Synthesis and Evaluation of a Novel Series of Pyrrolizine Derivatives as Dual Cyclooxygenase-1 and 5-Lipoxygenase Inhibitors☆

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Key Words: *Heteroarylpyrrolizines; NSAIDs: non-steroidal antiinflammatory drugs; COX-1: cyclooxygenase-1; 5-LOX: 5-lipoxygenase; ELISA: enzyme linked immunosorbent assay*

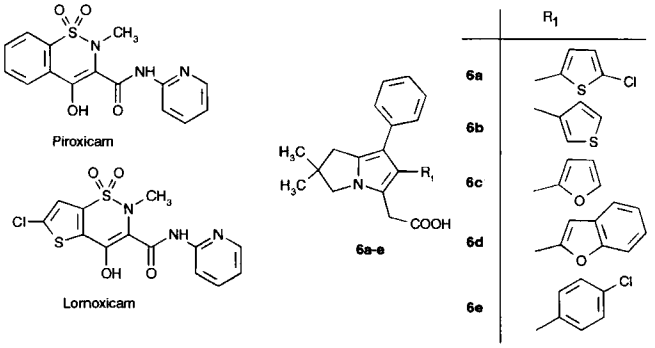
Summary

The aim of our study was to investigate structure activity relationship following the replacement of the 6-phenyl substituent at the 6,7-diaryl-2,3-dihydropyrrolizine template by various heteroaromatic residues. In this context we developed a new, efficient, and highly sensitive test method for the screening of dual cyclooxygenase-1 (COX-1) and 5-lipoxygenase (5-LOX) inhibitors. We used human platelets as a source of COX-1 and human PMNLs as a source of 5-LOX. Both cell types were isolated from the same volume of blood. PGE_2 and LTB_4 respectively were determined by highly selective and sensitive ELISA kits, using monoclonal antibodies. For a single determination at most 0.5 mL whole blood is needed.

Introduction

"Classical" non-steroidal antiinflammatory drugs (NSAIDs) are widely used remedies in the treatment of rheumatoid arthritis and other inflammatory diseases. The mechanism of action of almost all NSAIDs is the inhibition of cyclooxygenase, a pivotal enzyme of the arachidonic acid (AA) cascade[1]. COX transforms AA in a two-step reaction into PGH_2 which is metabolized in turn by specific synthases forming prostacyclin, thromboxanes, and prostaglandins[2]. However, the use of these efficacious drugs is limited by severe side-effects causing gastrointestinal irritations, kidney damage, and acute asthmatic reactions[3,4]. One theory accounting for these drawbacks focuses on a "shunt" to the second branch of the AA cascade, the 5-lipoxygenase side. There exists some evidence for complex feedback mechanisms in the control of eicosanoid biosynthesis, since the inhibiting influence of prostaglandins and prostacyclin on the leukotriene synthesis has been proven[5]. Under NSAID therapy a local decrease in prostaglandin concentrations occurs and simultaneously an increase in the amount of proinflammatory leukotrienes can be observed[6]. These leukotrienes exhibit the following physiological functions: LTB_4 is a chemotactic, chemokinetic, and degranulating agent of PMNLs[7]. LTC_4, LTD_4, and LTE_4 cause vaso- and bronchoconstrictions, smooth muscle contractions, and are "slow reacting substances of anaphylaxis" (SRS-A), e.g. in allergic events[8]. These metabolites might be responsible for the aforementioned side-effects[3,4], since they are postulated to be mediators in several human diseases including asthma and psoriasis[9]. Therefore, dual COX-1/5-LOX inhibitors should reduce some of these drawbacks that are associated with exclusive COX-1 inhibition and simultaneously maintain or improve the antiinflammatory effects.

