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(Article begins on next page)



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Synthesis and preliminary pharmacological characterization of new analogues of Zileuton endowed with dual 5-LO-inhibitor and NO-dependent activities.

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A novel class of NO-donor/5-lipoxygenase (5-LO) inhibitory agents was designed by joining Zileuton, a well known 5-LO inhibitor belonging to the family of hydroxyureas, to NO-donor nitrooxy or furoxan moieties. The products displayed a pharmacological profile both of NO-donor drugs and 5-LO inhibitors. Inhibition of LTB₄ production, induced in heparinized human whole blood by calcium ionophore A 23187, was observed, and the inhibitory potencies of the compounds were near that of the lead. In addition the products were capable of relaxing rat tracheal rings precontracted with 1 μM carbachol, with a mechanism NO-mediated, myorelaxing potencies lying in the μM range. According to their NO-donor nature, they were also able to relax rat aorta strips precontracted with phenylephrine, with varying potencies in a large concentration range. Finally compounds **18** and **25**, namely those with the lead substituted at 6-position with an alkoxy chain bearing a nitrooxy or a 3-carbamoylfuroxan moiety respectively, displayed anti-inflammatory activity when tested on carrageenan-induced paw edema in conscious rats. The family of the products described represents the first class of dual drugs with 5-LO inhibitor/NO-dependent activities.

Introduction

The leukotrienes (LTs) are a family of lipid derived autacoids which originate from arachidonic acid (AA). 5-Lipoxygenase (5-LO) is the key enzyme in this process. It transforms AA through a two steps process, first into 5-hydroperoxyeicosatetraenoic acid (5-HPETE), and then into unstable leukotriene A₄ (LTA₄). This intermediate can be transformed either by leukotriene B₄ synthase into leukotriene B₄ (LTB₄), or by leukotriene C₄ synthase, which is a specific glutathione-transferase enzyme, into peptide-lipid leukotrienes C₄, D₄, E₄ (LTC₄, LTD₄, LTE₄).¹⁻³LTs are involved in a variety of inflammatory and allergic disorders, in particular rheumatoid arthritis, inflammatory skin and bowel diseases. They also display potent bronchoconstrictor activity. Consequently, the treatment of allergic disorders and asthma are the classical indications of 5-LO inhibitors.^{4,5} Novel interesting potential indications are emerging for these products, for example an increasing amount of experimental evidence shows an involvement of 5-LO pathway in tumor cell proliferation.⁵ In particular, inhibition of 5-LO was found to induce apoptosis in different kind of cancer cells.⁶ The emerged evidence that LTs are involved in atherogenesis and arterial wall remodeling opens new interesting strategies in the treatment of atherosclerosis development and its progression.^{5, 7, 8} 5-LO inhibitors can be classified according to their action mechanism into four different classes of products: redox-active compounds, competitive reversible inhibitors, inhibitors of FLAP (5-lipoxygenase activating protein), iron chelating inhibitors. Many substances belonging to these classes were developed as potent 5-LO inhibitors, including natural products.⁹⁻¹¹ Among them, only 1-(1-benzo[b]thien-2-yl)ethyl]-1-

hydroxyurea (**1**) (Zileuton) (Chart), a hydroxyurea derivative of the iron ligand-type inhibitors class, entered into the market in 1996 as an antiasthmatic drug.^{12, 13} Commercially available product is a racemic mixture of *R* and *S*, both of which display in vitro 5-LO inhibitor activity. A number of studies have been addressed to design 5-LO inhibitors with dual activities. 5-LO/cyclooxygenase(COX) inhibitors have been received particular attention as anti-inflammatory agents, but also compounds either with dual 5-LO/thromboxane A₂ synthase inhibitory activity or with 5-LO inhibitor and platelet activating factor receptor (PAF) antagonist mixed properties have been developed.¹⁰ In our knowledge no documented example of nitric oxide (NO)-donor/5-LO inhibitor hybrids have been so far described, in spite of the undoubted interest of this combination. Indeed NO is a physiological messenger that triggers a variety of actions in different systems.¹⁴ In particular, in the cardiovascular system it plays very important roles in maintaining a number of homeostatic responses: preservation of endothelial integrity; arterial blood vessels dilation, including pulmonary arterial vasculature; inhibition of platelet adherence and aggregation, attenuation of leukocytes adherence and activation; and inhibition of vascular smooth muscle cell proliferation.¹⁵ NO also triggers relevant action in airways, inducing relaxation of airway smooth muscle, pro-inflammatory or anti-inflammatory effects, and regulation of mucociliary clearance.¹⁶ The use of NO-donors in the treatment of cardiovascular disease (CD) is well known, while the therapeutic potential of this kind of products in the field of respiratory diseases is still under examination.¹⁵⁻¹⁸ In this paper we propose new dual products, obtained by combining Zileuton with NO-donor nitrooxy or furoxan moieties. Synthesis, and preliminary pharmacological characterization of these compounds are described and discussed.

1. Results and Discussion

Chemistry. The synthesis of the final products required the preliminary preparation of a number of intermediates (Scheme). The substituted benzothiophene **3** was easily obtained treating 6-hydroxybenzothiophene (**2**) with *n*-butyllithium (*n*BuLi) and anhydrous acetaldehyde in THF at -20 °C. The triflates **8-11** were prepared by action of triflic anhydride in CH₂Cl₂ on the appropriate nitrooxy-substituted alcohols **4-7** in the presence of 2,6-lutidine at -40 °C and were immediately used. Treatment of **3** with NaH in THF, and then with a CH₂Cl₂ solution of the appropriate triflates **8-11** afforded the expected nitrates **14-17**. Action of 37% HCl on these products dissolved in THF/H₂O in the presence of hydroxyurea at 50 °C afforded the target compounds **18-21**. The furoxan derivatives **22** and **23** were prepared by reaction of **3** with 4-bromomethylfuroxan **12** or **13** respectively, in dry DMF, in the presence of K₂CO₃. The final furoxan models **24**, **25** were obtained from **22** or **23**, following the same procedure used to prepare the analogues nitric esters **18-21** from **14-17**.

