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Il Farmaco 60 (2005) 327–332
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Synthesis and biological evaluation of a new class of acyl derivatives of 3-amino-1-phenyl-4,5-dihydro-1*H*-pyrazol-5-one as potential dual cyclooxygenase (COX-1 and COX-2) and human lipoxygenase (5-LOX) inhibitors

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Received 11 November 2004; received in revised form 23 December 2004; accepted 27 December 2004

Available online 26 February 2005

Abstract

A series of acyl derivatives of 3-amino-1-phenyl-4,5-dihydro-1*H*-pyrazol-5-one as potential human 5-LOX and COX 1 and COX-2 inhibitors structurally related to the 1-phenyl-3-pyrazolidinone (phenidone, **1**) have been synthesized and the activity against COX-1, COX-2 and human 5-LOX enzymes has been evaluated. All the derivatives showed poor activity against enzymes. These data, together with our previous studies, indicated that phenidone and related compounds are not suitable as human 5-LOX inhibitors and that pyrazoline nucleus should not be considered a good scaffold for inhibitors of human 5-LOX enzyme, suggesting the necessity to revisit the proposed mechanism of action of phenidone (**1**) in human models.

Keywords: Cyclooxygenase; Lipoxygenase; Dual inhibitors; Phenidone derivatives

1. Introduction

Arachidonic acid (AA) is a polyunsaturated fatty acid stored in cell membranes. After its release from phospholipids, it is metabolized by two enzymatic families, the cyclooxygenases (COX-1, -2 and -3) and the lipoxygenases (5-, 8-, 12- and 15-LOX). These enzymes convert the arachidonic acid to prostaglandines, prostacyclines, and leucotrienes, which are involved in several physiological processes as inflammation and cancer development [1].

In the last decades, many COX inhibitors have been widely used for the treatment of inflammatory diseases. Moreover, COX inhibition leads to an up-regulation of the 5-LOX pathway, leading various adverse effects, especially in the gastroenteric tract and the kidney [2–5]. As a result, a new strategy has been considered: the simultaneous inhibition of 5-LOX and COX enzymes [6]. The simultaneous inhibition of COX and LOX produces increased levels of arachidonic acid, and this phenomenon induces tumor cell death through the activation of mitochondrial mechanisms such as by opening the transition pore [7–9]. In fact, simultaneous administration of COX-2 and 5-LOX inhibitors induces apoptosis in human prostate tumor cell line (PC3) and mouse liver tumor cells (MH1C1), permitting to consider these derivatives a new appealing approach to cancer treatment.

Recently, various chemical families of 5-LOX inhibitors have been designed, and they have been classified depending on their characteristics and mechanism of action. One of these chemical classes includes antioxidant agents, generally small lipophilic aromatic molecules, such as pyrazole derivatives [6]. Phenidone (**1**) could be considered the prototype of this

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doi:10.1016/j.farmac.2004.12.009

family, and its inhibiting action of rat 5-LOX is well described [10–13].

In our previous paper, we have described the activity of phenidone (**1**) and some structurally related derivatives (**2a–i**) in COX-1, COX-2 and human 5-LOX inhibition (Fig. 1) [14].

We have preliminarily observed that phenidone and some structural related compounds could not be considered human 5-LOX inhibitors, but they still retain a quite good COX-2 selective inhibition [14].

Nevertheless, due to the low number of previously reported compounds, it is not still clear if this biological profile should be attributed to the substitutions on the pyrazoline nucleus or if the heterocycle itself could be considered responsible for the inactivity versus the human 5-LOX. Moreover, if this behavior should be confirmed, the mechanism of action of phenidone and derivatives proposed for the rat 5-LOX inhibition, based on redox process, would not be applicable in the human model.

To have more data for speculating on this biological profile, we decided to synthesize and to study a new series of compounds, and we selected the 3-amino-1-phenyl-4,5-dihydro-pyrazol-5-one as lead compound (**3**), which is an isomer of amine **4** used in our previous work (Fig. 2).

