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Original article

Synthesis and biological evaluation of new phenidone analogues as potential dual cyclooxygenase (COX-1 and COX-2) and human lipoxygenase (5-LOX) inhibitors

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Abstract

A new series of potential human 5-LOX inhibitors structurally related to the 1-phenyl-3-pyrazolidinone (phenidone, **2**) has been synthesized and the activity against COX-1, COX-2, and human 5-LOX enzymes has been evaluated. In contrast with literature data, we observed that phenidone resulted to be inactive against human 5-LOX, while retains its activity against cyclooxygenases in a micromolar range. The present results suggest that the substitution of the amino function at the 4-position is detrimental in terms of activity toward COX-1 and COX-2, while the presence of a double bond at the 4,5-position does not alter the biological profile against COX. The absence of activity vs. human 5-LOX strongly suggests a re-consideration of phenidone and its analogs as 5-LOX inhibitors in humans.

Keywords: Cyclooxygenase; Lipoxygenase; Dual inhibitors; Phenidone derivatives

1. Introduction

Arachidonic acid is an important fatty acid present in cell membranes, being a precursor of several metabolites (leucotrienes, prostaglandines, prostacyclines), which are involved in several patho-physiological processes such as broncospasm and inflammation [1–4]. It is well known that the formation of these metabolites is catalyzed by cyclooxygenases (COX-1 and COX-2) and lipoxygenases (5-, 8-, 12- and 15-LOX), in fact their block is the most common approach for anti-inflammatory therapy [5–10].

Very recently, it has been demonstrated that the simultaneous inhibition of COX and LOX, which produces increased levels of arachidonic acid, is capable to induce tumor cell death through the activation of mitochondrial mechanisms such as by opening the transition pore [11–13]. These studies have been supported by several experimental details, 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) [14].

A better approach for increasing arachidonic acid levels was based on the administration of a compound capable to inhibit both COX and LOX. This approach has been confirmed by using a dual inhibitor derived from diclofenamic acid (**1**), which inhibits both COX and LOX at concentrations ranging from 0.1 to 0.5 μM [15], but its use in therapy is limited by the high toxicity (5–10 μM), which induces mitochondrial death (Fig. 1).

For this reason, the search for novel dual (COX and LOX) inhibitors, possessing low toxicity, represents an attractive tool for cancer chemotherapy. With the aim to discover new dual inhibitors, we selected phenidone (**2**) as lead compound. In fact, it is widely reported that this compound is a potent rat

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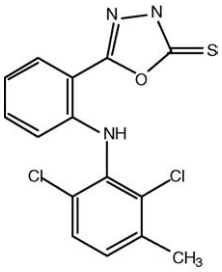

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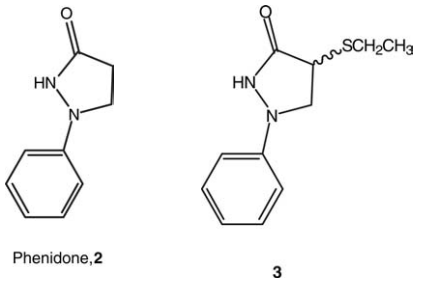
Fig. 1.

5-LOX inhibitor both in vitro and in vivo (0.1–0.5 μM) [16,17] and a quite potent COX-1 and COX-2 (7 μM tested in a mixture of enzymes) inhibitor [15,18,19]. Several modifications on this lead compound permitted to obtain a quite detailed structure–activity relationship profile, demonstrating that lipophilic substituents at the 4-position (compound **3**) could significantly increase 5-LOX inhibition potency (IC_{50} = 60 nM) [16] (Figs. 2 and 3).

