Bioorganic & Medicinal Chemistry 17 (2009) 1991–1996

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Inhibition of iNOS and COX-2 in human whole blood ex vivo and monocyte-macrophage J774 cells by a new group of aminothiopyrimidone derivatives

Venera Cardile [a,*], Laura Lombardo [a], Giuseppe Granata [b], Antonio Perdicaro [b], Michael Balazy [c], Andrea Santagati [b]

[a] Department of Physiological Sciences, University of Catania, V.le A. Doria 6, 95125 Catania, Italy
[b] Department of Pharmaceutical Sciences, University of Catania, V.le A. Doria 6, 95125 Catania, Italy
[c] Department of Pathology, New York Medical College, Valhalla, NY 10595, USA

ARTICLE INFO

Article history:
Received 17 October 2008
Revised 12 January 2009
Accepted 14 January 2009
Available online 20 January 2009

Keywords:
Nitric oxide
NSAIDs
Prostaglandins
Aminothiopyrimidone

ABSTRACT

We tested a series of 11 new aminothiopyrimidones on the activity of inducible nitric oxide synthase (iNOS) and prostaglandin G/H synthase-1 and 2 (COX-1 and COX-2) in the whole human blood and monocyte-macrophage J774 cell line. To induce COX-2 and iNOS, blood samples and J774 cells were stimulated with bacterial lipopolysaccharide (LPS) in the absence or presence of the test compounds. After incubation, the plasma and the supernatants of culture media were collected for the measurement of TxB_2 and PGE_2 by a specific enzyme-immunoassay and determination of nitrite by a colorimetric assay. Several phenylthieno derivatives of substituted pyrimidone inhibited formation of both COX-2 and iNOS derived products with one of the compounds (compound **11**, *N*-[2-[(2,4-dinitrophenyl)thio]-4-oxo-6-phenylthieno[2,3-*d*]pyrimidin-3(4*H*)-y]methanesulfonamide) showing a complete inhibition of LPS-stimulated formation of NO and PGE_2.

1. Introduction

Prostaglandin E_2 (PGE_2) is one of the principal mediators of inflammation, which is biosynthesised by the metabolism of the phospholipase A_2 (PLA_2)-derived arachidonic acid to PGG_2/PGH_2 via COX enzymes, and then followed by the action of PGE synthase. Two COX isozymes have been identified. COX-1 is a constitutively expressed isoform, which biosynthesizes physiologically relevant PGs involved in gastric and other organ cytoprotection. High levels of COX-1 are expressed in platelets,[1] vascular endothelial cells,[2] stomach,[3,4] and renal collecting tubules.[3,5,6] COX-1 levels remain fairly constant, but in some instances, 2- to 4-fold COX-1 protein increase has been observed following hormonal and/or growth factor stimulation.[7,8] COX-2 is an inducible isoform, which is almost undetectable in most tissues under physiological conditions, but its expression can be considerably increased (10- to 80-fold) following induction by many pro-inflammatory stimuli, cytokines, growth factors and mitogens.[3,5,6,9–12] Bacterial lipopolysaccharides (LPS) stimulate COX-2-dependent PG formation in monocytes[13] macrophages[14–16] and cause inflammation in synoviocytes[17] and follicles.[18]

While it has been long known that non-steroidal anti-inflammatory drugs (NSAIDs) inhibit PG formation, clinically useful NSAIDS are either fairly selective COX-1 inhibitors or dual COX-1/COX-2 inhibitors.[19–21] None of the traditional NSAIDs displays selective COX-2 inhibition, which explains some of the side effects of NSAIDS related to the removal of gastric cytoprotection.[22] This observation laid foundation for development of more specific COX-2 inhibitors as potentially better approach to inflammation therapeutics. However, recent placebo-controlled trials have revealed that COX-2 inhibitors pose a small risk of myocardial infarction and stroke, which resulted in the withdrawal from the market of some members of this class of drugs.[4] These inhibitors cause a suppression of COX-2-derived prostacyclin (PGI_2), which counteracts platelet COX-1-derived thromboxane (TxA_2), PGH_2, and other endogenous stimuli. The consumption of celecoxib, rofecoxib, and valdecoxib has raised concern that many patients may have suffered adverse cardiovascular reactions from these drugs. Thus, a new generation of safe COX-2 inhibitors would ideally not only spare COX-1-derived PGs but also PGI_2 to afford endothelial cytoprotection.

Another component of the inflammatory processes is the massive production of nitric oxide (NO) by inducible NO synthase (iNOS). Moreover, many inflammatory stimuli, induce expression of both iNOS and COX-2 proteins, which bind selectively.[23,24] These two enzyme systems appear to interact by a mechanism in which COX-2 activity increases via iNOS-stimulated nitration of the COX-2 cysteine residues. This provides a rationale for development of more refined dual COX-2/iNOS inhibitors that will block symptoms of inflammation related to PGs and NO formation.[23]

* Corresponding author. Tel.: +39 095 7384040; fax: +39 095 7384217.
E-mail address: cardile@unict.it (V. Cardile).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.01.029

Over the last few years, we have established new methods for the high yield synthesis of a number of derivatives containing the pyrimidin-4-one system with various functional groups.[25,26] The choice of the heterocyclic system was based on the remarkable analgesic and anti-inflammatory actions with no ulcerogenic effect of methyl and phenyl derivatives of the thieno-, indol- and benzopyrimidin-4-one systems.[25,26]

In this study, we tested the effects of novel aminothiopyrimidinone derivatives (**1–11**) on the generation of COX-2 and iNOS products in LPS-challenged human whole blood and monocyte-macrophage J774 cell line by measuring PGE_2 production by enzyme-immunoassay (EIA) system and formation of nitrite by a colorimetric assay. In addition, the compounds were tested on COX-1 activity in human whole blood and compared with the activity of indomethacin and celecoxib as references of COX-1 and COX-2 inhibitors, respectively.