Recently diarylpyrrolizines have been widely investigated in the inhibition of COX-1 and 5-LOX[10–12]. The general structure **6** was modified. For a dual inhibition of both COX-1 and 5-LOX, we found a 6,7-diphenyl substitution pattern is essential[12]. A compound from these series, ML3000 (**6e**, Scheme 1), is undergoing clinical trails. The replacement of phenyl groups by heteroaromatic residues is a general strategy in medicinal chemistry. Both pharmacodynamic and pharmacokinetic properties could be improved. As an example, in lornoxicam (Scheme 1) the benzene group of piroxicam (Scheme 1) is bioisosterically replaced by a chlorothiophen moiety, which increases the activity *in vivo* by a factor of 10[13] and decreases plasma $t_{1/2}$ from 20 to 4 h[14]. Therefore, we replaced the 6-(4-chlorophenyl) group of **6e** with 5-chlorothiophen-2-yl (**6a**), thiophen-3-yl (**6b**), furan-2-yl (**6c**), and benzo[*b*]furan-2-yl (**6d**) residues.



Scheme 1

Until now only a small number of *in vitro* assays have been developed, targeting the inhibitory influence of NSAIDs on both COX-1 and 5-LOX activity. For example, Dannhardt and Lehr[15] used bovine blood (0.5–1.0 L) as a source for platelets and PMNLs to measure the COX-1/5-LOX inhibition in a model of isolated, intact cells. Sweeney et al.[16] investigated COX-1/5-LOX activity in a whole blood test system. These assays worked for the investigation of dual COX-1/5-LOX inhibitors. However, flaws still exist in certain aspects. In our formerly evaluated test model[17] we

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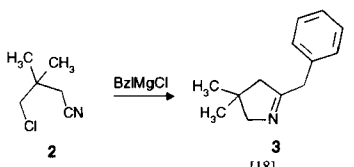
solved some of the problems of the aforementioned assays. To be as close as possible to the *in vivo* model, our assay was designed to work with freshly isolated, pooled human platelets and PMNLs of a definite cell count, avoiding the use of animal cells and the drawbacks of plasma protein binding found in whole blood systems. Yet, like all the previous mentioned test systems, we used the HPLC-UV method for the quantification of metabolites of the AA cascade. This method is limited by poor sensitivity, a low throughput of samples, sophisticated sample purification procedures and also the disadvantage of large quantities of blood needed. Hence, there are two main problems to be solved: First the low sensitivity of the HPLC-UV quantification method and secondly the limited number of samples that could be analyzed within one run. Therefore, our target was the use of a model, allowing the rapid assessment of low levels of compounds without prior extraction or chromatography. Due to its high sensitivity the ELISA quantification method should render the possibility to reduce the cell count. This alteration requires the adaptation and optimization of previously advanced enzyme kinetics.

Results and Discussion

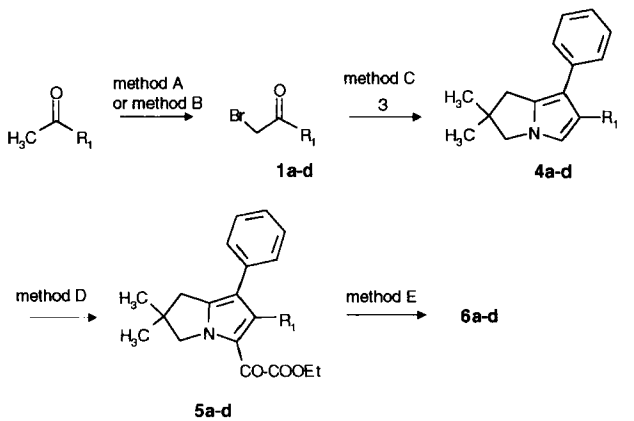
Chemistry

For preparation of the pyrrolizines we adapted the method described by G. Dannhardt and R. Obergrusberger[18]. We condensed 4-chloro-3,3-dimethyl-butyronitrile, obtained in several steps from 2,2-dimethylpropane-1,3-diol, with a commercially available benzyl-Grignard, and accomplished ring closure to 2-benzyl-4,4-dimethyl-1-pyrroline (**3**) *in situ* by heating (Scheme 2)[18].



2 **3**

Scheme 2. Synthetic pathway for compound 3[18].



