Multitarget Drugs: Synthesis and Preliminary Pharmacological Characterization of Zileuton Analogues Endowed with Dual 5-LO Inhibitor and NO-Dependent Activities

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(Article begins on next page)
Multitarget Drugs: Synthesis and Preliminary Pharmacological Characterization of Zileuton Analognes Endowed with Dual 5-LO Inhibitor and NO-Dependent Activities


The leukotrienes (LTs) are a family of lipid-derived autocoids that originate from arachidonic acid (AA). 5-Lipoxygenase (5-LO) is the key enzyme in this process. It transforms AA through a two-step 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 (5-HPETE), and then into unstable leukotriene A4 (LTA4). A two-step 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, into peptide–lipid leukotrienes C4, D4, and E4 (LTC4, LTD4, LTE4).[1–3] LTs are involved in a variety of inflammatory and allergic disorders, particularly rheumatoid arthritis and inflammatory skin and bowel diseases. They also display potent bronchoconstrictor activity. Consequently, the treatment of allergic disorders and asthma are the classical indications for 5-LO inhibitors.[4,5] Novel and interesting potential indications are emerging for these products; for example, an increasing amount of experimental evidence shows an involvement of the 5-LO pathway in tumor cell proliferation.[6] In particular, inhibition of 5-LO was found to induce apoptosis in various cancer cell types.[6] The evidence that LTs are involved in atherogenesis and arterial wall remodeling sets the stage for new strategies in treating the development and progression of atherosclerosis.[5,7,8] 5-LO inhibitors can be classified into four different classes according to their mechanism of action: redox-active compounds, competitive reversible inhibitors, inhibitors of 5-LO activating protein (FLAP), and iron-chelating inhibitors. Many substances that belong 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), a hydroxyurea derivative of the iron-ligand-type inhibitor class, entered into the market in 1996 as an anti-asthmatic drug.[12,13] The commercially available product is a racemic mixture of R and S enantiomers, both of which display in vitro 5-LO inhibitory activity.

A number of studies have been carried out in recent years to design 5-LO inhibitors with dual activity: 5-LO/cyclooxygenase (COX) inhibitors have received particular attention as anti-inflammatory agents, and compounds either with dual 5-LO/thromboxane A2 synthase inhibitory activity or with 5-LO inhibitor and platelet-activating factor (PAF) receptor antagonist mixed properties have also been developed.[16] These are examples of multitarget drugs, which should be able to modulate more than one target simultaneously; their development represents an alternative approach to using drug cocktails especially in the treatment of complex diseases such as atherosclerosis and inflammation. The most common strategy to obtain these products is the combination of two appropriate drugs, or their crucial parts, into a single molecule.[16] To our knowledge there has not yet been any documented examples of nitric oxide (NO) donor/5-LO inhibitor hybrids, despite the clear interest in such a combination. Indeed, NO is a physiological messenger that triggers a variety of actions in different systems.[15] In particular, it plays very important roles in the cardiovascular system in maintaining a number of homeostatic responses: preservation of endothelial integrity, arterial blood vessel dilatation including pulmonary arterial vasculature, inhibition of platelet adherence and aggregation, attenuation of leukocyte 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 these kinds of products in the field of respiratory disease is still under examination.[16–18] Herein we propose new dual-action products, obtained by combining zileuton with NO donor nitroxy or furoxan moieties.

The synthesis of the final products required the preliminary preparation of a number of intermediates (Scheme 1). The substituted benzo triophene 3 was easily obtained by treating 6-hydroxybenzothiophene (2) with n-butyl lithium and anhydrous acetaldehyde in THF at −20°C. The triflates 8–11 were prepared by the action of trifluoromethanesulfonyl anhydride in dichloromethane on the appropriate nitroxy-substituted alcohols 4–7 in the presence of 2,6-lutidine at −40°C and were immediately used. Treatment of 3 with sodium hydride in THF, followed by the appropriate triflates 8–11 in dichloromethane afforded the expected nitrates 14–17. The action of 37% hydrochloric acid on these products dissolved in THF/water in the presence of hydroxyurea at 50°C afforded the target com-
The dinitrooxy-substituted compounds 18–21. The furoxan derivatives 22 and 23 were prepared by reaction of 3 with the respective 4-bromomethylfuroxans 12 or 13 in dry DMF in the presence of potassium carbonate. The final furoxan models 24 and 25 were obtained from 22 and 23 by following the same procedure used to prepare the analogous nitric esters 18–21 from 14–17.

