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Synthesis and Effects on the COX-1 and COX-2 Activity in Human Whole Blood *ex vivo* of Derivatives Containing the [1]Benzothienol-[3,2-*d*]pyrimidin-4-one Heterocyclic System

Methyl and phenyl derivatives containing the [1]Benzothieno[3,2-*d*]pyrimidin-4-one system have been synthesized and tested as inhibitors of COX-1 and COX-2 activities in human whole blood (HWB) *ex vivo*; all compounds turned out to be weak inhibitors of COX-1 activity, as deduced from the TXB_2 (thromboxane B) generation; the acid phenyl derivative **11 b** was an interesting inhibitor of COX-2 activity, as deduced from the PGE_2 (prostaglandine E) generation.

Keywords: [1]Benzothieno [3,2-*d*] pyrimidin-4-one; Cyclooxygenase (COX-1, COX-2); NSAIDs (non-steroidal anti-inflammatory drugs); selective COX-2 inhibitors

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Introduction

For many years, we have been interested in the synthesis of derivatives containing the thienopyrimidine system in order to find compounds endowed with anti-inflammatory and analgesic activity; several of these tested *in vivo* [1] showed interesting effects, comparable to or slightly higher than those of phenylbutazone, with very low acute toxicity and no ulcerogenic activity. We have assumed that these derivatives act like non-steroidal anti-inflammatory drugs (NSAIDs), but we did not exclude other possible mechanisms of action.

NSAIDs act mainly as inhibitors of the cyclooxygenase (COX) activity of prostaglandin endoperoxide synthase (PGHS); COX catalyses the first committed step in the arachidonic acid metabolism, allowing the synthesis of prostanoids, as for instance PGG_2 and PGH_2, precursors of the prostaglandins (PGE_2, PGD_2, and PGF_2), prostacyclins (PGI_2), and thromboxane (TXA_2). Two COX isoforms, named COX-1 and COX-2 [2], have been characterized so far.

COX-1 is a constitutive isoform present in almost all cell types and tissues [3], including stomach, intestine, kidney, and platelets. It is thought to be involved in homeostatic functions of prostanoids, including gastric mucosal protection, control of kidney function, and platelet function [4]; thus, its inhibition in association with the clinical use of NSAIDs is supposed to cause side effects like ulcers and renal failure [5].

COX-2 is inducible and is stimulated by mediators such as growth factors, mitogens, and cytokines, and by inflammatory stimuli [6–12]; it is the predominant cyclooxygenase at the site of inflammation. COX-2 exhibits both the cyclooxygenase and the peroxidase activities and is thought to be involved in the prostanoid synthesis, mediating pathological processes like inflammation and fever; therefore, its inhibition alleviates inflammation effects [2].

Although COX-2 is considered an inducible enzyme, it has also been found to be constitutively expressed in the kidney [13], the spinal cord [14], the brain [15], and in numerous other organs [16]; moreover, recent clinical studies have proposed several roles for COX-2 in tumorigenesis [17] and in Alzheimer's disease [18].

Considering the above mentioned facts, selective COX-2 inhibitor substances could primarily provide anti-inflammatory agents missing the side effects associated with classical non-selective NSAID's [19]. However, the currently available selective COX-2 anti-inflammatory drugs are not devoid of undesirable or even lethal effects e.g., cardiovascular failure [20].

Recently, we have established new methods [21] for the high yield synthesis of a number of derivatives containing the above heterocyclic system with various functional groups; this allows us to test derivatives of several homologous series as COX inhibitors and to describe some structure-activity relations. In order to determine

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whether these compounds selectively interact with COX-1 or COX-2 we selected, after evaluating the available methods, the test of human whole blood (HWB), according to Patrono et al. [22] and Patrignani et al. [12].

The test evaluates the effects of a drug on COX-2 activity, after its induction by lipopolysaccharide (LPS) on samples of heparinized human peripheral blood drawn from healthy volunteers by measuring the generation of prostaglandin E_2 (PGE_2). The effects on COX-1 are evaluated in samples of human clotted blood, drawn from the same healthy volunteers, through the measurement of thromboxane B_2 (TXB_2) generation; this reflects maximally stimulated cyclooxygenase activity of platelet PGHS-1 (COX-1) by endogenously formed thrombin in clotting blood [12, 22].

This test gives an important indication on the selectivity of COX for a given compound [23], but its significance is obviously restricted to the test system [24], considering that the percentage of inhibition of COX-1 and COX-2 activity is determined by two different products and not through all the products of the above mentioned activities of COX, after only two different periods of incubation [12, 22], and that the COX-2 is induced artificially. Thus a factor of selectivity could be partial. However, according to Pairet and van Ryn [23], the plasma proteins present in whole blood also allow some representation of the *in vivo* interactions in the presence of NSAIDs.

