Potential effect of novel thiadiazole derivatives against radiation induced inflammation with low cardiovascular risk in rats

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Abstract
The aim of the present study is to explore new selective anti-inflammatory compounds with low cardiovascular risk. Twelve thiadiazole derivatives incorporating different amino acid moieties were newly synthesized (4–15) as potential anti-inflammatory agents with low cardiovascular risks through dual COX-2/MPO inhibition. Compounds were initially screened for their anti-inflammatory effect by assay of COX-2, the most potent (4–6, 8) were further tested for COX-1 inhibition, myeloperoxidase MPO activity as well as total nitric oxide content NO in heart of irradiated rats. Cardiac toxicity potential was evaluated by assay of creatine kinase-MB (CK-MB), troponin-I (Tn-I) and lactate dehydrogenase (LDH). Celcoxib was used as reference drug. S-(5-((4-Methoxybenzylidene)amino)-2,3-dihydro-1,3,4-thiadiazol-2-yl)2-amino propanethioate (5) was the most potent anti-inflammatory with the least cardiotoxicity effect. It exhibited IC\textsubscript{50} 0.09 µM on COX-2 inhibition with very low activity on COX-1. Troponin I was elevated by 11% using compound 5 in non-irradiated rats. Moreover, compound (5) showed 73% reduction in MPO level. Results were supported by molecular docking into the active sites of COX-2 and MPO enzymes to have more insights about the possible dual inhibition of compound 5 of both enzymes.

Graphical abstract

Keywords Thiadiazole · Amino acids · COX-2 · MPO · Irradiated rats

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Introduction

Cardiovascular disorders are the major causes of human morbidity and mortality worldwide [1]. Accumulating evidence that the inflammatory process and heart failure are inextricably linked [2]. Several studies have shown that cyclooxygenase-2 (COX-2) is easily detectable in cardiac myocytes of human suffering from heart failure [3]. Coxibs are a class of medicines that selectively inhibit COX-2 and have been developed as a safe alternative to other COX-2 inhibitors, which might cause major gastrointestinal problems as that observed with traditional NSAIDs [4]. Unfortunately, rofecoxib (Vioxx1, Merck & Co., USA), a COX-2 inhibitor, was taken off the market in 2004 because of the concerns about an increased risk for heart attack and stroke [5, 6]. The FDA, on the other hand, has decided to keep celecoxib (CEL) on the market since the benefit outweighs the potential cardiovascular risk [6]. Nevertheless, as observed in the Adenoma Prevention with Celecoxib trial, CEL may induce a considerable increase in major cardiovascular risks such as stroke, myocardial infarction or congestive heart failure, leading to the study’s early termination [6]. These serious complications may also occur in patients receiving radiotherapy for cancer treatment in the thoracic and mediastinal regions [7], reducing the therapeutic efficacy of radiotherapy and thereby, limiting the use of COX-2 inhibitors in such circumstances. Furthermore, exposure to radiation has been shown recently to be implicated in the early stages of atherosclerosis by causing vascular dysfunction, inflammation and fibrosis in the aorta [8], all of which may require the use of anti-inflammatory drugs as COX-2 inhibitors. Therefore, there is always a great effort done for developing new selective COX-2 inhibitors with a better cardiovascular profile.

Several serum cardiac markers, such as troponin I (TnI), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH), have been studied as a potential tool for assessing patients receiving adjuvant therapy who might be at increased risk of drug-induced cardiac dysfunction or cardiotoxicity [9]. Furthermore, new research suggests that myeloperoxidase (MPO), a heme-containing peroxidase enzyme released by activated neutrophils, monocytes, and some tissue macrophages (such as those found in atherosclerotic plaques), may play a role in the progression of coronary artery disease (CAD). MPO plays a vital role in the innate immune system via producing free radicals and reactive oxygen species in the first line of defense against pathogens through a pair of chlorination-peroxidation processes. MPO produces highly toxic hypochlorous acid (HOCl) by using chloride in the presence of hydrogen peroxide, and the enzyme is transformed to an inactive peroxide form, which then undergoes two sequential reductions back to the original form of the enzyme, releasing highly reactive free radicals. Together the produced highly toxic HOCl and the reactive species destroy pathogens [10]. However, overproduction is linked to a variety of inflammatory diseases as well as oxidative damage to host tissues at inflammatory locations, such as atherosclerotic plaques [11]. MPO has been linked to atherosclerosis by pathways involving its role in inflammation [11] and nitric oxide consumption, which results in endothelial dysfunction [12].

Based on the link between MPO and the development of CAD, it was hypothesized that developing novel COX-2 inhibitors with better MPO control would be a promising approach to improve anti-inflammatory efficacy of COX-2 inhibitors as well as having higher safety margins against cardiovascular risks.