Amine **3** has been chosen because of its significant inhibiting activity versus enzymes involved in the arachidonic acid pathway, as reported in the literature (15-LOX [IC_{50} = 6 μM], COX [IC_{50} = 43 μM] and thromboxane synthetase [IC_{50} = 71 μM]) [15], and, moreover, it is commercially available.

We prepared a new series of derivatives (see general formula **5**, Fig. 2) by acylation of amine **3**, using a variety of substituents with different steric and lipophilic properties, as already described for amine **4**.

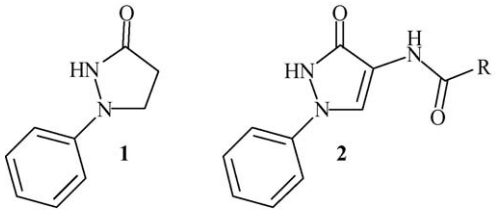


Fig. 1. Phenidone structure and general formula of the previously reported compounds.

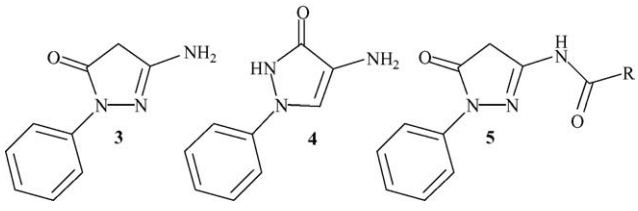


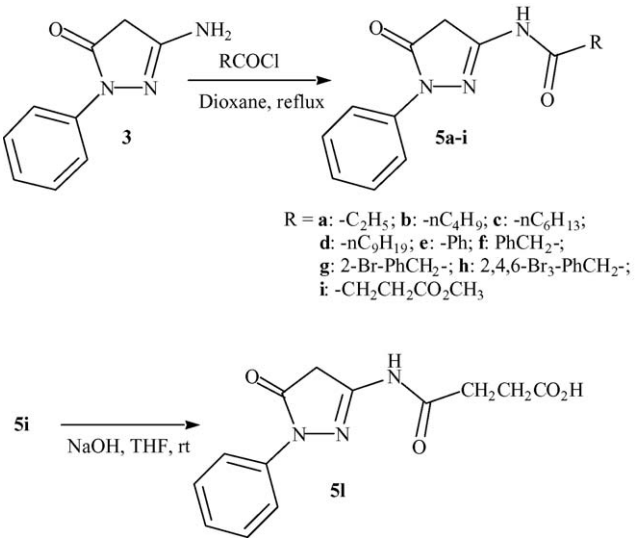
Fig. 2. Structures of compounds **3** and **4** and general formula of compound **5**.

2. Results and discussion

The designed compounds **5a–i** have been synthesized by acylation under standard conditions (dioxane, reflux) of amine **3** with the appropriate acyl chlorides (when not commercially available, the acyl chlorides were prepared from the corresponding acid by treatment with thionyl chloride). Compound **5l** was obtained by hydrolysis of compound **5i** by 1 M NaOH in THF (Scheme 1).

All the compounds (**3, 5a–l**) have been tested in inhibition assays of purified COX-1, COX-2 and human 5-LOX enzymes, using phenidone (**1**), amine **4**, indometacine (COX inhibitor), and Zileuton (5-LOX inhibitor) as internal controls.

The compounds showing significant activity in inhibition assay (**3, 5h**) are reported in Table 1. As clearly indicated, the derivatives do not display any significant activity versus human 5-LOX enzyme. As previously described, only the



Scheme 1. Synthesis of compounds **5a-l**

Table 1
Percentage of inhibition of reference compounds and derivatives **3** and **5h** against COX-1, COX-2 and 5-LOX at 10 μM concentration [a]

Compound	COX-1 [b] (% inhibition at 10 μM)	COX-2 [c] (% inhibition at 10 μM)	5-LOX [d] (% inhibition at 10 μM)
Phenidone, **1**	20.3 ± 4	80.2 ± 12	0
Amine **4**	12 ± 1	43.2 ± 6	0
Indometacine	50 ± 7	50 ± 3	ND
Zileuton	ND	ND	50 ± 4
3	11.4 ± 1.8	0.9 ± 0.6	0
5h	1.5 ± 0.3	0	0

ND: not determined. Values are reported as absolute percent inhibition of enzyme and ± standard error of the mean (SEM).

