}H_{18}N_6O_3S$: C, 54.26; H, 4.55; N, 21.09; S, 8.05. Found: C, 54.13; H, 4.59; N, 20.82; S, 8.18.

4.1.4.8. 7-(4-Methoxybenzyl)-3-methyl-5-((5-(ethylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (20). Obtained as a white solid (35% yield from acetone/water); mp 183–185 °C. IR; 3206 (N–H),

1687 (C=O). ^{1}H NMR (DMSO-d_6); δ 1.12 (3H, t, –CH_2CH_3, J: 6.8 Hz), 2.51 (3H, s, –CH_3), 3.22 (2H, m, –CH_2CH_3), 3.68 (3H, s, –OCH_3), 4.13 (2H, s, –CH_2–C_6H_5), 5.45 (2H, s, –N–CH_2–), 6.85 (2H, d, phenyl-H_3 and H_5, J: 8.8 Hz), 7.20(2H, d, phenyl-H_2 and H_6, J: 8.8 Hz), 7.71 (1H, t, –NH–CH_2CH_3, J: 5.2 Hz). ESI-MS (m/z); 435 $[M+Na]^+$(100%), 413 $[M+H]^+$. Anal. Calcd for $C_{19}H_{20}N_6O_3S$: C, 55.33; H, 4.89; N, 20.38; S, 7.77. Found: C, 54.85; H, 4.86; N, 19.82; S, 7.64.

4.1.4.9. 7-(4-Methoxybenzyl)-3-methyl-5-((5-(phenylamino)-1,3,4-thiadiazol-2-yl)methyl)isoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (21). Obtained as a white solid (90% yield from **12**); crystallized from acetone/water; mp 172–174 °C (dec). IR; 3203 (N–H), 1692 (C=O). ^{1}H NMR (DMSO-d_6, 400 MHz); δ 2.52 (3H, s, –CH_3), 3.67 (3H, s, –OCH_3), 4.14 (2H, s, –CH_2–C_6H_5), 5.56 (2H, s, –N–CH_2–), 6.83–7.56 (9H, m, –CH_2–C_6H_4 and –NH–C_6H_5), 10.34 (1H, s, NH–C_6H_5). ESI-MS (m/z); 483 $[M+Na]^+$ (100%), 461 $[M+H]^+$. Anal. Calcd for $C_{23}H_{20}N_6O_3S$: C, 59.99; H, 4.38; N, 18.25; S, 6.96. Found: C, 59.74; H, 4.34; N, 18.02; S, 7.02.

4.1.5. General procedure for the preparation of 7-benzyl-5-((4-substituted-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-ones (22–30)

12.5 mL of 1 M aqueous sodium bicarbonate solution was added to a solution of appropriately substituted thiosemicarbazide (**4**–**12**, 0.001 mol) in ethanol (7.5 mL); this mixture was stirred at room temperature for about 1 h and then poured into a beaker containing ice/water (100 mL). The solution was adjusted to pH 5–6 with 1 M HCl. The precipitated solid was filtered out, rinsed with water and purified by recrystallization from an appropriate solvent.

4.1.5.1. 7-Benzyl-5-((4-methyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (22). Obtained as a white solid (77% yield from **4**); crystallized from ethanol; mp 161–163 °C. IR; 3343 (N–H), 1680 (C=O), 1332 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.54 (3H, s, –CH_3), 3.43 (3H, s, –N–CH_3), 4.21 (2H, s, –CH_2–C_6H_5), 5.45 (2H, s, –N–CH_2–), 7.24–7.32 (5H, m, –CH_2–C_6H_5), 13.71 (1H, br s, –NH). ESI-MS (m/z); 391 $[M+Na]^+$ (100%), 369 $[M+H]^+$. Anal. Calcd for $C_{17}H_{16}N_6O_2S$: C, 55.42; H, 4.38; N, 22.81; S, 8.70. Found: C, 55.48; H, 4.24; N, 22.65; S, 8.77.