Rationale and design

Several studies had focused on designing MPO inhibitors and analyzing their binding modes in the catalytic site of MPO and this was facilitated by the availability of crystal structures of potent inhibitors. There are three main categories of MPO inhibitors: (1) Those that reversibly divert MPO from its chlorination cycle causing accumulation of the inactive peroxide form of the enzyme (e.g., indomethacin, tryptamine, 5-aminosalicylic acid, paracetamol and isoniazid). (2) Suicide or irreversible inhibitors which are oxidized by MPO leading to complete inactivation by destruction or covalent binding to heme (e.g., 4-aminobenzoic acid hydrazide (4-ABAH) and 2-thioxanthines). Inhibitors of both categories are prone to be oxidized by MPO and this is the main cause of their observed side effects, the generated reactive species cause toxic and damaging effect, besides, many have low bioavailability and are associated with gastrointestinal disorders [13]. (3) An alternate mechanism of inhibition is reversible inhibitors that compete with MPO substrates by binding into heme catalytic site (e.g., salicylhydroxamic acid (SHA)). This is an encouraging mode of inhibition that limits the oxidizing abilities of the enzyme without undesirable side effects [14].

Until now, only Verdiperstat (AZD3241) (I) is the only approved small molecule as MPO inhibitor. Verdiperstat is a selective, irreversible and orally active MPO inhibitor, with an IC_{50} of 630 nM for the neurodegenerative brain disorders [15].

Many efforts and SAR studies were performed in order to optimize MPO inhibition. Rivera-Antonio et al. reported new hydroxy-amoino-cinnammic acid derivatives (II) as potent antioxidants and MPO inhibitors, two structural features enable this activity; phenolic group and propenoic acid side chain leading to electron delocalization upon reaction with free radicals reducing their propagation.
Aldib et al. performed structure-based design of new inhibitors and introduced potent bis-aryl propylamine derivatives (III) and showed that replacement of one aryl group with heterocyclic ring as indole led to potent MPO inhibition \[17\]. Santos et al. reported a series of chalcones (IV) and showed the role of terminal amino group in binding to key amino acids in MPO active site \[18\]. Soubhye et al. synthesized new derivatives (V) based on structure-based docking of 5-fluorotryptamine and showed that increasing the amino alkyl side chain to 4- or 5-carbons led to potent MPO inhibition, also, the unsubstituted terminal amino group is important for activity as it carries positive charge at physiological pH and forms ionic interactions with Glu102 or propenoic heme group \[19\]. Forbes et al. identified new substituted aromatic hydroxamates (VI) as more potent reversible MPO inhibitors than SHA. They introduced pyrimidine ring that increased stability within the active site through interaction with heme propionate group (Fig. 1A) \[14\].

Amino acids are very important for living organisms as they are the basic units for enzymes and proteins, they are widely used as synthetic precursors in organic chemistry and drug design due to the presence of both amino and carboxylic groups \[20\]. Amino acid moieties are reported to be introduced to potential drugs as a strategy to maximize pharmacokinetics and pharmacodynamics \[21\] and due to the known antioxidant activity \[22\]. Etsè et al. reported a
series of benzodioxole amino acid derivatives as potent anti-peroxidase, radical scavenging and non-toxic agents [23].

In previous work [24], we reported novel thiadiazole derivatives as potent anti-inflammatory agents through selective COX-2 inhibition [24]. 5-(4-Methoxy/bromobenzylideneamino)-1,3,4-thiadiazole-2(3H)-thione (2, 3) were among the most potent compounds with high selectivity towards COX-2 (IC$_{50}$ = 0.24/0.19 µM), high anti-inflammatory and analgesic activities and high safety on gastric mucosa with no ulceration effect. Both compounds had similar docking scores in COX-2 active site, the 4-OCH$_3$ group was important for binding to His90 and Arg513, the key amino acids responsible for COX-2 selectivity. Accordingly, in continuation of this work, we modified previously reported thiadiazoles and introduced amino acid moieties on thione group, with perceiving structural features to act as MPO inhibitors and protect against cardiovascular side effects reported for potent COX-2 inhibitors. Terminal NH$_2$ group important for optimum ligand binding to MPO, α-side chain would increase the inhibitory potential by reaching the important amino acids in the heme binding site (Fig. 1B). The target compounds would be endowed with dual COX-2/MPO inhibition to act as anti-inflammatory and protective against MPO-mediated cardiovascular diseases.

**Results and discussion**

**Chemistry**

In this study, novel 12 thiadiazole derivatives bearing amino acid moieties have been synthesized as depicted in Scheme 1. The starting 5-amino-1,3,4-thiadiazole-2(3H)-thione (1) was synthesized from the reaction between thiosemicarbazide and carbon disulfide in alkaline medium as previously reported [25]. We reported compounds 5-(4-Methoxybenzylideneamino)-1,3,4-thiadiazole-2(3H)-thione (2) and 5-(4-Bromobenzylideneamino)-1,3,4-thiadiazole-2(3H)-thione (3) from most potent compounds among series of thiadiazole derivatives as selective COX-2 inhibitors [24]. They were then used in our current research as starting compounds for synthesis of more new derivatives as anti-inflammatory with low cardiovascular risk. Synthesis of target compounds 4–15 was carried by reaction of compounds 2 and 3 with different amino acids (glycine, alanine, glutamic acid, isoleucine, phenylalanine and proline) using water as solvent in green synthesis attempt. The proposed chemical structure of these derivatives was verified by their spectral and microanalytical data, as listed in details in material and methods section. IR spectra of all new compounds showed additional strong intensity absorption bands at range 1680–1720 cm$^{-1}$ ascribed for C=O of amino acid moieties introduced. IR spectra of compounds 4–15 revealed added bands for stretching NH & NH$_2$ of introduced amino acid moieties at range 3200–3330 cm$^{-1}$. $^1$H-NMR spectra of compounds 4–13, displayed extra upfield signal at δ 1.18–2.17 ppm attributed to NH$_2$, exchangeable with D$_2$O. Confirming reaction with different amino acids at specified positions. $^{13}$C spectra for all new compounds displayed signals for Cs at specified positions. Where most deshielded C appears at range 182.94–194.56 ppm ascribed for C=O of different amino acid moieties. Moreover, mass spectra and microanalytical data of compounds 4–15 were in agreement with their postulated structures.