On this basis, we planned to synthesize derivatives of several homologous series containing the thienopyrimidine system for testing *in vitro*, according to [12, 22], and selecting those derivatives with higher potency and specificity for COX-2 activity in the generation of PGE_2. These compounds will be subsequently screened *in vivo* for their anti-inflammatory activity (inhibition of carrageenin-induced rat paw oedema with behavioral and acute toxicity tests) with the aim of designing a lead molecule.

In this paper, we report on the synthesis and the effects on COX-1 and COX-2 in human whole blood of methyl and phenyl derivatives containing the [1]Benzothieno[3,2-*d*]pyrimidin-4-one system. The choice of this heterocyclic system to be tested as the first one of the above plan was based on the remarkable analgesic and interesting anti-inflammatory actions with no ulcerogenic effect of the ethyl thiadiazole derivative **5** [25]; we have prepared series of methyl and phenyl derivatives to elucidate the influence of the simplest alkyl or aromatic group.

Chemistry

The syntheses of the [1]Benzothieno[3,2-*d*] pyrimidine derivatives are demonstrated in Scheme 1. Intermediate key substances were the amino-thioxo derivatives **3** and **4**. They were obtained by starting from the isothiocyanate **2**, prepared without production of pollutants, in acetone at room temperature from the amino ester **1** and thiophosgene and subsequent dilution with water. The reaction with hydrazine hydrate at room temperature and subsequent treatment of the obtained mixture of thiosemicarbazide and amino-thioxo derivatives with KOH in refluxing ethanol afforded the potassium salt **3**, which was treated with HCl to yield the amino-thioxo derivative **4**. The structure of the amino-thioxo derivatives was confirmed by the independent preparations of the ethyl derivative **5**. The compound obtained from the condensation of amino-ester 1 with 2-chloro-5-phenyl-1,3,4-thiadiazole, as reported by Russo et al. [25], was identical to that prepared from amino-thioxo 4 and propionic acid under appropriate reaction conditions. Analytical and spectral data are in accordance with the proposed structures.

The methyl esters **6 a**, **b** and the cyclic amides **7 a**, **b** were prepared by starting from the potassium salt **3** and the appropriate methyl ester of the 2-bromo-acid derivative, respectively and subsequent alkaline hydrolysis in methanol.

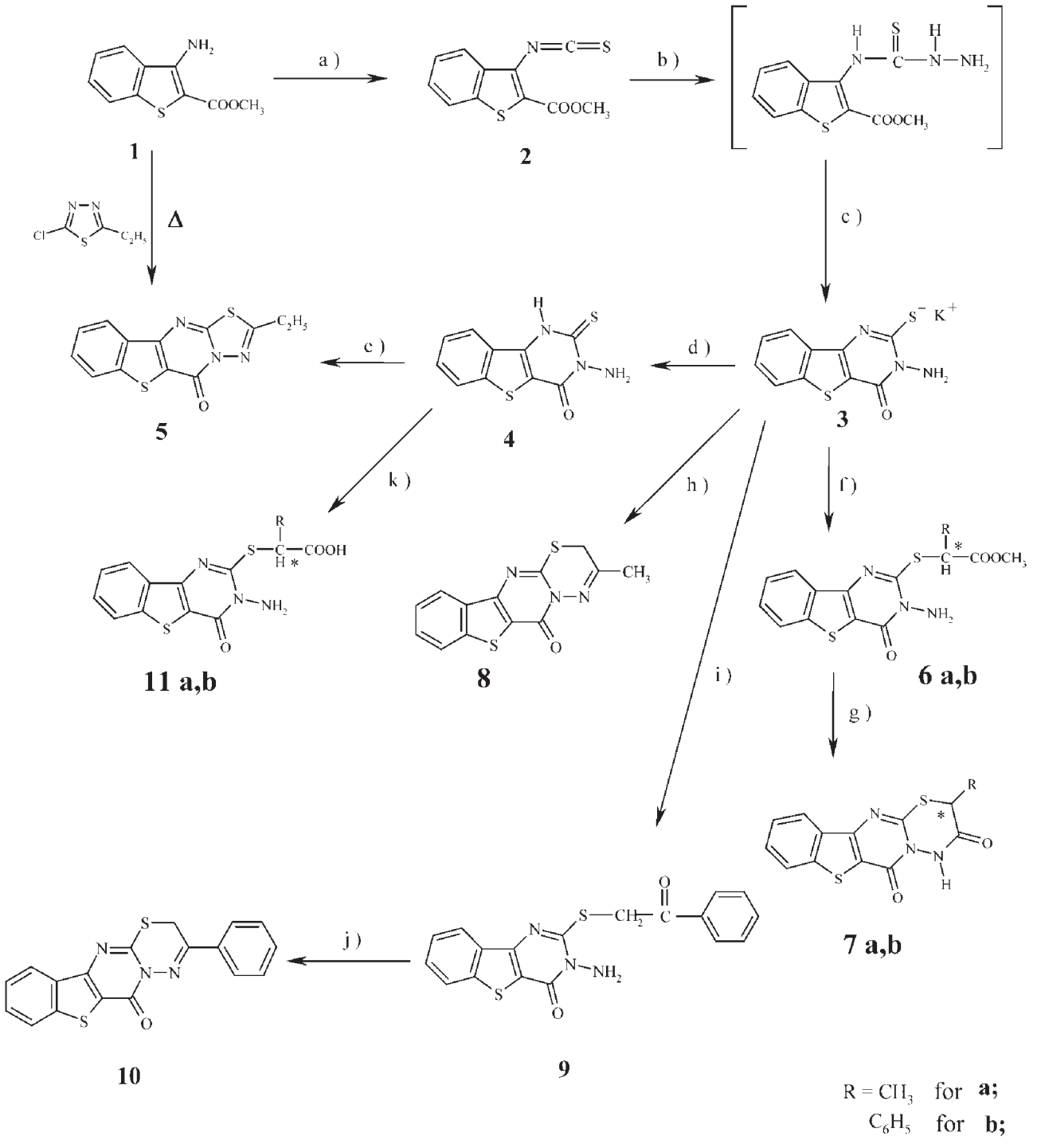
The methyl **8** and phenyl **10** derivatives of the new heterocyclic system [1]Benzothieno[3′,2′,4,5]pyrimido[2,1-*b*][1,3,4]thiadiazin-6-one were obtained by heating the potassium salt **3** with chloroacetone in ethanol and the thio-derivative **9** in presence of p-toluenesulfonic acid monohydrate (p-TSA), respectively.

