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FULL-LENGTH PAPER

Synthesis and biological evaluation of new benzo-thieno[3,2-d]pyrimidin-4-one sulphonamide thio-derivatives as potential selective cyclooxygenase-2 inhibitors

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Abstract The aim of this work was to evaluate the potential anti-inflammatory activity of eleven (**5–15**) new synthesized derivatives of benzo-thieno[3,2-d]pyrimidine on two cell models, namely human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774. For the synthesis of test compounds an efficient approach was developed: the key isothiocyanate was prepared through a simple and ecological method using di-2-pyridyl thionocarbonate (DPT) in substitution of thiophosgene, a highly toxic agent, and the cyclization reaction of benzo-thiosemicarbazide derivates was performed through Wamhoff methods. This procedure can be a new alternative method economically and environmentally advantageous by the simplicity of procedure, reduction of isolation and purification steps, time, costs, and waste production. The potential anti-inflammatory activity of **5–15** was evaluated by determining the expression of cyclooxygenase (COX)-2, inducible NO synthase (iNOS), and intercellular adhesion molecule-1 (ICAM-1), and the release of prostaglandins (PG)E_2 and interleukin-8 (IL-8). Our results demonstrate that the compounds **7**, **10**, **12**, **13**, **14**, and **15** act as a potent inhibitor of COX-2, iNOS, ICAM-1 expression while also suppressing the production of PGE_2 and IL-8 in human keratinocytes NCTC 2544 exposed to interferon-gamma (IFN-γ) and histamine and monocyte-macrophages J774 cells treated with lipopolysaccharides (LPS). In conclusion, some derivatives of benzo-thieno[3,2-d]pyrimidine could be developed as a novel class of anti-inflammatory agents.

Keywords Anti-inflammatory activity · Antipyrine · Benzo-thieno[2,3-d]pyrimidin-4-(3*H*)-ones · Cell cultures · COX-2 inhibitors · In vitro experiments

Electronic supplementary material The online version of this article (doi:10.1007/s11030-013-9443-0) contains supplementary material, which is available to authorized users.

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Introduction

Inflammation is a complicated response involving several cell types and many putative mediators and modulators [1]. It is well-documented that the majority of currently known non-steriodal anti-inflammatory drugs (NSAIDs) act peripherally by blocking the production of prostaglandins through inhibition of cyclooxygenase (COX) enzymes. COX is a membrane-bound heme protein which exists at least in two different isoforms, COX-1, and COX-2 [1,2]. It is responsible for the conversion of arachidonic acid to prostaglandins (PGs), and their metabolites play a pivotal role in multiple physiologic and pathophysiologic processes [3]. Constitutive COX-1, described as a "housekeeping" enzyme, is normally expressed in the gastrointestinal tract, kidneys, and platelets. Under the influence of COX-1, prostaglandins maintain the integrity of the gastric mucosa, mediate normal platelet function, and regulate renal blood flow [4]. In contrast, COX-2 is primarily associated with inflammation and induced in response to growth factors, such as endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2), cytokines, such

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as tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α), and interleukin-1β (IL-1β), and tumor promoters, such as v-src, v-Ha-ras, HER-2/neu, and Wnt [5–8]. These observations suggest that COX-1 and COX-2 serve different physiological and pathophysiological functions. However, this simplified paradigm of constitutive COX-1 and inducible COX-2 has many exceptions: COX-1 can be regulated during development [9], whereas COX-2 is constitutively expressed in the brain [10], reproductive tissues [11], and kidney [12–14].

Conventional NSAIDs inhibit both COX-1 and COX-2, their use is often accompanied by gastrointestinal side effects and renal function suppression. Therefore, molecular-based targeting strategies were employed to develop specific COX-2 inhibitors to circumvent the gastric and renal toxicities caused by mixed COX inhibitors [15,16]. Moreover, 1,2-diarylheterocyclic thienopyrimidine derivatives with sulphonamide functionality and other central ring pharmacophore templates have been extensively studied as important selective COX-2 inhibitors [17], with a high anti-inflammatory activity but lacking the undesirable side effects of most traditional NSAIDs [18].

Hence, several series of selective COX-2 inhibitors belonging to the 1,2-diaryl and thienopyrimidine classes of compounds containing different heterocyclic and carbocyclic moieties as a central scaffold have been developed. For optimal activity, all these tricyclic molecules possess a characteristic sulfonyl group such as a *para*-SO_2NH_2 or a *para*-SO_2Me substituent on one of the phenyl rings that interact with the COX-2 side pocket through slow, tight-binding kinetics. These tricyclic molecules play an important role on COX-2 selectivity [19]. Extensive literature review has been carried out to study the synthesis and biological effects of the thienopyrimidines that are potential bioactive molecules as they are structural analogs of biogenic purines and can be considered as potential nucleic acid antimetabolites. They are characterized by a broad spectrum of biological activities [18–22] and many thienopyrimidines have been found to exhibit a variety of pronounced activities, for example, as anti-inflammatory [18,19], antimicrobial [20], antiviral [21], and analgesic [22] agents.

Thus, we have focused our interests in the synthesis of a large number of thienopyrimidine derivatives in order to find compounds endowed with anti-inflammatory and analgesic activity and no or low ulcerogenic activity [23,24]. On the basis of recent developments in the research of NSAIDs without ulcerogenic effects, acting mainly as selective inhibitors of enzyme COX-2 [25,26], we have synthesized new derivatives of benzo-thieno[3,2-d]pyrimidine. These derivatives (**5**–**15**) with functional groups and substituents present in prototypical leads of above selective inhibitors of COX-2 [26], have been obtained through the methods of cyclization reported by Wamhoff, starting from heteroaromatic β-enaminoesters [27,28].

For the biological evaluation, the anti-inflammatory activity of new derivatives **5**–**15** was evaluated on in vitro human keratinocyte cell line NCTC 2544 exposed to interferon (IFN)-γ and histamine and on monocyte-macrophage J774 cell line stimulated with bacterial lipopolysaccharides (LPS), two cell models particularly useful for reproducing the mechanisms involved in inflammation and used by us in previous published studies [29,30].

Experimental section

Chemistry

General remarks

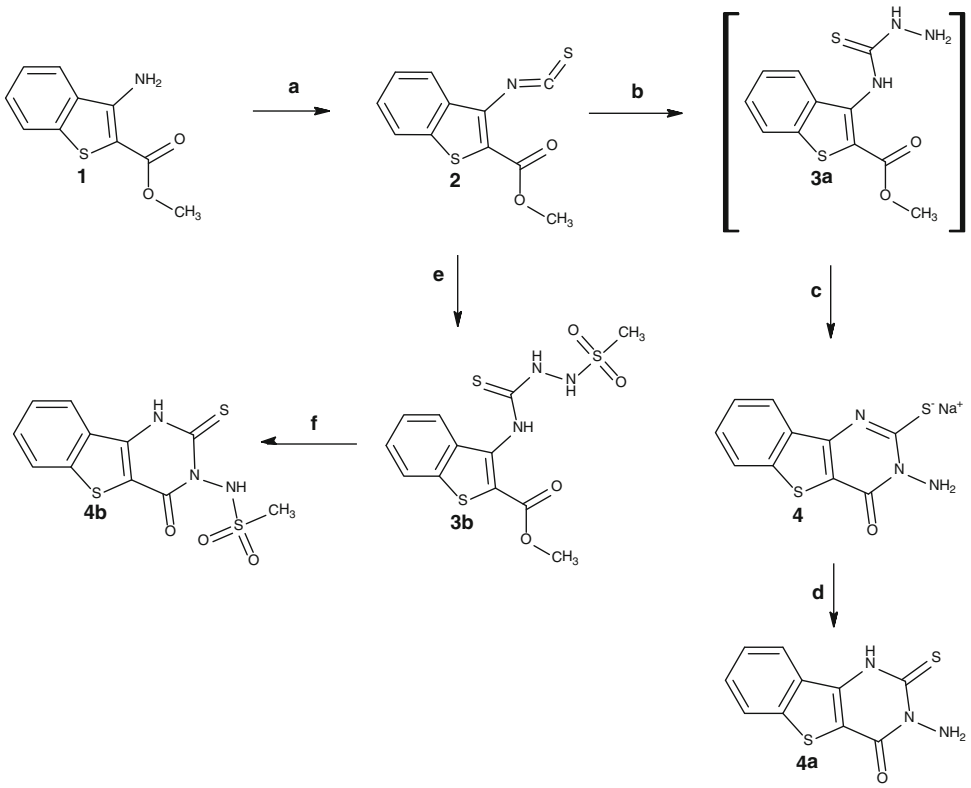
All commercial solvents and chemicals purchased from Sigma-Aldrich, Fluka, Merck, Lancaster, and Carlo-Erba were of analytic grade and used without further purification.