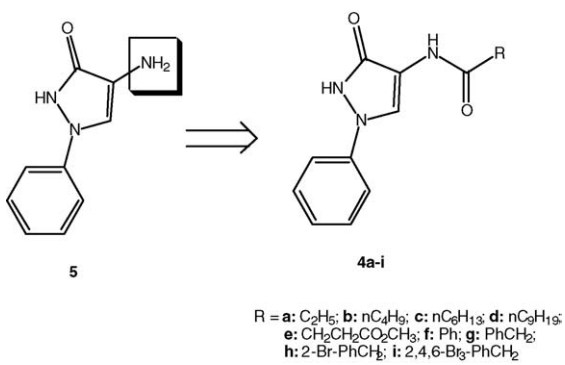
Taking into account these experimental observations, we decided to investigate a new series of phenidone analogues of general formula **4** (Scheme 1).

In particular, the introduction of an amino function at the 4-position on the pyrazoline ring (**5**) [20–22] could be an ideal appendage for linking different substituents possessing various steric and lipophilic properties, while the presence of a double bond confers more rigidity to the system, permitting to analyze new structural requirements indispensable for activity.



Phenidone,**2** **3**

Figs. 2 and 3.



Scheme 1.

2. Chemistry

The designed compounds **4a–i** have been prepared by acylation under standard conditions (dioxane reflux) of amine **5** with the appropriate acyl chlorides. Amine **5** was prepared by the known procedure [20–22] briefly summarized in Scheme 2.

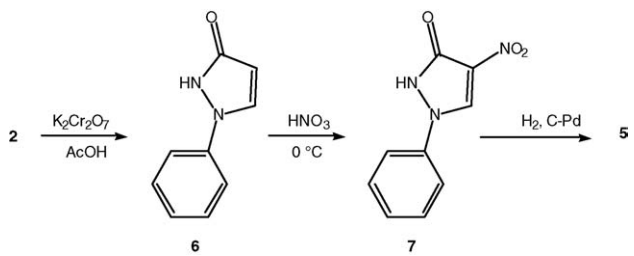
Oxidation with potassium dichromate in acetic acid [20], or $FeCl_3$ [22] of commercially available phenidone (**2**) afforded the corresponding dihydroderivate (**6**) which, after treatment with concentrated nitric acid at 0 °C, gave nitro derivative (**7**) [21]. The latter was then reduced with hydrogen in the presence of Pd/C to yield the desired amine (**5**) [21].

Amine **5** was coupled with the appropriate acyl chloride (**8a–i**) (when not commercially available, the acyl chlorides were prepared from the corresponding acid by treatment with thionyl chloride) to afford the final compounds **4a–i**. When the acyl chloride of succinic acid monomethyl ester was used, the compound of both *N*- and *O*-acylation (**9**) was also isolated (Scheme 3).

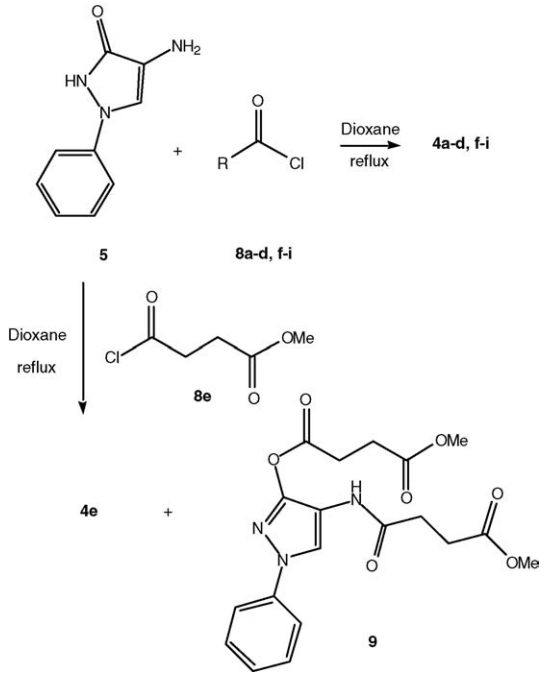
3. Results and discussion

All the synthesized compounds (**4a–i, 5, 9**) have been tested in inhibition assays against purified COX-1, COX-2, and human 5-LOX enzymes, using phenidone (**2**), indometacine (COX inhibitor), and zileuton (5-LOX inhibitor) as internal controls.