The acid derivatives **11 a** and **11 b** were obtained by reacting the above amino-thioxo derivative **4** with 2-bromopropionic or α-bromophenilacetic acid, respectively at room temperature in tetrahydrofuran in presence of trietylamine.

The analytical and spectral data are in accordance with the proposed structure for all the above methyl and phenyl derivatives; in particular, the methyl substituted **6 a**, **7 a**, and **11 a** show the expected multiplet pattern in the ^{1}H NMR spectra.

Pharmacology

We have evaluated the ability of compounds **5**, **7 a**, **7 b**, **8**, **10**, **11 a**, and **11 b** at concentrations of 1, 10, and 100 μM to inhibit the cyclooxygenase activity of prostaglandin endoperoxide synthase-2 (PGHS-2) or (COX-2) in peripheral whole blood versus prostaglandin endoperoxide synthase-1 (PGHS-1) or (COX-1) *in vitro* monitoring the production of PGE_2 and TXB_2, respectively [12, 22] Indomethacin was tested as reference drug and as a positive control, being most active on both COXs. The methyl ester derivatives **6 a** and **6 b** were not tested, because



Scheme 1. Synthesis of [1]Benzothieno[3,2-*d*]pyrimidin-4-one derivatives. Reagents and conditions: a) Cl_2CS in CH_3COCH_3, room temperature, stirring 1 h; b) $NH_2NH_2 \cdot H_2O$ in CH_2Cl_2,room temperature, stirring 2 h; c) KOH in C_2H_5OH, reflux 30 min; d) H_2O/HCl, room temperature, stirring 30 min; e) P_2O_5, CH_3CH_2COOH, CH_3SO_3H, 115 °C, stirring 60 min; f) $CH_3CH(Br)COOCH_3$ for ***6 a***, $C_6H_5CH(Br)COOCH_3$ for **6 b** in C_2H_5OH, reflux 1 h; g) NaOH in CH_3OH, room temperature, stirring 1 h., HCl; h) $ClCH_2COCH_3$ in ethanol, reflux 2 h; i) $BrCH_2COC_6H_5$ in C_2H_5OH, room temperature, stirring 2 h, H_2O, j) p-TSA in C_2H_5OH, reflux, stirring 2 h; k) $CH_3CH(Br)COOH$ for **11 a**, $C_6H_5CH(Br)COOH$ for **11 b**, in THF, Et_3N, room temperature, stirring 48 h.

their structurally analogous derivatives showed low anti-inflammatory activities *in vivo* [1] and *in vitro* [26].

Results

Compounds **7 a**, **b** and **11 a**, **b** were tested as racemates. The results in per cent (%) of inhibition are summarized in Table 1. The ethyl thiadiazole derivative **5** was tested as reference substance, because of its interesting analgesic and anti-inflammatory activities, and no ulcerogenic activity, when tested *in vivo* [25]. The methyl and phenyl derivatives **7 a**, **7 b**, **8**, and **10** are, in contrast with the compound **5**, thiadiazine derivatives. The methyl **11 a** and phenyl **11 b** are acid derivatives, therefore they can interact with the guanidinic group of arginine at position120 (arginine ^{120}) of the active COX site of the enzyme [27].