NMR spectra (^{1}H NMR were recorded at 500 MHz, ^{13}C NMR were recorded at 125 MHz) on a Varian Instruments; chemical shifts (δ) are reported in ppm from tetramethylsilane as an 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 were within $\pm$0.4 % of the theoretical values. Melting points were determined in open capillary tubes on a SMP1 apparatus (Stuart Scientific Staffordshire) and are uncorrected. The purity of substances was checked by thin layer chromatography on Merck silica gel 60 F-254 plates. Mass spectra were recorded by Perkin Elmer Turbo Mass Clarus 560 Mass Spectrometer, with a 70 eV working ionization energy, source temperature 250 °C, trap current 90 µA.

General procedure

The synthetic routes to prepare the benzo-thieno[3,2-d] pyrimidine derivates **5**–**15** by up-to-date methods are outlined in Schemes 1 and 2 that describe a facile and efficient domino one-pot methodology. Thus, the 3-amino-2-thiophene carboxylic ester starting materials **1** is easily prepared following the well-established procedure reported in the literature [31,32], but some modifications in the use of reactants and in the conditions of reaction that furnished the experimental benzothieno derivates and intermediates with high yields and without polluting residues were implemented.

The key isothiocyanate **2** was prepared in dichloromethane at room temperature through a simple method by the reaction of amino-ester **1** with di-2-pyridyl thionocarbonate (DPT) in substitution of thiophosgene, a highly toxic agent. The



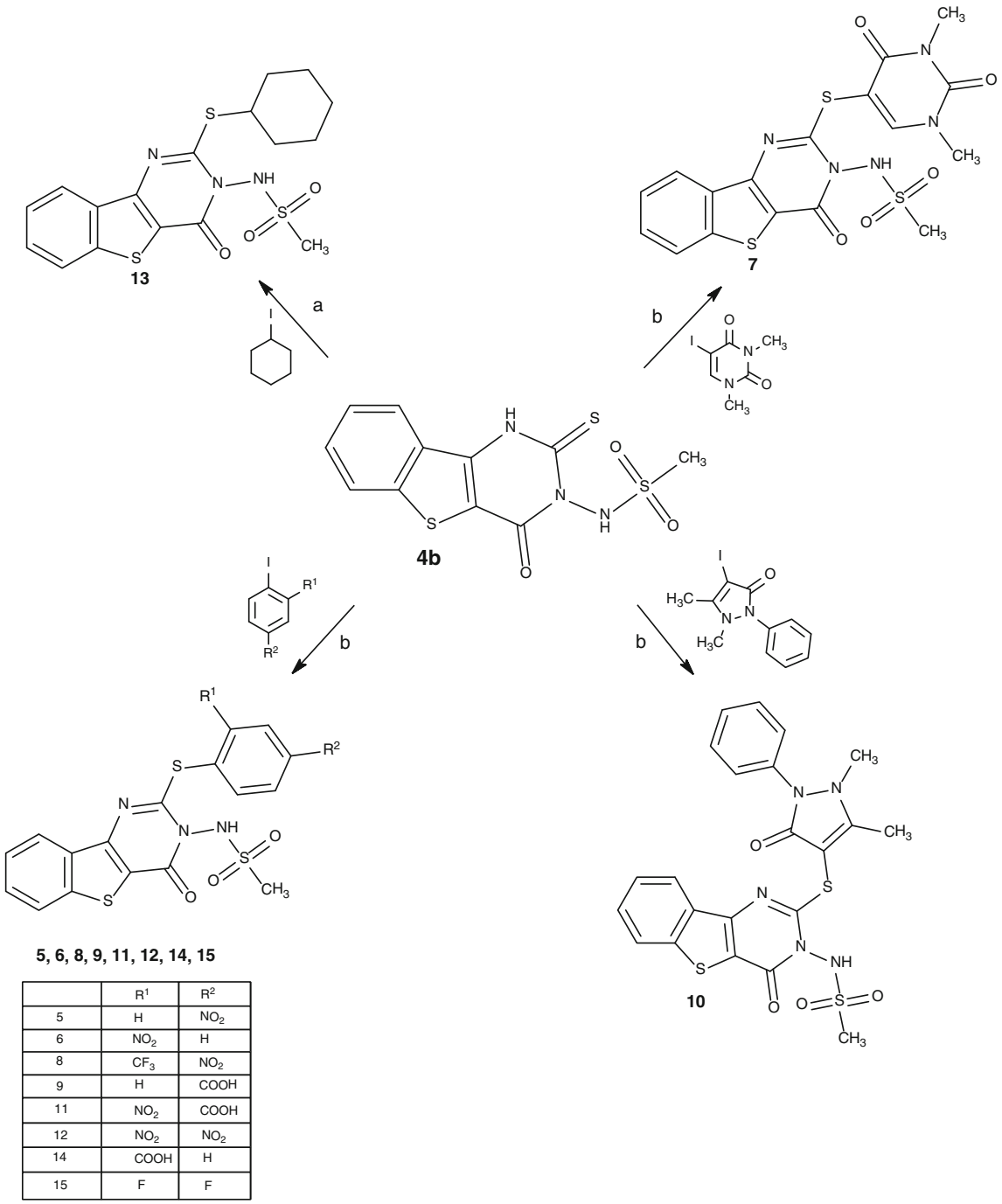
Scheme 1 Reagents and conditions: *a* DPT, CH_2Cl_2, r.t. stirring. *b* NH_2NH_2, CH_2Cl_2, r.t. stirring. *c* NaOH/EtOH, reflux. *d* H^+, r.t. *e* $NH_2NHSO_2CH_3$, toluene, reflux or CH_2Cl_2, r.t. stirring. *f* 1. NaOH/H_2O, reflux, 2. H^+, r.t.

infrared spectra of the product **2** confirmed the band in the regions 2140-2090 of the N=C=S group. The mild and simple reaction conditions (no dry solvent and inert atmosphere) make this procedure well suitable for the generation of new isothiocyanates. Isothiocyanate acts as electrophile with the carbon atom as the electrophilic center; the reaction at room temperature of isothiocyanate **2** in dichloromethane with hydrazine or mesylhydrazide gave the thiosemicarbazide **3a** and methylsulfonyl hydrazino **3b**, respectively. Alternatively, the compound **3b** has also been prepared in toluene under refluxing.

The cyclization of these thiosemicarbazide derivates, according to the methods reported by Wamhoff [27,28], was accomplished by treatment of compounds **3a** and **3b** with a solution of sodium hydroxide for 3 h and subsequently acidificated with hydrochloric acid to afford versatile intermediates benzo-aminothioxo **4a** and benzo-thioxo-methanesulphonamide **4b** of the heterocyclic system. Analytical and spectral data of all key intermediates were in agreement with the proposed structures and were also confirmed by independent preparations. The reactions were always carried out in the presence of a ligand and a base, under relatively mild condition and the catalytic system used for the synthesis of substituted benzo-thieno pyrimidine derivates was simple, inexpensive, and efficient. Therefore, the reaction of benzo-methanesulphonamid e-thioxo derivative **4b** with a variety of aryl and heterocyclic iodides in water/ ethanol, under refluxing in basic conditions and in the presence of catalytic quantitative of copper powder and copper iodide for 6 h, gave the corresponding benzo-thio-aryl derivatives (**5**–**15**) in acceptable good yields.

Alternatively, the benzo-thio-cycloesyl derivative **13** was obtained in dimethylformamide at 80 °C in the presence of potassium carbonate, from the reaction of benzo-sulfolnylamide derivative **4b** with cycloexyl iodide. Further experiments were performed to find the optimal reaction temperature and reaction time. An excellent yield of 95 % was obtained after 6 hours of reaction at 70–80 °C. The importance of the formation of carbon–sulfur bonds is well known in synthetic organic chemistry and represent a key step in the synthesis of many molecules that are of biological, pharmaceutical, and materials interest.