In Table 1 are reported only the compounds showing significant activity in COX-1 and COX-2 inhibition assays (**4i, 5**). As clearly indicated, the not substituted derivative (**5**) presented considerable activity against both COX-1 and COX-2 enzymes, comparable to the reference compound (**2**), giving 43% of inhibition toward COX-2 with a selectivity ratio COX-1/COX-2 of 0.27, quite similar to that of the reference compound (**2**). In contrast, the introduction of acyl groups at the 4-position seems to be detrimental in terms of activity. Only compound **4i**, which bears a bulky and highly lipo-



Scheme 2.



Scheme 3.

Table 1
Inhibition percentage of compounds **4i**, **5** against COX-1, COX-2, and 5-LOX at 10 μM concentration[a]

Compound	COX-1[b]	COX-2[c]	5-LOX[d]
Phenidone, 2	20.3 ± 4	80.2 ± 12	0
Indometacine	50 ± 7	50 ± 3	ND
Zileuton	ND	ND	50 ± 4
4i	8.3 ± 2	3.2 ± 0.8	0
5	12 ± 1	43.2 ± 6	0

ND: not determined.

[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. Values are reported as absolute percent inhibition of enzyme and ± standard error of the mean (S.E.M).

philic substituent at the 4-position, displays poor activity (3–8% inhibition at 10 μM). These results seems to suggest that substituents at the 4-position on the pyrazoline nucleus highly decrease the COX-1 and COX-2 inhibition, while the presence of the double bond does not influence significantly the activity with respect to the reference compound (**2**). To further validate these experimental data, the exact IC_{50} values against COX-1 and COX-2 enzymes for compound (**5**) were measured (Table 2).

As reported, derivative (**5**) showed IC_{50} values very similar to that of the reference compound (**2**) with a slight improved COX-1/COX-2 selectivity (2.7 vs. 2.2).

Instead, unexpected data were obtained in the evaluation of the human 5-LOX inhibition. In fact, all the synthesized compounds resulted to be inactive against 5-LOX, but surprisingly, while the reference compound zilutron showed inhibitory activity, phenidone (**2**), in contrast to literature data, proved to be ineffective (7% inhibition at 10 μM concentration). These results seem to suggest that this total inactivity could not be attributed to the structural modifications performed on the phenidone (**2**) nucleus, but to the choice of the lead compound. The discrepancy with literature data [16,17,19] strongly supports a significant difference in the sequence homology between murine and human 5-LOX.

Table 2
Estimation of IC_{50} values in inhibition assay against COX-1 and COX-2 of selected compounds

Compound	COX-1[a] (IC_{50}, μM)	COX-2[b] (IC_{50}, μM)	COX-1/COX-2
Phenidone, 2	20.2 ± 2.3	9.2 ± 0.8	2.2
5	36.7 ± 4.2	13.4 ± 2.3	2.7

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

[b] COX-2 is from sheep placental cotyledones (Cayman Chemical Company, Ann Arbor, MI, USA). $n = 2–4$ experiments in duplicate. Values are reported as absolute percent inhibition of enzyme and ± standard error of the mean (S.E.M).

4. Conclusions

The present work reports a new investigation of phenidone (**2**) and its structurally related analogs as dual inhibitors for COX and human 5-LOX enzymes. Only not substituted derivative (**5**) showed significant activity against both COX-1 and COX-2, while resulted to be inactive against human 5-LOX.

Nevertheless, the inactivity against human 5-LOX of this compound could be attributed not only to the kind of substitutions, but, most probably, to the choice of phenidone (**2**) as a lead compound. In fact, while phenidone and its derivative proved to be potent rat 5-LOX inhibitors, we found it totally inactive in human 5-LOX inhibition assay, suggesting that phenidone (**2**) could not be considered a 5-LOX inhibitor in human. Nevertheless, further studies are in course in our laboratories to support these preliminary experimental observations.