Table 1. % inhibition of platelet COX-1 and LPS-induced monocyte COX-2 by the tested compounds in human whole blood (HWB).

Compound	% inhibition[a] of TXB_2 generation (COX-1)[b] at	% inhibition[a] of PGE_2 generation (COX-2)[c, d] at	
	100 μM	10 μM	100 μM
5	n.s.[e]	23.5	56.0
7 a	n.s.[e]	28.5	96.0
7 b	n.s.[e]	n.s[e]	n.s[e]
8	n.s.[e]	23.0	34.0
10	n.s.[e]	19.5	40.0
11 a	n.s.[e]	23.5	46.0
11 b	n.s.[e]	54.0[f]	58.5

[a] Assays were performed in duplicate or repeated, so standard errors are within ±10 %.
[b] Indomethacin at 1 μM: 42 %, at 10 μM: 85 %, and at 100 μM: 100 %.
[c] Indomethacin at 1 μM: 100 %, at 10 μM: 100 %, and at 100 μM: 100 %.
[d] except for compound **11 b**, the % of inhibition achieved at 1 μM was low and has not significant value.
[e] n.s. (not significant): the % of inhibition achieved was low and has not significant value.
[f] at 1 μM 43 %.

All compounds in the TXB_2 production test did not show a significant inhibition of the COX-1 activity, even at 100 μM, thus the percentages of inhibition are given only for this concentration (100 μM); they are not significant (n.s.).

Indomethacin showed percentages of inhibition of COX-1 from the TXB_2 test of 42, 85, and 100 % at 1, 10, and 100 μM, respectively, whereas it reached 100 % inhibition of COX-2 from the PGH_2 production test at concentrations of 1, 10, and 100 μM, respectively. These data confirmed that in this test, in accordance with [12], the affinity of indomethacin for COX-2 seems to be superior to that for COX-1, in contrast with a stated higher affinity for COX-1 [23, 24].

Ethyl thiadiazole **5**, the compound previously tested *in vivo* [25], exhibited an interesting inhibition value (56 %) of COX-2 activity only at 100 μM.

The amide derivative with methyl group **7 a** inhibited the production of PGE_2 from COX-2 activity by 96 % at 100 μM, and had a negligible effects on COX-1.

The amide derivative with phenyl group **7 b** showed negligible effects on both above COX-1 and COX-2 productions. The methyl acid derivative **11 a** showed a substantial inhibition of COX-2 activity only at 100 μM.

The phenyl acid derivative **11 b** exhibited low effects on COX-1 and a noticeable percentage of inhibition of COX-2 at all concentrations tested, although nearly constant.

Discussion

The methyl **8** and phenyl **10** thiadiazine substituted compounds show modest inhibition in the production of PGE_2 and also the anti-inflammatory activities of the corresponding thiadiazole derivatives [25] were as modest *in vivo*. These results could indicate that substitution on a larger ring does not influence the activity.

The inhibition the of PGE_2 production from COX-2 activity by the acid derivatives **11 a** and **11 b** could indicate that the acid group plays a role in COX-2 inhibition and that the phenyl group of compound **11 b** favors the activity. The nearly constant values of phenyl derivative acid **11 b** could be due to the saturation of the active site and/or the formation of a salt bond with the guanidinic group of arginine ^{120} in the active site [27] and/or competitive mechanisms, time-dependent and slowly reversible as proposed for the anti-inflammatory acidic agents Indomethacin, Flurbiprofen, and Diclofenac [27]. However, the range of percentage of inhibition of the above mentioned acid 11b is in the same order as of that for the acid drugs in the same tests [28]: Flurbiprofen IC_{50} 6.4 μM, Naproxen IC_{50} 73.7 μM, and Ibuprofen IC_{50} >30 μM.

Conclusions

The above data for all the derivatives show data low affinity for COX-1, thus indicating a role of the heterocyclic system. The percentages of COX-2 inhibition from production of PGE_2 do not indicate any trend in relation to the nature of substitute group. In the case of the ethyl

derivative **5** its interesting anti-inflammatory activity *in vivo* has been confirmed in part. The racemate phenyl-acid derivative **11 b** needs to be tested *in vivo* before being selected as a candidate lead compound; also, the reaction mechanism needs to be investigated.