2. Results

2.1. Effects on COX-2

The effects of pyrimidinone derivatives (**1–11**) were tested on the formation of PGE_2 and NO in human whole blood ex vivo and mouse monocyte-macrophage J774 cells in culture. The COX-2 activity was evaluated by measurement of PGE_2 levels in LPS-treated whole blood.[27] The control blood samples contained 0.28 ± 0.02 ng/ml (n = 9) of PGE_2, which increased by 35.8 ± 5.5 ng/ml (n = 9) following the LPS stimulation (10 μg/ml for 24 h) (12,785% increase). Stimulation of the J774 cells with LPS (5 μg/ml for 72 h) increased the PGE_2 levels to 30.1 ± 3.5 pg/ml (n = 9) from the control levels of 10 ± 0.6 pg/ml (n = 9) (301% increase). We observed that the compounds synthesized by us either inhibited (Table 2) or stimulated (Table 3) PGE_2 formation by COX-2. Compounds **4**, **8**, **9**, **10**, and **11** inhibited LPS-induced COX-2 both in blood and J774 cells (Table 2) by 70–100%. The IC_{50} values for the derivatives **4**, **8** and **11** were 7 ± 0.4, 5.8 ± 0.2 and 3.4 ± 0.2 μM, respectively. Compound **11** was the most potent among these inhibitors showing potency comparable to celecoxib (Table 2). Compounds **4**, **8**, **9** and **10** were relatively less potent but showed preferential specificity for blood (70–90% inhibition) as compared to J774 cell-derived PGE_2 (0–50% inhibition).

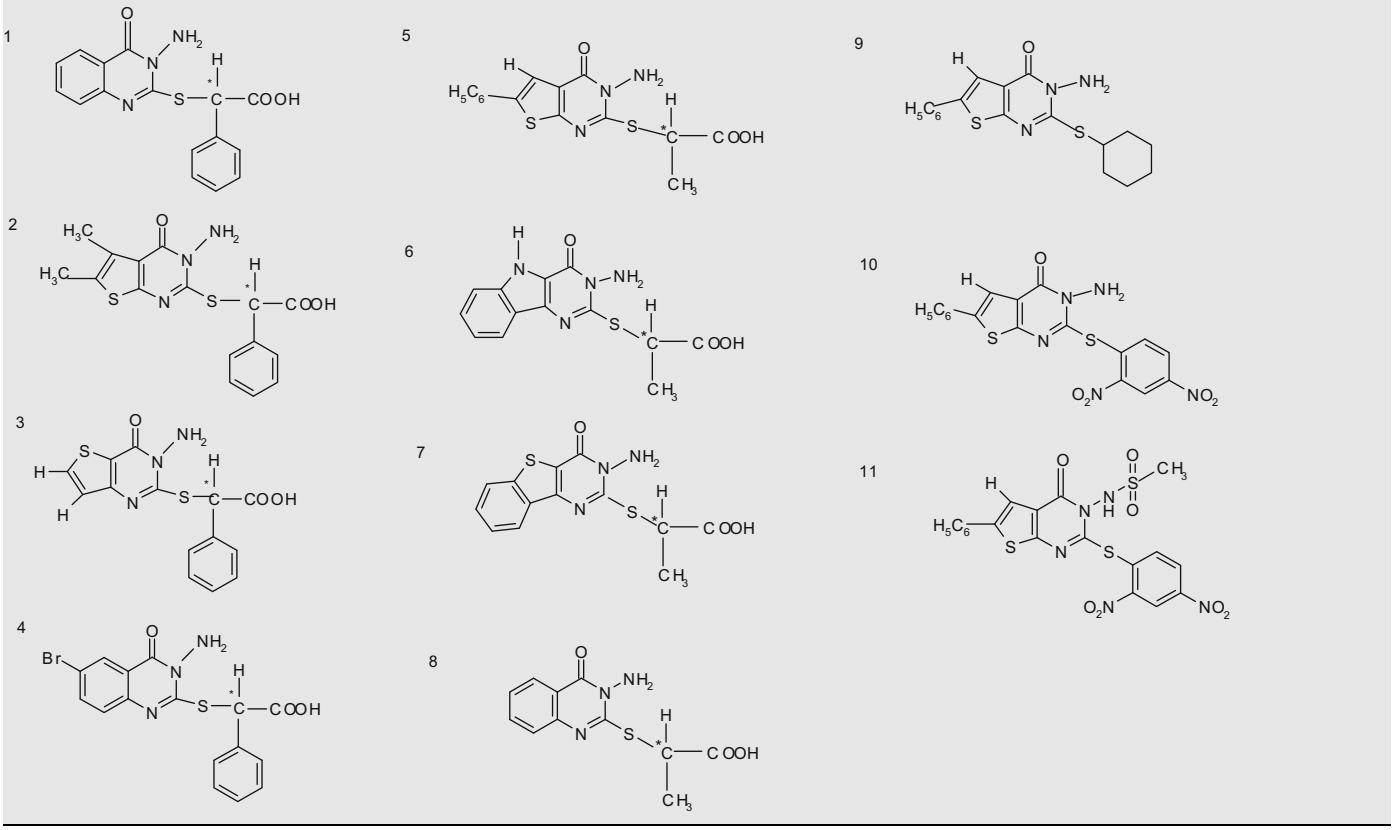
The effects of the compounds on COX-1 were evaluated in samples of human clotted blood drawn from the same healthy volunteers by measurement of TxB_2 generation, which reflects maximally stimulated cyclooxygenase activity of platelet COX-1 by endogenously formed thrombin in clotting blood. Similar to celecoxib, compound **11** did not show a significant inhibition of the COX-1 activity (4 ± 0.5%). In this assay, indomethacin (10 μM) inhibited COX-1 by 85%. Compounds **3** and **10** (10 μM) inhibited COX-1 by 3% and 36%, respectively.