It is of added interest to mention that the newly synthesized compounds closely fulfilling Lipinski’s rule of drug likeness.

![Scheme 1 Synthetic routes for compounds 4–15](image)
**In vitro COX-2/COX-1 inhibition**

Compounds 4–15 underwent selectivity test for COX-2 compared to celecoxib. IC$_{50}$ was calculated and presented in Table 1. Compounds 5 and 6 were the most potent inhibitors (IC$_{50}$ = 0.09 and 0.16 µM) and even more potent than celecoxib (IC$_{50}$ = 0.22 µM). Compounds 4 and 8 were of equal potency as celecoxib (IC$_{50}$ = 0.24 and 0.26 µM). COX-2 inhibition for the starting materials 4 and 6 were previously reported to be 0.24 and 0.19 µM, respectively [24].

Studying the effect of para-substituting the terminal aromatic ring with either methoxy or bromine group, we can conclude that 4-methoxy phenyl substituents were more potent, this emphasize our previous finding that the methoxy group stabilized the compounds in the side pocket of COX-2 by binding to the two main amino acids responsible for selectivity (His90 and Arg513). Whereas, the bulky bromine group could lead to different orientation in the active site.

Introduction of amino acid moiety ameliorated the activity of the methoxy derivatives 5 and 6, retained similar activities as the starting material for compounds 4 and 8, but unfortunately led to decrease in the activity for the rest of compounds.

With regard to SAR of the amino acids derivatives, the alanine derivative 5 was the most active and we can conclude that a short side chain like methyl group is optimum for activity as elongation of the side chain or introduction of either carboxylic or aromatic ring decreased the activity.

In addition, the inhibitory activity of the most potent compounds (4–6, 8) were tested against COX-1 and they showed remarkable lower activity compared to that on COX-2. Accordingly, compounds 4, 5, 6 and 8 were chosen for in vivo examination because of their high selectivity to COX-2 which resembles celecoxib.

**In vivo studies**

**Determination of acute toxicity**

Acute toxicity of the most potent compounds 4, 5, 6 and 8 was evaluated by i.p injection of selected doses [100, 200, 400, 800 and 1000 mg/kg] of each compound dissolved in 5% Tween 80 (in saline). No mortality was recorded within 48 h. indicating that these compounds were practically safe in vivo (Table 2).

**Cardiovascular risk for compounds 4–6, 8: assessment of CK-MB, troponin I and LDH**

Annual CVD mortality is expected to reach more than 23.3 million people by 2030; thereby there is a great concern about this rising incidence of CVDs [26]. As a result, significant efforts should be made to minimize the risks of CVDs. If proper preventive measures are not in place, the increasing use of IR for cancer treatment and diagnostic purposes may result in cardiovascular damage. Many accumulating studies have shown that there is a link between cancer and inflammation where inflammation in the tumor microenvironment has a great role in promoting tumor growth [27]. Selective COX-2 inhibitors have been utilized extensively as potent anti-inflammatory medications in combination with radiotherapy in patients with COX-2-expressing malignancies, where they have been shown to improve tumor radio-sensitivity in cell and animal models [28]. Unfortunately, administration of Celecoxib, alone or combined with doxorubicin, was previously shown to cause cardiotoxicity in rats, as manifested by highly significant increase in the serum LDH, CK-MB, and Troponin level [29]. Therefore, it was of great importance to synthesize new COX-2 inhibitors with the lowest cardiovascular risk.

In the present study, the possible cardiovascular risks of the most active compounds 4, 5, 6 and 8 were evaluated using the radiation induced cardiotoxicity in rats compared to that induced by celecoxib. The response of the heart towards the tested compounds was expressed as the change

| Compound number | COX-2 IC$_{50}$ (µM) | COX-1 IC$_{50}$ (µM) |
|-----------------|----------------------|----------------------|
| 4               | 0.24 ± 0.51          | 30.43 ± 0.31         |
| 5               | 0.09 ± 0.77          | 46.83 ± 0.12         |
| 6               | 0.16 ± 2.64          | 41.05 ± 0.43         |
| 7               | 0.98 ± 0.51          | –                    |
| 8               | 0.26 ± 2.82          | 44.12 ± 0.33         |
| 9               | 1.47 ± 0.05          | –                    |
| 10              | 5.08 ± 0.08          | –                    |
| 11              | 0.98 ± 0.12          | –                    |
| 12              | 0.80 ± 0.41          | –                    |
| 13              | 5.42 ± 0.13          | –                    |
| 14              | 1.23 ± 1.83          | –                    |
| 15              | 3.51 ± 0.64          | –                    |
| Celecoxib       | 0.22 ± 0.21          | 55.05 ± 0.10         |