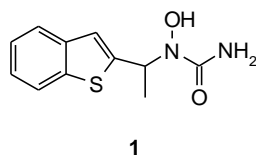
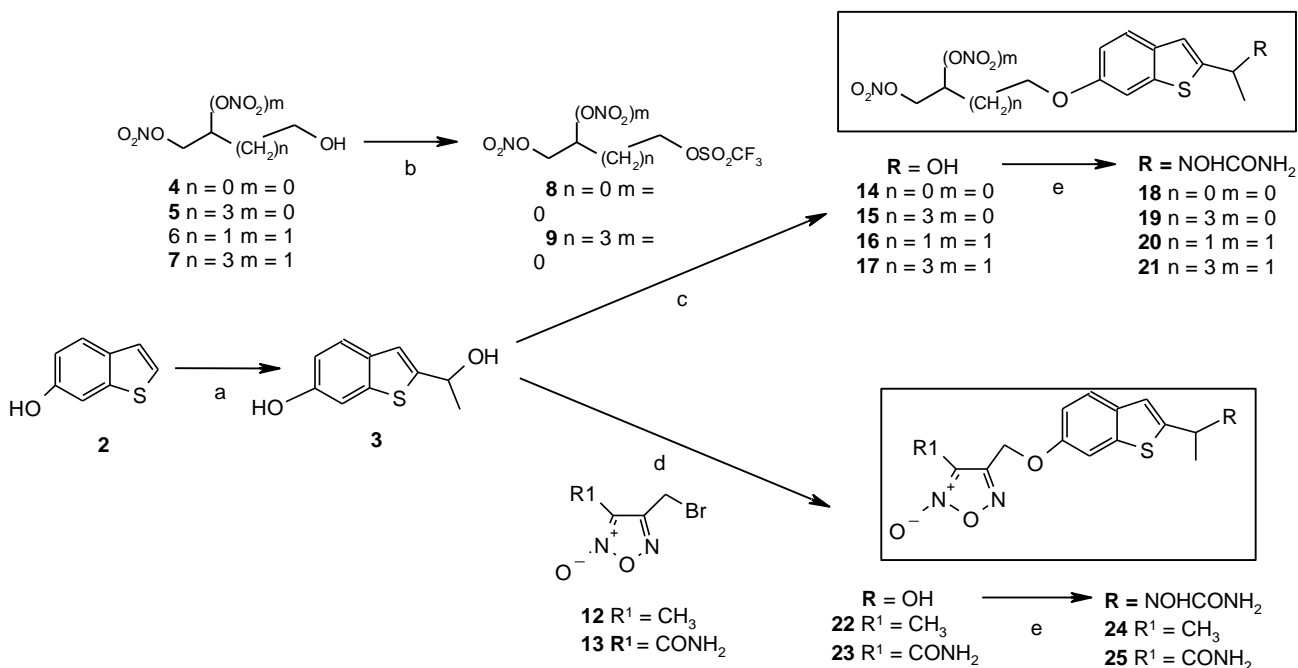


Figure 1. Zileuton **1**



Scheme. Reagents and conditions: (a) *n*BuLi, acetaldehyde, dry THF, -20 °C; (b) Triflic anhydride, 2,6-lutidine, dry CH₂Cl₂, -40 °C, N₂; (c) NaH, dry THF, -10 °C, N₂, **8-11** dissolved in dry CH₂Cl₂; (d) **12** or **13**, K₂CO₃, dry DMF, r.t.; (e) hydroxyurea, 37% HCl, H₂O/THF, 50 °C.

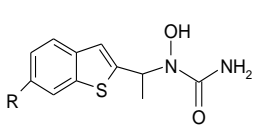
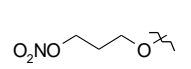
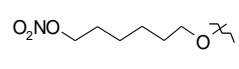
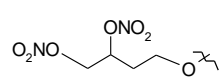
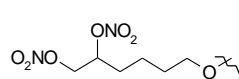
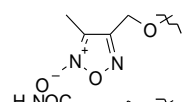
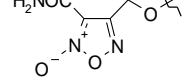
5-LO Inhibition. The ability of the target products **18-21**, **24**, **25**, and of the reference compound Zileuton, to inhibit 5-LO enzyme, was assessed by incubating each compound in heparinized human whole blood. After a fixed time, LTs biosynthesis was initiated by adding calcium ionophore A 23187, and terminated by rapid cooling of the blood. After centrifugation, the level of LTB₄ in plasma was analysed by enzyme immunoassay (ELISA). All the products were able to inhibit LTB₄ production in concentration dependent manner. The potencies of inhibition expressed as IC₅₀ cover the concentration range 2-18 μM (Table). Detailed analysis of data shows that the most potent inhibitor was the mononitrooxy substituted compound **18**. Its IC₅₀ was the same as that of Zileuton, within the experimental error. For the homologue **19**, in which the length of the nitrooxyalkoxy lateral chain is increased, the inhibitory activity is lower, although it maintains a good level. Also the dinitrooxy substituted compound **20** triggers inhibitory action near that of Zileuton, and this activity is largely retained in its higher homologue **21**. The furoxancarboxamide **25** is the less active product of the series, showing a potency about ten folds lower than Zileuton, while its methyl analogue **24** is only four folds less potent than the reference. Altogether the data indicate that hybridization of Zileuton with appropriate NO-donor moieties affords compounds with good or rather good levels of 5-LO inhibition.