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Experimental

Chemistry

Melting points were determined in open capillary tubes on a SMP1 apparatus (Stuart Scientific, Staffordshire, UK) and are uncorrected. IR spectra were recorded on a Perkin Elmer 1600 Series FT-IR in KBr disks (Perkin Elmer, Überlingen, Germany). Elemental analyses for C, H, N, and S were obtained on a Fisons-Carlo Erba EA1108 elemental analyzer (Milan, Italy) and were within ±0.4 of the theoretical values. ^{1}H NMR spectra were recorded at 200 MHz on a NMR Varian Gemini 200 spectrometer (Varian, Darmstadt, Germany) in DMSO-d_6 solution; chemical shifts (δ) are reported in ppm from TMS as internal standard; coupling constant (J) are in Hertz (Hz). The mp's of all crude compounds were within –3 °C, if compared with the pure product; therefore, the synthetic intermediates could be used without further purification. The purity of compounds was checked by TLC on Merck silica gel 60 F-254 plates (Merck, Darmstadt, Germany). All commercial chemicals were purchased from Aldrich (Gillingham-Dorset, UK), Fluka (Buchs, Switzerland), Merck, and Carlo Erba and were used without further purification.

Methyl of 3-isothiocyanate-[1]benzothiophene-2-carboxylate **2**

A solution of amino-ester **1** [29] (3.0 g, 14.0 mmol) in acetone (40 mL) was added dropwise at room temperature to a stirred solution of thiophosgene (1.2 mL, 97 %, d = 1.508) in acetone (15 mL). After 20 min of stirring at room temperature, water was added (200 mL): the solid separated, it was collected, washed with water until pH 7, dried, and crystallized from ethanol/water to give the isothiocyanate **2** as pale yellow microcrystals (2.45 g, 9.8 mmol, 70 %); m.p. 117–120 °C; IR (cm^{-1}): 2140 and 2105 (N=C=S); 1715 (C=O) as in other compounds; ^{1}H NMR (DMSO-d_6): δ 3.99 (s, 3 H, OCH_3), 7.40–8.15 (m, 4 H, aromatic).

Anal. Calcd. for $C_{11}H_7NO_2S_2$: C, 53.00; H, 2.80; N, 5.60; S 25.70 Found: C, 52.80; H, 2.65; N, 5.70; S, 25.30.

3-Amino-2-thioxo-[1]benzothieno[3,2-d]pyrimidin-4(1H)-one **4** *from its potassium salt* **3**

Isothiocyanate **2** (2.5 g, 10.0 mmol) dissolved in CH_2Cl_2 (30 mL) was added dropwise at room temperature to a stirred solution of $NH_2NH_2 \cdot H_2O$ (0.5 mL, 10.0 mmol) in CH_2Cl_2 (50 mL). The mixture was stirred at room temperature for 2 h; the resulting solid was collected, washed with CH_2Cl_2 and dried to give a white powder (2.3 g); this white powder, a mixture of the thiosemicarbazide derivative and the amino-thioxo derivative, was heated for 1 h under reflux while stirring in a solution of KOH (0.53 g, 9.5 mmol) in ethanol (190 mL). The resulting solid was collected, washed with warm dioxane, and dried to give the potassium salt **3** as a white amorphous powder (1.98 g, 7.0 mmol, 67 %); m.p. >310 °C dec.

IR (cm^{-1}): 3230 (br, NH_2); 3135 (NH_2,); 1640 (C=O).

Anal. Calcd. for $C_{10}H_6KN_3OS_2$: C, 41.80; H, 2.10; N, 14.60; S, 22.30. Found: C, 42.00; H, 2.00; N, 14.35; S, 22.25

To a suspension of the potassium salt of **3** (1.98 g, 7.0 mmol) in water (200 mL), conc. HCl was added dropwise under stirring until pH 3–4 was reached; the mixture was stirred for 30 min; the resulting solid was collected, washed with water, dried, and crystallized from dioxane to give the amino-thioxo derivative **4** as a white microcrystalline powder (0.87 g, 3.5 mmol, 50 %); m.p. >280 °C dec.;

IR (cm^{-1}): 3320 (NH_2); 3235 (NH_2); 3160 (NH); 1690 (C=O).

Anal. Calcd. for $C_{10}H_7N_3OS_2$: C, 48.20; H, 2.80; N, 16.85; S, 25.70. Found: C, 48.35; H, 2.95; N, 17.00; S, 25.55.

2-Ethyl-5H-[1]benzothieno[3,2-d]-1,3,4-thiadiazolo[3,2-a]pyrimidin-5-one **5**

A mixture of amino-thioxo derivative 4 (0.37 g, 1.5 mmol), P_2O_5 (0.3 g), propionic acid (1.0 mL, 99.5 %, d = 0.993), and methanesulfonic acid (0.6 mL, d = 1.481) was heated under stirring at 115 °C for 60 min. After cooling to room temperature, the reaction mixture was treated with ice/water and 5 % NaOH solution was added, to pH 7. The resulting solid was collected, washed with water, dried, and crystallized from ethanol to give compound **5** as pale yellow needles (0.26 g, 0.9 mmol, 60 %); m.p. 204–207 °C.

The analytical and spectral data of the ethyl derivative **5** were identical with those of a sample obtained according to Russo et al. [25].