**Table 1** In vitro COX-2 enzyme inhibition assay for compounds 4–15 and COX-1 inhibition assay for compounds 4–6, 8

**Table 2** Acute toxicity test for compounds 4–6, 8

| Compound number | ALD$_{50}$ (mg/kg) |
|-----------------|--------------------|
| Celecoxib       | >1000              |
| 4               | >1000              |
| 5               | >1000              |
| 6               | >1000              |
| 8               | >1000              |
compared to normal control, rats (the tested compounds (Tn-I and LDH) as compared to normal control. Whereas, all nontoxic biomarkers of myocardial damage levels (CK-MB, aggravates the increase induced by radiation in the dia-
gusted and non-irradiated rats including CK-MB, Tn-I and LDH. The results revealed that celecoxib administration
aggravates the increase induced by radiation in the dia-
godic biomarkers of myocardial damage levels (CK-MB, Tn-I and LDH) as compared to normal control. Whereas, all
test compounds (4, 5, 6 and 8) exhibited better cardio protective profile signified by low level of induction in these
biochemical levels when compared to that induced by cel-
coxib either given alone or combined with radiation
(Figs. 2–4).

In non-irradiated rats, compounds 4, 6 and 8 caused an increase in the levels of CK-MB by 25% whereas com-
ound 5 elevated its level by only 5% compared to a dra-
amic increase reaching to four folds induced by celecoxib
(Fig. 2A). Moreover, TnI in non-irradiated rats was elevat-
ed by nearly 30% using compounds 4, 6 and 8 and by 11% using compound 5 whereas celecoxib increased its level by 64% (Fig. 3A). In addition, LDH in non-irradiated rats was increased after treatment with compounds 4, 6 and 8 by nearly 11% and was kept within normal range with comp-
ound 5 whereas celecoxib led to an increase by 47.7%
(Fig. 4A). Fortunately, compound 5 nearly maintained these
cardiac biomarkers within the normal range proofing its
protective cardiovascular activity. The leakage of these
cardiac enzymes into the blood stream might be due to
rupture of cardiac membrane [30]. Previous studies con-
cluded that COX-2 inhibition favors prothrombotic events
by tipping the balance of prostacyclin/thromboxane in favor
of thromboxane, a prethrombotic eicosanoid and thereby
developing thrombotic cardiovascular events [31]. Similar
findings by [32] revealed that celecoxib administration
induced significant increase in the diagnostic biomarkers of
myocardial damage including CK-MB, TnI and LDH
levels, as compared to normal control.

Exposure to IR caused highly significant increase in
CK-MB, TnI and LDH by four folds, two folds and three
folds respectively (Figs. 2B, 3B and 4B) which reflect the
extent of damage in myocardial musculature. These
results agree with Elkady et al. [33] who reported that
whole body gamma irradiation showed a significant
increase in the level of serum enzyme CK-MP, TnI and
LDH due to the damage in the heart. The mechanism of
radiation-induced cardiotoxicity has been reported to be
through formation of superoxide anions and highly reac-
tive and damaging hydroxyl radicals, which induces
Fig. 4 Effect of thiadiazole derivatives and celecoxib on lactate dehydrogenase LDH activity (U/dl) in serum of normal (A) and irradiated (B) rats (n = 8). All compounds were delivered orally in a dose of (25 mg/kg/day), in irradiated group treatment starts after 1 h of irradiation (6 Gy) and treatment continued for 2 days further. Values are plotted as means ± SEM. *p ≤ 0.05 compared to normal control, †p ≤ 0.05 compared to irradiated rats.

peroxidation of cell membrane lipid [34]. Radiation-induced cardiotoxicity signified by elevated level of CK-MP, TnI and LDH in irradiated rats was significantly ameliorated by compounds 4, 6, 8 and particularly compound 5 whereas they were exaggerated by celecoxib, suggesting their possible low cardiovascular risk if delivered as an adjuvant anti-inflammatory agents with radiotherapy (Figs. 2B, 3B and 4B).

Anti-inflammatory markers evaluation for compounds 4–6, 8

The anti-inflammatory potency of compounds 4, 5, 6 and 8 was investigated by measuring the MPO activity as well as total nitrite content in heart of irradiated rats.

MPO inhibitory activity for compounds 4–6, 8

MPO is a hemoperoxidase found in abundance in neutrophils, which serve as the first line of defense against microbes via phagocytosis. MPO recognizes the produced hydrogen peroxide (H₂O₂), generating a substrate-enzyme complex with high oxidative capability for destroying microorganisms. Under certain circumstances, however, MPO’s excessive synthesis of reactive species causes oxidation of intracellular proteins, lipids, and nucleic acids, leading to additional damage to host tissues at sites of inflammation, such as atherosclerotic plaques [35, 36]. As a result of MPO’s elevated activity during inflammation and oxidative stress in a variety of disorders, it is regarded as a highly significant biomarker [37].