Interestingly, compounds **1**, **2**, **5**, **6**, **7** increased the LPS-induced PGE_2 formation. The J744 cells were more sensitive than blood to these derivatives. Compound **2** was the most potent among these derivatives and stimulated PGE_2 formation 2- to 3.5-fold in blood and J744 cells, respectively.

2.2. Effects on iNOS

The nitrite levels produced by J744 cells before and after LPS stimulation were 4 ± 0.2 μM and 21 ± 3 μM (525% increase),

Table 1
Structures of compounds **1–11**



An asterisk indicates the optically active carbon in the thiopropanoic and thiophenylacetic acid derivatives.

Table 2
Comparison of the inhibitory activity of aminothiopyrimidone derivatives with indomethacin and celecoxib in human whole blood and mouse monocyte-macrophage J774 cells stimulated with LPS

Inhibitors 10 μM concentration	Inhibition of PGE_2 generation (%)		Inhibition of NO generation (%) in J774 cells
	Human whole blood	J774 Cells	
3	None inhibition	None inhibition	55 ± 2*
4	70 ± 2*	50 ± 4*	38 ± 2*
8	88 ± 6*	23 ± 1*	50 ± 7*
9	90 ± 2*	20 ± 5*	70 ± 5*
10	75 ± 5*	None inhibition	60 ± 5*
11	100 ± 5*	100 ± 6*	100 ± 3*
Indomethacin	100 ± 4*	100 ± 3*	90 ± 6*
Celecoxib	100 ± 5*	100 ± 2*	96 ± 8*

The values are the mean ± SEM of three experiments performed in triplicate.
* Denotes significant difference of control vs LPS treatment ($P < 0.01$).

Table 3
Stimulatory activity of aminothiopyrimidone derivatives in human whole blood and mouse monocyte-macrophage J774 cells stimulated with LPS

Activators 10 μM concentration	Activation of PGE_2 generation (%)		Activation of NO generation (%) in J774 cells
	Human whole blood	J774 Cells	
1	21 ± 3*	30 ± 5*	5 ± 1*
2	205 ± 22*	346 ± 17*	11 ± 2*
5	90 ± 12*	250 ± 22*	52 ± 7*
6	28 ± 3*	180 ± 14*	35 ± 6*
7	6 ± 0.5*	52 ± 12*	10 ± 3*

The values are the mean ± SEM of three experiments performed in triplicate.
* Denotes significant difference of control vs LPS stimulation ($P < 0.01$).

respectively. The same compounds **4**, **8**, **9**, **10** and **11** inhibiting COX-2 reduced NO production with the exception of compound **3**, which was a iNOS inhibitor but not active toward COX-2 (Table 2). COX-2 activators (**1**, **2**, **5**, **6** and **7**) were also moderate activators of iNOS in J744 cells (Table 3).

3. Discussion

In this study, a number of heterocyclic derivatives containing aminothiopyrimidone system with various functional substituents were tested on COX-2 activity. Our efforts identified several compounds that were excellent COX-2 as well as iNOS inhibitors. One of these compounds (**11**) inhibited COX-2-generated PGE_2, and iNOS generated nitrite, and showed similar potency to celecoxib.

There are several lines of rationale for development of dual COX-2/iNOS inhibitors as a group of more effective anti-inflammatory and analgesic drugs. Increased levels of both prostaglandins and NO-derived products such as peroxynitrite and nitrogen dioxide mediate the inflammatory reactions and pain.[28,29] While binding of NO to COX heme does not appear to alter its activity,[30] several mechanisms may be involved in the activation of COX-2 by NO and iNOS.[23,31] These involve stimulation of COX-2 by NO that involves MAP kinase and superoxide.[31] In human colon and breast cancers, increased expression of iNOS and elevated levels of prostanoids are associated with COX-2 overexpression, in both cancer cells and stroma,[32] suggesting a close association between these two enzymes.[33] More recently, Kim, Huri and Snyder[23] have reported that iNOS specifically binds to COX-2 in a continuous macrophage cell line (RAW264.7) treated with LPS and IFN-γ. iNOS binding causes S-nitrosylation of COX-2 cysteines, which enhances COX-2 catalytic activity.[23] Selective disruption of the iNOS-COX-2 binding prevents NO-mediated activation of COX-2. This synergistic molecular interaction between two inflammatory systems provides a strong rationale for development of dual COX-2/iNOS inhibitors rather than selective COX-2 inhibitors as a novel class of anti-inflammatory drugs.