[a] All the compounds not included in the table resulted to be completely inactive.

[b] COX-1 is from ram seminal vesicles (Cayman Chemical Company, Ann Arbor, MI, USA).

[c] COX-2 is from sheep placental cotyledones (Cayman Chemical Company, Ann Arbor, MI, USA).

[d] For the test with 5-LOX, we have used the leukocytes suspension from human blood of volunteers. n = 2–4 experiments in duplicate.

unmodified derivative **3** presents a COX-1 inhibition activity comparable to the reference compound **4**. In fact, the inhibition percentage of **3** toward COX-1 is 11%, while the activity against COX-2 is significantly diminished with respect to reference compounds **1** and **4**.

These results suggest that the shift of the double bond is detrimental in terms of activity versus COX-2 while a slight retention of action against COX-1 is conserved with respect to amine **4**. Also, in this case, there are not improvements in the inhibition of human 5-LOX.

3. Conclusions

The present work reports a new investigation of pyrazoline analogs as potential dual inhibitors for COX and 5-LOX enzymes. All the derivatives showed poor activity against enzymes. Only unsubstituted derivative **3** showed poor activity against COX-1 while resulted to be totally inactive against COX-2 and human 5-LOX.

These data suggest that phenidone and related compounds are not suitable as human 5-LOX inhibitors and that pyrazoline nucleus should not be considered a good scaffold for inhibitors of the arachidonic acid pathway, in particular for the human 5-LOX enzyme. In addition, we could speculate about the proposed mechanism of action of phenidone (**1**), which most probably should be better clarified considering human 5-LOX.

4. Experimental section

4.1. Chemistry

General Remarks: Reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated F_{254} Merck plates) and products visualized with iodine or aqueous potassium permanganate. Infrared spectra (IR) were measured on a Jasco FT-IR instrument using NaCl cells (oils) or KBr powder (DRIFT system). UV-spectra were recorded on a Perkin-Elmer Lambda 20 spectrophotometer. ^{1}H and ^{13}C NMR were determined in DMSO-d_6 solutions, unless otherwise noted, at 200 and 50 MHz, respectively, with a Varian Gemini 200 spectrometer, peak positions are given in parts per million (δ) downfield, and J values are given in Hz. All the compounds have been crystallized by ethanol. Melting points were determined on a Buchi-Tottoli instrument and are uncorrected. EI-MS spectra were recorded on a VG7070 H spectrometer using electron energy 70 eV. Chromatography was performed with Merck 60-200 mesh silica gel. All products reported showed IR and NMR spectra in agreement with the assigned structures. Organic solutions were dried over anhydrous magnesium sulfate. All final compounds showed a purity >99% checked by HPLC using reverse phase column (Luna 5 μ, C18(2) 250 × 4.60 mm), eluting with a mixture of CH_3CN (0.35 ml/min) and H_2O (0.15 ml/min).

*4.2. General procedure for the preparation of compounds **5a–l***

To a solution of amine (**3**) (2 mmol) in dry dioxane (10 ml), the appropriate acyl chloride (1 mmol) dissolved in dry dioxane (10 ml) was added. The resulting mixture was stirred overnight, heating to reflux. The solvent was then removed under reduced pressure and the crude material was purified by flash chromatography to give the final product as a white solid (compound **5i**), or by crystallization in diethyl ether/ethanol. The compound **5l** was obtained by basic hydrolysis (NaOH 1 M, 4 ml) of compound **5i** (0.1 mmol), in THF (4 ml). The solvent was removed under reduced pressure, then HCl (1 M, 4 ml) was added to the crude material. Derivative **5i** was crystallized by ethanol.