Myorelaxing Activity. The myorelaxing effects of the target products were assessed on rat tracheal rings precontracted with 1 μM carbachol. All the products were capable of relaxing the contracted tissue in a concentration-dependent manner, and the maximum response was determined with (-) isoprenaline 10 μM. The potencies of the products, expressed as EC₅₀, are reported in Table. Analysis of the Table shows that these figures fall in the narrow concentration range 15-37 μM. In the nitric acid ester series the mononitrooxy derivatives **18**, **19** display the same activity and are half as potent as the equipotent dinitrooxy analogues **20**, **21**. Between the two furoxan derivatives, only the furoxancarboxamide **25** displays

an EC₅₀ value in the μM range, while the methyl substituted furoxan **24** does not trigger any myorelaxing effect when tested up to concentration of 3x10⁻⁵M. As expected, Zileuton also does not trigger any effect on the contracted tissue and confirms that the myorelaxing effects of all the active products we detected are mediated by NO. To confirm this hypothesis the activity of the products on the precontracted tracheal rings was assessed in the presence of 10 μM ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), a well known inhibitor of the soluble guanylate cyclase (sGC). This is the key enzyme in mediating tracheal relaxation induced by NO and NO-related compounds, through the elevation of the intracellular concentration of guanosine 3',5'-cyclic monophosphate (c-GMP).¹⁹ The suppression of the activity, when the products were tested up to 30 μM concentration, is in keeping with the involvement of this messenger in their myorelaxing action. The NO-dependent myorelaxing potency range of the products is near their 5-LO inhibition potency range. This means that the hybrids in vitro display these two activities in fairly well balanced manner.

Vasodilator activity. The vasodilator effects of target hybrids were assessed on denuded rat aorta strips precontracted with phenylephrine. The vasodilator potencies of the products, expressed as EC₅₀, are collected in Table. The nitrooxy substituted compounds are very potent vasodilators, showing their EC₅₀ values in a submicromolar range. In the nitric ester series, the products display similar potencies which rank the order **20** > **19** ≥ **21** > **18**. The furoxancarboxamide **25** is also a quite potent vasodilator, about ten folds less than dinitrooxy esters and one hundred folds more than the methyl furoxan **24**. The strong decrease in the activities which occurred when the experiments were repeated in the presence of 1 μM ODQ, is in keeping with a NO-mediated vasodilator mechanism. Altogether the data indicate that in vitro NO-mediated vasodilator effects of the tested compounds prevail on their 5-LO inhibitor capacity. The only exception is the methylfuroxan **24** in which these two activities occur at similar concentrations.

table 1: Vasodilating, myorelaxing and inhibition of 5-lipoxygenase data for zileuton **1** and target products **18-21**, **24**, **25**

compound		vasodilating activity ^a	myorelaxing activity ^b	inhibition of 5-lipoxygenase ^c
		EC ₅₀ ± SE μM (+ ODQ 1 μM)	EC ₅₀ ± SE μM (+ ODQ 10 μM)	IC ₅₀ (95%CL) μM
1	H	- ^d	- ^d	1.6 (1.3-2.0)
18		0.039 ± 0.007 (> 30)	37 ± 5 (- ^d)	2.0 (1.6-2.5)
19		0.023 ± 0.003 (> 30)	36 ± 8 (- ^d)	5.8 (4.3-7.7)
20		0.015 ± 0.004 (23 ± 3)	16 ± 3 (- ^d)	2.8 (2.0-4.1)
21		0.027 ± 0.004 (14 ± 4)	15 ± 1 (- ^d)	3.5 (2.8-4.3)
24		28 ± 2 (- ^d)	- ^d	6.3 (4.9-8.3)
25		0.18 ± 0.04 (6.4 ± 0.7)	24 ± 2 (- ^d)	17.9 (11.5-28.0)

^a Determined on rat thoracic aorta precontracted with 1 μM phenylephrine.

^b Determined on rat tracheal rings precontracted with 1 μM carbachol.

^c Measured as the ability of the compound to inhibit biosynthesis of LTB_4 in human whole blood challenged with calcium ionophore A23187

^d The product was inactive at maximal concentration tested (30 μM)

Antiinflammatory activity.

All the target products, including Zileuton **1** as reference, were tested on carrageenan-induced paw edema in conscious rats. The injection of carrageenan into rat hind paw produced a paw swelling which reached its maximum at 5-6 hours. Zileuton (30 mg/kg i.g.) significantly reduced paw edema at 3, 4 and 5 h from carrageenan injection. Maximum inhibition was achieved at 3h, being $41.01\% \pm 5.40\%$, as compared with corresponding control values. The inhibitory activity of the compound was well maintained throughout the duration of the experiment (Figure 2). This is in line with previous data showing that both 5-LO inhibitors and LT receptor antagonists are effective against carrageenan-induced inflammation and pain.^{20, 21} Inflammatory reactions to carrageenan involves activation of neutrophils and mast cells, which both are the predominant source of chemotactic LTB_4 and peptide-leukotrienes.^{22, 23}

Compound **18**, administered at a dose (45.12 mg/kg i.g.) equimolar to Zileuton, induced a significant inhibition of paw edema, displaying the same activity as the lead. (Fig.). Compound **25** (49.94 mg/kg i.g.) significantly reduced (approximately 20%) edema at 4 h only (Fig.), while at the other time periods it showed a trend towards inhibition, although its effect did not reach statistical significance. All the other analogues proved to be ineffective. The dose-dependence of the activity of zileuton in carrageenan-induced paw edema test²¹ indicates that higher doses of the present series of zileuton analogues should be tested. However these experiments were precluded by the low solubility of the products in the vehicle.

The observed differences in anti-inflammatory activity among the compounds at the tested dose, compared with their similar in vitro 5-LO inhibitor potency, could indicate a different pharmacokinetic profile for the compounds.

It is to be noted that while the compounds, at the doses tested, differed in anti-inflammatory activity, they displayed comparable in vitro 5-LO inhibitor potency, suggesting a different pharmacokinetic profile for the compounds.