Interestingly, several of our thiopyrimidone derivatives stimulated COX-2 activity and, to a lesser extent, iNOS activity. This observation suggests that a combination of structural elements of the pyrimidone derivatives may lead to a more refined COX-2 inhibitor that at the same time inhibits iNOS.

The compounds tested contained a basic 2-thio-3-amino-pyrimidone-4-on structure that was modified at key atoms based on molecular modeling and our previous study.[26] We identified several structural elements that contributed to modulation of the COX-2 and iNOS activities in blood and J744 cells stimulated with LPS. These modifications included substitutions at the thiol group, fusion of the pyrimidone ring and a substitution of the amino group. Both S substituted phenylacetic and propanoic acids derivatives activated COX-2 with exception of derivatives **4** and **8**, which were mild inhibitors. Synthesis of dinitrophenylthio derivatives resulted in strong inhibitory properties of compounds **10** and **11**. Another major effect was observed with the dihydrothieno fusion to the pyrimidone ring. The dimethyl-dihydrothieno derivative **2** showed potent COX-2 stimulatory effect whereas phenyl substituted dihydrothieno derivatives **9**, **10**, and **11** displayed strong inhibitory effects. A striking effect was observed by creation of the methanesulfonamide derivative of the N-amino group in compound **10**. This modification generated compound **11**, which substantially increased the ability of compound **11** to inhibit COX-2 resulting in 100% of COX-2 inhibition relatively to compound **10** (Table 2) in blood and J744 cells, respectively. While celecoxib and valdecoxib are sulfonamines, the effect of the methanesulfonamide group in compound **11** suggests an important contribution of the amino group to the modulatory effect on COX-2 in the studied compounds.

COX-2 inhibitors are believed to position themselves inside the critical catalytic pocket of the enzyme whereby blocking the access of the substrate, arachidonic acid. The spatial position of many inhibitors relative to the COX-2 tertiary protein structure is not known, and, therefore, trial and error approaches have revealed various chemical structures that can produce an inhibitory effect. It has been suggested that the preference or selectivity toward the COX-2 enzyme of the methylsulfonamide derivatives does not depend solely on the presence of these moieties in their molecular structures, but also on the fact that they possess an overall molecular profile which allows these groups to occupy a favorable spatial position during the interaction of the inhibitor with catalytic site, which permits interactions with amino acidic residues specific for the COX-2 active site.[34] In J774 cells, in fact, the compounds **3**, **8**, **9**, and **10**, well inhibiting NO production, did not reduce in the same manner the PGE_2 release.

In conclusion, our work further supports our original finding that the aminothio derivatives of pyrimidone could be a novel promising group of anti-inflammatory drugs and emphasises the significance of the methylsulfonamide substitution of the aminopyrimidine ring. We believe that these new derivatives offer a new direction for development of safer COX-2 inhibitors having additional anti-inflammatory effects derived from iNOS inhibition.

4. Experimental

4.1. Chemistry

The structures of the compounds used in this study are summarized in Table 1. The syntheses of amino acid derivatives [(3-amino-4-oxo-3,4-dihydroquinazolin-2-yl)thio](phenyl)acetic acid **1**, [(3-amino-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-yl)thio](phenyl)acetic acid **2**, [(3-amino-4-oxo-3,4-dihydrothie-

no[3,2-*d*]pyrimidin-2-yl)thio](phenyl)acetic acid **3**, [(3-amino-6-bromo-4-oxo-3,4-dihydroquinazolin-2-yl)thio] (phenyl)acetic acid **4**, 2-[(3-amino-4-oxo-6-phenyl-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-yl)thio]propanoic acid **5**, 2-[(3-amino-4-oxo-4,5-dihydro-3*H*-pyrimido[5,4-*b*]indol-2-yl)thio]propanoic acid **6**, 2-[(3-amino-4-oxo-3,4-dihydro[1]benzothieno[3,2-*d*]pyrimidin-2-yl)thio]propanoic acid **7**, 2-[(3-amino-4-oxo-3,4-dihydroquinazolin-2-yl)thio]-propanoic acid **8**, has been accomplished according to methods reported by Santagati et al.[25] Amino-thio derivatives 3-amino-2-(cyclohexylthio)-6-phenylthieno[2,3-*d*]pyrimidin-4(3*H*)-one **9**, 3-amino-2-[(2,4-dinitrophenyl)thio]-6-phenylthieno[2,3-*d*]pyrimidin-4(3*H*)-one **10** and *N*-[2-[(2,4-dinitrophenyl)thio]-4-oxo-6-phenylthieno[2,3-*d*]pyrimidin-3(4*H*)-y]methanesulfonamide **11** have been synthesized according to methods reported by Granata et al.[26] The purity and structures of compounds **1–11** was accomplished as described.[25,26] The compounds **1–8** were tested as racemic mixtures. Indomethacin was purchased from Sigma Chemical (Milan, Italy) whereas celecoxib was isolated by extraction from Celebrex (Pharmacia or Pfizer) followed by purification on a chromatographic column. Stock solutions of the reference standards or test compounds were prepared in dimethyl sulfoxide, and an equivalent amount of dimethyl sulfoxide was included in control samples.