IR (cm^{-1}): 1695 (C=O); ^{1}H NMR (DMSO-d_6): δ 1.35 (t, J = 6.2 Hz, 3 H, CH_3), 3.10 (q, J = 6.2 Hz, 2 H, CH_2), 7.35–8.30 (m, 4 H, aromatic).

Anal. Calcd. for $C_{13}H_9N_3OS_2$: C, 54.35; H, 3.15; N, 14.65; S 22.30 Found: C, 54.70; H, 3.35; N, 14.70; S, 22.50.

Methyl (±)-2-[(3-amino-3,4-dihydro-4-oxo-[1]benzothieno[3,2-d]pyrimidin-2-yl)thio]-propionate **6 a**

A mixture of potassium salt **3** (0.75 g, 2.6 mmol) and methyl 2-bromopropionate (0.3 mL, 2.6 mmol, 98 % d = 1.497) in ethanol (40 mL) was refluxed under stirring for 2 h and cooled to room temperature: the reaction mixture was poured into water (300 mL): the solid separated was collected, dried, and recrystallized from ethanol to give the methyl ester **6 a** as a white powder (0.74 g, 2.2 mmol, 85 %); m.p. 202–205 °C;

IR (cm^{-1}): 3295 and 3195 (NH_2); 1720 (C=O); 1670 (C=O); ^{1}H NMR (DMSO-d_6): δ 1.60 (d, J = 6.7 Hz, 3 H, CH_3), 3.65 (s, 3 H, OCH_3), 4.40 (q, J = 6.7 Hz, 1 H, CH), 6.00 (s, 2 H, NH_2) 7.55–8.15 (m, 4 H, aromatic).

Anal. Calcd. for $C_{14}H_{13}N_3O_3S_2$: C, 50.15; H, 3.90; N, 12.55; S 19.10. Found: C, 49.85; H, 4.00; N, 12.50; S, 18.95.

Methyl (±)-α-[(3-amino-3,4-dihydro-4-oxo-[1]benzothieno[3,2-d]pyrimidin-2-yl)thio]-phenylacetate **6 b**

A mixture of potassium salt **3** (0.63 g, 2.2 mmol) and methyl α-bromophenylacetate (0.4 mL, 97 %, d = 1.455) in ethanol (40 mL) was refluxed under stirring for 3 h and then cooled to room temperature. The mixture was poured into water; the resulting solid was collected, dried, and recrystallized from dioxane/water to give methyl ester **6 b** as a white powder (0.74 g, 1.9 mmol, 85 %); m.p. 230–232 °C dec.

IR (cm^{-1}): 3320 and 3270 (NH_2), 1735 (C=O), 1675 (C=O); ^1H NMR (DMSO-d_6): δ 3.70 (s, 3 H, OCH_3), 5.55 (s, 1 H, CH), 6.05 (s, 2 H, NH_2) 7.35–8.20 (m, 9 H, aromatic).

Anal. Calcd. for $C_{19}H_{15}N_3O_3S_2$: C, 57.43; H, 3.78; N, 10.58; S 16.12. Found: C, 57.70; H, 3.50; N, 10.75; S, 16.30.

(±)-2-Methyl-2H,6H-[1]benzothieno[3′,2′:4,5]pyrimido[2,1-b]-[1,3,4]thiadiazin-3,6-(4H)-dione **7 a**

A suspension of methyl ester **6 a** (0.39 g, 1.2 mmol) in a solution of NaOH (50 mg, 1.2 mmol) in methanol (10 mL) and water (2 mL) was stirred at room temperature for 24 h; the mixture was poured into water (100 mL) and filtered: by acidification of the resulting solution with conc. HCl to pH 4–5 a white solid separated; this solid was collected, washed with water, dried, and recrystallized from dioxane/water to give the dione **7 a** as a white amorphous powder (0.29 g, 0.96 mmol, 80 %); m.p. 263–265 °C dec.

IR (cm^{-1}): 3180 (br. NH), 1690 (br. C=O); ^1H NMR (DMSO-d_6): δ 1.45 (d, J = 7.2 Hz, 3 H, CH_3), 4.30 (q, J = 7.2 Hz, 1 H, CH), 7.55–8.25 (m, 4 H, aromatic), 12.05 (br s, 1 H, NH).

Anal. Calcd. for $C_{13}H_9N_3O_2S_2$: C, 51.48; H, 2.75; N, 13.85; S 21.10 Found: C, 51.70; H, 2.65; N, 13.70; S, 21.30.