The ability of the target products 18–21, 24, 25, and of the reference compound zileuton to inhibit 5-LO was assessed by incubating each compound in heparin-treated human whole blood. After a fixed time, LT biosynthesis was initiated by addition of the calcium ionophore A23187, and terminated by rapid cooling of the blood. After centrifugation, the plasma level of LTB₄ was analyzed by enzyme-linked immunosorbent assay (ELISA). All the products were able to inhibit LTB₄ production in a concentration-dependent manner. The potencies of inhibition, expressed as IC₅₀, cover the concentration range of 2–18 μM (Table 1). Detailed data analysis shows that the most potent inhibitor is the mononitrooxy-substituted compound 18. Its IC₅₀ value is same as that of zileuton, within experimental error. The inhibitory activity still good, but a bit lower for homologue 19, in which the length of the nitrooxyalkoxy lateral chain is increased. The dinitrooxy-substituted compound 20 is also nearly equipotent to zileuton, and this activity is largely retained in its higher homologue 21. The furoxan-(-)-isoprenaline at 10 μM. The potencies of the products, expressed as EC₅₀, are listed in Table 1; these figures fall in the narrow concentration range of 15–37 μM. In the nitric acid ester series, the mononitrooxy derivatives 18 and 19 display the same activity and are half as potent as the equipotent dinitrooxy analogues 20 and 21. Between the two furoxan derivatives, only the furoxancarboxamide 25 shows an EC₅₀ value in the micromolar range, while the methyl-substituted furoxan 24 does not elicit any myorelaxing effect when tested up to 30 μM. As expected, zileuton also does not trigger any effect.

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**Table 1. Vasodilation, myorelaxation, and 5-LO inhibition properties of zileuton (1) and compounds 18–21, 24, and 25.**

<table>
<thead>
<tr>
<th>Compd</th>
<th>R</th>
<th>Vasodilation[+]</th>
<th>Myorelaxation[+]</th>
<th>5-LO Inhibition[+]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>EC₅₀ ± SE (μM)</td>
<td>IC₅₀ ± SE (μM)</td>
<td>IC₅₀ (μM)</td>
</tr>
<tr>
<td>18</td>
<td>O₂NO</td>
<td>0.039 ± 0.007 (10)</td>
<td>37 ± 5 (10)</td>
<td>1.6 (1.3–2.0)</td>
</tr>
<tr>
<td>19</td>
<td>O₂NO</td>
<td>0.023 ± 0.003 (30)</td>
<td>36 ± 8 (10)</td>
<td>2.0 (1.6–2.5)</td>
</tr>
<tr>
<td>20</td>
<td>O₂NO</td>
<td>0.015 ± 0.004 (23)</td>
<td>16 ± 3 (10)</td>
<td>5.8 (4.3–7.7)</td>
</tr>
<tr>
<td>21</td>
<td>O₂NO</td>
<td>0.027 ± 0.004 (14)</td>
<td>15 ± 1 (10)</td>
<td>2.8 (2.0–4.1)</td>
</tr>
<tr>
<td>24</td>
<td>O₂NO</td>
<td>28 ± 2 (10)</td>
<td>6.3 (4–8.3)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>O₂NO</td>
<td>0.18 ± 0.04 (6.4 ± 0.7)</td>
<td>24 ± 2 (10)</td>
<td>17.9 (11.5–28.0)</td>
</tr>
</tbody>
</table>