N-(5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-propionamide (**5a**)

$C_{12}H_{13}N_3O_2$ (231 MW); yield: 43%; white solid, m.p.: 217–219 °C; IR-DRIFT: cm^{-1} 4416, 4313, 4089, 2977, 2674, 2556, 2389, 2150, 1965, 1879, 1711, 1633, 1499, 1359, 1177, 1123, 1071, 1021, 938, 701, 577, 506, 427; ^{1}H NMR: δ 10.35 (s, 1H), 7.68 (d, 2H, J = 7.6 Hz), 7.41 (t, 2H, J = 6.8 Hz), 7.18 (t, 1H, J = 7.4 Hz), 5.95 (s, 2H), 2.28 (q, 2H, J = 7.0 Hz), 1.02 (t, 3H, J = 7.6 Hz); ^{13}C NMR: δ 171.23, 168.02, 152.00, 147.00, 138.66, 128.76, 124.80, 120.22, 117.67, 80.57, 28.74, 9.58; EI-MS: m/z 231 (M^+, 14%), 174 (M^+—COC_2H_5, 60%), 91 (PhN^+, 18%), 77 (Ph^+, 63%), 57 ($COC_2H_5^+$, 100%); UV–Vis (EtOH): λ_{max} 260, 199.

Pentanoic acid (5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-amide (**5b**)

$C_{14}H_{17}N_3O_2$ (259 MW); yield: 67%; white solid, m.p.: 188–190 °C; IR-DRIFT: cm^{-1} 3164, 3066, 2951, 2868, 1684, 1632, 1594, 1498, 1448, 1412, 1345, 1266, 1199, 1162, 1102, 973, 902, 754, 689, 642; ^{1}H NMR: δ 10.35 (s, 1H), 7.68 (d, 2H, J = 8.0 Hz), 7.41 (t, 2H, J = 7.7 Hz), 7.19 (t, 1H, J = 7.3 Hz), 5.94 (s, 2H), 2.26 (t, 2H, J = 7.3), 1.52 (quin, 2H, J = 7.4 Hz), 1.33–1.21 (m, 2H), 0.87 (t, 3H, J = 7.3 Hz); ^{13}C NMR: δ 171.66, 170.55, 152.04, 147.00, 138.70, 128.79, 124.84, 120.28, 117.70, 80.62, 35.26, 27.17, 21.78, 13.76; EI-MS: m/z 259 (M^+, 25%), 174 (M^+—COC_4H_9, 100%), 77 (Ph^+, 38%), 57 ($C_4H_9^+$, 46%); UV–Vis (EtOH): λ_{max} 259, 206, 198.

Heptanoic acid (5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-amide (**5c**)

$C_{16}H_{21}N_3O_2$ (287 MW); yield: 63%; white solid, m.p.: 188–190 °C; IR-DRIFT: cm^{-1} 3163, 3065, 2951, 2851, 1684, 1634, 1592, 1497, 1453, 1411, 1345, 1199, 1159, 1114, 976, 899, 754, 688, 644, 581, 505, 415; ^{1}H NMR: δ 10.35 (s, 1H), 7.69 (d, 2H, J = 8.0 Hz), 7.41 (t, 2H, J = 8.0 Hz), 7.19 (t, 1H, J = 7.4 Hz), 5.94 (s, 2H), 2.26 (t, 2H, J = 7.1 Hz), 1.53 (quint, 2H, J = 7.1 Hz), 1.34–1.18 (m, 6H), 0.85 (t, 3H, J = 6.6 Hz); ^{13}C NMR: δ 170.53, 166.75, 152.18, 147.06, 138.70, 128.75, 124.76, 120.21, 117.75, 80.63, 35.54, 31.02, 28.29, 24.97, 21.99, 13.96; EI-MS: m/z 287 (M^+, 55%), 174 (M^+—COC_6H_{13}, 100%), 113 ($COC_6H_{13}^+$, 12%), 85 ($C_6H_{13}^+$, 18%), 77 (Ph^+, 80%), 57 ($C_4H_9^+$, 30%); UV–Vis (EtOH): λ_{max} 260, 201, 193.