Scheme 3. Synthetic pathway. Method A: Br_2, CH_2Cl_2, –10 °C, room temp. Method B: NBS, DBP, CCl_4 reflux. Method C: a) **3**, CH_2Cl_2, room temp., 4 h; b) $NaHCO_3$ (5%), 3 h. Method D: ClCO-COOEt, CH_2Cl_2, –10 °C, room temp., 1/2 h. Method E: a) $N_2H_4 \cdot H_2O$ (80%), $(CH_2OH)_2$, 60 °C, 1/2 h; b) KOH, 140 °C, 2 h.

The new aryl-heteroaryl-pyrrolizine-acetic acids have been prepared from corresponding aryl-heteroaryl-pyrrolizines according to Scheme 3. Instead of phenacyl bromide the heteroaromatic 1-bromo-1-ethanones **1a–d** were used. Pyrrolinium intermediates have not been isolated.

Pharmacology

In order to achieve a more sensitive and efficient testing method, we modified the previously evaluated HPLC quantification method[17] using ELISA assays.

Compared with the detection limit of the HPLC method (ng/mL) the ELISA system is highly sensitive and is able to detect even in the lower pg/mL range. This fact makes it possible to reduce the number of cells per sample and also the calcium ionophore A 23187 concentration. In the ELISA system the number of platelets was reduced to 0.3×10^8 cells/mL and the count of PMNLs was decreased to 0.125×10^7 cells/mL. This is a 75% reduction of cells, when compared with the cell count required for the HPLC method. The calcium ionophore A 23187 concentration was halved from formally 20 µM to 10 µM. Due to the reduced cell count and lower levels of calcium ionophore A 23187, it was necessary to recalculate the stimulation times. The newly optimized enzyme kinetic of leukotriene B_4 (LTB_4), as seen in Figure 1, resulted in an increase in metabolites during the first 3 min and thereafter reached a plateau at 140 pg/mL. Therefore, we fixed the new incubation time for all future experiments at 1.5 min, since the stimulation time should be in the linear part of the kinetic curve.

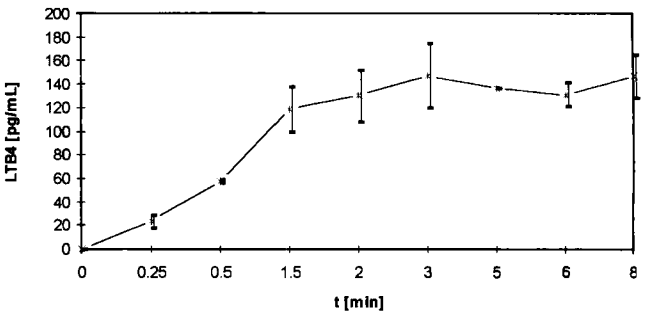


Figure 1. Time course of LTB_4 production by human PMNLs, stimulated with 10 µM calcium ionophore A 23187. Values are reported as mean ± SD from four separate experiments.

The stimulation of human platelets required some new assay conditions, because of the fact that no available commercial ELISA kit can determine 12-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT), which was earlier quantified in our HPLC method[17]. We decided to determine another metabolite of platelets, prostaglandin E_2 (PGE_2)[19]. To suppress the formation of thromboxanes and allow the shift to PGE_2, 10 µL thromboxane synthase inhibitor (*E/Z*)-7-phenyl-1-(3-pyridyl)-6-heptenoic acid[20] (1 µM) was added to all samples. This step was necessary to ensure only the involvement of cyclooxygenase and exclude any participation of thromboxane synthase in the test system. The PGE_2 formation reached a maximum after 45 s at 400 pg/mL. After this point in time, the PGE_2 release was discontinuous until 2 min and thereafter reached a plateau at 300 pg/mL. Thus, we

adjusted the stimulation time for all further experiments at 45 s. The time course is shown in Figure 2.