4.2. Synthesis and characterization of derivatives

^{1}H NMR-spectra were recorded at 200 MHz on a Varian Gemini 200 spectrometer; chemical shifts (δ) are reported in ppm from tetramethylsilane as internal standard; coupling constants (J) are in Hertz (Hz). IR spectra were recorded on a Perkin Elmer 1600 Series FT-IR in potassium bromide disks. Microanalyses for C, H, N and S were obtained from an EA 1108 elemental analyzer Fisons Carlo-Erba instrument. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values. Melting points are uncorrected and were determined in open capillary tubes on a SMP1 apparatus (Stuart Scientific Staffordshire). The mp's of all crude compounds were within −3 °C, if compared with the pure product: therefore, as synthetic intermediates they could be used without further purification. The purity of compounds was checked by thin layer chromatography on Merck silica gel 60 F-254 plates. All commercial chemicals were purchased from Aldrich, Fluka, Merck, Lancaster and Carlo-Erba and were used without further purification.

The synthesis of [(3-amino-4-oxo-3,4-dihydroquinazolin-2-yl)thio](phenyl)-acetic acid **1** was reported in Santagati et al.[35]

The synthesis of [(3-amino-5,6-dimethyl-4-oxo-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-yl)thio](phenyl)acetic acid **2** was reported in Santagati et al.[36]

[(3-Amino-4-oxo-3,4-dihydrothieno[3,2-*d*]pyrimidin-2-yl)thio] (phenyl)acetic acid **3** was prepared according to the same procedure adopted for compound **2**; yield 60%, as a white powder, pure at TLC, mp 113–15 °C dec.; IR: 3313 and 3200 (NH_2), 1729 and 1659 (C=O) cm^{-1}.

[(3-Amino-6-bromo-4-oxo-3,4-dihydroquinazolin-2-yl)thio] (phenyl)acetic acid **4**, as compound **3**; yield 25%, as white powder, pure at TLC; mp > 125 °C dec.; IR: 3324 and 3210 (NH_2), 1721 and 1678 (C=O) cm^{-1}.

2-[(3-Amino-4-oxo-6-phenyl-3,4-dihydrothieno[2,3-*d*]pyrimidin-2-yl)thio]propanoic acid **5** was prepared according to the same method adopted for compound **7**; yield 35%, as a white powder, pure at TLC; mp > 265 °C dec.; IR: 3301 and 3191 (NH_2), 1715 and 1680 (C=O) cm^{-1}; ^{1}H NMR: δ 5.35 (s, 1H, CH), 5.92 (s, 2H, NH2), 7.32–7.78 (m, 11H, Ar–H and H-thiophene), 13.00 (s, 1H, COOH).

2-[(3-Amino-4-oxo-4,5-dihydro-3H-pyrimido[5,4-b]indol-2-yl) thio]propanoic acid **6**, as compound **5**; yield 65%, as a powder, pure at TLC; mp 239–241 °C dec.; IR: 3324, 3271 and 3185 (NH and NH_2), 1700 and 1661 (C=O) cm^{-1}; ^{1}H NMR: δ 1.57 (d, J = 7.6, CH_3), 4.36 (q, J = 7.6, CH), 5.89 (s, 2H, NH_2), 7.17–7.95 (m, 4H, Ar–H), 11.99 (s, 1H, NH).

The synthesis of 2-[(3-amino-4-oxo-3,4-dihydro[1]benzothieno[3,2-*d*]pyrimidin-2-yl)thio]propanoic acid **7** was reported in Santagati et al.[37]

2-[(3-Amino-4-oxo-3,4-dihydroquinazolin-2-yl)thio]propanoic acid **8**, as compound **5**, yield 10%, as a white powder, pure at TLC, mp 260–63 °C dec.; IR: 3313 and 3204 (NH_2), 1710 and 1694 (C=O).

3-Amino-2-(cyclohexylthio)-6-phenylthieno[2,3-d]pyrimidin-4(3H)-one **9**: A mixture of sodium salt of 3-amino-2,3-dihydro-6-phenyl-2-thioxothieno[2,3-*d*]pyrimidin-4(1*H*)-one (200 mg, 0.67 mmol), cyclohexyl iodide (0.1 ml, 98% d = 1.624, 0.75 mmol) in dimethylformamide (2 ml) was heated at 80 °C under stirring for 5 h.[38] After cooling to room temperature the mixture was poured in to water (100 ml): a solid separated. The solid was collected, treated with sodium hydroxide, collected, washed with water, dried first at room temperature and then under vacuum to give **9** as a green-yellow powder, pure at TLC, mp: 208–10 °C dec.; yield 50%; IR: 3301 and 3191 (NH_2), 2928 [$(CH_2)_6$], 1679 (C=O) cm^{-1}; ^{1}H NMR (dimethylsulfoxide-d_6): δ 1.22–2.03 (m, 10H, cyclohexyl), 3.40–3.79 (m, 1H, S–CH), 5.76 (s, 2H, NH_2), 7.34–7.84 (m, 6H, Ar–H and thiophene-H). Anal. Calcd for $C_{18}H_{19}N_3OS_2$ (MM 357): C, 60.50; H, 5.30; N, 11.75; S, 17.92. Found: C, 60.15; H, 5.00; N, 12.00; S, 18.75.