5. Experimental section

5.1. Chemistry

5.1.1. 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 Hertz. Melting points were determined on a Buchi-Tottoli instrument and are uncorrected. Electrospray mass spectra were recorded on a Perkin-Elmer PE SCIEX API 1 spectrometer, and compounds were dissolved in methanol/tetrahydrofuran 4/1, unless otherwise noted. 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 MeCN (0.35 ml/min) and H_2O (0.15 ml/min).

*5.1.2. General procedure for the preparation of phenidone analogues **4a–i, 9***

To a solution of amine (**5**) (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, which was crystallized by diethyl ether/ethanol.

5.1.2.1. N-*(3-Oxo-1-phenyl-2,3-dihydro-1*H-*pyrazol-4-yl)-propionamide (**4a**).* $C_{16}H_{13}N_3O_2$ (279 MW); yield: 32%; (chromatography eluent: petroleum ether/ethyl acetate, 1:1); white solid, m.p.: 236–238 °C; IR-DRIFT: cm^{-1} 3263, 3167, 3066, 2538, 2019, 1639, 1544, 1406, 1332, 1208, 1069, 952, 904, 806, 753, 696, 512, 426; ^{1}H NMR: δ 10.49 (bs, 1H), 8.90 (bs, 1H), 8.16 (d, 2H, J = 7.3 Hz), 7.94–7.37 (m, 8H), 7.13 (t, 1H, J = 7.5 Hz); ^{13}C NMR: δ 164.77, 155.16, 139.68, 133.65, 131.62, 129.39, 128.37, 127.58, 124.44, 121.21, 116.37, 109.01; ES-MS (MeOH–THF): m/z 279 (M^+), 302 $(M + Na^+)^+$, 318 $(M + K^+)^+$; EI-MS: m/z 279 (M^+, 33%), 105 (PhN_2^+, 100%), 77 (Ph^+, 78%); UV-Vis (EtOH): λ_{max} 199, 203, 220.

*5.1.2.2. Pentanoic acid (3-oxo-1-phenyl-2,3-dihydro-1*H-*pyrazol-4-yl)-amide (**4b**).* $C_{14}H_{17}N_3O_2$ (259 MW); yield: 56%; (chromatography eluent: CH_2Cl_2/ethyl acetate, 8:2); white solid, m.p.: 173–174 °C; IR-DRIFT: cm^{-1} 3287, 2957, 2867, 2407, 1939, 1863, 1727, 1651, 1601, 1508, 1450, 1409, 1384, 1289, 1226, 1196, 1107, 1075, 1055, 965, 938, 891, 831, 778, 747, 687; ^{1}H NMR: δ 10.75 (bs, 1H), 9.63 (bs, 1H), 8.37 (s, 1H), 7.61 (d, 2H, J = 7.9 Hz), 7.40 (t, 2H, J = 7.3 Hz), 7.13 (t, 1H, J = 7.3 Hz), 2.32 (t, 2H, J = 7.3 Hz), 1.54 (m, 2H,), 1.29 (m, 2H,), 0.88 (t, 3H, J = 7.3 Hz); ^{13}C NMR: δ 170.63, 154.13, 139.74, 129.33, 124.09, 119.12, 116.15, 109.65, 34.74, 27.43, 21.83, 13.78; EI-MS: m/z 259 (M^+, 27%), 175 (M^+—C_4H_9CO, 100%), 104 (PhN_2^+, 48%), 77 (Ph^+, 60%), 57 ($C_4H_9^+$, 50%), 41 ($C_3H_7^+$, 49%); UV-Vis (EtOH): λ_{max} 198, 295.