The proposed structures were confirmed by elemental analysis, IR, ^{1}HNMR, and mass spectrum. Specifically, ^{1}H NMR spectra in the region of 11.0–11.6 ppm showed a typical singlet attributable to NH of methanesulfonamide group; ^{1}H NMR spectra of thio-aryl derivatives exhibited the chemical shift of multiplet aromatic signals and ^{1}H NMR spectrum of cycloesyl derivative **13** showed at 1.22–1.99 ppm the multiplet due to ten methylenes and at 3.4–3.8 ppm the multiplet due to proton bonded to carbon adjacent to sulphur at position 2. The mass spectrum showed a prominent



	R^1	R^2
5	H	NO_2
6	NO_2	H
8	CF_3	NO_2
9	H	COOH
11	NO_2	COOH
12	NO_2	NO_2
14	COOH	H
15	F	F

Scheme 2 Reagents and conditions: *a* 1. DMF, K_2CO_3, reflux; 2. H^+, r.t.; b 1. H_2O/EtOH, Cu, CuI, reflux; 2. H^+, r.t.

molecular ion signal [M^+] as the base signal and a fragmentation pattern consistent with their structures.

Celecoxib was isolated by extraction from Celebrex(R) (Pfizer Inc.) followed by column chromatography. Stock solutions of the reference standard or test compounds were prepared in dimethyl sulfoxide (DMSO), and an equivalent amount of dimethyl sulfoxide was included in control samples.

Synthesis and characterization of derivatives

*Methyl 3-isothiocyanato-[1]-benzothiophene-2-carboxylate (**2**)*

A solution of amino ester (3.0 g, 14.4 mmoles) in dichlorometane (40 mL) was added slowly drop-wise at room temperature to a stirred solution of di-2-pyridyl thionocarbonate

(DPT) (3.3 g, 14.4 mmoles) in dichlorometane (40 mL). The mixture was stirred at room temperature for 24 h. The resulting solution was concentrated under reduced pressure to give a residue that was treated with HCl, the resulting solid was collected, washed with water, dried under reduced pressure, and treated according to Kienzle et al. [23] to give the isothiocyanate **2** as yellowish microcrystals. Yield: 2.5 g, mp: 117–120 °C; the isothiocyanate **2** resulted identical to the sample obtained according to previous papers. The unreported spectra data are now reported. ^{1}H NMR (500 MHz, [D6]DMSO): $\delta = 3.99$ (s, 3H), 7.40–8.15 (m, 4H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 14.29, 22.01, 26.78, 27.01, 59.78, 100.24, 128.37, 129.88, 134.78, 163.30, 164.28 ppm; IR (KBr): ν = 2140, 2090, 1710 cm^{-1}; HRMS m/z calcd for $C_{11}H_7NO_2S_2$ $[M]^+$: 248.99127, found: 248.99125.

*Methyl3-({[2-(methylsulphonyl)hydrazino]carbonothioyl} amino)1-benzothiophene-2-carboxylate (***3b***)*

To stirred solution of methanesulphonyl hydrazide (0.48 g, 98 %, 4.9 mmoles) in dichloromethane (20 mL) isothiocyanate (1.1 g, 4.1 mmoles) in dichloromethane (10 mL) was added drop-wise; the mixture was stirred at room temperature for 2 h. The resulting solid was collected, washed with dichloromethane and crystallized from ethanol to give **3** as white micro-needles; yield 77 %; mp: 223–25 °C dec; ^{1}H NMR (500 MHz, [D6]DMSO): δ=1.26 (t, J = 7.0 Hz, 3H), 3.08 (s, 3H), 7.40–8.15 (m, 4H), 9.95, 10.67, and 12.55 (s, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 14.02, 22.48, 24.35, 25.62, 40.92, 60.77, 125.35, 134.67, 140.34, 150.29, 163.34, 177.99 ppm; IR (KBr): ν=3294, 3243, 1657, 1324, and 1146 cm^{-1}; HRMS m/z calcd for $C_{12}H_{13}N_3O_4S_3$ $[M]^+$: 359.00626, found: 359.00525.

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

Isothiocyanate **2** (2.5 g, 10.0 mmol) dissolved in dichloro methane (30 mL) was added drop-wise at room temperature to a stirred solution of hydrazine hydrate (0.5 mL, 10.0 mmol) in dichloromethane (50 mL). The mixture was stirred at room temperature for 2 h; the resulting solid was collected, washed with dichloromethane and dried to give a white powder (2.3 g); a mixture of the thioxosemicarbazide derivate and the amino-thioxo derivate, was heated for 1 h under reflux while stirring in a solution of sodium hydroxide (0.53 g, 9.5 mmol) in ethanol (190 mL). The resulting solid was collected, washed with warm dioxane, and dried to give the sodium salt **4** as a white amorphous powder. Yield: 1.98 g, 67 %; mp: > 310 °C dec.

^{1}H NMR (500 MHz, [D6]DMSO): $\delta = 6.33$ (s, 2H), 7.40–8.15 (m, 4H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.40, 22.31, 24.06, 24.80, 115.32, 129.45, 130.90, 154.87, 161.85, 168.80 ppm; IR (KBr): ν = 3230, 3135, 1640 cm^{-1}.

To a suspension of the sodium salt of **4** (1.98 g, 7.0 mmol) in water (200 mL), HCl was added drop-wise 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 **4a** as a white microcrystalline powder. Yield: 0.87 g, 50 %; mp: > 280 °C.

The analytical and spectral data were identical to those of a sample obtained according to previous paper.

*N-(4-oxo-2-thioxo-1,4-dihydro[1]benzothieno[3,2-d]pyrimidin-3(2H)yl-methanesulfonamide (***4b***)*

A solution of mesylthiosemicarbazide (1.0 g, 2.65 mmol) and sodium hydroxide (0.24 g, 6.0 mmol) in water (40 mL) was refluxed under stirring for 3 h; the solution, containing the disodium salt was filtered, quenched, and acidified with concentrated hydrochloric acid to pH 3–4: the resulting solid was collected, washed with water, dried, and crystallized from dimethylformamide/water to give **4b** as a white powder; yield 40 %; mp: 268–70 °C dec.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 3.26 (s, 3H), 8.87–7.56 (m, 4H), 10.28 (s, 1H), 14 (brs, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.46, 22.40, 23.97, 24.76, 44.51, 115.28, 129.38, 131.10, 149.00, 155.50, 174.53 ppm; IR (KBr): ν = 3214, 1708, 1345, 1158 cm^{-1}; HRMS m/z calcd for $C_{11}H_9N_3O_3S_3$ $[M]^+$: 326.98005, found: 326.88765.

*N-[2-[(4-nitrophenyl)thio]-4-oxo[1]benzothieno[3,2-d]pyrimidin-3(4H)yl]methanesulfonamide (***5***)*

To a solution of methane sulphonamide-thioxo derivate **4b** (0.14 g, 0.43 mmol) and potassium hydroxide (40 mg) in ethanol/water 1:1 (40 mL) 1-iodo-4-nitrobenzene (0.106 g, 98 %, 0.43 mmol), dissolved in a small amount of 10 % potassium hydroxide and powdery copper (50 mg) were added. The mixture was heated at 100 °C under stirring for 5 h. The mixture was filtered while hot and after cooling to room temperature the resulting solution was acidified to pH 3–4: the resulting solid was collected, washed with water, dried, and crystallized from ethanol/water to give **5** as a yellow powder; yield 90 %; mp: 255 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 3.41(s, 1H), 7.50–8.59 (m, 8H), 11.63 (s, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): δ=21.54, 22.28, 24.47, 24.97, 43.83, 119.66, 120.64, 127.34, 130.91, 131.14, 133.96, 138.36, 148.07, 150.80, 155.68, 157.015, 159.53 ppm; IR (KBr): ν=3310, 1690, 1340, 1150 cm^{-1}; HRMS m/z calcd for $C_{17}H_{12}N_4O_5S_3$ $[M]^+$: 448.98005, found: 448.95005.