Figure 2

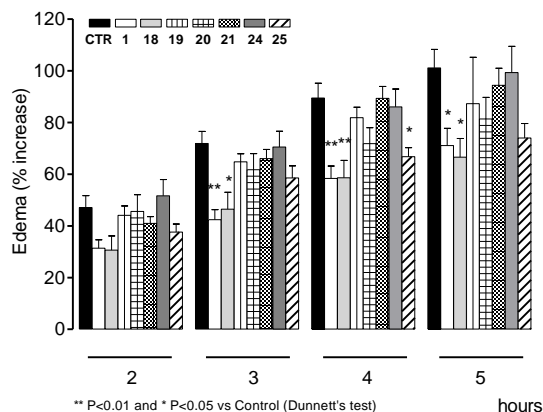


Figure 2 Percentage of increase in carrageenan-induced paw edema in rat. The compounds were administered by intragastric route at dose equimolar with zileuton **1**, 30 mg/kg, and their effects were evaluated at 2, 3, 4 and 5 hours from carrageenan injection.

Conclusion. A number of hitherto unknown hybrid products, obtained by combining Zileuton with either NO-donor nitrooxy or furoxan moieties were designed and evaluated as dual 5-LO inhibitors, rat tracheal ring myorelaxing agents, and vasodilators of rat aorta strips precontracted with phenylephrine. The products displayed 5-LO inhibition activity in micromolar range, close to where NO-dependent myorelaxing effects are displayed. By contrast, their NO-dependent vasodilator effects occur in a submicromolar range, with the sole exception of the methyl substituted furoxan derivative **24** which was active at micromolar concentrations. Altogether the in vitro results reported in this work indicate that this new class of dual 5-LO inhibitors/NO-donors could find interesting applications in the treatment of airways and inflammation diseases, as well as in the management of atherosclerosis development and its progression.

Preliminary characterization of in vivo pharmacological activity evidenced that products **18**, namely Zileuton substituted at 6-position with the simple 3-nitrooxypropyloxy chain, exhibited anti-inflammatory activity near that of the lead, when tested in the carrageenan-induced paw edema assay in the rat. Also the product **25**, bearing as 6-substituent (3- carboxamide furoxan-4-yl)methoxy group was able to display significant activity after 4 h. Since solubility limitations preclude administration of higher doses in this experimental model, additional in vivo studies are necessary to fully evaluate the potential of this series of compounds.

Experimental Part

Experimental Part.

Chemistry. M.p. were measured with a capillary apparatus (*Büchi 540*). M.p. with decomposition were determined after placing the sample in a bath at a temperature 10° below the M.p.; a heating rate of 3° min⁻¹ was used. All compounds were routinely checked by FT-IR (*PerkinElmer SPECTRUM BXII*), ¹H and ¹³C-NMR (*Bruker Avance 300*) and mass spectrometry (*Finnigan-Mat TSQ-700*). Flash column chromatography was performed on silica gel (*Merck Kieselgel 60, 230-400 mesh ASTM*) using the eluents indicated. Thin layer chromatography (TLC) was carried out on 5 x 20 cm plates with 0.25 mm layer thickness. Anhydrous MgSO₄ was used as drying agent for the organic phases. Analysis (C, H, N) of the new compounds was performed by REDOX (*Monza*): the results are within ± 0.4% of the theoretical values. Compounds **2**,²⁴ **4**,²⁵ **5**,²⁵ **6**,²⁶ **7**,²⁷ **12**,²⁸ **13**,²⁸ were synthesized following methods described in the literature. Tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone.

1-(6-hydroxybenzo[b]thiophen-2-yl)ethanol (3): To a stirred solution of the compound **2** (2.00 g, 13 mmol) in anhydrous THF (80 mL) under nitrogen, 1.6 M n-BuLi in hexane (25 mL, 3 equiv) was added below -20 °C, and the mixture was stirred for 3 h at 0 °C. Then, anhydrous acetaldehyde (1.72 g, 2.2 mL, 40 mmol) was added below -20 °C, and the solution was allowed to warm to r. t. in 2 h. The mixture was quenched with saturated NH₄Cl solution (80 mL). After separation of the forming layers, the water layer was extracted with EtOAc (3x 75 mL). The combined organic layers were washed with water (50 mL) and brine (30 mL), dried over anhydrous MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography (CH₂Cl₂ /EtOAc 9:1) to give 1.00 g of unreacted starting compound **2** and 1.26 g of the title compound **3** as a white solid (1.26 g, 50%): mp: 146–147 °C, dec.; ¹H NMR (300 MHz, [D₆]DMSO): δ=1.44 (d, ³J = 6 Hz, 3H, CH₃), 4.94 (m, 1H, CH), 5.56 (d, 1H, ³J = 5 Hz, OH), 6.81 (dd, 1H, ⁴J = 2 Hz, ³J = 9 Hz, H-5), 7.04 (s, 1H, H-3), 7.19 (d, 1H, ⁴J = 2 Hz, H-7) 7.52 (d, 1H, ³J = 9 Hz, H-4), 9.46 ppm (s, 1H, 6-OH); ¹³C NMR (75 MHz, [D₆]DMSO): δ=25.1, 64.4, 106.9, 113.8, 117.8, 123.5, 131.9, 139.7, 148.3, 154.2 ppm; IR (KBr): ν̃=3376, 1611, 1571, 1542, 1429, 1349, 1257, 1216, 1150, 1073, 1048, 1004, 985, 915, 848, 806 cm⁻¹; MS (EI, 70 eV) m/z (%): 194 (94) [M]⁺, 179 (94), 151 (100); Anal. calcd. for C₁₀H₁₀O₂S (194.25): C 61.83, H 5.19; found: C 61.87, H 5.23.

General method for preparation of triflates 8-11. Triflic anhydride (0.44 mL, 2.6 mmol, 1.3 equiv) was added dropwise to a solution of the appropriate nitrate **4-7** (2 mmol, 1.0 equiv.) and 2,6-lutidine (0.30 mL, 2.6 mmol, 1.3 equiv) in dry dichloromethane (10 mL) at -40 °C under nitrogen, and the resulting solution was stirred at this temperature for 1 h. Then the solution was filtered on a small pad of silica gel (eluent CH₂Cl₂) and the filtered solution was evaporated under vacuum to give an oil that was immediately used in the next step.