Whole body exposure to IR led to an increase in MPO activity by three folds (Fig. 5). Despite Celecoxib inhibited the induced elevation in MPO activity by 42% in irradiated rats, yet it still has been associated with cardiovascular risk (Figs. 2B, 3B and 4B). Thus, it is interesting to have other possible MPO inhibitors that exhibit less cardiovascular risk than celecoxib to treat inflammatory diseases, such as the novel thiadiazole derivatives in this study, which could be used in future as a better alternative for treatment of inflammatory disorders. The tested compounds 4, 6 and 8 inhibited the increase in MPO induced by radiation by nearly 63–68% and compound 5 showed a 73% reduction in its level, thereby representing more promising anti-inflammatory agents than celecoxib with less cardiovascular risk (Fig. 5).

NO content evaluation for compounds 4–6, 8

MPO also is capable of using nitric oxide (NO) as a physiological substrate, perhaps contributing to endothelial dysfunction. Therefore, the increase in MPO activity is associated with an increase in total nitrite content which was nearly doubled in irradiated rats (Fig. 5). Similarly, an increase in NO content was previously observed in mouse intestine after radiation exposure [38]. In response to pro-inflammatory stimuli, cyclooxygenase-2 (COX-2) and
inducible isoform of nitric oxide synthase (iNOS) gets regulated by NF-κB that mediates subsequent inflammation process [39]. Thus agents that inhibit COX-2, iNOS expression and subsequent NO production can be a potential target for protection against radiation due to its anti-inflammatory role.

In accordance, the present study showed that compounds 4, 6 and 8 inhibited the increase in total Nitric oxide content by nearly 30% whereas celecoxib caused an inhibition by 17% (Fig. 6). In particular, Compound 5 normalizes the total NO content signifying the best anti-inflammatory activity compared to the other tested compounds (Fig. 6).

**Docking study**

In order to interpret the preliminary MPO inhibition results for the synthesized compounds, in silico evaluation using molecular docking was performed with human MPO (PDB: 1DNW), using MOE software. Docking study was performed for the most active compound (5), within the active site of MPO to have more insights about the binding mode based on the description and characterization of heme binding site reported by Fiedler et al. [40].

The methoxy phenyl group is positioned almost parallel to the plane of heme and bound with arene-cation interaction to Arg239 at the opening of the heme distal cavity. The thiadiazole ring occupies the center of the heme distal cavity and participates with two hydrogen bonds with MPO residues. One of the nitrogens of thiadiazole is bound to Arg333 while the sulfur forms an important H-bond with Thr100 which is interacted with the propionate group of heme. The amino acid chain provides the main interaction with the key residue Glu102 through the terminal NH2 group. Moreover, compound 5 is more stabilized within the active site by another H-bond with Arg424 through the carbonyl group with docking score equal to −9.32 kcal/mol (Fig. 7).

Compound (5) was also docked into the active site of COX-2 (PDB: 1CX2) in order to monitor the effect of alanine amino acid substitution on the orientation of the compound within the active site. The methoxy phenyl moiety is directed towards the primary hydrophobic pocket defined by Tyr385 and Tyr348, the thiadiazole ring is oriented towards the carboxylate site defined by Arg120 and Thr355, while the alanine chain led the compound to enter the side pocket bordered with Val523 where it is involved with two hydrogen bonds with Arg513 and His90, the unique residues for COX-2 which are not present in COX-1 [41]. The docking score was equal to −12.67 kcal/mol (Fig. 8).
Conclusion

In Conclusion, our interest in developing novel COX-2 inhibitors arises from the high risk of cardiovascular toxicity associated with the use of this class. Patients undergoing radiotherapy or exposed to hazards sources of radiation are at great risk of normal tissue toxicity and they were using the anti-inflammatory agents especially COX-2 inhibitors on a large scale. Therefore, efforts have been undertaken to develop novel synthesized agents that protect normal tissues with the lowest possible cardiovascular toxicity. In this work, we performed structural modification of previously reported selective COX-2 inhibitors, different amino acids were introduced on thione group of thiadiazole leading to more potent COX-2 inhibition and low cardiovascular risk than that induced by celecoxib or radiation with nearly the same anti-inflammatory activity. The alanine derivative 5 in particular, showed the most promising anti-inflammatory activity with the lowest cardiovascular risk. The obtained activity was supported with docking of compound (5) into COX-2 and MPO active sites suggesting possible dual inhibition of both enzymes.

Materials and method

Chemistry

A Stuart melting point apparatus (Stuart Scientific, Redhill, UK) was used for recording of uncorrected melting points and were carried in open capillary tubes. FTIR Shimadzu spectrometer (Shimadzu, Tokyo, Japan) was used for recording Infrared (IR) spectra of the compounds. A Bruker (400 MHz for 1H-NMR and 100 MHz for 13C-NMR) spectrometer (Bruker Bioscience, MA, USA) was used for recording 1H NM R and 13C NMR spectra, using TMS as an internal Standard and DMSO-d6 as solvent. Mass spectra were run on HP Model MS-5988 (Hewlett Packard, Palo Alto, California, USA). A Carlo Erba 1108 Elemental Analyzer (Heraeus, Hanau, Germany), was used for obtaining microanalyses values. Pre-coated SiO2 gel (HF254, 200 mesh) aluminum plates (Merk, Darmstadt, Germany) were used as TLC for checking of reactions completion, washed with water, dried and crystallized from ethanol to give the assigned products (4–15).