Decanoic acid (5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-amide (**5d**)

$C_{19}H_{27}N_3O_2$ (329 MW); yield: 52%; white solid, m.p.: 168–170 °C; IR-DRIFT: cm^{-1} 3162, 3068, 2920, 2854, 2637, 1687, 1634, 1494, 1409, 1349, 1201, 1159, 1115, 971, 895, 749, 687, 647, 573, 503; ^{1}H NMR: δ 10.31 (s, 1H), 7.66 (d, 2H, J = 8.1 Hz), 7.38 (t, 2H, J = 7.9 Hz), 7.16 (t, 1H, J = 7.3 Hz), 5.92 (s, 2H), 2.23 (t, 2H, J = 7.2 Hz), 1.62–1.41 (m, 2H), 1.31–1.15 (m, 12H), 0.82 (t, 3H, J = 5.9 Hz); ^{13}C NMR: δ 170.55, 168.02, 152.03, 147.00, 138.68, 128.78, 124.83, 120.26, 117.69, 80.61, 35.54, 31.30, 28.90, 28.80, 28.70, 28.63, 25.02, 22.13, 13.99; EI-MS: *m/z* 329 (M^+, 80%), 174 (M^+—COC_9H_{19}, 100%), 155 ($COC_9H_{19}^+$, 8%), 85 ($C_6H_{13}^+$, 22%), 77 (Ph^+, 82%), 55 ($C_4H_7^+$, 80%); UV–Vis (EtOH): λ_{max} 261, 204.

N-(5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-benzamide (**5e**) [16]

$C_{16}H_{13}N_3O_2$ (279 MW); yield: 75%; white solid, m.p.: 209–220 °C; IR-DRIFT: cm^{-1} 4316, 4064, 3937, 3311, 3067, 2973, 2724, 2542, 2291, 2144, 1963, 1681, 1615, 1478, 1350, 1257, 1199, 1112, 1024, 920, 803, 758, 699, 622, 503, 412; ^{1}H NMR: δ 10.87 (s, 1H), 8.00 (d, 2H, J = 6.5 Hz), 7.73 (d, 2H, J = 8.7 Hz), 7.60–7.36 (m, 5H), 7.20 (t, 1H, J = 7.3 Hz), 6.11 (s, 2H); ^{13}C NMR: δ 169.24, 165.42, 152.78, 147.67, 139.24, 138.72, 134.48, 133.29, 132.45, 129.61, 129.55, 129.24, 129.08, 128.78, 128.41, 125.99, 125.13, 121.42, 118.84, 82.93; EI-MS: *m/z* 279 (M^+, 20%), 105 ($PhCO^+$, 100%), 77 (Ph^+, 58%); UV–Vis (EtOH): λ_{max} 274, 200.

N-(5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-2-phenyl-acetamide (**5f**)

$C_{17}H_{15}N_3O_2$ (293 MW); yield: 63%; white solid, m.p.: 221–223 °C; IR-DRIFT: cm^{-1} 3068, 2965, 1675, 1625, 1493, 1441, 1353, 1257, 1200, 1127, 1031, 989, 898, 807, 728, 690, 580, 512, 423; ^{1}H NMR: δ 10.69 (s, 1H), 7.68 (d, 2H, J = 8.0 Hz), 7.41 (t, 2H, J = 8.0 Hz), 7.39-7.06 (m, 6H), 5.92 (s, 2H), 3.66 (s, 2H); ^{13}C NMR: δ 168.39, 152.12, 146.93, 138.65, 135.92, 129.09, 128.84, 128.22, 126.45, 124.95, 120.34, 117.73, 80.53, 42.59; EI-MS: m/z 293 (M^+, 53%), 202 (M^+—CH_2Ph, 7%), 174 (M^+—$COCH_2Ph$, 100%), 91 ($PhCH_2^+$, 80%), 77 (Ph^+, 23%); UV–Vis (EtOH): λ_{max} 262, 201, 195.