(±)-2-Phenyl-2H,6H-[1]benzothieno[3′,2′:4,5]pyrimido[2,1-b]-[1,3,4]thiadiazin-3,6-(4H)-dione **7 b**

A suspension of methyl ester **6 b** (0.47 g, 1.2 mmol) in a solution of NaOH (50 mg, 1.2 mmol) in methanol (10 mL) and water (2 mL) was stirred at room temperature for 24 h; the mixture was poured into water (100 mL) and then filtered; by acidification of the resulting solution with conc. HCl to pH 4–5 a white solid separated; this solid was collected, washed with water, dried, and recrystallized from dioxane/water to yield the dione **7 b** as a white amorphous powder (0.32 g, 0.88 mmol, 73 %); m.p. 285–88 °C dec.

IR (cm^{-1}): 3200 (br NH), 1685 (br C=O); ^1H NMR (DMSO-d_6): δ 5.60, (s, 1 H, CH), 7.30–8.20 (m, 9 H, aromatic), 12.50 (s, 1 H, NH).

Anal. Calcd. for $C_{18}H_{11}N_3O_2S_2$: C, 59.17; H, 3.10; N, 11.50; S 17.55. Found: C, 59.45; H, 3.35; N, 11.70; S, 17.50.

3-Methyl-2H,6H-[1]benzothieno[3′,2′:4,5]pyrimido[2,1-b]-[1,3,4]thiadiazin-6-one **8**

A mixture of potassium salt **3** (0.37 g, 1.3 mmol) and chloroacetone (0.15 mL, 96 %, d = 1.16) in ethanol (20 mL) was refluxed for 2 h and then cooled to room temperature; the resulting precipitate was collected, washed with water, dried, and crystallized from ethanol to give the methyl derivative **8** as pale yellow needles (0.19 g, 0.65 mmol, 50 %); m.p. 258–261 °C dec.

IR (cm^{-1}): 1685 (C=O); ^1H NMR (DMSO-d_6): δ 2.40 (s, 3 H, CH_3), 3.85 (s, 2 H, CH_2), 7.55–8.20 (m, 4 H, aromatic).

Anal. Calcd. for $C_{13}H_9N_3OS_2$: C, 54.35; H, 3.15; N, 14.65; S 22.30 Found: C, 54.45; H, 3.05; N, 14.45; S, 22.30.

3-Amino-2-[(2-phenyl-2-oxo-ethyl)thio][1]benzothieno[3,2-d]-pyrimidin-4(1H)-one **9**

A mixture of potassium salt **3** (0.37 g, 1.3 mmol) and of 2-bromoacetophenone (0.26 g, 1.3 mmol) in ethanol (30 mL) was stirred at room temperature for 2 h. The resulting mixture was poured into water (300 mL); the solid separated was collected after 24 h, dried,and crystallized from ethanol/water to give compound **9** as pale yellow powder (0.37 g, 1.04 mmol, 80 %); m.p. 232–235 °C dec.

IR (cm^{-1}): 3275 and 3190 (NH_2); 1675 (br C=O); ^1H NMR (DMSO-d_6): δ 4.75 (s, 2 H, CH_2), 6.05 (s, 2 H, NH_2), 7.10–8.20 (m, 9 H, aromatic).

Anal. Calcd. for $C_{18}H_{13}N_3O_2S_2$: C, 58.85; H, 3.55; N, 11.45; S 17.45. Found: C, 59.05; H, 3.70; N, 11.65; S, 17.30.

3-Phenyl-2H,6H-[1]benzothieno[3′,2′:4,5]pyrimido[2,1-b][1,3,4]thiadiazin-6-one **10**

To a refluxing suspension of derivative **9** (0.30 g, 0.85 mmol) in ethanol (25 mL) *p*-toluenesulfonic acid monohydrate (p-TSA) (50 mg) was added. The mixture was refluxed for 1.5 h and then cooled to room temperature. The resulting solid was collected, washed with warm ethanol, dried, and recrystallized from dioxane/ethanol to give the phenyl derivative **10** as yellow microcrystals (0.22 g, 0.64 mmol, 75 %); m.p. 248–250 °C dec.;

IR (cm^{-1}): 1680 (C=O); ^1H NMR (DMSO-d_6): δ 4.40 (s, 2 H, CH_2), 7.55–8.20 (m, 9 H, aromatic).

Anal. Calcd. for $C_{18}H_{11}N_3OS_2$: C, 61.90; H, 3.15; N, 12.00; S 18.30. Found: C, 61.80; H, 3.00; N, 11.90; S, 18.45.

(±)-2-[(3-Amino-3,4-dihydro-4-oxo-[1]benzothieno[3,2-d]pyrimidin-2-yl)thio]-propionic acid **11 a**

A mixture of amino-thioxo derivative **4** (0.25 g, 1.0 mmol), 2-bromopropionic acid (0.1 mL, 99.0 %, d = 1.7), triethylamine (0.3 mL) in tetrahydrofuran (THF) (20 mL) was stirred at room temperature for 48 h. The resulting mixture was treated with 5 % $NaHCO_3$ solution and after filtration, the resulting solution was acidified with conc. HCl to pH 5–6; a white solid separated it was collected, washed with water, and dried to give the acid derivative **11 a** as an amorphous solid (0.16 g, 5 mmol, 50 %); tlc pure; m.p. 260–263 °C dec.