General procedure for the preparation of compounds (4–15)

In a 250-ml round bottom flask, Na2CO3 (0.01 mol) was dissolved in 10 ml water and then different L-amino acids was added; glycine, alanine, glutamic acid, isoleucine, phenylalanine and proline (0.02 mol). To this mixture, addition of 5-(4-Methoxybenzylideneamino)-1,3,4-thiadiazole-2(3H)-thione (2) (0.02 mol) and/OR 5-(4-Bromobenzylideneamino)-1,3,4-thiadiazole-2(3H)-thione (3) (0.02 mol) with continuous stirring was done for half an hour. Then the mixture was stirred at reflux for 6–9 h. After the completion of the reaction (checked by TLC), a precipitate was formed, filtered while hot. The product obtained, washed with water, dried and crystallized from ethanol to give the assigned products 4–15, respectively.

A mixture of 5-Amino-3H-[1,3,4]thiadiazole-2-thione 1 (0.53 g, 0.004 mol) and the appropriate aldehydes (0.004 mol) was refluxed in ethanol containing (1 ml) glacial acetic acid for 5 h. The reaction mixture was cooled, poured onto ice water and the precipitated solid was collected by filtration, dried, and crystallized from methanol to give 2 and 3, respectively (as reported).

S-(5-((4-Methoxybenzylidene)amino)-2,3-dihydro-1,3,4-thiadiazol-2-yl)2-amino ethanethioate (4)

Yield, 86%, mp 200–202 °C. IR (KBr, cm−1): 3320, 3230, 3200 (NH, NH2), 2939, 2870 (CH aliph.), 3048 (CH arom.), 1720 (C=O), 1626 (C=N). 1H-NMR (DMSO-d6, ppm): 1.62 (s, 2H, NH2, exchangeable with D2O), 3.76 (s, 2H, CH2), 3.81 (s, 3H, OCH3), 4.98 (s, 1H, CH-thiadiazole), 6.96 (d, 2H, J = 7.5 Hz, Ar-H), 7.27 (d, 2H, J = 7.5 Hz, Ar-H), 7.70 (s, 1H, N=CH), 9.44 (s, 1H, NH, exchangeable with D2O).13C-NMR (DMSO-d6, ppm): 48.67 (CH2), 56.04 (OCH3), 104.05 (CH-thiadiazole), 114.49, 128.31, 132.32 (5C-aromatic), 164.06 (C=O), 165.84 (C-thiadiazole), 168.15(C=N), 182.94 (C=O). MS ml/z: 310 [M+]. Analysis calculated for C12H14N4O2S2: C, 46.43; H, 4.57; N, 18.02. Found: C, 46.45; H, 4.57; N, 18.02.

S-(5-((4-Methoxybenzylidene)amino)-2,3-dihydro-1,3,4-thiadiazol-2-yl)2-amino propanethioate (5)

Yield, 82%, mp 200–202 °C. IR (KBr, cm−1): 3230, 3200 (NH, NH2), 2939, 2870 (CH aliph.), 3048 (CH arom.), 1720 (C=O), 1626 (C=N). 1H-NMR (DMSO-d6, ppm): 1.45 (s, 2H, NH2, exchangeable with D2O), 1.37 (d, 3H, CH3), 3.59 (q, 1H, C=H), 4.98 (s, 1H, CH-thiadiazole), 9.76 (s, 1H, N=CH), 9.44 (s, 1H, NH, exchangeable with D2O).13C-NMR (DMSO-d6, ppm):
4-Amino-5-((5-(4-methoxybenzylidene)amino)-2,3-dihydro-1,3,4-thiadiazol-2-yl)thio-5-oxopentanoic acid (6) Yield, 89%, mp 245–247 °C. IR (KBr, cm\(^{-1}\)): 3420 (OH), 3319, 3232, 3202 (NH, NH\(_3\)), 2939, 2870 (CH aliph.), 3047 (CH arom.), 1720 (C=O), 1629 (C=N). \(^1\)H-NMR (DMSO-d\(_6\), ppm): 1.93 (s, 2H, NH\(_2\), exchangeable with D\(_2\)O), 2.16 (td, 2H, CH\(_2\), J = 8.1, 0.6 Hz), 2.31 (t, 2H, CH\(_2\)), 7.53 (t, 1H, CH, J = 7.4 Hz), 3.88 (s, 3H, OCH\(_3\)), 4.92 (s, 1H, CH-thiadiazole), 6.82 (s, 1H, NH, exchangeable with D\(_2\)O), 7.09 (d, 2H, J = 7.5 Hz, Ar-H), 7.14 (d, 2H, J = 7.5 Hz, Ar-H), 7.38 (1H, C=H), 9.50 (s, 1H, OH, exchangeable with D\(_2\)O), 3.09 (2CH\(_2\)), 56.24 (OCH\(_3\)), 104.25 (CH-thiadiazole), 114.58, 128.41, 132.39 (5C-aromatic), 164.46 (C-OCH\(_3\)), 165.74 (C-thiadiazole), 168.15 (C=N), 176.74 (COOH), 194.56 (C=O). MS m/z: 382 [M\(^+\)]. Analysis calculated for C\(_{19}\)H\(_{20}\)N\(_4\)O\(_2\)S\(_2\): C, 56.98; H, 5.03; N, 15.21.