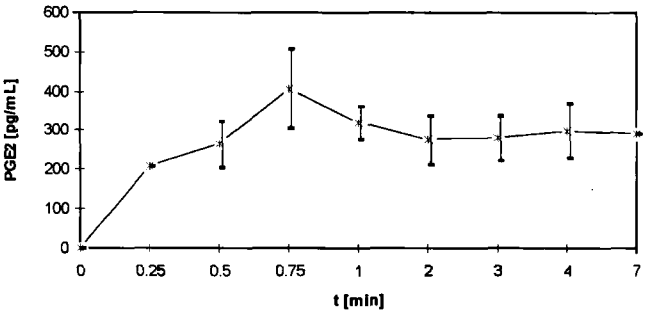


Figure 2. Time course of PGE_2 production by human platelets, stimulated with 10 µM calcium ionophore A 23187. Values are reported as mean ± SD from four separate experiments.

Since organic solvents would interfere with the ELISA components, the termination of the cell stimulation phase had to be changed. Therefore, the PGE_2 and LTB_4 formation respectively were terminated through ultra-sound treatment of the cells for 5 min in an ice bath and the simultaneous addition of ice-cold water (containing nordihydroguajaretic acid [NDGA] as an oxygen scavenger). To remove cell fragments, the samples were centrifuged at 4 °C for 12 min at 46,000 × *g*. After the centrifugation the sample supernatants were removed and immediately stored at –20 °C. After an additional 1:50 dilution these samples could be directly pipetted without further purification procedures on the ELISA assay.

Finally we can conclude that we accomplished the aforementioned targets. The optimized test method for the evaluation of dual COX-1/5-LOX inhibitors is working with as low as 0.5 mL human whole blood per sample. The greatly decreased cell count led to improved IC_{50} values of the tested inhibitors, which represents a better correlation with the *in vivo* animal model. Furthermore, this method has the additional advantage that one can test a large number of samples per assay within a short period of time.

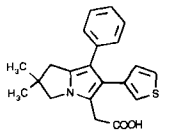
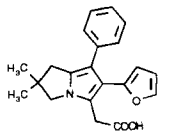
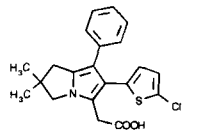
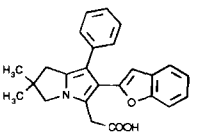
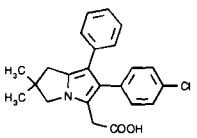
Data obtained for reference compounds are comparable to other test systems and listed in Table 1.

Table 1. IC_{50} values of reference compounds.

Inhibitor	IC_{50} values [µM] ± SEM COX		5-LOX		other test systems
Diclofenac	0.05 ± 0.02	$n = 3$			0.04[21]
Piroxicam	4.0 ± 1.4	$n = 4$			2.8[22]
Indomethacin	0.002 ± 0.001	$n = 2$			0.032[23]
BAY-X-1005			0.8 ± 0.007	$n = 2$	0.22[24]
BAY-Y-1015			0.01 ± 0.0005	$n = 2$	0.07[25]

The compounds **6a, 6b, 6c, 6d** and ML3000 were tested in the validated test model.

Table 2. Effects of compounds **6a, 6b, 6c**, and **6d** on COX-1 and 5-LOX in isolated human platelets and PMNLs, respectively.

	IC_{50}-values [µM] ± SEM COX-1		5-LOX	
 **6b**	0.06 ± 0.007	$n = 2$	1.7 ± 1.1	$n = 3$
 **6c**	0.13 ± 0.02	$n = 3$	2.4 ± 0.2	$n = 3$
 **6a**	0.21 ± 0.09	$n = 3$	0.25 ± 0.008	$n = 3$
 **6d**	0.02 ± 0.006	$n = 2$	0.03 ± 0.02	$n = 2$
 ML3000 (**6e**)	0.22 ± 0.07	$n = 4$	0.37 ± 0.01	$n = 4$

The replacement of the 6-phenyl residue of ML3000 by 2-furanyl or 3-thiophenyl residues led to equal or improved COX-1 activities, whereas the 5-LOX activities decreased. Thus, the exchange resulted in an unbalanced dual inhibition of COX-1/5-LOX. Compound **6a** with the chlorothiophenyl-moiety showed a balanced activity on COX-1 and 5-LOX and similar IC_{50} values compared with ML3000. The most potent compound in our novel series of pyrrolizine derivatives was the benzofuranyl substituted inhibitor **6d** with a 10-fold improved activity on both COX-1 and 5-LOX [IC_{50} = 0.02 µM (COX-1); IC_{50} = 0.03 µM (5-LOX)]. Therefore, a well-balanced dual inhibitor with a highly improved COX-1 and 5-LOX activity could be obtained through the substitution of the 6-phenyl group by a benzofuranyl residue.