[a] Determined on rat thoracic aorta pre-contracted with 1 μM phenylephrine. [b] Determined on rat tracheal rings pre-contracted with 1 μM carbachol. [c] Measured as the ability of the compound to inhibit biosynthesis of LTB₄ in human whole blood challenged with the calcium ionophore A23187. [d] Inactive at the maximum concentration tested (30 μM).
on the contracted tissue, suggesting that the myorelaxing effects observed for all the active products are mediated by NO. To confirm this hypothesis, the activity of the products on pre-contraction tracheal rings was assessed in the presence of 10 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinolinoxaline-1-one (ODQ), a well-known inhibitor of soluble guanylate cyclase (sGC). This is the key enzyme in mediating tracheal relaxation induced by NO and NO-related compounds through elevation of the intracellular concentration of guanosine 3',5'-cyclic monophosphate (cGMP).\textsuperscript{[20]} The suppression of sGC activity, when the products were tested up to a concentration of 30 μM, is in keeping with the involvement of the NO messenger in their myorelaxing action. The NO-dependent myorelaxing potency of the products is near their 5-LO inhibition potency range. This means that the hybrids display these two activities in vitro in a fairly well-balanced manner.

The vasodilator effects of the target hybrids were assessed with denuded rat aorta strips pre-contracted with phenylephrine. The vasodilator potencies of the products, expressed as EC\textsubscript{50}, are also listed in Table 1. The nitrooxy-substituted compounds are very potent vasodilators, with EC\textsubscript{50} values in the sub-micromolar range. In the nitric ester series, the products display similar potencies, ranked in the order: 20 > 19 ≥ 21 > 18. The furoxancarboxamide 25 is also quite a potent vasodilator, about tenfold less potent than ditinotroyloxy ester 20, the most potent derivative of the series, and 150-fold more potent than the methyl furoxan 24. The significant decrease in activities observed when the experiments were repeated in the presence of 1 μM ODQ is in keeping with a NO-mediated vasodilator mechanism. Altogether, these data indicate that in vitro NO-mediated vasodilator effects of the tested compounds dominate their 5-LO inhibitor capacity. The only exception is methylfuroxan 24, for which these two activities occur at similar concentrations.

All the target products, including zileuton (1) as reference, were tested on carrageenan-induced paw edema in conscious rats. The injection of carrageenan into the hind paws produced swelling, which reached its maximum at 5–6 h. Zileuton (30 mg kg\textsuperscript{-1} i.g.) significantly decreased paw edema at 3, 4, and 5 h from carrageenan injection. Maximum inhibition was achieved at 3 h: 41.01 ± 5.40% relative to corresponding control values. The inhibitory activity of the compound was well maintained throughout the duration of the experiment (Figure 1). 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.\textsuperscript{[21,22]} The inflammatory reaction to carrageenan involves activation of neutrophils and mast cells, both of which are the predominant source of chemotactic LTB\textsubscript{2} and peptide-leukotrienes.\textsuperscript{[21,24]} Compound 18, administered at a dose (45.12 mg kg\textsuperscript{-1} i.g.) equimolar to zileuton, induced a significant inhibition of paw edema, displaying the same activity as the lead (Figure 1). Compound 25 (49.94 mg kg\textsuperscript{-1} i.g.) significantly decreased (~20%) edema at 4 h only, while at the other time points it showed a trend toward 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 the carrageenan-induced paw edema test\textsuperscript{[22]} 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 (Figure 1).

Notably, while the compounds at the doses tested differ in their anti-inflammatory activity, they display similar in vitro 5-LO inhibition potency, suggesting a different pharmacokinetic profile between the compounds.

In 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 pre-contrasted with phenylephrine. The products display 5-LO inhibitory activity in the micromolar range, close to where NO-dependent myorelaxing effects are observed. In contrast, their NO-dependent vasodilator effects occur in the sub-micromolar range, with the sole exception of the methyl-substituted furoxan derivative 24, which is active at micromolar concentrations. Altogether, the in vitro results reported herein indicate that this new class of dual 5-LO inhibitors/NO donors could find interesting applications in the treatment of airway and inflammatory diseases, as well as in the management of atherosclerosis development and its progression.