*N-[2-[(2-nitrophenyl)thio]-4-oxo[1]benzothieno[3,2-d]pyrimidin-3(4H)yl]methanesulfonamide (**6**)*

To a solution of methane sulphonamide-thioxo derivate **4b** (0.118 g, 0.36 mmol) and potassium hydroxide (40 mg) in ethanol/water 1:1 (40 mL) 1-iodo-2-nitrobenzene (89 mg, 98 %, 0.36 mmol), dissolved in a small amount of 10 % potassium hydroxide and powdery copper (50 mg) were added. The mixture was heated at 100 °C under stirring for 5 h. The mixture was filtered while hot and after cooling to room temperature the resulting solution was acidified to pH 3–4: the resulting solid was collected, washed with water, dried, and crystallized from ethanol /water to give **6** as a yellow powder; yield 90 %; mp: 235 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 3.41(s, 1H), 7.50–8.59 (m, 8H), 11.63 (s, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.54, 22.28, 24.47, 24.97, 43.83, 119.66, 120.64, 127.34, 130.91, 131.14, 133.96, 138.36, 148.07, 150.80, 155.68, 157.015, 159.53 ppm; IR (KBr): ν=3310, 1690, 1340, 1150 cm^{-1}; HRMS m/z calcd for $C_{17}H_{12}N_4O_5S_3$ $[M]^+$: 448.98005, found: 448.95005.

*N-[2-[(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)thio]-4-oxo[1]benzothieno[3,2-d]pyrimidin-3(4H)yl]methanesulfonamide (**7**)*

Methane-sulfonamide **4b** (0.167 g, 0.51 mmol) was dissolved in ethanol/water (16 mL/16 mL) and KOH (28 mg, 0.51 mmol). 5-Iodo-1,3-dimethyluracil (136 mg, 99 %, 0.51 mmol) and powdery copper (15 mg)/copper(I) iodide (10 mg) were added to the stirred solution. The mixture was heated at reflux under stirring for 5 h and filtered while hot; after cooling to room temperature, the solution was poured in water (150 mL). The solution was filtered and acidified with concentrated hydrochloric acid until pH 3–4: a light brown solid mass separated that was collected, washed with water, dried, and crystallized from ethanol/water to give 7 as a light brown powder. Yield 90 %; mp: 275 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 3.25 (s, 3H), 3.39 (s, 3H), 3.43 (s, 3H), 7.56–8.42 (m, 6H), 11.58 (s, 1H);^{13}C NMR (125 MHz, [D6]DMSO): δ = 22.28, 24.47, 36.48, 42.30, 119.15, 125.17, 125.99, 130.83, 131.14, 133.96, 138.67, 148.34, 154.10, 156.64, 156.83, 157.42, 159.33 ppm; IR (KBr): ν=3059, 1690, 1640, 1600, 1351, 1154 cm^{-1}; HRMS m/z calcd for $C_{17}H_{15}N_5O_5S_3$ $[M]^+$: 465.02298, found: 465.12998.

*N-[2-{[4-nitro-2-(trifluoromethyl)pheny]thio}-4-oxo[1]benzothieno[3,2-d]pyrimidin-3(4H)yl]methanesulfonamide (**8**)*

To a solution of methane sulphonamide-thioxo derivate **4b** (0.133 g, 0.41 mmol) and potassium hydroxide (40 mg) in ethanol/water 1:1 (40 mL) 1-iodo-4-nitro-2(trifluoromethyl) benzene (0.128 g, 98 %, 0.41 mmol), dissolved in a small amount of 10 % potassium hydroxide and powdery copper (50 mg) were added. The mixture was heated at 100 °C under stirring for 5 hours. The mixture was filtered while hot and after cooling to room temperature the resulting solution was acidified to pH 3–4: the resulting solid was collected, washed with water, dried, and crystallized from ethanol/water to give **8** as a orange powder; yield 80 %; mp: 125 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 3.41 (s, 1H), 7.52–8.46 (m, 7H), 11.69 (s, 1H);^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.01, 23.71, 24.88, 25.77, 44.12, 115.12, 125.16, 128.33, 130.01, 132.01, 133.44, 135.01, 135.66, 140.01, 158.22, 163.27, 168.01, 169.79 ppm; IR (KBr): ν = 3227, 1700, 1350, 1150 cm^{-1}; HRMS m/z calcd for $C_{18}H_{11}F_3N_4O_5S_3$ $[M]^+$: 516.49449, found: 516.59558.

*4-({3-[(Methylsulfonyl)amino]-4-oxo-3,4-dihydro[1]benzothieno[3,2-d]pyrimidin-2-yl}thio)benzoic acid (**9**)*

To a solution of methane sulphonamide-thioxo derivate **4b** (0.34 g, 1.03 mmol) and sodium hydroxide (72 mg, 1.8 mmol) in water (40 mL) 4-iodobenzoic acid (0.257 g, 98 %, 1.03 mmol) in ethanol (20 mL) and powdery copper (30 mg)/copper(I) iodide were added. The mixture was heated at 100 °C under stirring for 5 h. The mixture was filtered while hot and after cooling to room temperature, the resulting solution was acidified to pH 3–4: the resulting solid was collected, washed with water, dried, and crystallized from dioxane/water to give **9** as a white powder; yield 95 %; mp: 236–238 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 3.28 (s, 3H), 7.21–8.05 (m, 8H), 11.37 (s, 1H), 13.01 (s, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.01, 23.71, 24.88, 25.77, 44.12, 115.12, 125.16, 128.33, 130.01, 132.01, 133.44, 135.01, 135.66, 140.01, 158.22, 163.27, 168.01, 169.79 ppm; IR (KBr): ν = 3494, 1695, 1350, 1156 cm^{-1}; HRMS m/z calcd for $C_{18}H_{13}N_3O_5S_3$ $[M]^+$: 447.00990, found: 447.99879.

*N-[2-[(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)thio]-4-oxo[1]benzothieno[3,2-d]pyrimidin-3(4H)yl]methanesulfonamide (**10**)*

Methane-sulfonamide **4b** (0.130 g, 0.40 mmol) was dissolved in ethanol/water (16 mL/16 mL) and KOH (22 mg, 0.40 mmol). Iodoantipyrine (127 mg, 99 %, 0.40 mmol) and powdery copper (15 mg)/copper(I) iodide (20 mg) were added to the stirred solution. The mixture was heated at reflux under stirring for 5 h and filtered while hot; after cooling to room temperature, the solution was poured in water (150 mL). The solution was filtered and acidified with

concentrated hydrochloric acid until pH 3–4: a green solid mass separated that was collected, washed with water, dried, and crystallized from ethanol/water to give **10** as a green powder. Yield 95 %; mp: 185–190 °C; ^{1}H NMR (500 MHz, [D6]DMSO): δ = 2.36 (s, 3H), 3.38 (s, 3H), 3.81 (s, 3H), 7.39–8.14 (m, 7H), 11.51 (s, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.01, 23.71, 24.88, 34.37, 42.30, 99.83, 119.15, 121.72, 124.08, 125.17, 125.99, 128.29, 130.01, 132.83, 134.21, 135.01, 138.67, 143.05, 154.61, 156.80, 157.10, 167.37 ppm IR (KBr): ν = 3224, 3038, 1694, 1650, 1344, 1155 cm^{-1}; HRMS m/z calcd for $C_{22}H_{19}N_5O_4S_3$ $[M]^+$: 513.05936, found: 513.14598.

*4-({3-[(Methylsulfonyl)amino]-4-oxo-3,4-dihydro[1] benzothieno[3,2-d]pyrimidin-2-yl}thio)-3-nitrobenzoic acid (**11**)*

To a solution of methane sulphonamide-thioxo derivate **4b** (0.156 g, 0.47 mmol) and potassium hydroxide (40 mg) in ethanol/water 1:1 (40 mL) 4-iodo-3-nitrobenzoic acid (0.139 g, 98 %, 0.47 mmol), dissolved in a small amount of 10 % potassium hydroxide and powdery copper (50 mg) were added. The mixture was heated at 100 °C under stirring for 5 h. The mixture was filtered while hot and after cooling to room temperature the resulting solution was acidified to pH 3–4: the resulting solid was collected, washed with water, dried, and crystallized from ethanol/water to give **11** as a yellow powder; yield 80 %; mp: 215 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 3.42 (s, 1H), 7.53–8.62 (m, 7H), 11.69 (s, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.01, 23.71, 24.88, 25.77, 44.12, 115.12, 125.16, 128.33, 130.01, 132.01, 133.44, 135.01, 135.66, 140.01, 158.22, 163.27, 168.01, 169.79 ppm; IR (KBr): ν=3225, 1695, 1345, 1155 cm^{-1}; HRMS m/z calcd for $C_{18}H_{12}N_4O_7S_3$ $[M]^+$: 492.53778, found: 492.50609.