*5.1.2.3. Heptanoic acid (3-oxo-1-phenyl-2,3-dihydro-1*H-*pyrazol-4-yl)-amide (**4c**).* $C_{16}H_{21}N_3O_2$ (287 MW); yield: 64%; (chromatography eluent: CH_2Cl_2/ethyl acetate, 8:2); white solid, m.p.: 166–168 °C; IR-DRIFT: cm^{-1} 3283, 2923, 2853, 2399, 1863, 1651, 1600, 1507, 1461, 1233, 1195, 1117, 1055, 966, 938, 892, 830, 775, 750, 689, 587, 503, 449, 416; ^{1}H NMR: δ 10.75 (bs, 1H), 9.62 (bs, 1H), 8.37 (s, 1H), 7.61 (d, 2H, J = 8.0 Hz), 7.39 (t, 2H, J = 7.6 Hz), 7.13 (t, 1H, J = 7.3 Hz), 2.31 (t, 2H, J = 7.3 Hz), 1.55 (m, 2H), 1.35–1.17 (m, 6H), 0.85 (t, 3H, J = 6.2 Hz); ^{13}C NMR: δ 170.61, 154.09, 139.72, 129.31, 124.08, 119.07, 116.16, 109.64, 35.01, 31.05, 28.35, 25.25, 22.02, 13.97; EI-MS: m/z 287 (M^+, 17%), 175 (M^+—C_4H_9CO, 100%), 130 ($C_6H_{13}CONH^+$, 12%), 104 (PhN_2^+, 52%), 77 (Ph^+, 78%); UV-Vis (EtOH): λ_{max} 194, 201, 295.

*5.1.2.4. Decanoic acid (3-oxo-1-phenyl-2,3-dihydro-1*H-*pyrazol-4-yl)-amide (**4d**).* $C_{19}H_{27}N_3O_2$ (329 MW); yield: 59%; (chromatography eluent: petroleum ether/ethyl acetate, 1:1); white solid, m.p.: 159–161 °C; IR-DRIFT: cm^{-1} 3283, 2917, 2848, 2279, 1776, 1654, 1601, 1528, 1465, 1372, 1233, 1099, 1055, 918, 810, 747, 687, 607, 437; ^{1}H NMR: δ 10.73 (bs, 1H), 9.62 (bs, 1H), 8.37 (s, 1H), 7.61 (d, 2H, J = 8.0 Hz), 7.39 (t, 2H, J = 7.7 Hz), 7.13 (t, 1H, J = 7.4 Hz), 2.31 (t, 2H, J = 7.4 Hz), 1.64–1.46 (m, 2H), 1.34–1.14 (m, 12H), 0.84 (t, 3H, J = 6.7 Hz); ^{13}C NMR: δ 170.62, 154.07, 139.73, 129.32, 124.09, 119.06, 116.15, 109.64, 35.01, 31.30, 28.93, 28.83, 28.71, 25.29, 22.13, 13.99; EI-MS: m/z 329 (M^+, 50%), 218 (M^+—C_8H_{17}, 15%), 202 (M^+—C_9H_{19}, 11%), 176 (M^+—$C_9H_{19}CO$, 100%), 104 (PhN_2^+, 15%), 77 (Ph^+, 12%); UV-Vis (EtOH): λ_{max} 296, 199.

*5.1.2.5. N-(3-Oxo-1-phenyl-2,3-dihydro-1*H-*pyrazol-4-yl)-benzamide (**4e**).* $C_{16}H_{13}N_3O_2$ (279 MW); yield: 31%; (chromatography eluent: petroleum ether/ethyl acetate, 1:1); white solid, m.p.: 236–238 °C; IR-DRIFT: cm^{-1} 3263, 3167, 3066, 2538, 2019, 1639, 1544, 1406, 1332, 1208, 1069, 952, 904, 806, 753, 696, 512, 426; ^{1}H NMR: δ 10.49 (bs, 1H), 8.90 (bs, 1H), 8.16 (d, 2H, J = 7.3 Hz), 7.94–7.37 (m, 8H), 7.13 (t, 1H, J = 7.5 Hz); ^{13}C NMR: δ 164.77, 155.16, 139.68, 133.65, 131.62, 129.39, 128.37, 127.58, 124.44, 121.21, 116.37, 109.01; ES-MS: m/z 279 (M^+), 302 $(M + Na^+)^+$, 318 $(M + K^+)^+$; EI-MS: m/z 279 (M^+, 33%), 105 (PhN_2^+, 100%), 77 (Ph^+, 78%); UV-Vis (EtOH): λ_{max} 199, 203, 220.