3-((2-(1-carbamoyl(hydroxyamino)ethyl)-1-benzo[b]thiophen-6-yl)oxy)propyl nitrate (18). To a solution of **3** (291 mg, 1.5 mmol, 1.0 equiv) in dry THF (5 mL), under nitrogen and at -15 °C, 60% sodium hydride in mineral oil (78 mg, 1.95 mmol, 1.3 equiv) was added portion wise and the solution was stirred until no more gas was liberated. At this solution was added by gas-tight syringe, a solution of the triflate **8** (506 mg, 2 mmol, 1.3 equiv) in dry CH₂Cl₂. The reaction mixture was stirred at -15 °C for 30 min then was quenched with saturated NH₄Cl solution. After separation of the forming layers, the water layer was

extracted with EtOAc. The combined organic layers were washed with water and brine, dried over anhydrous MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography (PE/EtOAc 8.5:1.5) to give 3-((2-(1-hydroxyethyl)-1-benzothien-6-yl)oxy)propyl nitrate (**14**) as a transparent oil that was used in the next step (54%); NMR (300 MHz, [D₆]DMSO): δ=1.47 (d, ³J = 6 Hz, 3H, CH₃), 2.21-2.12 (m, 2H, CCH₂C), 4.11 (t, ³J = 6 Hz, 2H, CH₂OC), 4.71 (t, ³J = 6 Hz, 2H, CH₂ON), 5.01-4.94 (m, 1H, CHO), 5.63 (d, ³J = 5 Hz, 1H, OH), 6.94 (dd, ⁴J = 2 Hz, ³J = 9 Hz, 1H, H-5), 7.11 (s, 1H, H-3), 7.49 (d, ⁴J = 2 Hz, 1H, H-7) 7.63 ppm (d, ³J = 9 Hz, 1H, H-4); ¹³C NMR (50 MHz, [D₆]DMSO): δ=25.8, 26.7, 64.7, 65.1, 71.4, 103.1, 106.4, 114.6, 118.6, 124.3, 133.9, 140.3, 150.6, 155.9 ppm.

A solution of hydroxyurea (112 mg, 1.5 mmol, 1.5 equiv.) in water (7 mL) was added to a solution of **14** (300 mg, 1.0 mmol, 1 equiv) in THF (10 mL) and this reaction mixture was warmed to 50 °C and added of 37% HCl (3.00 g, 2.61 mL, 30 mmol, 30 equiv.). The reaction mixture was warmed to 50 °C for 1.5 h and then was quenched with saturated NH₄Cl solution. After separation of the forming layers, the water layer was extracted with EtOAc. The combined organic layers were washed with water and brine, dried over anhydrous MgSO₄ and concentrated under reduced pressure. The residue was purified by flash chromatography (CH₂Cl₂/i-PrOH 7:3) to give **18** as a white solid (309 mg, 58%): m. p. 153-156° C dec. (MeOH); ¹H NMR (300 MHz, [D₆]DMSO): δ=1.48 (d, ³J = 7 Hz, 3H, CH₃), 2.20-2.12 (m, 2H, CCH₂C), 4.11 (t, ³J = 6 Hz, 2H, CH₂OC), 4.70 (t, ³J = 6 Hz, 2H, CH₂ON), 5.51 (q, ³J = 7 Hz, 1H, CHN), 6.43 (s, 2H, NH₂), 6.94 (dd, ⁴J = 2 Hz, ³J = 9 Hz, 1H, H-5), 7.15 (s, 1H, H-3), 7.47 (d, ⁴J = 2 Hz, 1H, H-7) 7.64 (d, ³J = 9 Hz, 1H, H-4), 9.19 ppm (s, 1H, OH); ¹³C NMR (50 MHz, [D₆]DMSO): δ=17.9, 26.4, 52.4, 64.4, 71.1, 105.9, 114.3, 120.9, 124.0, 133.2, 140.6, 143.5, 155.8, 161.5 ppm; IR (KBr): ν̃=3462, 3186, 2891, 1654, 1609, 1542, 1460, 1290, 1263, 1215, 1153, 1091, 1059, 956, 838; MS (EI, 70 eV) *m/z* (%): 355 (1)[M]⁺, 338 (7), 280 (37), 234 (49), 176 (100); Anal. calcd. for C₁₄H₁₇N₃O₆S (355.37): C 47.32, H 4.82, N 11.82; found: C 47.52, H 4.84, N 11.84.

6-((2-(1-carbamoyl(hydroxyamino)ethyl)-1-benzo[b]thiophen-6-yl)oxy)hexyl nitrate (19). The title compound was obtained as **18** starting from **9** with the only difference that the intermediate **15** was directly used, without further purification, in the reaction with hydroxyurea. The title compound was purified by flash chromatography (PE/EtOAc 8:2) to give a white solid (37%): m. p. 135-136 °C dec. (EtOAc); ¹H NMR (300 MHz, [D₆]DMSO): δ=1.26-1.45 (m, 4H), 1.65-1.76 (m, 4H) (CH₂ chain), 1.48 (d, ³J = 7 Hz, 3H, CH₃), 4.02 (t, ³J = 6 Hz, 2H, CH₂OC), 4.52 (t, ³J = 6 Hz, 2H, CH₂ON), 5.51 (q, ³J = 6 Hz, 1H, CHN), 6.43 (s, 2H, NH₂), 6.93 (dd, ⁴J = 2 Hz, ³J = 9 Hz, 1H, H-5), 7.13 (s, 1H, H-3), 7.43 (d, ⁴J = 2 Hz, 1H, H-7) 7.63 (d, ³J = 9 Hz, 1H, H-4), 9.19 ppm (s, 1H, OH); ¹³C NMR (50 MHz, [D₆]DMSO): δ=17.7, 24.7, 25.0, 25.9, 28.4, 52.2, 67.5, 73.7, 105.5, 114.1, 120.7, 123.7, 132.7, 140.4, 143.0, 155.9, 161.3 ppm; IR (KBr): ν̃=3463, 3321, 3177, 2989, 2939, 1657, 1572, 1543, 1470, 1388, 1367, 1280, 1263, 1215, 1153, 1081, 1025, 992, 950, 870, 838 cm⁻¹; MS (EI, 70 eV) *m/z* (%): 397 (1)[M]⁺, 380 (3), 352 (20), 307 (40), 207 (90), 176 (100); Anal. calcd. for C₁₇H₂₃N₃O₆S (397.45): C 51.37, H 5.83, N 10.57; found: C 51.28, H 5.89, N 10.49.