*N-[2-[(2,4-nitrophenyl)thio]-4-oxo[1]benzothieno[3,2-d]pyrimidin-3(4H)yl]methanesulfonamide (**12**)*

To a solution of methane sulphonamide-thioxo derivate **4b** (0.14 g, 0.43 mmol) and potassium hydroxide (40 mg) in ethanol/water 1:1 (40 mL) 2,4-dinitroiodobenzene (0.13 g, 98 %, 0.43 mmol), dissolved in a small amount of 10 % potassium hydroxide and powdery copper (50 mg) were added. The mixture was heated at 100 °C under stirring for 5 h. The mixture was filtered while hot and after cooling to room temperature the resulting solution was acidified to pH 3–4: the resulting solid was collected, washed with water, dried, and crystallized from ethanol /water to give **12** as a orange powder; yield 69 %; mp: 123–26 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ=3.39 (s, 3H), 7.26–7.80 (m, 7H), 11.43 (s, 1H). ^{13}C NMR (125 MHz, [D6] DMSO): δ = 21.54, 22.28, 24.47, 24.97, 43.83, 119.66, 120.64, 127.34, 130.91, 131.14, 133.96, 138.36, 148.07, 150.80, 155.68, 157.015, 159.53 ppm; IR (KBr): ν=3220, 1700, 1343, 1156 cm^{-1}; calcd for $C_{17}H_{11}N_5O_7S_3$ HRMS m/z[M]$^+$: 493.49463, found: 493.69699.

*N-[2-(cyclohexylthio)-4-oxo[1]benzothieno[3,2-d]pyrimidin-3(4H)yl]methanesulfonamide (**13**)*

A mixture of methane sulfonamide **4b** (0.180 g, 0.55 mmol), cyclohexyl iodide (0.115 g, 98 %, 0.55 mmol, 0.07 mL, d = 1.625), and potassium carbonate (76 mg, 0.55 mmol) in dimethylformamide (2 mL) was heated at 80 °C under stirring for 12 h. After cooling to room temperature, the mixture was acidified with hydrochloric acid and then poured in water (100 mL). The resulting solid was collected, washed with water, dried, and crystallized from petroleum ether to give **13** as a white power. Yield 86 %; mp: 106–108 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 1.45, 2.05 (m, 10H), 3.29 (s, 3H), 3.72 (s, 1H), 7.38, 8.10 (m, 4H), 11.14 (s br, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.54, 22.28, 24.47, 24.97, 43.83, 119.66, 120.64, 127.34, 130.91, 131.14, 133.96, 138.36, 148.07, 150.80, 155.68, 157.015, 159.53 ppm; IR (KBr): ν = 3195, 1695, 1350, 1150 cm^{-1}; calcd for $C_{17}H_{19}N_3O_3S_3$ HRMS m/z[M]$^+$: 409.05830, found: 409.09887.

*2-({3-[(Methylsulfonyl)amino]-4-oxo-3,4-dihydro[1]benzothieno[3,2-d]pyrimidin-2-yl}thio)benzoic acid (**14**)*

To a solution of methane sulphonamide-thioxo derivate **4b** (0.34 g, 1.03 mmol) and sodium hydroxide (72 mg, 1.8 mmol) in water (40 mL) 2-iodobenzoic acid (0.257 g, 98 %, 1.03 mmol) in ethanol (20 mL) and powdery copper (30 mg)/ copper(I) iodide were added. The mixture was heated at 100 °C under stirring for 5 h. The mixture was filtered while hot and after cooling to room temperature, the resulting solution was acidified to pH 3–4: the resulting solid was collected, washed with water, dried, and crystallized from dioxane/water to give **15** as a white powder; yield 55 %; mp: 236–38 °C.

^{1}H NMR (500 MHz, [D6]DMSO): δ = 3.28 (s, 3H), 7.21–8.05 (m, 8H), 11.37 (s, 1H), 13.01 (s, 1H);^{13}C NMR (125 MHz, [D6]DMSO): δ = 21.01, 23.71, 24.88, 25.77, 44.12, 115.12, 125.16, 128.33, 130.01, 132.01, 133.44, 135.01, 135.66, 140.01, 158.22, 163.27, 168.01, 169.79 ppm; IR (KBr): ν = 3494, 1695, 1350, 1156 cm^{-1}; HRMS m/z calcd for $C_{18}H_{13}N_3O_5S_3$ $[M]^+$: 447.00990, found: 447.99879.

*N-[2-[(2,4-difluorophenyl)thio]-4-oxo[1]benzothieno[3,2-d]pyrimidin-(4H)yl]methanesulfonamide (**15**)*

To a stirred of methane sulphonamide-thioxo derivate **4b** (0.180 g, 0.55 mmol) and sodium hydroxide (22 mg, 0.55 mmol) in ethanol/water 1:1 (40 mL) 2,4-difluoroiodobenzene (0.241 g, 0.12 mL, 98 %, 0.55 mmol, $d = 2.0$) and powder copper (30 mg) were added; the mixture was heated at reflux under stirring for 4 h; after cooling to room temperature the mixture was filtered and the filtrate poured in water (150 mL); the resulting solution was filtered and acidified with hydrochloric acid to pH 3–4: the solid separated was collected, washed with water, dried, and crystallized from water/dioxane to give **14** as a grayish powder; yield 85 %; mp: 235–236 °C.

^{1}H NMR (500 MHz, [D6]DMSO): $\delta = 3.37$ (s, 3H), 7.2–8.10 (m, 7H), 11.46 (s, 1H); ^{13}C NMR (125 MHz, [D6]DMSO): $\delta = 21.49$, 22.23, 24.32, 24.91, 43.97, 105.12 (t, $J = 26.15$ Hz), 112.68 (d, $J = 21.2$ Hz), 130.88, 132.70, 138.77 (d, $J = 10.3$) 139.21, 160.22, 161.50, 165.51, 166.50, 184.99, 188.44 .

IR (KBr): ν=3200, 3095, 1695, 1350, 1155 cm^{-1}; calcd for $C_{17}H_{11}F_2N_3O_3S_3$ HRMS m/z[M]$^+$:438.99250, found: 438.65455.

Biology

Cell cultures

The normal human keratinocyte cell line NCTC 2544 was provided by Interlab Cell Line Collection (Genoa, Italy) and routinely maintained in Minimum Essential Medium (MEM) (Sigma-Aldrich, Italy) supplemented with 10 % fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin, and incubated at 37 °C in a humidified, 95 % air/5 % CO_2 atmosphere.

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/Lglucose, 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 media were changed every 2–3 days. For experiments, 24 h before the cells were trypsinized, and plated in 96-wells (for MTT assays), or 6-well plates (for ELISA tests) or in 100 mm Petri dishes (for Western blot).

After 24 h, experimental keratinocytes were stimulated or not (untreated controls) with 200 U/mL of IFN-γ and 10^{-4} M of histamine, used to reproduce the mechanisms involved in the pathogenesis of inflammatory processes, in the absence or presence of the derivatives (**5**–**15**) (10 μM) or celecoxib (10 μM), used as a reference anti-inflammatory drug.

Table 1 iNOS and COX-2 IC_{50} values (μM) of human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774 treated with interferon-γ plus histamine and lipopolysaccharides, respectively

Compounds	NCTC2544		J774	
	iNOS	COX-2	iNOS	COX-2
5	35 ± 0.5	32 ± 2	33 ± 0.8	35 ± 1
6	38 ± 2	36 ± 0.9	35 ± 3	37 ± 2
7	7 ± 0.9	6.7 ± 0.3	6.5 ± 0.5	6.9 ± 0.5
8	32 ± 3	31 ± 2	29 ± 3	33 ± 0.9
9	22 ± 2	25 ± 3	23 ± 0.5	25 ± 1
10	5 ± 0.2	4.5 ± 0.5	4.8 ± 0.3	5 ± 0.1
11	34 ± 2	35 ± 0.5	31 ± 2	34 ± 0.9
12	8 ± 0.3	8 ± 1	7.8 ± 0.9	8.2 ± 0.4
13	7 ± 0.2	6.8 ± 0.5	7.3 ± 0.3	6.9 ± 0.9
14	6.5 ± 1.3	6.2 ± 0.8	6.5 ± 1.5	6.2 ± 0.5
15	5.8 ± 0.3	6.1 ± 1.5	6.5 ± 0.9	6.2 ± 0.6

Experimental J774 cells were stimulated with LPS (1 mg/mL) to induce COX-2 in the absence (DMSO alone) or presence of the test compounds at concentration of 10 μM.