*5.1.2.6. N-(3-Oxo-1-phenyl-2,3-dihydro-1*H-*pyrazol-4-yl)-2-phenyl-acetamide (**4f**).* $C_{17}H_{15}N_3O_2$ (293 MW); yield: 42%; (chromatography eluent: CH_2Cl_2/ethyl acetate, 9:1); white solid, m.p.: 224–226 °C; IR-DRIFT: cm^{-1} 3280, 3033, 1664, 1571, 1509, 1408, 1318, 1199, 1067, 959, 904, 813, 691, 562, 488, 429; ^{1}H NMR: δ 10.82 (bs, 1H), 9.92 (bs, 1H), 8.39 (s, 1H), 7.60 (d, 2H, J = 7.4 Hz), 7.52–7.18 (m, 7H), 7.12 (t, 1H, J = 7.3 Hz), 3.66 (s, 2H); ^{13}C NMR: δ 168.29, 154.09, 139.72, 136.07, 129.34, 129.03, 128.18, 126.40, 124.16, 119.20, 116.19, 109.47, 41.89; EI-MS: m/z 293 (M^+, 100%), 175 (M^+—$PhCH_2CO$, 78%), 104 (PhN_2^+, 32%), 91 ($PhCH_2^+$, 88%), 77 (Ph^+, 52%); UV-Vis (EtOH): λ_{max} 295, 193.

*5.1.2.7. 2-(2-Bromo-phenyl)-*N*-(3-oxo-1-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-acetamide (**4g**).* $C_{17}H_{14}BrN_3O_2$ (372 MW); yield: 61%; (chromatography eluent: CH_2Cl_2/ethyl acetate, 9:1); white solid, m.p.: 218–219 °C; IR-DRIFT: cm^{-1} 3266, 3025, 1655, 1565, 1511, 1414, 1320, 1205, 1063, 956, 814, 754, 687, 565, 500, 431; ^{1}H NMR: δ 10.81 (bs, 1H), 9.96 (bs, 1H), 8.40 (s, 1H), 7.59 (d, 2H, J = 8.0 Hz), 7.45–7.26 (m, 4H), 7.32–7.05 (m, 3H), 3.86 (s, 2H); ^{13}C NMR: δ 166.55, 153.55, 139.23, 135.28, 131.69, 131.48, 128.86, 128.15, 127.02, 123.97, 123.64, 118.60, 115.66, 109.07, 41.53; EI-MS: *m/z* 371 (M^+, 16%), 291 (M^+—Br, 25%), 176 (M^+—$BrPhCH_2CO$, 100%), 169 ($BrPhCH_2^+$, 14%), 130 ($PhCH_2CON^+$, 53%), 104 (PhN_2^+, 50%), 77 (Ph^+, 98%); UV-Vis (EtOH): λ_{max} 295, 202, 193.

5.1.2.8. N*-(3-Oxo-1-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-2-(2,4,6-tribromo-phenyl)-acetamide (**4h**).* $C_{17}H_{12}Br_3N_3O_2$ (530 MW); yield: 15%; (chromatography eluent: CH_2Cl_2/ethyl acetate, 9:1); white solid, m.p.: >300 °C; IR-DRIFT: cm^{-1} 3258, 3070, 2624, 1661, 1597, 1565, 1534, 1436, 1412, 1338, 1240, 1188, 1152, 1111, 1056, 965, 920, 856, 752, 729, 671; ^{1}H NMR: δ 10.85 (bs, 1H), 10.07 (bs, 1H), 8.39 (s, 1H), 7.95 (s, 2H), 7.59 (d, 2H, J = 8.3 Hz), 7.38 (t, 2H, J = 7.5 Hz), 7.12 (t, 1H, J = 7.9 Hz); ^{13}C NMR: δ 165.02, 153.92, 139.75, 134.95, 133.85, 129.32, 126.49, 124.14, 120.88, 119.04, 116.19, 109.48, 42.59; EI-MS: *m/z* 530 (M^+, 3%), 451 (M^+—Br, 16%), 330 ($Br_3PhCH_2^+$, 5%), 294 (M^+—3Br, 3%), 248 ($Br_2PhCH_2^+$, 3%), 175 (M^+—Br_3PhCH_2CO, 68%), 104 ($PheN_2^+$, 47%), 77 (Ph^+, 100%); UV-Vis (EtOH): λ_{max} 334, 296, 213.