4-((2-(1-carbamoyl(hydroxyamino)ethyl)-1-benzo[b]thiophen-6-yl)oxy)buthan-1,2-diyl dinitrate (20). The title compound was obtained as **19** starting from **10**, through the crude intermediate **16**. The title product was purified by flash chromatography (CH₂Cl₂/MeOH 9.8:0.2) to give a white solid (17%): m. p. 115-116 °C dec. (EtOAc/Hexane); ¹H NMR (300 MHz, [D₆]DMSO): δ=1.48 (d, ³J = 6 Hz, 3H, CH₃), 2.22-2.25 (m, 2H, CCH₂C), 4.04 (m, 2H, CH₂OC), 4.80 (dd, ²J = 13 Hz, ³J = 6 Hz, 1H, CH₂ON), 5.02 (dd, ²J = 13 Hz, ³J = 2 Hz, 1H, CH₂ON), 5.51-5.53 (m, 1H, CHONO₂), 5.66 (m, 1H, CHNOH), 6.43 (s, 2H, NH₂), 6.93 (dd, ⁴J = 2 Hz, ³J = 9 Hz, 1H, H-5), 7.15 (s, 1H, H-3), 7.47 (d, ⁴J = 2 Hz, 1H, H-7), 7.64 (d, ³J = 9 Hz, 1H, H-4), 9.19 ppm (s, 1H, OH); ¹³C NMR: 17.7, 28.2, 52.2, 63.7, 72.0, 77.9, 105.7, 114.0, 120.7, 123.8, 133.1, 140.3, 143.4, 155.4, 161.3; IR (KBr/cm⁻¹): 3480, 3350, 2941, 1659, 1646, 1601, 1578, 1466, 1273, 1227, 1150, 1064, 845 cm⁻¹; MS

(EI, 70 eV) m/z (%): 385 (17), 340 (30) 295 (27), 263 (100), 207 (75). Anal. calcd. for $C_{15}H_{18}N_4O_9S$ (430.39): C 41.86, H 4.22, N 13.02; found: C 42.33, H 4.23, N 12.70.

6-((2-(1-carbamoyl(hydroxyamino)ethyl)-1-benzo[b]thiophen-6-yl)oxy)hexan-1,2-diyl dinitrate (21). The title compound was obtained as **19** starting from **11**, through the crude intermediate **17**. The title compound was purified by flash chromatography ($CH_2Cl_2/EtOAc$ 7:3) to give a white solid (30%): m. p. 105-107 °C dec. ($EtOAc/Hexane$); 1H NMR (300 MHz, $[D_6]DMSO$): δ =1.48 (d, $^3J = 7$ Hz, 3H, CH_3), 1.54-1.60 (m, 2H) 1.73-1.83 (m, 4H), (CCH_2C), 4.02 (t, $^3J = 6$ Hz, 2H, CH_2OC), 4.72 (dd, $^2J = 13$ Hz, $^3J = 6$ Hz, 1H, CH_2ON), 4.95 (dd, $^2J = 13$ Hz, $^3J = 2$ Hz, 1H, CH_2ON), 5.42-5.47 (m, 1H, $CHONO_2$), 5.50 (q, $^3J = 7$ Hz, 1H, $CHNOH$), 6.43 (s, 2H, NH_2), 6.93 (dd, $^4J = 2$ Hz, $^3J = 9$ Hz, 1H, $H-5$), 7.14 (s, 1H, $H-3$), 7.44 (d, $^4J = 2$ Hz, 1H, $H-7$), 7.63 ppm (d, $^3J = 9$ Hz, 1H, $H-4$); ^{13}C NMR (75 MHz, $[D_6]DMSO$): δ =17.7, 21.1, 27.9, 28.1, 52.2, 67.3, 71.9, 80.2, 105.5, 114.1, 120.7, 123.7, 132.8, 140.4, 143.1, 155.9, 161.3; IR (KBr): $\tilde{\nu}$ =3465, 3196, 2939, 2870, 1659, 1572, 1465, 1270, 1225, 1152, 844 cm^{-1} ; MS (CI) m/z (%): 459 (1)[$M+H$] $^+$, 414 (100). Anal. calcd. for $C_{17}H_{22}N_4O_9S$ (458.45): C 44.54, H 4.84, N 12.22; found: C 44.69, H 4.80, N 11.98.

1-hydroxy-1-(1-(6-((4-methyl-furoxan-3-yl)methoxy)benzo[b]thiophen-2-yl)ethanyl)urea (24). Compound **12** (289 mg, 1.5 mmol, 1.5 equiv.) was added portion wise to a solution of **3** (194 mg, 1.0 mmol, 1 equiv.) and K_2CO_3 (552 mg, 4.0 mmol, 4.0 equiv.) in dry DMF (1.5 mL) and the reaction mixture was stirred at r.t. for 1 h. Then ice water was added and the formed solid was triturated and filtrated to give crude **22** that was directly used in the next step. 1H NMR (300 MHz, $[D_6]DMSO$): δ =1.47 (d, $^3J = 6$ Hz, 3H, CH_3), 2.20 (s, 3H, 4- CH_3 -fx), 5.00 (m, 1H, CH), 5.35 (s, 2H, CH_2O), 5.68 (d, $^3J = 4$ Hz, 1H, OH), 7.07 (dd, $^4J = 2$ Hz, $^3J = 9$ Hz, 1H, $H-5$), 7.15 (s, 1H, $H-3$), 7.67 (s, 1H, $H-7$), 7.70 ppm (s, 1H, $H-4$); ^{13}C NMR (75 MHz, $[D_6]DMSO$): δ =7.4, 25.4, 61.1, 64.6, 107.0, 113.0, 114.3, 118.0, 123.9, 134.3, 139.7, 150.3, 154.4, 155.5 ppm; MS (EI, 70 eV) m/z (%): 306 (30)[M] $^+$, 288 (30), 193 (70), 175 (100).