After 48 h each sample was tested for the expression of iNOS, COX-2, and ICAM-1, and the release of PGE_2 and IL-8.

To measure the effectiveness of each compound in inhibiting the biological functions, for some evaluated parameters (iNOS and COX-2) the half maximal inhibitory concentration (IC_{50}) was determined. In this experiments, the compounds were used at the concentrations of 0.5, 5, 10, 20, and 50 μM. The results were reported in Table 1.

Western blot

The expression of iNOS, COX-2, and ICAM-1 was evaluated by Western blot analysis. Briefly, the untreated and treated NCTC 2544 and J774 cells were washed twice with ice-cold PBS and resuspended with lysis buffer (M-PER® Mammalian Protein Extraction Reagent, Thermo scientific, PIERCE Biotechnology) supplemented with a cocktail of protease inhibitor (complete, Mini, Protease Inhibitor Cocktail Tablets, Roche) according to manufacturer's instructions. Sixty micrograms of total protein, present in the supernatant, was loaded on each lane and separated by 4–12% Novex Bis-Tris gel electrophoresis (NuPAGE, Invitrogen, Italy). Proteins were then transferred to nitrocellulose membranes (Invitrogen, Italy) in a wet system. The transfer of proteins was verified by staining the nitrocellulose membranes with Ponceau S and the Novex Bis-Tris gel with Brilliant blue R. The membranes were blocked in Tris buffered saline containing 0.01 % Tween-20 (TBST) and 5 % non-fat dry milk at 4 °C overnight. Mouse monoclonal anti-ICAM-1 (1H4: sc-51632, Santa Cruz Biotechnology) (1:200 dilution),

-NOS2 (N-20, sc-651, Santa Cruz Biotechnology) (1:300 dilution), -COX-2 (N-20, sc-1746, Santa Cruz Biotechnology) (1:100 dilution) and -α tubulin (T9026; Sigma-Aldrich) (1:5,000 dilution) antibodies were diluted in TBST and the membranes incubated for 2 h at room temperature. Antibodies were detected with horseradish peroxidase conjugated secondary antibody using the enhanced chemiluminescence detection Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL). The signal intensity of primary antibody binding was quantitatively analyzed with ImageJ software and was normalized to a loading control α-tubulin. Values were expressed as arbitrary densitometric units (A.D.U.) corresponding (proportional) to signal intensity.

ELISA assay

The concentration of PGE_2 was measured in keratinocytes 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. In brief, 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 λ = 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).

The production of interleukin-8 (IL-8) was measured on cell-free supernatants collected after 48 h treatment by specific sandwich enzyme-linked immunosorbent assay (ELISA) kit (Amersham Biosciences, Switzerland). Keratinocyte and J774 cultures were carried out in triplicate for each condition. All assays were performed as specified by the manufacturer of the kit. A standard curve was produced for each assay using known concentrations of IL-8 provided in the kit. The sensitivity of the ELISA kit of IL-8 was <5 pg/mL. The results were given as mean pg/mL ± SEM.

Cell viability

The MTT proliferation assay is based on the conversion by mitochondrial dehydrogenases of a substrate containing a tetrazolium ring into blue formazan, detectable spectrophotometrically. The level of blue formazan is then used as an index of cell viability. Briefly, cell cultures (8×10^3 cells/microwell) were set up in flat-bottomed 200 µL microplates, incubated at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2 and after 24 h (60–70 % confluence) treated with 0.5, 5, 10, 20, and 50 µM of each experimental derivative, or celecoxib (10 µM) for 48 h. Four hours before the end of the culture period, 20 µL of 0.5 % MTT in PBS was added to each microwell. After incubation with the reagent, the supernatant was removed and replaced with 100 µL of DMSO. The optical density of each sample was measured using a microplate spectrophotometer (Titertek Multiskan; DAS) at λ = 550 nm. Each sample was tested in quadruplicate.

Statistical analysis

Each experiment was repeated at least three times in triplicate and the mean ± SEM for each value was calculated. Statistical analysis of results [Student's *t* test for paired and unpaired data; variance analysis (ANOVA)] was performed using the statistical software package SYSTAT, version 11 (Systat Inc., Evanston IL, USA). A difference was considered significant at $p < 0.05$.

Results and discussion

The aim of this work was to evaluate the potential anti-inflammatory activity of eleven (**5**–**15**) new synthesized derivatives of benzo-thieno[3,2-d]pyrimidine on two cell models, such as human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774. For the synthesis of compounds, some efficient modifications were used by the preparation of isothiocyanate using di-2-pyridyl thionocarbonate (DPT) in substitution of thiophosgene and the execution of the cyclization reaction of benzo-thiosemicarbazide derivates through the methods reported by Wamhoff [27,28]. The results demonstrated that this procedure, in a one-pot process, can be a new alternative method economically and environmentally advantageous by the simplicity of procedure, reduction of isolation and purification steps, time, costs, and waste production.

To prove this biological propriety of new compounds, the expression of COX-2, inducible NO synthase (iNOS), immuno-modulatory membrane molecules such as intercellular adhesion molecule-1 (ICAM-1), and the release of PGE_2 and interleukin-8 (IL-8) were determined. These medi-

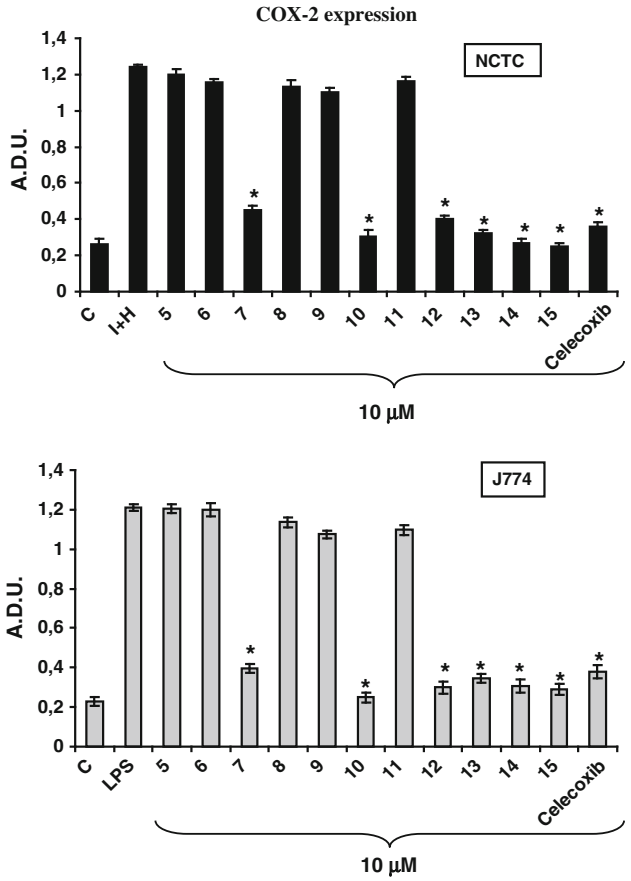


Fig. 1 Effects of benzo-thieno[3,2-d]pyrimidine derivatives **5**–**15** and celecoxib on COX-2 expression induced by interferon-γ (I) plus histamine (H) and lipopolysaccharides (LPS) on human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774, respectively, determined by Western blot analysis. Data show the relative expression (mean ± SEM) of COX-2 calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments. $^*p < 0.05$ compared to interferon-γ (I) plus histamine (H)- or lipopolysaccharides (LPS)-induced COX-2