Preliminary characterization of in vivo pharmacological activity showed that compound 18, namely zileuton substituted at the 6-position with the simple 3-nitrooxynpropoxy chain, exhibits anti-inflammatory activity near that of the lead 1, when tested in the carrageenan-induced rat paw edema assay. Compound 25, bearing a (3-carbamoylfuroxan-4-yl)methoxy group at the 6-position, also displays significant activity after 4 h. Because 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 Section

Melting points (mp) were measured on a capillary apparatus (Büchi 540); mp with decomposition was determined after placing the sample in a bath at a temperature 10 °C below the mp: a heating rate of 3 °C min−1 was used. All compounds were routinely checked by FTIR (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 × 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 theoretical values. Compounds 2, 4, 5, 6, 7, 12, 13 and 18 were synthesized by following published methods. Tetrahydrofuran (THF) was distilled immediately before use from sodium and benzophenone.

1-(6-Hydroxybenzyl)[thiophen-2-yl]ethanol (3). nBuLi (1.6 M in hexane, 25 mL, 3 eqv) was added at −20 °C to a stirred solution of compound 2 (2.00 g, 13 mmol) in anhydrous THF (80 mL) under N2, and the mixture was stirred for 3 h at 0 °C. Anhydrous acetaldehyde (1.72 g, 2.2 mL, 40 mmol) was then added dropwise to a solution of the appropriate nitrate (15 mmol) at −20 °C, and the solution was allowed to warm to room temperature over 2 h. The mixture was quenched with saturated NH4Cl (30 mL). After separation of the layers, the aqueous layer was extracted with EtOAc. The combined organic layers were washed with H2O and brine, dried over anhydrous MgSO4, and concentrated under reduced pressure. The residue was purified by flash chromatography (CH2Cl2/MeOH, 9:1) to give a pure (0.1 g of unreacted starting compound 2 and 1.26 g of the title compound as a white solid (12.6 g, 50%): mp = 146–147 °C (dec). 1H NMR (300 MHz, [D6]DMSO): δ = 1.44 (d, J = 6 Hz, 3H, CH3), 4.94 (m, 1H, CH), 5.56 (dd, J = 5 Hz, 6 Hz, 2H, CH2), 6.81 (dd, J = 2 Hz, 3 Hz, J = 9 Hz, H-5), 7.04 (s, 1H, H-3), 7.19 (d, J = 2 Hz, J = 7 Hz, H-7), 5.25 (d, J = 12 Hz, H-4), 9.46 ppm (s, 1H, 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, 1517, 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. calc. for C9H13NO2 (194.25): C 61.83, H 5.19; found: C 61.87, H 5.23.

General method for the preparation of triflates 8–11. Trifluoromethanesulfonil anhydride (0.44 mL, 2.6 mmol, 1.3 eqv) was added dropwise to a solution of the appropriate nitrate 4–7 (2 mmol, 1.0 eqv) and 2,6-lutidine (0.30 mL, 2.6 mmol, 1.3 eqv) in CH2Cl2 (anhyd, 10 mL) at −40 °C under N2, and the resulting solution was stirred at this temperature for 1 h. The solution was then filtered on a small pad of silica gel (eluent CH2Cl2) and evaporated under vacuum to give an oil that was immediately used in the next step.