S-(5-(4-Bromobenzylidene)amino)-2,3-dihydro-1,3,4-thiadiazol-2-yl)2-amino propanethioate (10) Yield, 78%, mp 261–262 °C. IR (KBr, cm\(^{-1}\)): 3232, 3205 (NH, NH\(_3\)), 2939, 2875 (CH aliph.), 3049 (CH arom.), 1716 (C=O), 1628 (C=N). \(^1\)H-NMR (DMSO-d\(_6\), ppm): 1.18 (s, 2H, NH\(_2\), exchangeable with D\(_2\)O), 1.41 (d, 3H, CH\(_3\)), 6.59 (q, 1H, CH\(_2\), J = 6.5 Hz), 4.8 (s, 1H, CH-thiadiazole), 7.11 (d, 2H, J = 7.5 Hz, Ar-H), 7.65 (d, 2H, J = 7.5 Hz, Ar-H), 7.63 (s, 1H, N=CH), 8.18 (s, 1H, NH, exchangeable with D\(_2\)O), 13C-NMR (DMSO-d\(_6\), ppm): 22.59 (CH\(_3\)), 57.71 (CH), 104.35 (CH-thiadiazole), 114.49, 128.21, 132.22 (5C-aromatic), 164.06 (C-OCH\(_3\)), 165.74 (C-thiadiazole), 168.15 (C=N), 192.23 (C=O). MS m/z: 373 [M\(^+\)]. Analysis calculated for C\(_{14}\)H\(_{15}\)BrN\(_4\)OS\(_2\): C, 38.61; H, 3.51; N, 15.01, found: C, 38.71; H, 3.41; N, 15.21.
S-(5-(4-Bromobenzylidene)amino)-2,3-dihydro-1,3,4-thiadiazone-2-yl)amo-no-3-methylpentanethioate (12) Yield, 85%, mp 267–269 °C. IR (KBr, cm⁻¹): 3322, 3223, 3210 (NH, NH₂), 2949, 2881 (CH aliph.), 3046 (CH arom.), 1722 (C=O). MS m/z: 431 [M⁺], 433 [M + 2]. Analysis calculated for C₁₈H₁₇BrN₄O₂S₂: C, 48.11; H, 3.81; N, 12.47, found: C, 38.88; H, 3.61; N, 12.79.

S-(5-(4-Bromobenzylidene)amino)-2,3-dihydro-1,3,4-thiadiazone-2-yl)pyrrolidine-2-carbothioate (13) Yield, 82%, mp 232–234 °C. IR (KBr, cm⁻¹): 3240, 3209 (NH), 2937, 2870 (CH aliph.), 3044 (CH arom.), 1687 (C=O), 1628 (C=N). ¹H-NMR (DMSO-d₆, ppm): 1.15 (s, 1H, NH, exchangeable with D₂O), 1.71–2.01 (m, 4H, 2CH₂-pyrrolidine), 2.70–2.87 (m, 2H, CH₂-pyrrolidine), 3.68 (t, 1H, CH-pyrrolidine), 4.98 (s, 1H, CH-thiadiazole), 7.10 (d, 2H, J = 7.5 Hz, Ar-H), 7.55 (d, 2H, J = 7.5 Hz, Ar-H), 7.62 (s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (DMSO-d₆, ppm): 11.56 (CH₃), 16.19(CH₃), 24.33 (CH₃), 39.13 (CH), 104.05 (CH-thiadiazole), 128.75 (C-Br), 131.29, 132.50, 133.87 (5C-aromatic), 165.64 (C-thiadiazole), 168.35 (C=N), 193.36 (C=O). MS m/z: 415 [M⁺], 417 [M + 2]. Analysis calculated for C₁₅H₁₉BrN₄S₂: C, 51.51; H, 5.38; N, 15.79.

S-(5-(4-Methoxybenzylidene)amino)-2,3-dihydro-1,3,4-thiadiazone-2-yl)pyrrolidine-2-carbothioate (14) Yield, 79%, mp 262–264 °C. IR (KBr, cm⁻¹): 3231, 3205 (NH), 2941, 2880 (CH aliph.), 3049 (CH arom.), 1721 (C=O), 1629 (C=N). ¹H-NMR (DMSO-d₆, ppm): 1.86 (s, 1H, NH, exchangeable with D₂O), 1.73–2.09 (m, 4H, 2CH₂-pyrrolidine), 3.32–3.41 (m, 2H, CH₂-pyrrolidine), 3.68 (s, 1H, CH-thiadiazole), 7.69 (s, 1H, CH=O), 9.46 (s, 1H, NH, exchangeable with D₂O). ¹³C-NMR (DMSO-d₆, ppm): 24.68, 33.34, 46.90 (3CH₂-pyrrolidine), 56.04 (OCH₃), 67.49 (CH-pyrrolidine), 104.05 (CH-thiadiazole), 114.49, 128.61, 132.72 (5C-aromatic), 164.76 (C=OCH₃), 165.84 (C-thiadiazole), 189.36 (C=N), 193.36 (C=O). MS m/z: 350 [M⁺]. Analysis calculated for C₁₅H₁₃BrN₄O₂S: C, 51.41; H, 5.18; N, 15.99, found: C, 51.51; H, 5.38; N, 15.79.