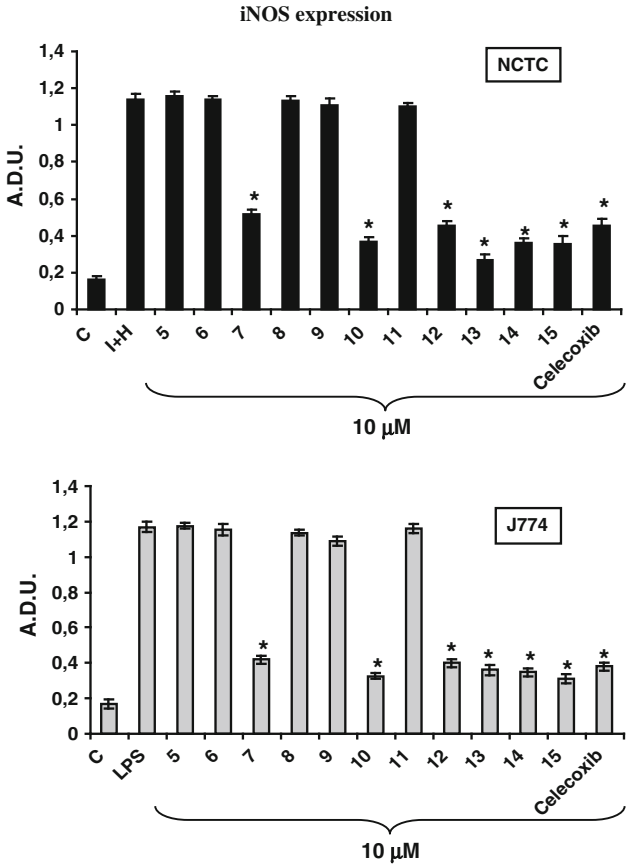


Fig. 2 Effects of benzo-thieno[3,2-d]pyrimidine derivatives **5**–**15** and celecoxib on iNOS expression induced by interferon-γ (I) plus histamine (H) and lipopolysaccharides (LPS) on human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774, respectively, determined by Western blot analysis. Data show the relative expression (mean ± SEM) of iNOS calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments. $^*p < 0.05$ compared to interferon-γ (I) plus histamine (H)- or lipopolysaccharides (LPS)-induced iNOS

ators are involved in the inflammation and their reduction has been associated with the amelioration of pyrexia, inflammation, and pain [33].

Our results demonstrate that, among the derivatives **5**–**15**, *N*-[2-[(1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)thio]-4-oxo[1]benzothieno[3,2-d]pyrimidin-3(4*H*)yl] methanesulfonamide (**7**), *N*-[2-[(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazol-4-yl)thio]-4oxo[1]benzothieno [3,2-d]pyrimidin-3(4*H*)yl]methanesulfonamide (**10**), *N*-[2-[(2, 4-nitrophenyl)thio]-4-oxo[1]benzothieno[3, 2-d]pyrimidin-3(4*H*)yl]methanesulfonamide (**12**), *N*-[2-(cyclohexylthio)-4-oxo[1]benzothieno[3, 2-d]pyrimidin-3 (4*H*)yl]methanesulfonamide (**13**), *N*-[2-[(2,4-difluorophenyl)thio]-4-oxo [1] benzothieno[3, 2-d] pyrimidin-(4*H*)yl] methanesulfonamide (**15**), and 2-({3-[(methylsulfonyl)amino]-4-oxo-3,4-dihydro[1]benzothieno[3, 2-d]pyrimidin-2-yl}thio)benzoic acid (**14**) act as a potent inhibitor of COX-2 (Fig. 1), iNOS (Fig. 2), ICAM-1 (Fig. 3) expression while also suppressing the production of PGE_2 (Fig. 4) and IL-8 (Fig. 5) in human keratinocytes NCTC 2544 exposed to interferon-gamma (IFN-γ) and histamine (H) and monocyte-macrophages J774 cells treated with lipopolysaccharides (LPS). As assessed by MTT assay, all the derivatives **5**–**15** were well tolerated by both keratinocytes and monocyte-macrophages (Fig. 6). Therefore, the inhibition of the IFN-γ plus H-, or LPS-stimulated expression of pro-inflammatory molecules in keratinocytes and monocyte-macrophages, respectively, by derivatives **7**, **10**, **12**, **13**, **14**, and **15** was not due to cytotoxicity,

Heterocycles containing pyrimidine as purine analogues, especially pyrido[2,3-d]pyrimidines, an important biologically significant annulated pyrimidines connected with purine pteridines system, are of great synthetic interest due to their wide biological and pharmacological activities [34].

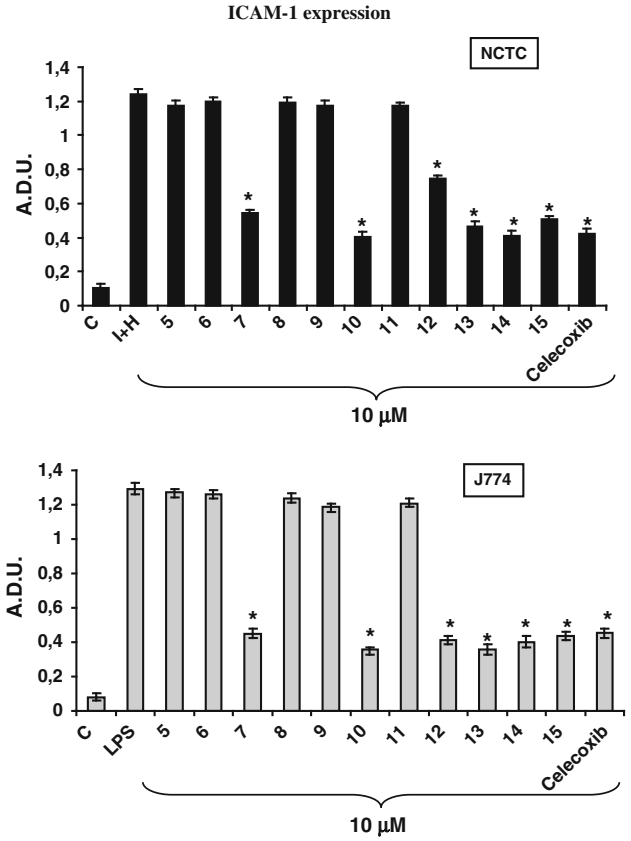


Fig. 3 Effects of benzo-thieno[3,2-d]pyrimidine derivatives **5**–**15** and celecoxib on ICAM-1 expression induced by interferon-γ (I) plus histamine (H) and lipopolysaccharides (LPS) on human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774, respectively, determined by Western blot analysis. Data show the relative expression (mean ± SEM) of ICAM-1 calculated as arbitrary densitometric units (A.D.U.) collected from three independent experiments. $^*p < 0.05$ compared to interferon-γ (I) plus histamine (H)- or lipopolysaccharides (LPS)-induced ICAM-1

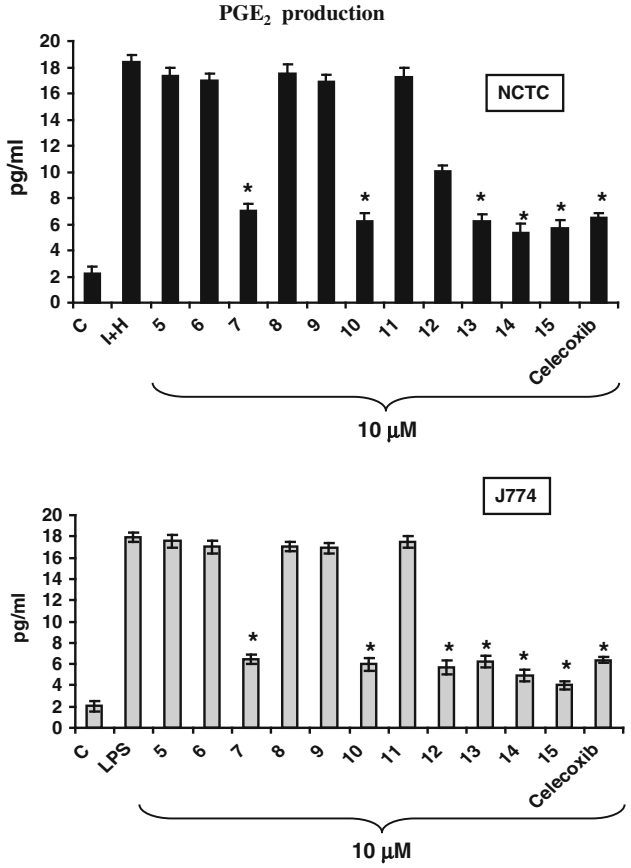


Fig. 4 Effects of benzo-thieno[3,2-d]pyrimidine derivatives **5**–**15** and celecoxib on interferon-γ (I) plus histamine (H) or lipopolysaccharides (LPS)-induced prostaglandin E_2 (PGE_2) production in on human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774, respectively, measured in the culture media by enzyme-linked immunosorbent assay (ELISA). Data were derived from three independent experiments and are expressed as means ± S.E.M. $^*p < 0.05$ indicates significant differences from the interferon-γ (I) plus histamine (H)- or the lipopolysaccharides (LPS)-treated groups

Insights into the differences between the binding sites of COX-1 and COX-2 obtained from X-ray crystal structure data provided useful guidelines that facilitated the design of the selective COX-2 inhibitors [35,36].