3-[2-(1-Carbamoyl[hydroxymino]ethyl)-1-benzo[thiophen-6-yl]oxy]propyl nitrate (18). NaOH (60% in mineral oil, 78 mg, 1.95 mmol, 1.3 eqv) was added portion-wise to a solution of 3 (291 mg, 1.5 mmol, 1.0 eqv) in THF (anhyd, 5 mL), under N2 at −15 °C, and the solution was stirred until no more gas developed. A solution of triflate 8 (506 mg, 2 mmol, 1.3 eqv) in anhydrous CH2Cl2 was added to this solution via gas-tight syringe. The reaction mixture was stirred at −15 °C for 30 min, and then it was quenched with saturated NH4Cl. After separation of the layers, the aqueous layer was extracted with EtOAc. The combined organic layers were washed with H2O 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-benzo[thiophen-6-yl]oxy]propyl nitrate (18). A solution of the purified oil (18) (61 mg) in THF (10 mL) was added to a solution of AlBN (5 mol%, 1 eqv) in THF (10 mL), and this reaction mixture was warmed to 50 °C. After 2.5 h, the reaction mixture was warmed to 50 °C for 30 min, and then it was quenched with saturated NH4Cl. After separation of the layers, the aqueous layer was extracted with EtOAc. The combined organic layers were washed with H2O 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-benzo[thiophen-6-yl]oxy]propyl nitrate (18). The title compound was obtained as was 18 by starting from 9, with the only difference that the intermediate 15 was directly used, without further purification, in the reaction with hydroxurea. The title compound was purified by flash chromatography (PE/EtOAc 8:2) to give a white solid (37%): mp = 135–136 °C (dec., EtOAc); 1H NMR (300 MHz, [D6]DMSO): δ = 1.26–1.45 (m, 4H, CH2), 1.45–1.71 (m, 4H, CH2CH2), 4.02 (t, J = 6 Hz, 2H, CH2O), 4.52 (t, J = 6 Hz, 2H, CH2O), 5.51 (q, J = 7 Hz, 1H, CH2CH2), 6.43 (s, 2H, NH), 6.93 (dd, J = 2 Hz, J = 9 Hz, 1H, H-5), 7.15 (s, 1H, H-3), 7.47 (d, J = 2 Hz, 1H, H-7), 7.63 ppm (d, J = 9 Hz, 1H, H-4); 13C NMR (75 MHz, [D6]DMSO): δ = 25.8, 26.7, 64.7, 65.1, 71.4, 106.4, 114.6, 118.4, 123.9, 140.3, 150.6, 155.9 ppm.

A solution of hydroxurea (112 mg, 1.5 mmol, 1 equiv) in H2O (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. After 2.5 h, the reaction mixture was warmed to 50 °C for 30 min, and then it was quenched with saturated NH4Cl. After separation of the layers, the aqueous layer was extracted with EtOAc. The combined organic layers were washed with H2O and brine, dried over anhydrous MgSO4, and concentrated under reduced pressure. The residue was purified by flash chromatography (CH2Cl2/iPrOH 7:3) to give 18 as a white solid (309 mg, 58%): mp = 153–156 °C (dec., MeOH); 1H NMR (300 MHz, [D6]DMSO): δ = 1.48 (d, J = 7 Hz, 3H, CH3), 2.20–2.12 (m, 2H, CH2), 4.11 (t, J = 6 Hz, 2H, CH2O), 4.71 (t, J = 6 Hz, 2H, CH2ON), 5.01–4.94 (m, 1H, CHO), 5.63 (d, J = 5 Hz, 1H, OH), 6.94 (dd, J = 2 Hz, J = 9 Hz, 1H, H-5), 7.11 (s, 1H, H-3), 7.49 (d, J = 2 Hz, 1H, H-7) 7.63 ppm (d, J = 9 Hz, 1H, H-4); 13C NMR (75 MHz, [D6]DMSO): δ = 25.8, 26.7, 64.7, 65.1, 71.4, 106.4, 114.6, 118.4, 123.9, 140.3, 150.6, 155.9 ppm.