2-(2-Bromo-phenyl)-*N*-(5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-acetamide (**5 g**)

$C_{17}H_{14}BrN_3O_2$ (372 MW); yield: 53%; white solid, m.p.: 191–193 °C; IR-DRIFT: cm^{-1} 3066, 2964, 1947, 1682, 1628, 1491, 1434, 1350, 1234, 1139, 1026, 985, 901, 791, 745, 692, 577, 501, 430; ^{1}H NMR: δ 10.73 (s, 1H), 7.69 (d, 2H, J = 8.1 Hz), 7.59 (d, 1H, J = 8.0 Hz), 7.51–7.31 (m, 4H), 7.20 (t, 2H, J = 7.3), 5.91 (s, 2H), 3.81 (s, 2H); ^{13}C NMR: δ 167.16, 152.16, 146.91, 138.67, 135.63, 132.22, 132.17, 128.83, 128.69, 127.51, 124.92, 124.51, 120.32, 80.53, 42.51; EI-MS: *m/z* 372 (M^+, 8%), 174 (M^+—$COCH_2PhBr$, 100%), 170 ($BrCH_2Ph^+$, 16%), 77 (Ph^+, 27%); UV–Vis (EtOH): λ_{max} 261, 209, 206, 196.

N-(5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-2-(2,4,6-tribromo-phenyl)-acetamide (**5 h**)

$C_{17}H_{12}Br_3N_3O_2$ (530 MW); yield: 18%; white solid, m.p.: 255–257 °C; IR-DRIFT: cm^{-1} 3248, 3072, 2934, 1768, 1659, 1572, 1536, 1503, 1482, 1437, 1413, 1389, 1327, 1203, 1143, 1108, 1017, 964, 922, 860, 816, 758, 731, 694; ^{1}H NMR: δ 10.85 (s, 1H), 8.03–7.97 (m, 3H), 7.49–7.33 (m, 4H), 6.60 (s, 2H), 4.08 (s, 2H); ^{13}C NMR: δ 165.87, 164.58, 149.63, 142.63, 136.96, 134.46, 134.33, 133.91, 132.17, 129.34, 127.09, 126.53, 126.12, 122.22, 122.15, 121.13, 88.72, 43.03, 41.55; EI-MS: *m/z* 530 (M^+, 6%), 356 ($Br_3PhCH_2CO^+$, 18%), 328 ($Br_3PhCH_2^+$, 12%), 249 ($Br_2PhCH_2^+$, 40%), 174 (M^+—Br_3PhCH_2CO 100%), 77 (Ph^+, 31%); UV–Vis (EtOH): λ_{max} 265, 225, 213, 194.

N-(5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-succinamic acid methyl ester (**5i**)

$C_{14}H_{15}N_3O_4$ (289 MW); yield: 61% (chromatography eluent: petroleum ether/ethyl acetate, 7:3); white solid, m.p.: 199–200 °C; IR-DRIFT: cm^{-1} 3166, 3080, 2967, 1717, 1681, 1627, 1500, 1438, 1351, 1228, 1160, 1061, 994, 958, 912, 844, 760, 712, 653, 585, 504, 479; ^{1}H NMR: δ 10.47 (s, 1H), 7.69 (d, 2H, J = 7.5 Hz), 7.42 (t, 2H, J = 7.7 Hz), 7.20 (t, 1H, J = 7.4 Hz), 5.91 (s, 2H), 3.58 (s, 3H), 2.56 (s, 4H); ^{13}C NMR: δ 172.70, 169.09, 152.07, 146.87, 138.67, 128.81, 124.87, 120.29, 117.71, 80.53, 51.36, 30.13, 28.35; EI-MS: *m/z* 289 (M^+, 17%), 258 (M^+—OCH_3, 100%), 202 (M^+—$C_2H_4COOCH_3$, 8%), 176 (M^+—$C_2OCH_2COOCH_3$, 82%), 91 (PhN^+, 67%), 77 (Ph^+, 88%), 56 ($C_2H_4CO^+$, 85%); UV–Vis (EtOH): λ_{max} 260, 202.