3-Amino-2-[(2,4-dinitrophenyl)thio]-6-phenylthieno[2,3-d]pyrimidin-4(3H)-one **10**: To a solution of sodium salt of 3-amino-2,3-dihydro-6-phenyl-2-thioxo-thieno[2,3-*d*]pyrimidin-4(1*H*)-one (200 mg, 0.67 mmol) in water (10 ml), 2,4-dinitroiodobenzene (200 mg, 98%, 0.67 mmol) in ethanol (10 ml) and powdery copper (20 mg) were added; the mixture was heated at 80 °C under stirring for 2 h.[38] An orange precipitate formed. The mixture was filtered while warm. The precipitate was washed with warm ethanol, treated with sodium hydroxide, collected, washed with water and dried first at room temperature and then at 100 °C to give **10** as an yellowish powder; yield 40%; mp 213–15 °C dec.; IR: 3305 and 3189 (NH_2), 1681 (C=O) cm^{-1}. Anal. Calcd for $C_{18}H_{11}N_5O_5S_2{\cdot}2H_2O$ (MM 477): C, 45.28; H, 3.14; N, 14.67; S, 13.41. Found: C, 45.31; H, 3.00; N, 14.30; S, 13.80.

N-[2-[(2,4-dinitrophenyl)thio]-4-oxo-6-phenylthieno[2,3-d]pyrimidin-3(4H)-yl]methanesulfonamide **11**: To a solution of *N*-(4-oxo-6-phenyl-2-thioxo-1,4-dihydrothieno[2,3-*d*]pyrimidin-3(2*H*)methanesulfonamide (100 mg, 0.28 mmol), and sodium hydroxide (30 mg, 0.75 mmol) in water (20 ml) 2,4-dinitroiodobenzene (90 mg, 98%, 0.3 mmol) in ethanol (5 ml) and powdery copper (20 mg) were added and the mixture was heated at reflux under stirring;[38] after 6 h the mixture was filtered while hot and the filtrate was cooled to room temperature and poured in to water; the solution was acidified with concentrated hydrochloride acid to pH 3–4: the resulting solid was collected, washed with water, dried first at room temperature and then under vacuum and crystallized from ethanol/water to give **11** as an orange powder, pure at TLC; yield 60%; mp < 130 °C dec.; IR: 3210 br (NH), 3100 (C–H benzene), 1705 (C=O), 1345 and 1160 (N–SO_2) cm^{-1}; ^{1}H NMR (dimethylsulfoxide-d_6): d 3.39 (s, 3H, CH_3), 7.32–8.89 (m, 9H, Ar–H and H-thiophene), 11.68 (s, 1H, NH). Anal. Calcd for C_{19} H_{13} N_5 0_7 S_3 (MM 519.5): C, 43.88; H, 2.50; N, 13.47; S, 18.47. Found: C, 43.77; H, 2.47; N, 13.75; S, 18.58.

4.3. Determination of COX-1 activity in human whole blood

Aliquots of 1 ml of whole blood drawn from the volunteers, students at the University of Catania, prior to aspirin administration, were immediately transferred into glass tubes and allowed to clot

at 37 °C for 60 min. The test derivatives were added in 2 μl of DMSO, to attain the final concentration of 10 μM—blanks received DMSO. Serum was separated by centrifugation (10 min at 2000 rpm) and kept at −70 °C until assayed for TxB_2 production by specific enzyme-immunoassay kit (RPN 220 Amersham Biosciences, USA). TxB_2 was determined according to the instructions provided by the manufacturer of the kit. The results were expressed as percent of inhibition of TxB_2 production relative to control (blanks) incubation containing DMSO (vehicle). The detection limit was 29 pg/ml.

4.4. COX-2 induction and human whole blood assay

Aliquots of 1 ml of whole blood samples drawn from young healthy volunteers, students at the University of Catania (both male and female), 48 h after oral administration of 300 mg of aspirin, to suppress the contribution platelet, PGHS-1 (COX-1), and containing 10 U.I. of sodium heparin, were incubated either in the absence or in the presence of LPS (10 μg/ml) for 24 h at 37 °C, to obtain the maximum production of PGE_2.[39] The test compounds were added in 2 μl of DMSO before stimulation by LPS to attain the final concentration of 10 μM except blanks, which received DMSO. After incubation, the plasma was separated by centrifugation (10 min at 2000 rpm) and kept at −70 °C until assayed for PGE_2 by a specific enzyme-immunoassay kit (Prostaglandin E_2, Biotrak, EIA System, Amersham Biosciences, USA).