In this study, we synthesized derivatives, potential COX-2 inhibitors, where the substitution pattern was selected so as to confer different electronic environment that would affect the lipophilicity, and hence the activity of the target molecules. The objective of forming these hybrids was an attempt to reach an active anti-inflammatory agent with potentiated activity and selectivity toward COX-2. Thus, the introduction of 1,3-dimethyluracil (**7**), antipyrine (**10**), NO_2 in ortho and para (**12**), cyclohexyl (**13**), COOH in ortho (**14**), and 2,4-difluorophenyl (**15**) produced compounds with effective anti-inflammatory proprieties. In particular, the compound **10**-containing antipyrine more efficiently than celecoxib blocks some pro-inflammatory actions of IFN-γ plus histamine and LPS on keratinocytes and monocyte-macrophages, respectively.

Antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) was the first pyrazolone derivative used in the management of pain and inflammation, and their derivatives have attracted the attention of several research groups due to their potential activities [34–36]. In this context, broad spectra of bioactive antipyrine derivatives have been investigated and diversities of bioactivities such as analgesic, anti-inflammatory, antimicrobial, and anticancer activity have been reported [36]. These classes of compounds also act as strong inhibitors of cycloxygenase isoenzymes, platelet thromboxane synthesis, and prostanoid synthesis [35]. Antipyrine has been found to be effective in scavenging peroxyradicals ($ROO^\bullet$) and inhibiting the synthesis of prostaglandins in thermo regulator hypothalamic centers [34]. The pharmacological properties of the compounds containing antipyrine and sulfonilamidothiopyrimidone system moieties have prompted us to design and synthesize hybrid molecules incorporating those scaffolds in a single molecule. So, we have synthe-

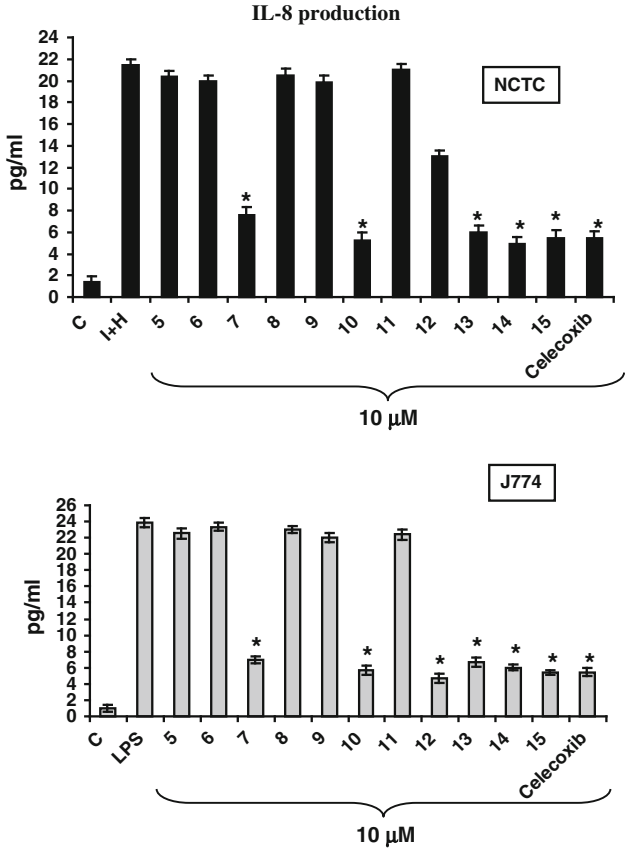


Fig. 5 IL-8 production (means ± SEM) measured in the culture media by enzyme-linked immunosorbent assay (ELISA) from human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774 48 h after the addition of benzo-thieno[3,2-d]pyrimidine derivatives **5**–**15** or celecoxib with interferon-γ (I) plus histamine (H)- and lipopolysaccharides (LPS). Values are expressed as pg/mL. *Significantly different from interferon-γ (I) plus histamine (H)- or lipopolysaccharides (LPS)-treated samples ($p < 0.05$)

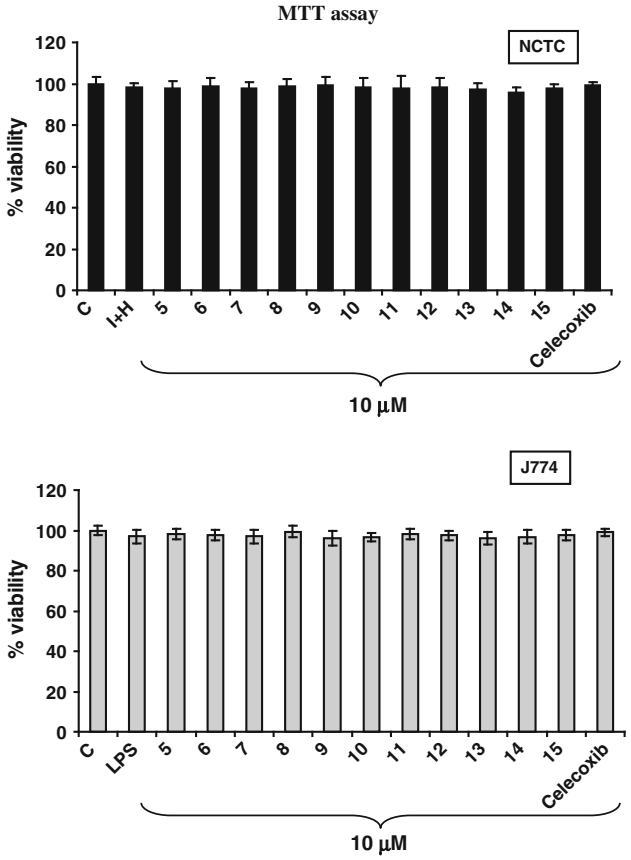


Fig. 6 Human keratinocytes NCTC 2544 and mouse monocyte-macrophages J774 cell viability measured with tetrazolium salt assay (MTT) after 48 h of treatment with benzo-thieno[3,2-d]pyrimidine derivatives **5**–**15** or celecoxib. The values of optical density measured at $\lambda = 550$ nm are reported as percentage with respect to the optical density registered for untreated control, the latter considered as 100 % of cell viability. The values are the mean ± SEM of three experiments performed in triplicate. All values with $p < 0.05$ were considered significantly different. *Significant versus untreated control

sized the compound **10** comprising basically the antipyrine moiety attached to the benzo-thioxo-methane-sulphonamide through various linkages as described above.

The linker between the uracil and the anti-inflammatory moiety was investigated too. Since it was reported that a methyl group was found to be the most favorable substituent at the uracil N(3) position and the N(1) substituent was found to have a great influence on the inflammation inhibitory activities, the linker between the uracil and the anti-inflammatory moiety was investigated achieving improvements in the anti-inflammatory propriety.

The presence of electron withdrawing groups like $-NO_2$, –COOH, and F attached to benzo-thienopyrimidine compounds showed to be useful in the anti inflammatory activity. The nonsteroidal anti-inflammatory drugs generally possess an aromatic group and/or heteroaromatic rings (such as purine units) in their structure. From the point of view of the degree of anti-inflammatory action, ionic substituents, such as a nitro group on the aromatic ring of the molecule, enhance the activity [37]. The presence of such groups enables the drug to interact with the bi-layer cell membrane [38]. Examination of our biological results indicated that the structural modifications carried out produce remarkable changes of the activity in terms of anti-inflammation.

In conclusion, the results of the present research indicate that, compared to celecoxib, the derivatives **7**, **10**, **12**, **13**, **14**, and **15** exhibit more interesting anti-inflammatory properties, expressed by their capacity to counteract some pro-inflammatory effects induced by IFN-γ plus histamine in normal human keratinocyte NCTC 2544 or LPS in monocyte-macrophage J774 cells.

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