IR (cm^{-1}): 3305 and 3190 (NH_2); 1680 and 1660 (C=O);); ^1H NMR (DMSO-d_6): δ 1.60 (d, J = 7.6, 3 H, CH_3), 4.33 (q, J = 7.6, 1 H, CH), 6.00 (s, 2 H, NH_2) 7.54–8.23 (m, 4 H, aromatic).

Anal. Calcd. for $C_{13}H_{11}N_3O_3S_2$: C, 48.60; H, 3.45; N, 13.10; S 19.95. Found: C, 48.55; H, 3.70; N, 12.90; S, 19.75.

(±)-α-[(3-Amino-3,4-dihydro-4-oxo-[1]benzothieno[3,2-d]pyrimidin-2-yl)thio]-phenylacetic acid **11 b**

A mixture of amino-thioxo **4** (0.37 g, 1.5 mmol), α-bromo-phenylacetic acid (0.32 g, 1.5 mmol), triethylamine (0.2 mL) in tetrahydrofuran (THF) (20 mL) was stirred at room temperature for 2 days; the mixture was treated with 5 % $NaHCO_3$ (50 mL); after filtration the resulting solution was acidified with conc. HCl to pH 5–6. The separating solid was collected, washed with water, and dried to give the acid derivative **11 a** as a white amorphous powder (0.17 g, 4.5 mmol, 30 %); tlc pure; m.p. >235 °C dec.

IR (cm^{-1}): 3310 and 3190 (NH_2); 1710 and 1675 (C=O);); ^1H NMR (DMSO-d_6): δ 5.45 (s, 1 H, CH), 6.05 (s, 2 H, NH_2) 7.30–8.30 (m, 9 H, aromatic).

Anal. Calcd. for $C_{18}H_{13}N_3O_3S_2$: C, 56.40; H, 3.40; N, 10.95; S 16.70. Found: C, 56.55; H, 3.70; N, 10.90; S, 16.45.

Pharmacology

Subjects

Healthy volunteers (aged 27–32) were asked to consent to the drawing of blood from their peripheral veins and to take aspirin. The study protocol was in a accordance with the Italian laws (D.L.116/92, art. 5).

Peripheral venous blood samples were drawn at 10 am, before and 48 h after the oral administration of 300 mg of aspirin. The

donors had not taken any NSAIDs during 2 weeks preceding the study.

Materials

Heparin from porcine intestinal mucosa, lipopolysaccharide (LPS) from *Escherichia coli,* indomethacin and aspirin were purchased from Sigma Chemical (St. Louis, MO, USA) and dimethylsulfoxide (DMSO) was purchased from Aldrich. Prostaglandin E_2 enzyme immunoassay system and Thromboxane B_2 enzyme immunoassay system were purchased from Amersham Pharmacia Biotech Ltd. (Little Chalfont, UK).

Thromboxane 2 (TBX_2) and prostaglandin E_2 (PGE_2) levels were determined according to the instructions provided by the manufacturer of the kits. All assays were performed in duplicate and repeated; standard errors should be less than 10 %.

The results were expressed as percent of inhibition of TBX_2 or PGH_2 production relative to control (blanks) incubation containing DMSO (vehicle).

Test compounds were prepared as stock solutions in DMSO, and appropriate dilutions were made in DMSO. Preliminary experiments indicated that the addition of 2 and 10 µl of DMSO per mL of human blood had no effect on the release of TBX_2 and PGE_2. Thus, DMSO was used as the vehicle for all the tested compounds.

COX-1 activity in human whole blood

Aliquots of 1 mL of whole blood drawn from the volunteer, before aspirin administration, were immediately transferred into glass tubes and allowed to clot at 37 °C for 60 min. The test compounds were added in 2 µl of DMSO, to attain the final concentrations (1.0, 10, and 100 µM) 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).

Whole blood TXB_2 production was measured as a reflection of maximally stimulated cyclo-oxygenase activity (COX-1) of platelet PGHS-1 by endogenously formed thrombin during the clotting [12, 22].

COX-2 induction and activity in human whole blood

Aliquots of 1 mL of whole blood samples drawn from the same volunteer 48 h after oral administration of 300 mg of aspirin, to suppress the contribution of platelet PGHS-1 (COX-1), and containing 10 I.U. of sodium heparin, were incubated either in the absence or in the presence of lipopolysaccharide (LPS) (10 µg/mL) for 24 h at 37 °C, to obtain the maximum production of PGE_2 [12, 22]. The test compounds were added in 2 mL of DMSO before stimulation by LPS to attain the final concentrations (1.0, 10, and 100 µM).

After incubation, 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 (RPN 222, Amersham).

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