4.5. Cell cultures

The J774 cells, a mouse monocyte-macrophage cell line, were obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in DMEM containing 10% fetal calf serum, 4.5 g/l glucose, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml fungizone (InVitrogen, UK) and incubated at 37 °C and 5% CO_2. The medium was changed every three days and subcultures were performed every 8–10 days. Experimental J774 cells were plated in 24-well cultures plated at a density of 2.5×10^5 cells/ml and allowed to adhere at 37 °C in 5% CO_2/95% O_2 for 24 h. After this time, the cells were stimulated with LPS (5 μg/ml) for 72 h to induce COX-2 in the absence (DMSO alone) or presence of the test compounds with concentrations of 10 μM. The supernatants were collected for the measurement of PGE_2 by a specific enzyme-immunoassay kit (Prostaglandin E_2, Biotrak, EIA System, Amersham Biosciences, USA), and the determination of NO by colorimetric assay.

4.6. PGE_2 determination

The concentration of PGE_2 was measured in the human whole blood and J774 culture media by biotrak enzyme-immunoassay (EIA) system according to the manufacturer's instructions (Amersham Biosciences, USA). The assay is based on competition between unlabeled PGE_2 and a fixed quantity of peroxidase-labeled PGE_2 for a limited number of binding sites on a PGE_2 specific antibody. Briefly, 50 μl of supernatant was dispensed into 96 wells microplate and added of 50 μl of diluted antibody and 50 μl of diluted conjugate. The plate was incubated at room temperature for 1 h. Afterwards, all wells were washed four times with wash buffer and 150 μl of room temperature equilibrate enzyme substrate was added to each well. The plate was mixed on a microtitre plate shaker for 30 min at room temperature. The reaction was stopped by the addition of 100 μl of sulfuric acid 1 M to each well. The optical density of each sample was measured with a microplate spectrophotometer reader (Titertek Multiskan, DAS, Italy) at $\lambda = 450$ nm within 30 min. A calibration curve was generated by using as a PGE_2 standard, part of the commercial kit and ready for use. This opportunely diluted provides the top standard of 320 pg/50 μl employed for the serial dilutions (8 standard levels). PGE_2 was measured in the range 2.5–320 pg/well (50–6400 pg/ml).

4.7. NO determination

The concentration of nitrite and nitrate in the supernatant of J774 culture media was quantified by colorimetric assay based on the Griess[40] reaction, as described by Ding et al.[41] Briefly, 0.1 ml of supernatant from untreated or treated J774 cultures was mixed with an equal volume of Griess reagent (a 1:1 mixture of 0.1% naphthylenediamine dihydrochloride and 1% sulfanilamide in 5% H_3PO_4) at room temperature for 10 min. The absorbance was measured at λ 550 nm in a microplate spectrophotometer reader (Titertek Multiskan, DAS, Italy). Sodium nitrite was used as a standard. The detection limit was 1 μM.

As control, to verify the effect of an inhibitor of NO generation on levels of nitrite/nitrate, N^G-monomethyl-L-arginine (N^GMMA) was used, dissolved in the medium and employed at the concentration of 500 μM (124 μg/ml).

4.8. Statistical analysis

Each experiment was repeated at least three times in triplicate and the mean ± SEM for each value was calculated. Statistical analysis of the results was performed using Student's *t*-test and one-way ANOVA by the statistical software package SYSTAT, version 9 (Systat Inc., Evanston IL, USA). A difference was considered significant at $P < 0.01$.

Acknowledgment

This work was supported in part by grants from the project of Ministero Istruzione Università Ricerca (MIUR).