Experimental

Chemistry

Melting points were determined on Mettler FP 51 and were not corrected.– IR spectra were measured on Shimadzu R 470 (beam splitter), or Nicolet Impact 410 (FT).– ^{1}H-NMR spectra were measured on Bruker AC 200

(200 MHz against TMS as an internal standard.– Chemicals: 1-(5-Chlorothiophen-2-yl)-ethanone, 1-(furan-2-yl)-ethanone, 1-(thiophen-3-yl)-ethanone, and 1-(benzo[*b*]furan-2-yl)-ethanone were commercially available, corresponding 2-bromoethanones are known [26-29].– CC material: SiO_2 Geduran (MERCK, order no 7734), Alumina ICN TSC, order no. 04511.

General procedure A for bromination (2-bromo-1-(5-chlorothien-2-yl)-ethanone, **1a***)*

A solution of 1-(5-chlorothien-2-yl)-ethanone (12 g, 75 mmol) in CH_2Cl_2 (50 mL) was stirred vigorously at room temp. A solution of Br_2 (12 g, 75 mmol) in CH_2Cl_2 (40 mL) was added in small portions (1 mL) in such a way that decoloration was complete before addition of the next portion. Generally after a delay of 15 min complete addition of Br_2 took 1 h. HBr gas was evolved by further vigorous stirring for an additional hour. The reaction mixture was diluted with CH_2Cl_2 before neutralization and extraction with a saturated $NaHCO_3$ solution (2 × 100 mL) followed by brine (100 mL) and drying with Na_2SO_4 sicc.

Evaporation of solvent CH_2Cl_2 left 15 g of crude product as a brown yellow oil. The oil crystallized after trituration with *n*-hexane (10 mL). Recrystallization from *n*-hexane gave 13.5 g 2-bromo-1-(5-chlorothien-2-yl)-ethanone, **1a** (75% yield) as lachrymatory, pale yellow crystals of mp 72 °C.

General procedure B for bromination (2-bromo-1-(furan-2-yl)-ethanone, **1c***)*

To 2-acetylfuran (2.2 g, 20 mmol) dissolved in CCl_4 and a catalytic amount of DBP (dibenzoyl peroxide, 50 mg) was added NBS (*N*-bromosuccinimide, 3.9 g, 22 mmol) in one portion. The suspension was heated under reflux for 2 h. After that time NBS was consumed quantitatively and succinimide was separated on the surface of the solution. Chilling in an ice bath and filtering left a solution of two products. Separation by CC on SiO_2, *n*-hexane:CH_2Cl_2 = 1:3 afforded 2-bromo-1-(5-bromo-furan-2-yl)-ethanone and 2-bromo-1-(furan-2-yl)-ethanone **1c** in equal amounts, both as lachrymatory substances.

2-Bromo-1-(5-chlorothiophen-2-yl)-ethanone [26] **(1a)**

For preparation see general procedure A: from 1-(5-chloro-thiophen-2-yl)-ethanone (Aldrich) in 75% yield after recrystallization from *n*-hexane with mp 72 °C.

$C_6H_4BrClOS$, MW = 239.52.

IR (KBr): 1/λ = 1675 (C=O), 1418, 1390 (sh), 1198, 1014, 795, 647, 506.– ^{1}H NMR ($CDCl_3$): δ = 4.28 (s, 2H, CH_2), 7.00 (d, *J* = 4.0 Hz, thien.), 7.60 (d, *J* = 4.0 Hz, thien.).