N-(5-Oxo-1-phenyl-4,5-dihydro-1*H*-pyrazol-3-yl)-succinamic acid (**5 l**)

$C_{13}H_{13}N_3O_4$ (275 MW); yield: 92%; white solid, m.p.: 242–243 °C; IR-DRIFT: cm^{-1} 3168, 3086, 2980, 1696, 1627, 1495, 1431, 1341, 1163, 919, 758, 684, 625, 576, 514; ^{1}H NMR: δ 11.97 (bs, 1H), 10.44 (s, 1H), 7.68 (d, 2H, J = 12.1 Hz), 7.40 (t, 2H, J = 7.1 Hz), 7.18 (t, 1H, J = 7.7 Hz), 5.92 (s, 2H), 2.51 (s, 4H); ^{13}C NMR: δ 173.75, 169.42, 152.22, 147.02, 138.73, 128.84, 124.88, 120.30, 117.78, 80.62, 30.35, 28.70; EI-MS: *m/z* 257 (M^+—H_2O, 88%), 202 (M^+—C_2H_4COOH, 12%), 174 (M^+—COC_2H_4COOH, 30%), 158 (M^+—$NH_2COC_2H_4COOH$, 14%), 105 (PhN_2^+, 30%), 91 (PhN^+, 78%), 77 (Ph^+, 80%), 56 ($C_2H_4CO^+$, 100%); UV–Vis (EtOH): λ_{max} 259, 202.

4.3. Biology

4.3.1. COX-1 and COX-2 inhibition assays

The assays were carried out in a 96 microwell titre plate (Bibby Sterilin, Staffordshire, UK), as elsewhere described [22], and using purified COX-1 from ram seminal vesicles and purified COX-2 from sheep placental cotyledones (both: Cayman Chemical Company, Ann Arbor, MI, USA). The incubation mixture contained 180 μl 0.1 M TRIS/HCl buffer (pH 8.0) (Roth, Karlsruhe, Germany), 5 μM hematin (porcine, ICN, Aurora, Ohio, USA), 18 mM epinephrin-hydrogentartrate (Fluka, Buchs, Switzerland), 0.2 U of

enzyme preparation and 50 μM Na_2EDTA (only COX-2 assay, Titriplex III, Merck, Darmstadt, Germany). After adding 10 μl of synthesized compound dissolved in EtOH p.a. or DMSO for compound **5h** (final concentration 10 μM) or 10 μl of EtOH p.a. in control wells, the system was preincubated for 5 min at room temperature.

The addition of 5 μM arachidonic acid (10 μl) (Cayman Chemical Company) triggered the enzymatic conversion of arachidonic acid to PGE_2 metabolite. After incubation for 20 min at 37 °C in darkness, the reaction is stopped by addition of 10% HCOOH (10 μl).

4.3.2. Determination of PGE_2 by enzyme immunoassay

After dilution of the incubation mixture by EIA buffer, the concentration of PGE_2 was determined utilizing a competitive enzyme immunoassay PGE2-EIA-kit (R&D Systems, Minneapolis, MN, USA), used according to the company's instruction.

The EIA evaluation was performed with an ELISA reader ″rainbow″ (TECAN) and determined as elsewhere described [22]. Inhibition was inferred from the reduction of PGE_2 formation in comparison to a blank run without inhibitor. The positive controls were indomethacin (ICN) and NS-398 (Cayman Chemical Company).