In vitro COX-2/COX-1 enzyme inhibition assay

The in vitro ability of the 12 synthesized compounds and celecoxib to inhibit the COX-2/COX-1 isozymes was carried out using Cayman colorimetric COX (ovine) inhibitor screening assay kit (kit catalog number 560101, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instructions. Percent inhibition was calculated by the comparison of compound treated to various control incubations. The concentration of the test compound causing 50% inhibition (IC₅₀, μM) was calculated from the concentration-inhibition response curve following triplicate determinations.

In vivo studies

Animals

Male Wistar rats (150–180 g) and mice (25 ± 5 g) purchased from the animal breeding unit of the National Research Centre, Giza, Egypt, and acclimatized in the animal facility of the National Centre for Radiation Research and Technology (NCRRT)-Atomic Energy Authority, Cairo, Egypt for 1 week before being used. Animals were housed at a temperature of 25 ± 5 °C, humidity of 60 ± 5% and 12/12-h light-dark cycle. They were fed standard pellet diet obtained from the National Research Centre, Dokki, Cairo and...
enabled free access to water ad libitum. All animal experiments comply with the ARRIVE guidelines. The study was conducted under U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines set by EU Directive (2010/63/EU) for animal experiments.

Irradiation

Animals were exposed to whole body irradiation at an acute dose level of 6 Gy at the NCRRT using the Gamma Cell-40 biological irradiator with a Caesium137 source (Atomic Energy of Canada Ltd; Sheridan Science and Technology Park, Mississauga, Ontario, Canada). The radiation dose level was 6 Gy with a dose rate of 0.643 Gy/min.

Determination of acute toxicity

The most potent compounds (4, 5, 6 and 8) were chosen after in vitro COX-2 inhibition assay for in vivo studies. The approximate 50% lethal dose (ALD50) of the compounds (4, 5, 6 and 8) was determined. Adult male Swiss albino mice (3–4 months) weighing 25–35 g were obtained from the animal breeding unit of the National Research Centre, Giza, Egypt. A total of 160 mice were randomly divided into four main groups (corresponding to compounds 4, 5, 6 and 8), each eight mice received I.p. one acute dose of each compound as follows: 100, 200, 400, 800 and 1000 mg/kg.b.wt. All mice were observed for 48 h during which any mortality in each group was recorded. After drug administration, all the animals had free access to food and water. The LD50 was calculated from the data obtained [42].

Experimental design

Rats were blindly allocated into 12 groups, consisting of 8 rats each (n = 8). Group 1: served as control non-irradiated animals, Group 2: rats were irradiated at 6 Gy [43, 44] but left untreated, Group 3: irradiated rats were given celecoxib (25 mg/kg) as a reference drug [45], Group 4, 5, 6, 7: irradiated rats were given the newly synthesized compounds 4, 5, 6 and 8 respectively in a dose of (25 mg/kg) similar to the dose of celecoxib. All compounds were dissolved in normal saline and delivered orally after 1 h of irradiation and treatment continued for 2 days further. Group 8: non-irradiated rats were given celecoxib (25 mg/kg). Group 9, 10, 11 and 12: non-irradiated rats were given the newly synthesized compounds 4, 5, 6 and 8 respectively in a dose of (25 mg/kg). On the third day, the rats were sacri-

Inflammatory markers

(A) Myeloperoxidase (MPO) assay

MPO activity was determined in heart tissue homogenates prepared in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (1:1 ratio) The assay method was that described by [47]. Values were expressed as U/g tissue.

(B) Nitric oxide assay

Total nitrate/nitrite (NOx) was measured according to the method described by [48] using vanadium tri-chloride (VCl3), sulfanilamide N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD). Pink color chromophore of the end product was measured at 540 nm using double beam...
Docking study

Molecular docking study was performed utilizing Molecular Operating Environment (MOE) software, version 2014.090. Structure of compound 5 was drawn on MOE. Hamiltonian-Force Field-MMFF94x was used to minimize compound’s energy. The forcefield partial charges were calculated. Default settings were utilized for the analysis of the conformational stochastic of the compound. X-ray crystal structure of MPO in complex with isothiocyanate (PDB ID: 1DNW) and that of COX-2 in complex with SC-558 (PDB ID: 1CX2) were downloaded from http://www.rcsb.org/pdb. The protein–ligand complex obtained from the Protein Data Bank (pdb) was prepared for docking, the enzyme was protonated three-dimensionally and then the system was optimized. Repeated chains of the protein and co-crystallized water molecules were removed. Determination and isolation of the binding pocket were performed and then the backbone was hidden. MOEDOCK was used to determine flexible docking of the ligand-rigid receptor of the most stable conformers. Scoring was performed using the alpha triangle placement method and London dG as a function. Forcefield refinement was performed on the obtained poses using the same scoring function. Retention of 50 of the most stable docking models of the ligand with the best-scored conformation was performed.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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