2-Bromo-1-(thiophen-3-yl)-ethanone [27] **(1b)**

Prepared analogously to the general procedure A: from 1-(thiophen-3-yl)-ethanone (Aldrich) a 39% yield of 2-bromo-1-(thiophen-3-yl)-ethanone was obtained after CC (SiO_2, *n*-hexane:CH_2Cl_2 = 1:2) as colourless crystals with mp 61.6 °C. The brown oily crude product (89% g.c., obtained by removal of solvent CH_2Cl_2) can be used directly in ring synthesis.

C_6H_5BrOS, MW = 205.07.

IR (KBr): 1/λ = 3085 (CH), 1676, 1503,1411 (sh), 1395, 1378 (sh), 1272, 1217, 1179, 1141, 1065, 1001, 897, 796, 704, 660, 616.– ^{1}H NMR ($CDCl_3$): δ = 4.340 (s, 2H, CH_2), 7.366 (dd, J_1 = 2.8 Hz, J_2 = 5.1 Hz, thien.), 7.585 (dd, J_1 = 1.2 Hz, J_2 = 5.1 Hz, thien.), 8.180 (dd, J_1 = 1.3 Hz, J_2 = 2.9 Hz, thien.).

2-Bromo-1-(furan-2-yl)-ethanone [28] **(1c)**

Using general procedure A at 0–5 °C instead of procedure B: from 1-(furan-2-yl)-ethanone (Aldrich) a 79% yield of 2-bromo-1-(furan-2-yl)-ethanone was obtained without byproduct 2-bromo-1-(5-bromofuran-2-yl)-ethanone, having mp 43.7 °C after recrystallization from pentane.

$C_6H_5BrO_2$, MW = 189.01.

IR (KBr): 1/λ = 3130 (CH), 1670, 1562, 1459,1418 (sh), 1388, 1295, 1222, 1159, 1129 (sh), 1079, 1037, 1015, 911, 879, 763.– ^{1}H NMR ($CDCl_3$): δ = 4.331 (s, 2H, CH_2), 6.610 (dd, J_1 = 1.6 Hz, J_2 = 3.7 Hz, furan), 7.347 (dd, J_1 = 3.6 Hz, J_2 = 0.8 Hz, furan), 7.655 (dd, J_1 = 0.8 Hz, J_2 = 1.6 Hz, furan).

2-Bromo-1-(benzo[b]furan-2-yl)-ethanone [29] **(1d)**

Prepared analogously to the general procedure A: from 1-(benzo[*b*]furan-2-yl)-ethanone (Aldrich) 2-bromo-1-(benzo[*b*]furan-2-yl)-ethanone can be isolated in 88% yield as crystals from *n*-hexane having a pale yellow colour and mp 84.3 °C.

$C_{10}H_7BrO_2$, MW = 239.07.

IR (KBr): 1/λ = 1685, 1606, 1504, 1543, 1377, 1340 (sh), 1325, 1241, 1153, 1130, 1011, 745, 663, 563.– ^{1}H NMR ($CDCl_3$): δ = 4.457 (s, 2H, CH_2), 7.390–7.350 (m, 1H, benzofuran.), 7.592–7.527 (m, 2H, benzofuran.), 7.673 (d, *J* = 0.7 Hz, 1H, H-3), 7.767–7.727 (m, 1H, benzofuran.).

General procedure C:

The synthesis of 6-heteroaryl-7-phenyl-2,3-dihydro-1*H*-pyrrolizines (**4a–d**) was adapted from a procedure described by Dannhardt and Obergrusberger[18]:

To a solution of the heteroaromatic 2-bromo-1-ethanone (**1a–d**) in CH_2Cl_2 an equimolar amount of 2-benzyl-4,4-dimethyl-1-pyrroline (**3**) dissolved in CH_2Cl_2 was added. The pyrroline was prepared by Grignard reaction of benzyl-magnesium chloride in toluene with 3-chloro-2,2-dimethylbutyronitrile (**2**), following the method of Dannhardt and Obergrusberger. The mixture of pyrroline and bromo-ketone was stirred for 4 h at room temp., then a solution (5%) of $NaHCO_3$ was added and stirring continued for 3 h. After addition of water to form a clear aqueous layer, the organic layer was separated and dried (Na_2SO_4 sicc.) and the solvent evaporated.