4.4. 5-LOX inhibition assays

4.4.1. Isolation of human neutrophile granulocytes

Human blood (30 ml) from volunteers is collected with Vacutainer™ system with preanalytical citric acid solution to prevent clogging. The blood is immediately transferred to a falcon tube containing 20 ml of Dextran solution (1.9 g NaCl, 12.0 g Dextran T-500, H_2O to 200.0 ml) and incubated for 60 min at 4 °C; then it is centrifuged at 1600 rpm at 4 °C for 10 min. Leukocytes precipitated forming a pellet while thrombocytes and plasma remain in the supernatant, which is discarded. The pellet is then suspended in 10 ml of washing buffer (1.48 g $CaCl_2 \cdot 2\ H_2O$ p.a., 0.2 g anhydrous D-Glucose, 0.04 g $MgCl_2 \cdot 6\ H_2O$, 0.08 g KCl, 3.5 g TRIS p.a., H_2O to 200.0 ml, pH 7.6). After centrifugation at 1400 rpm at 4 °C for 10 min and removal of the supernatant, the resulting pellet is suspended in 10 ml of hypotonic lysis buffer (0.17 g NH_4Cl, 0.2 g TRIS, H_2O to 100.0 ml, pH 7.2) and gently shaken for 5 min at room temperature to destroy remaining erythrocytes. The suspension is centrifuged at 1400 rpm at 4 °C for 5 min. The pellet is dissolved in 10 ml of washing buffer and then centrifuged at 1400 rpm at 4 °C for 15 min. The resulting pellet, which now mainly contains neutrophile granulocytes, is suspended in 2 ml of TRIS buffer (5.25 g TRIS p.a., 2.7 g NaCl, H_2O to 300.0 ml, pH 7.4).

4.4.2. Cell vitality test and determination of cell concentration

50 μl of leukocytes suspension and 10 μl of 0.4% Trypan blue solution (Sigma Chemical Co. Steinheim, Germany) are mixed on a glass object carrier and examined with a light microscope at 1000× magnification. Dead cells appear larger and dark because of the absorption of Trypan blue solution, whereas vital cells remain smaller, lighter and more granulose. The vitality of the cells must be over 90%.

Leukocytes concentration was determined using cell diluted suspension distributed into a Neubauer chamber (Assistant, Germany) and a light microscope with 100× magnification.

For the bioassay, the cell concentration must be 5000 cells/μL with TRIS buffer.

4.5. The 5-LOX bioassay

The assay was performed in the 96 microwell titre plate (Bibby Sterilin, Staffordshire, UK). The incubation mixture consists of 225 μl of leukocytes suspension (5000 cells/μL), 10 μl $CaCl_2$ 2 mM, 10 μl eicosatetraenoic acid 10 μM as 12-LOX pathway inhibitor, 5 μl of synthesized compound dissolved in EtOH p.a. (DMSO for compound **5h**) to the final concentration of 10 μM or 5 μl of EtOH p.a. in control wells. The addition of Ca Ionophore A23187 (10 μl, 17 μM) and 5 μl of 120 μM arachidonic acid (Cayman Chemical Company) triggers the enzymatic conversion of arachidonic acid to LTB_4 metabolite. After incubation for 10 min at 37 °C in darkness, the reaction is stopped by addition of 20 μl 10% HCOOH. The microplate is centrifuged for 15 min at 1400 rpm to separate the free LTB_4 from cellular particles. The supernatant is then diluted 50-fold with TRIS buffer.

4.6. Determination of LTB_4 by enzyme immunoassay

The concentration of LTB_4 was measured utilizing a competitive enzyme immunoassay LTB_4-EIA-kit (Cayman Chemical Company, Ann Arbor, USA) used according to the company's instruction.

The EIA evaluation was performed with a photometric ELISA plate reader (TECAN) at $\lambda = 412$ nm. Inhibition was inferred from the reduction of LTB_4 formation in comparison to a blank run without inhibitor. The positive control was Zileuton.

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