A solution of hydroxyurea (112 mg, 1.5 mmol, 1.5 equiv.) in water (7 mL) was added to a solution of **22** (306 mg, 1.0 mmol, 1 equiv.) in THF (10 mL) and this reaction mixture was warmed to 50 °C and added of 37% HCl (3.00 g, 2.61 mL, 30 mmol, 30 equiv.). The reaction mixture was warmed to 50 °C for 2 h and then the reaction mixture was concentrated under reduced pressure and the formed solid was triturated and filtrated. The crude **24** was purified by crystallization from $EtOAc/Hexane$ to give a white solid (146 mg, 40 %): m. p. 143-144 °C dec. ($EtOAc/Hexane$); 1H NMR (300 MHz, $[D_6]DMSO$): δ =1.49 (d, $^3J = 7$ Hz, 3H, CH_3), 2.19 (s, 3H, 4- CH_3 -fx), 5.35 (s, 2H, CH_2O), 5.52 (q, $^3J = 7$ Hz, 1H, CH), 6.44 (s, 2H, NH_2), 7.07 (dd, $^4J = 2$ Hz, $^3J = 9$ Hz, 1H, $H-5$), 7.18 (s, 1H, $H-3$), 7.65 (d, $^4J = 2$, 1H, $H-7$), 7.70 (d, $^3J = 9$ Hz, 1H, $H-4$), 9.21 ppm (s, 1H, OH); ^{13}C NMR (75 MHz, $[D_6]DMSO$): δ =7.6, 17.9, 52.4, 61.3, 107.0, 113.2, 114.4, 120.9, 124.1, 134.0, 140.4, 144.3, 154.8, 155.7, 161.5 ppm; IR (KBr): $\tilde{\nu}$ =3482, 3340, 2826, 1664, 1628, 1578, 1475, 1375, 1258, 1229, 1150, 1026, 847 cm^{-1} ; MS (CI) m/z (%): 365 (40)[$M+H$] $^+$, 320 (100). Anal. calcd. for $C_{15}H_{16}N_4O_5S$ (364.38): C 49.44, H 4.43, N 15.38; found: C 49.02, H 4.46, N 15.03.

4-((2-(1-carbamoyl(hydroxyamino)ethyl)-1-benzo[b]thiophen-6-yl)oxy)methyl)-furoxan-3-carboxamide (25). Compound **13** (222 mg, 1.5 mmol, 1.5 equiv.) was added portion wise to a solution of **3** (194 mg, 1.0 mmol, 1 equiv.) and K_2CO_3 (552 mg, 4.0 mmol, 4.0 equiv.) in dry DMF (1.5 mL) and the reaction mixture was stirred at r.t. for 1 h. Then ice water was added and after separation of the forming layers, the water layer was extracted with $EtOAc$. The combined organic layers were washed with water and brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography ($CH_2Cl_2/EtOAc$ 8:2) to give **23** as a white solid (201 mg, 60 %): 1H NMR (300 MHz, $[D_6]DMSO$): δ =1.46 (d, $^3J = 6$ Hz, 3H, CH_3), 4.99 (m, 1H, CH), 5.46 (s, 2H, CH_2O), 5.66 (d, $^3J = 5$ Hz, 1H, OH), 7.06 (dd, $^4J = 2$ Hz, $^3J = 9$ Hz, 1H, $H-5$), 7.14 (s, 1H, $H-3$), 7.63 (d, $^4J = 2$ Hz, 1H, $H-7$), 7.66 (d, $^3J = 9$ Hz, 1H, $H-$

4), 7.85, 8.50 ppm (2s, 2H, NH₂); ¹³C NMR (75 MHz, [D₆]DMSO): δ=26.3, 62.5, 65.6, 107.7, 111.4, 115.2, 118.9, 124.8, 135.0, 140.7, 151.7, 155.7, 156.2, 156.6 ppm; MS (EI, 70 eV) *m/z* (%): 335 (30) [M]⁺, 317 (2), 193 (100), 175 (60).

A solution of hydroxyurea (112 mg, 1.5 mmol, 1.5 equiv.) in water (7 mL) was added to a solution of **23** (335 mg, 1.0 mmol, 1 equiv) in THF (10 mL) and this reaction mixture was warmed to 50 °C and added of 37% HCl (3.00 g, 2.61 mL, 30 mmol, 30 equiv.). The reaction mixture was warmed to 50 °C for 2 h and then the reaction mixture was concentrated under reduced pressure. From the cooled aqueous solution precipitated a solid that was triturated and filtrated. The obtained crude **25** was purified by flash chromatography (CH₂Cl₂/i-PrOH 9:1) to give a white solid (160 mg, 46 %): m. p. 175-176 °C dec. (EtOH/EtOAc); ¹H NMR (300 MHz, [D₆]DMSO): δ=1.49 (d, ³J = 7 Hz, 3H, CH₃), 5.46 (s, 2H, CH₂O), 5.52 (q, ³J = 7 Hz, 1H, CH), 6.44 (s, 2H, NH₂), 7.05 (dd, ⁴J = 2 Hz, ³J = 9 Hz, 1H, H-5), 7.18 (s, 1H, H-3), 7.65 (d, ⁴J = 2, 1H, H-7), 7.68 (d, ³J = 9 Hz, 1H, H-4), 7.85, 8.50 (2s, 2H, 4-NH₂CO-fx), 9.20 ppm (s, 1H, OH); ¹³C NMR (75 MHz, [D₆]DMSO): δ=17.7, 52.2, 61.5, 106.4, 110.4, 114.2, 120.7, 123.8, 133.6, 140.2, 143.9, 154.8, 155.2, 155.6, 161.3 ppm; IR (KBr): ν̃=3466, 3427, 3190, 1688, 1670, 1572, 1469, 1443, 1265, 1219, 1153, 1067, 838, 776 cm⁻¹; MS (EI, 70 eV) *m/z* (%): 348 (35), 334 (60), 318 (95), 232 (50), 190 (82), 177 (100). Anal. calcd. for C₁₅H₁₅N₅O₆S (393.38): C 45.80, H 3.84, N 17.80; found: C 45.81, H 4.03, N 17.53.