*6-(5-Chloro-thiophen-2-yl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizine (***4a***)*

Following method C, purified by CC on SiO_2 with *n*-hexane:diethyl ether = 6:1, obtained as an oil in 46% yield.

$C_{19}H_{18}ClNS$, MW = 327.87, mp: – (oil).

IR (film): 1/λ = 2950, 1656, 1596, 1444, 1414, 1382, 792, 759, 697.– ^{1}H NMR ($CDCl_3$): δ = 7.29–7.17 (m, 5H, arom.); 6.71 (s, 1H, N-CH-); 6.70/6.49 (AB, *J* = 3.5 Hz, thien.); 2.72 (s, 2H, -CH_2-N); 2.75 (s, 2H, -CH_2-); 1.27 (s, 6H, -CH_3).

*2,2-Dimethyl-7-phenyl-6-(thiophen-3-yl)-2,3-dihydro-1H-pyrrolizine (***4b***)*

Following method C, purified by CC on Al_2O_3 with *n*-hexane:diethyl ether = 9:1, obtained as a pale yellow oil in 34% yield.

$C_{19}H_{19}NS$, MW = 293.43, mp: – (oil).

IR (film): 1/λ = 2945, 1597, 1551, 1460, 1416, 1363, 1156, 757, 696.– ^{1}H NMR ($CDCl_3$): δ = 7.27–7.17 (m, 6H, arom. + thien.); 6.97/6.95 (m, 2H, thien.); 6.72 (s, 1H, =CH-N-); 3.73 (s, 2H, -CH_2N-); 2.78 (s, 2H, pyr.); 1.28 (s, 6H, -CH_3).

*2,2-Dimethyl-6-(furan-2-yl)-7-phenyl-2,3-dihydro-1H-pyrrolizine (***4c***)*

Following method C, purified by CC on Al_2O_3 with *n*-hexane:diethyl ether = 6:1 and recrystallization from MeOH, light yellow crystals were obtained in 19% yield.

$C_{19}H_{19}NO$, MW = 277.37, mp: 107.7 °C.

IR (KBr): 1/λ = 2940, 1600, 1478, 1368, 1176, 1008, 968, 753, 721, 698.– ^{1}H NMR ($CDCl_3$): δ = 7.33–7.17 (m, 6H, arom., =CH-O); 6.88 (s, 1H, =CH-N-); 6,30 (dd, 1H, *J* = 1.8 Hz); 6.01 (d, 1 H, *J* = 3.2 Hz); 3.73 (s, 2H, -CH_2-N-), 2.74 (s, 2H, -CH_2-); 1.29 (s, 6H, -CH_3).

*6-(Benzo[b]furan-2-yl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizine (***4d***)*

Following method C, purified by CC on Al_2O_3 with *n*-hexane:diethyl ether = 9:1 and recrystallization from MeOH, light yellow crystals were obtained in 27.5% yield.

$C_{23}H_{21}NO$, MW = 327.43, mp: 145.2 °C.

IR (KBr): 1/λ = 2945, 1601, 1462, 1276, 1249, 1162, 970, 790, 740, 698.– ^{1}H NMR ($CDCl_3$): δ = 7.4–7.11 (m, 10H, benzofuran.); 6.31 (d, 1H, =CH-N, *J* = 0.76 Hz); 3.77 (s, 2H, -CH_2-N-); 2.74 (s, 2H, -CH_2-); 1.28 (s, 6H, -CH_3)

General procedure D:

*Synthesis of ethyl (6-heteroaryl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl)-2-oxoacetate (**5a–d**)*

To a stirred solution of a slight excess (10%) of ethyloxalyl chloride in CH_2Cl_2 abs. a solution of 6-heteroaryl-7-phenyl-2,3-dihydro-1*H*-pyrrolizines (**4a–d**) in CH_2Cl_2 abs. was added dropwise at room temp. Stirring was continued for further 20 min. For work-up water was added carefully and the organic layer separated and dried (Na_2SO_4 sicc.) and the solvent evaporated. The residual oil crystallized after treatment with diisopropyl ether. The crystals were collected.