4.1.5.2. 7-Benzyl-5-((4-ethyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (23). Obtained as a white solid (85% yield from **5**); crystallized from ethanol; mp 172–174 °C. IR; 3365 (N–H), 1688 (C=O), 1350 (C=S). ^{1}H NMR (DMSO-d_6); δ 1.12 (3H, t, –CH_2CH_3, J: 7.2 Hz), 2.54 (3H, s, –CH_3), 4.01 (2H, q, –CH_2CH_3, J: 7.2 Hz), 4.22 (2H, s, –CH_2–C_6H_5), 5.48 (2H, s, –N–CH_2–), 7.27–7.32 (5H, m, –CH_2–C_6H_5), 13.74 (1H, br s, –NH). ESI-MS (m/z); 405 $[M+Na]^+$ (100%), 383 $[M+H]^+$. Anal. Calcd for $C_{18}H_{18}N_6O_2S$: C, 56.53; H, 4.74; N, 21.97; S, 8.38. Found: C, 56.12; H, 4.96; N, 21.01; S, 8.07.

4.1.5.3. 7-Benzyl-5-((4-phenyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (24). Obtained as a white solid (95% yield from **6**); crystallized from ethanol; mp 240–242 °C (dec). IR; 3361 (N–H), 1691 (C=O), 1307 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.42 (3H, s, –CH_3), 4.10 (2H, s, –CH_2–C_6H_5), 5.29 (2H, s, –N–CH_2–), 7.17–7.36 (10H, m, –CH_2–C_6H_5 and –NH–C_6H_5), 13.93 (1H, br s, –NH). ESI-MS (m/z); 453 $[M+Na]^+$ (100%), 431 $[M+H]^+$. Anal. Calcd for $C_{22}H_{18}N_6O_2S$: C, 61.38; H, 4.21; N, 19.52; S, 7.45. Found: C, 61.55; H, 3.92; N, 19.43; S, 7.23.

4.1.5.4. 7-(4-Nitrobenzyl)-5-((4-methyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (25). Obtained as a white solid (75% yield from **7**); crystallized from ethanol/acetone; mp 209–211 °C (dec). IR; 3360 (N–H), 1685 (C=O), 1330 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.56 (3H, s, –CH_3), 3.38 (3H, s, –N–CH_3), 4.40 (2H, s, –CH_2–C_6H_5), 5.42 (2H, s, –N–CH_2–), 7.55(2H, d, phenyl-H_2 and H_6, J: 8.8 Hz), 8.19 (2H, d, phenyl-H_3 and H_5, J: 8.8 Hz), 13.71 (1H, br s, –NH). ESI-MS (m/z); 436 $[M+Na]^+$ (100%), 414 $[M+H]^+$. Anal. Calcd for $C_{17}H_{15}N_7O_4S$: C, 49.39; H, 3.66; N, 23.72; S, 7.76. Found: C, 49.50; H, 4.03; N, 23.33; S, 7.43.

4.1.5.5. 7-(4-Nitrobenzyl)-5-((4-ethyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (26). Obtained as a white solid (89% yield from **8**); crystallized from ethanol/acetone; mp 180–182 °C (dec). IR; 3359 (N–H), 1686 (C=O), 1320 (C=S). ^{1}H NMR (DMSO-d_6); δ 1.11 (3H, t, –CH_2CH_3, J: 7.2 Hz), 2.56 (3H, s, –CH_3), 3.97 (2H, q, –CH_2CH_3, J: 7.2 Hz), 4.42 (2H, s, –CH_2–C_6H_5), 5.45 (2H, s, –N–CH_2–), 7.56(2H, d, phenyl-H_2 and H_6, J: 8.8 Hz), 8.20 (2H, d, phenyl-H_3 and H_5, J: 8.8 Hz), 13.74 (1H, br s, –NH). ESI-MS (m/z); 450 $[M+Na]^+$ (100%), 428 $[M+H]^+$. Anal. Calcd for $C_{18}H_{17}N_7O_4S$: C, 50.58; H, 4.01; N, 22.94; S, 7.50. Found: C, 50.74; H, 3.71; N, 22.06; S, 7.18.