Leukotriene Assay. Study of 5-LO Inhibition was carried out following a procedure similar to that reported in literature.²⁹ Blood samples were obtained from healthy volunteers who had not taken any drug for at last two weeks. Methanolic solutions of the tested compounds at different concentrations were prepared, 10 μL aliquots were distributed in incubation polystyrene tubes and the solvent was evaporated. The residues were dissolved by vortexing in 1mL heparinized (20 IU/mL) venous blood and the tubes were preincubated for 15 min at 37°C.

Eicosanoid biosynthesis was initiated by adding calcium ionophore A23187 at 50 μM concentration (final 0.05% v/v DMSO) and terminated after 30 min by rapid cooling of the blood in an ice bath and centrifuging at 3°C for 10 min at 2500 x *g*. Plasma samples were further centrifuged at 12000 x *g* at 3 °C for 3 min and then supernatants were ready to be tested for Leukotriene B₄ production. Basal LTB₄ production in blood untreated with A23187 was subtracted and % inhibition in samples incubated with tested compounds was evaluated in comparison with control samples with maximal LTB₄ production.

LTB₄ production was evaluated by competitive enzyme immunoassay, according to specific instructions provided by Cayman Chemical, based on a competition between LTB₄ and a LTB₄-acetylcholinesterase conjugate (LTB₄ tracer). At the end of an overnight incubation at 4°C the amount of tracer (added in each well at a constant concentration) bound to antiserum is inversely proportional to the added concentrations of LTB₄, produced by 5-LO. This antibody-LTB₄ complex binds to an unspecific antibody that had been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent, which contains a substrate for acetylcholinesterase, is added to the well. The product of this enzymatic reaction gives a distinct yellow colour that absorbs at 405 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of Leukotriene-tracers present in the well, which is inversely proportional to the amount of leukotrienes present in the well during the incubation. Standard curves with known concentrations of LTB₄ is used to obtain concentrations in the sample wells. Percent inhibition in compound-treated samples were calculated by comparison with control untreated samples. The concentration of the tested compounds causing 50% inhibition (IC₅₀) was calculated from the concentration-inhibition response curve (3-4 experiments).

Myorelaxing activity. Tracheas and aortas were isolated from male Wistar rats weighing 180-200 g. The animals, treated humanely in accordance with recognised guidelines on experimentation, were anaesthetised with CO₂ and killed by decapitation. As few animals as possible were used. The purposes and the protocols of our studies have been approved by the Ministero della Salute, Rome, Italy. The tissues were mounted in organ baths containing 30 mL of Krebs-bicarbonate buffer of the following composition (mM): NaCl 111.2, KCl 5.0, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.0, NaHCO₃ 12, glucose 11.1. The solution was maintained at 37°C and continuously gassed with 95% O₂-5% CO₂ (pH = 7.4). The tracheal tube was quickly excised and cleaned free of excess tissues, then was cut into single rings. Three to four rings were joined together by thread to form a chain and mounted in organ baths. After 120 min equilibration period 1 μM carbachol was added to the organ bath to induce a spasm of the trachea rings and when a constant level was reached, cumulative concentration–response curves to compounds were determined. Effects of 10 μM ODQ were evaluated in separate series of experiments in which it was added 20 min before the contraction. With this protocol the inhibitor is preincubated for at least 40 minutes before the start of the curve. Results are expressed as EC₅₀ ± SE (μM); EC₅₀ values are the mean of 4-6 determinations. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab.

Vasodilator activity. The aorta endothelium was removed and the vessels were cut helically: three strips were obtained from each aorta. The tissues were mounted under 1.0 g of tension in organ baths. The aortic strips were allowed to equilibrate for 120 min and then contracted with 1 μM phenylephrine. When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. The effects of 1 μM ODQ on relaxation were evaluated in separate series of experiments in which it was added 5 min before the contraction. With this protocol the inhibitor is preincubated for at least 30 minutes before the addition of the vasodilator compound. Results are expressed as EC₅₀ ± SE (μM); EC₅₀ values are the mean of 4-8 determinations

Antiinflammatory activity. The antiinflammatory activity of compounds under study was tested in the carrageenan-induced paw edema in conscious rats. Acute edema was induced in the right hind paw of rats by injecting into the plantar region 0.1 ml of freshly prepared solution of 1% carrageenan. The volume of the paw was measured using a plethysmometer (Basile, Italy) at 2, 3, 4 and 5 hours after carrageenan challenge. Inflammation was expressed as the percentage change in paw volume.³⁰ Compounds under study were administered intragastrically (i.g.) in a volume of 10 ml/kg immediately before carrageenan injection.

Acknowledgments

Key words

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The compounds, designed by joining Zileuton to NO-donor nitrooxy or furoxan moieties, represent the first class of dual drugs with 5-LO inhibitor/NO-dependent activities. The products inhibited LTB₄ production with a potencies near that of the Zileuton. In addition they were capable of relaxing rat tracheal rings and rat aorta strips and that one substituted with an alkoxy chain bearing a nitrooxy or a 3-carbamoylfuroxan moiety, displayed anti-inflammatory activity.