*5.1.2.9. N-(3-Oxo-1-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-succinamic acid methyl ester (**4i**).* $C_{14}H_{15}N_3O_4$ (289 MW); yield: 42%; (chromatography eluent: CH_2Cl_2/ethyl acetate, 8:2); white solid, m.p.: 187–189 °C; IR-DRIFT: cm^{-1} 3345, 3244, 3153, 3106, 2925, 2852, 1776, 1716, 1683, 1595, 1559, 1503, 1438, 1388, 1322, 1253, 1169, 1122, 1053, 1001, 964, 946, 883, 853, 794, 758, 684, 645; ^{1}H NMR: δ 10.74 (bs, 1H), 9.74 (bs, 1H), 8.38 (s, 1H), 7.61 (d, 2H, J = 8.3 Hz), 7.39 (t, 2H, J = 8.1 Hz), 7.12 (t, 1H, J = 7.3 Hz), 3.63 (s, 3H), 2.61 (m, 4H); ^{13}C NMR: δ 172.71, 169.00, 153.98, 139.73, 129.33, 124.09, 119.03, 116.14, 109.52, 51.34, 29.68, 28.63; EI-MS: *m/z* 289 (M^+, 2%), 257 (M^+—OCH_3, 100%), 176 (M^+—$COC_2H_4COOCH_3$, 50%), 130 ($NH_2COC_2H_4COOCH_3^+$, 20%), 104 (PhN_2^+, 17%), 77 (Ph^+, 28%), 55 ($C_2H_4CO^+$, 40%); UV-Vis (EtOH): λ_{max} 201, 292.

*5.1.2.10. Succinic acid 4-(3-methoxycarbonyl-propionyl-amino)-1-phenyl-1*H*-pyrazol-3-yl ester methyl ester (**9**).* $C_{19}H_{23}N_3O_7$ (405 MW); yield: 32%; (chromatography eluent: CH_2Cl_2/ethyl acetate, 7:3); white solid, m.p.: 137–139 °C; IR-DRIFT: cm^{-1} 3286, 3158, 2925, 2575, 1734, 1657, 1623, 1580, 1521, 1440, 1412, 1359, 1325, 1203, 1168, 1062, 1003, 982, 957, 918, 848, 808, 755, 719, 691; ^{1}H NMR: δ 9.76 (bs, 1H), 8.67 (bs, 1H), 7.73 (d, 2H, J = 8.3 Hz), 7.46 (t, 2H, J = 8.1 Hz), 7.27 (t, 1H, J = 7.3 Hz), 3.63 (s, 3H), 3.59 (s, 3H), 2.96 (t, 4H, J = 6.5 Hz), 2.71 (t, 4H, J = 7.0 Hz), 2.61 (m, 4H); ^{13}C NMR: δ 172.71, 172.68, 1689.90, 168.82, 146.52, 139.12, 129.52, 125.93, 120.11, 117.43, 113.57, 51.65, 51.39, 29.80, 28.58, 28.49, 28.29; ES-MS: *m/z* 404 (M^+), 427 (M + Na^+), 443 (M + K^+); UV-Vis (EtOH): λ_{max} 193, 200, 277.

5.2. Biology

5.2.1. 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 [23], 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, OH, 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 **4i** (final concentration 10 μM) or 10 μl of EtOH p.a. in control wells, the system was pre-incubated 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).

5.2.2. 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 [23]. 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).

5.3. 5-LOX inhibition assays

5.3.1. 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).

5.3.2. Cell vitality test and determination of cell concentration

A 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 1000x 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 100x magnification.

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

5.3.3. 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 **4i**) 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.

5.3.4. 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 zilutron.

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