References and notes

1. Funk, C. D.; Funk, L. B.; Kennedy, M. E.; Pong, A. S.; Fitzgerald, G. A. *FASEB J.* **1991**, *5*, 2304.
2. Goppelt-Struebe, M. *Biophys. Acta* **1995**, *1256*, 257.
3. Smith, W. L.; Meade, E. A.; DeWitt, D. L. *Ann. N.Y. Acad. Sci.* **1994**, *744*, 50.
4. Grosser, T.; Fries, S.; Fitzgerald, G. A. *J. Clin. Invest.* **2006**, *116*, 4.
5. Sanchez, T.; Moreno, J. J. *Prostaglandins Other Lipid Mediators* **1999**, *57*, 119.
6. Seibert, K.; Zhang, Y.; Leahy, K.; Hauser, S.; Masferrer, J.; Perkins, W.; Lee, L.; Isakson, P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12013.
7. DeWitt, D. L. *Biochim. Biophys. Acta* **1991**, *1083*, 121.
8. Oshima, T.; Yoshimoto, T.; Yamamoto, S.; Kumegawa, M.; Yokoyama, C.; Tanabe, T. *J. Biol. Chem.* **1991**, *266*, 13621.
9. Seibert, K.; Masferrer, J. L. *Receptor* **1994**, *4*, 17.
10. Crofford, L. J.; Wilder, R. L.; Ristimaki, A. P.; Sano, H.; Remmers, E. F.; Epps, H. R.; Hla, T. *J. Clin. Invest.* **1994**, *93*, 1095.
11. Kujubu, D. A.; Fletcher, B. S.; Varnum, B. C.; Lim, R. W.; Herschman, H. R. *J. Biol. Chem.* **1991**, *266*, 12866.
12. O'Banion, M. K.; Winn, V. D.; Young, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4888.
13. Fu, J. Y.; Masferrer, J. L.; Seibert, K.; Raz, A.; Needleman, P. *J. Biol. Chem.* **1990**, *265*, 16737.
14. Masferrer, J. L.; Zweifel, B. S.; Seibert, K.; Needleman, P. *J. Clin. Invest.* **1990**, *86*, 1375.
15. Lee, S. H.; Soyoola, E.; Chanmugam, P.; Hart, S.; Sun, W.; Zhong, H.; Liou, S.; Simmons, D.; Hwang, D. *J. Biol. Chem.* **1992**, *267*, 25934.
16. O'Sullivan, M. G.; Huggins, E. M., Jr.; Meade, E. A.; DeWitt, D. L.; McCall, C. E. *Biochem. Biophys. Res. Commun.* **1992**, *187*, 1123.
17. Sano, H.; Hla, T.; Maier, J. A.; Crofford, L. J.; Case, J. P.; Maciag, T.; Wilder, R. L. *J. Clin. Invest.* **1992**, *89*, 97.
18. Sirois, J.; Richards, J. S. *J. Biol. Chem.* **1992**, *267*, 6382.
19. Masferrer, J. L.; Seibert, K.; Zweifel, B.; Needleman, P. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 3917.
20. Masferrer, J. L.; Reddy, S. T.; Zweifel, B. S.; Seibert, K.; Needleman, P.; Gilbert, R. S.; Herschman, H. R. *J. Pharmacol. Exp. Ther.* **1994**, *270*, 1340.
21. Vane, J. R.; Bakhle, Y. S.; Botting, R. M. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *38*, 97.
22. Bateman, D. N. *Lancet* **1994**, *343*, 1051.
23. Kim, S. F.; Huri, D. A.; Snyder, S. H. *Science* **2005**, *310*, 1966.
24. Appleton, I.; Tomlinson, A.; Willoughby, D. A. *Adv. Pharmacol.* **1996**, *35*, 27.

25. Santagati, A.; Granata, G.; Marrazzo, A.; Santagati, M. *Arch. Pharm. (Weinheim)* **2003**, *336*, 429.
26. Granata, G.; Barbagallo, S.; Marrazzo, A.; Santagati, A.; Lombardo, L.; Cardile, V. *J. Heterocyl. Chem.* **2006**, *43*, 1099.
27. Brideau, C.; Kargman, S.; Liu, S.; Dallob, A. L.; Ehrich, E. W.; Rodger, I. W.; Chan, C. C. *Inflamm. Res.* **1996**, *45*, 68.
28. Balazy, M.; Poff, C. D. *Curr. Vasc. Pharmacol.* **2004**, *2*, 81.
29. Kermorvant-Duchemin, E.; Sennlaub, F.; Sirinyan, M.; Brault, S.; Andelfinger, G.; Kooli, A.; Germain, S.; Ong, H.; d'Orleans-Juste, P.; Gobeil, F.; Zhu, T.; Boisvert, C.; Hardy, P.; Jain, K.; Falck, J. R.; Balazy, M.; Chemtob, S. *Nat. Med.* **2005**, *11*, 1339.
30. Tsai, A. L.; Wei, C.; Kulmacz, R. J. *Arch. Biochem. Biophys.* **1994**, *313*, 367.
31. Yang, T.; Zhang, A.; Pasumarthy, A.; Zhang, L.; Warnock, Z.; Schnermann, J. B. *Am. J. Physiol. Renal Physiol.* **2006**, *291*, F891.
32. Bing, R. J.; Miyataka, M.; Rich, K. A.; Hanson, N.; Wang, X.; Slosser, H. D.; Shi, S. R. *Clin. Cancer Res.* **2001**, *7*, 3385.
33. Gasparini, G.; Longo, R.; Sarmiento, R.; Morabito, A. *Lancet Oncol.* **2003**, *4*, 605.
34. Balsamo, A.; Mancini, F.; Milanese, C.; Orlandini, E.; Ortore, G.; Pinza, M.; Rapposelli, S.; Rossello, A. *Il Farmaco* **2003**, *58*, 707.
35. Santagati, A.; Modica, M.; Monsù Scolaro, M.; Santagati, M. *J. Chem. Res. (S)* **1999**, *5*, 86.
36. Santagati, A.; Modica, M.; Santagati, M. *J. Heterocycl. Chem.* **2000**, *37*, 1161.
37. Santagati, A.; Granata, G.; Marrazzo, A.; Santagati, M. *Arch. Pharm. Pharm. Med. Chem.* **2003**, *336*, 429.
38. Santagati, A.; Marrazzo, A.; Granata, G. *J. Heterocycl. Chem.* **2003**, *40*, 869.
39. Patrignani, P.; Panara, M. R.; Greco, A.; Fusco, O.; Natoli, C.; Iacobelli, S.; Cipollone, F.; Ganci, A.; Creminon, C.; Maclouf, J. *J. Pharmacol. Exp. Ther.* **1994**, *271*, 1705.
40. Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. *Anal. Biochem.* **1982**, *126*, 131.
41. Ding, A. H.; Nathan, C. F.; Stuehr, D. J. *J. Immunol.* **1988**, *141*, 2407.