4.1.5.6. 7-(4-Nitrobenzyl)-5-((4-phenyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (27). Obtained as a white solid (84% yield from **9**); crystallized from ethanol/acetone; mp 202–204 °C (dec). IR; 3357 (N–H), 1698 (C=O), 1333 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.45 (3H, s, –CH_3), 4.33 (2H, s, –CH_2–C_6H_5), 5.28 (2H, s, –N–CH_2–), 7.20–8.21 (9H, m, –CH_2–C_6H_4 and –NH–C_6H_5), 13.95 (1H, br s, –NH). ESI-MS (m/z); 498 $[M+Na]^+$ (100%), 476 $[M+H]^+$. Anal. Calcd for $C_{22}H_{17}N_7O_4S$: C, 55.57; H, 3.60; N, 20.62; S, 6.74. Found: C, 55.54; H, 3.75; N, 20.85; S, 6.68.

4.1.5.7. 7-(4-Methoxybenzyl)-5-((4-methyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (28). Obtained as a white solid (91% yield from **10**); crystallized from ethanol; mp 195–197 °C. IR; 3359 (N–H), 1684 (C=O), 1320 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.54 (3H, s, –CH_3), 3.44 (3H, s, –N–CH_3), 3.72 (3H, s, –OCH_3), 4.13 (2H, s, –CH_2–C_6H_5), 5.45 (2H, s, –N–CH_2–), 6.88(2H, d, phenyl-H_3 and H_5, J: 8.8 Hz), 7.18 (2H, d, phenyl-H_2 and H_6, J: 8.8 Hz), 13.71 (1H, br, –NH). ESI-MS (m/z); 421 $[M+Na]^+$(100%), 399 $[M+H]^+$. Anal. Calcd for $C_{18}H_{18}N_6O_3S$: C, 54.26; H, 4.55; N, 21.09; S, 8.05. Found: C, 53.89; H, 4.33; N, 20.74; S, 7.93.

4.1.5.8. 7-(4-Methoxybenzyl)-5-((4-ethyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (29). Obtained as a white solid (82% yield from **11**); crystallized from ethanol; mp 190–192 °C. IR; 3365 (N–H), 1684 (C=O), 1326 (C=S). ^{1}H NMR (DMSO-d_6); δ 1.12 (3H, t, –CH_2CH_3, J: 7.2 Hz), 2.54 (3H, s, –CH_3), 3.72 (3H, s, –OCH_3), 4.01 (2H, q, –CH_2CH_3, J: 7.2 Hz), 4.14 (2H, s, –CH_2–C_6H_5), 5.47 (2H, s, –N–CH_2–), 6.87 (2H, d, phenyl-H_3 and H_5, J: 8.8 Hz), 7.19(2H, d, phenyl-H_2 and H_6, J: 8.8 Hz), 13.72 (1H, br s, –NH). ESI-MS (m/z); 435 $[M+Na]^+$ (100%), 413 $[M+H]^+$. Anal. Calcd for $C_{19}H_{20}N_6O_3S$: C, 55.33; H, 4.89; N, 20.38; S, 7.77. Found: C, 55.16; H, 4.75; N, 20.15; S, 7.72.

4.1.5.9. 7-(4-Methoxybenzyl)-5-((4-phenyl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)methyl)-3-methylisoxazolo[4,5-*d*]pyridazin-4(5*H*)-one (30). Obtained as a white solid (86% yield from **12**); crystallized from ethanol); mp 174–176 °C. IR; 3360 (N–H), 1689 (C=O), 1304 (C=S). ^{1}H NMR (DMSO-d_6); δ 2.43 (3H, s, –CH_3), 3.72 (3H, s, –OCH_3), 4.04 (2H, s, –CH_2–C_6H_5), 5.30 (2H, s, –N–CH_2–), 6.87–7.35 (9H, m, –CH_2–C_6H_4 and –NH–C_6H_5), 10.94 (1H, br s, –NH). ESI-MS (m/z); 483 $[M+Na]^+$ (100%), 461 $[M+H]^+$. Anal. Calcd for $C_{23}H_{20}N_6O_3S$: C, 59.99; H, 4.38; N, 18.25; S, 6.96. Found: C, 59.66; H, 4.44; N, 18.07; S, 6.78.