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A solution of hydroxurea (112 mg, 1.55 mmol, 1 equiv) in H₂O (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. HCl (37%, 3.00 g, 26.1 ml, 30 mmol, 30 equiv) was added. The reaction mixture was warmed to 50 °C for 2 h, and then concentrated under reduced pressure. The formed solid was triturated and filtered. The obtained crude 24 was purified by flash chromatography (CH₂Cl₂/iPrOH 9:1) to give a white solid (160 mg, 46%): mp: 175–176 °C (dec., Et₂O/CH₂Cl₂); 1H NMR (300 MHz, [D₆]DMSO): δ = 1.49 (d, J = 7 Hz, 3H, CH₃), 2.84 (s, 3H, 4-CH₃-fx), 5.00 (m, 1H, CH), 5.35 (s, 2H, CH₂O), 5.68 (d, J = 4 Hz, 1H, CH₂), 7.07 (dd, J = 2Hz, J = 9 Hz, 1H, H-5), 7.15 (s, 1H, H-3), 7.67 (s, 1H, H-7), 7.70 ppm (s, 1H, H-4); 13C NMR (75 MHz, [D₆]DMSO): δ = 53.5, 61.7, 115.7, 121.4, 123.9, 131.3, 135.1, 139.7, 150.4, 155.5 ppm; MS (EI, 70 eV) m/z (%): 306 (30 [%]), 288 (30), 193 (70), 175 (100).

Leukotriene assay. Studies of 5-L0 inhibition were carried out by following a procedure similar to that reported previously.[6] Blood samples were obtained from healthy volunteers who had not taken any drug for at least two weeks. Volunteers gave their informed and signed consent to the use of blood samples for research purposes. Methanolic solutions of the tested compounds at various concentrations were prepared, 10 μL aliquots were distributed in incubation polystyrene tubes, and the solvent was evaporated. The residues were dissolved by vortexing in 1 mL heparinized (20 IU/mL) venous blood, and the tubes were pre-incubated for 15 min at 37 °C. Eicosanoid biosynthesis was initiated by adding the calcium ionophore A23187 at 50 μM (final 0.05% v/v DMSO) and terminated after 30 min by rapid cooling of the blood in an ice bath and centrifuging at 5 °C for 10 min at 2500 g. Plasma samples were further centrifuged at 12000 g at 3 °C for 3 min, and then supernatants were ready to be tested for LTB₄ production. Basal LTB₄ production in blood untreated with A23187 was subtracted, and percent inhibition in samples incubated with tested compounds was evaluated in comparison with control samples with maximal LTB₄ production. LTB₄ production was evaluated by competitive ELISA according to specific instructions provided by Cayman Chemical, based on a competition between LTB₄ and an 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-L0.

This antibody–LTB₄ complex binds to an unspecified 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 color that absorbs at λ 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of LT-tracers present in the well, which is inversely proportional to the amount of LTs present in the well during the incubation. A standard curve with known concentrations of LTD4 is used to obtain concentrations in the sample wells. Percent inhibition in compound-treated samples was calculated by comparison with control untreated samples. The concentration of the tested compound effects 50% inhibition (IC50) was calculated from the concentration-inhibition response curve (3–4 experiments).

**Myorelaxing activity.** Tracheas and aortas were isolated and cut into single rings. Three to four rings were joined together by thread to form a chain and mounted under tension (1.0 g) in organ baths. After an equilibration period of 120 min, 1 μM carbobihexylylephrine was added to induce a plateau in the tracheal 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 experiments in which the concentration of the tested compound was increased in the sample wells. Percent inhibition in the amount of LTs present in the well during the incubation. A standard curve with known concentrations of LTD4 was used to obtain concentrations in the sample wells. Percent inhibition in compound-treated samples was calculated by comparison with control untreated samples. The concentration of the tested compound effects 50% inhibition (IC50) was calculated from the concentration-inhibition response curve (3–4 experiments).

**Antifibrotic activity.** The protective effect against collagean-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% collagenase. The volume of the paw was measured using a plethysmograph (Basile, Italy) at 2, 3, 4, and 5 h after collagenase challenge. Inflammation was expressed as the percentage change in paw volume.[31] Compounds under study were administered intragastrically (i.g.) in a volume of 10 mL kg−1 immediately before carrageenan injection.

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