4.2. Biological assays

4.2.1. Cyclooxygenase inhibition assay (in vitro)

The synthesized compounds were evaluated for their ability to inhibit human recombinant COX-2 and ovine COX-1 using a fluorescent inhibitor screening assay in vitro (catalog number 700100, Cayman Chemical, Ann Arbor, MI, USA) following the procedure suggested by the manufacturer (excitation wavelength = 530–540 nm, emission wavelength of 585–595 nm). DuP-697 (selective COX-2 inhibitor) and SC-560 (selective COX-1 inhibitor) were used as reference compounds.

4.2.2. 5-lipoxygenase inhibition assay

The ability of the test compounds (listed in Table 2) to inhibit potato 5-LOX (catalog number 60401, Cayman Chemical, Ann Arbor, MI, USA) (IC_{50} values, μM) was determined using an enzyme immuno assay kit (catalog number 760700, Cayman Chemical) according to the experimental procedure described previously.[48]

4.2.3. Anti-inflammatory assay

The test compounds, as well as the reference drug ibuprofen, were evaluated using the in vivo carrageenan-induced rat foot paw edema model reported previously.[39] All compounds were administered orally (30 mg/kg) suspended in methylcellulose (1% aqueous solution) as vehicle. This assay was carried out using protocols approved by the Health Sciences Animal Welfare Committee at the University of Alberta.

4.2.4. Ulcer index assay

Ulcerogenic activity was evaluated after oral administration of test compounds (0.14 mmol/kg) or ibuprofen (0.14 mmol/kg). All drugs were suspended and administered in 1.0 mL of a 1% methylcellulose solution. Control rats received oral administration of vehicle (1.0 mL of 1% methylcellulose solution). Food, but not water, was removed 24 h before administration of test compounds. Six hours after oral administration of the drug, rats were euthanized in a CO_2 chamber and their stomachs were removed, cut out along the greater curvature of the stomach, gently rinsed with water and placed on ice. The number and the length of ulcers observed in each stomach were determined using magnifier lenses. The severity of each gastric lesion was measured along its greatest length (1 mm = rating of 1, 1–2 mm = rating of 2, >2 mm = rating according to their length in mm). The 'ulcer index' (UI) for each test compound was calculated by adding the total length (L, in mm) of individual ulcers in each stomach and averaging over the number of animals in each group ($n = 4$): $UI = (L_1+L_2+L_3+L_4)/4$.

4.3. Molecular modeling studies

Docking experiments were performed using Discovery Studio Client v2.5.0.9164 (2005–09), Accelrys Software Inc. running on a HP xw4600 workstation (Processor x86 family 6 model 23 stepping 10 GenuineIntel 2999 ~Mhz). The coordinates for the X-ray crystal structure of the enzyme COX-2[49], COX-1[50] and 5-LOX[51] were obtained from the RCSB Protein Data Bank and hydrogens were added. The ligand molecules were constructed using the Build Fragment tool and energy-minimized for 1000 iterations reaching a convergence of 0.01 kcal/mol Å. The docking experiment on COX-2 was carried out by using the energy minimized ligand in the PDB file 1cx2 after deleting the ligand SC-558. The coordinates for COX-1 was obtained from PDB file 1prh and the energy minimized ligand was used for docking experiments. The coordinates for 5-LOX were obtained from PDB file 1lox, and the energy minimized ligand was superimposed on the inhibitor RS75091 after which RS75091 was deleted. In all these experiments the resulting ligand–enzyme complex was subjected to docking using the Libdock command in the receptor–ligand Interactions protocol of Discovery Studio after defining subsets of the enzyme within 10 Å sphere radius of the ligand. The force field, Chemistry at HARvard Macromolecular Mechanics (CHARMM) was employed for all docking purposes. The ligand–enzyme assembly was then subjected to a molecular dynamics (MD) simulation using Simulation protocol at a constant temperature of 300 K with a 100 step equilibration for over 1000 iterations and a time step of 1 fs using a distance dependent dielectric constant 4r. The optimal binding orientation of the ligand–enzyme assembly obtained after docking was further minimized for 1000 iterations using the conjugate gradient method until a convergence of 0.001 kcal/mol Å was reached after which $E_{intermolecular}$ (kcal/mol) of the ligand–enzyme assembly was evaluated.

4.4. Statistical analysis

Data for the ulcer index assay are presented as the mean ± SEM, with sample sizes of 4 rats/group. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Holm–Sidak method).

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