*Ethyl [6-(5-chloro-thiophen-2-yl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl]-2-oxoacetate (**5a**)*

Following method D, crystals with greenish, pale yellow colour obtained from diisopropyl ether in 38.9% yield.

$C_{23}H_{22}ClNO_3$, MW = 427.95, mp: 133.0 °C.

IR (KBr): 1/λ = 2955, 1736, 1619, 1467, 1426, 1373, 1241, 1179, 1049, 701.– ^{1}H NMR ($CDCl_3$): δ = 7.26–7.10 (m, 5H, arom.); 6.82 (AB, *J* = 3.7, -CH=); 6.77 (AB, *J* = 3.7, -CH=); 4.22 (s, 2H, $-CH_2N-$); 3.87 (q, 2H, *J* = 7.0, ethyl); 2.82 (s, 2H, $-CH_2-$); 7.31 (s, 6H, $-CH_2$); 1.19 (t, 3H, *J* = 7.0, ethyl).

*Ethyl [2,2-dimethyl-7-phenyl-6-(thiophen-3-yl)-2,3-dihydro-1H-pyrrolizin-5-yl]-2-oxoacetate (**5b**)*

Following method D, crystals of light yellow colour obtained from diisopropyl ether in 60% yield.

$C_{23}H_{23}CNO_3S$, MW = 393.5, mp: 136.4 °C.

IR (KBr): 1/λ = 2950, 1732, 1609, 1450, 1420, 1249, 1132, 1062, 743.– ^{1}H NMR ($CDCl_3$): δ = 7.26–6.88 (m, 8H, arom. = ABX-thien.); 4.23 (s, 2H, $-CH_2N-$); 3.75 (q, 2H, *J* = 7.0, ethyl); 2.85 (s, 2H, $-CH_2-$); 1.32 (s, 6H, $-CH_3$); 1.12 (t, 3H, *J* = 7.0, ethyl).

*Ethyl [2,2-dimethyl-6-(furan-2-yl)-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl]-2-oxoacetate (**5c**)*

Following method D, crystals with light green colour obtained from diisopropyl ether in 67% yield.

$C_{23}H_{23}NO_4$, MW = 377.44, mp: 129 °C.

IR (KBr): 1/λ = 2975, 1719, 1616, 1443, 1262, 1181, 1062, 757, 692.– ^{1}H NMR ($CDCl_3$): δ = 7.43–7.10 (m, 6H, arom. =CH-O); 6.41 (dd, 1H, *J* = 1.9,); 6.23 (d, 1H, *J* = 3.2); 4.21 (s, 2H, $-CH_2N-$); 3.95 (q, 2H, *J* = 7.1, ethyl); 2.84 (s, 2H, $-CH_2-$); 1.32 (s, 6H, $-CH_3$); 1.15 (t, 3H, *J* = 7.1, ethyl).

*Ethyl [6-(benzo[b]furan-2-yl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl]-2-oxoacetate (**5d**)*

Following method D, brown solid recrystallized from diisopropyl ether gave a solid of pale yellow colour in 78% yield.

$C_{27}H_{25}NO_4$, MW = 427.5, mp: 161 °C.

IR (KBr): 1/λ = 2950, 1625, 1739, 1451, 1423, 1370, 1306, 1241, 1199, 1060.– ^{1}H NMR ($CDCl_3$): δ = 7.53–7.17 (m, 9H, arom., benzofuran.); 6.58 (d, 1H, *J* = 0.75 Hz, ArC*H*=C-); 4.23 (s, 2H, $-CH_2$-N); 3.65 (q, 2H, *J* = 7.2 Hz, O-CH_2-CH_3); 2.83 (s, 2H, $-CH_2-$); 1.33 (s, 6H, $C(CH_3)_2$); 0.98 (t, 3H, *J* = 7.2 Hz, O-CH_2-CH_3).

General procedure E:

*Synthesis of 6-heteroaryl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl-acetic acids (**6a-d**)*

In a mixture of hydrazine hydrate (80% aqueous solution) in diethylene glycol was suspended an ethyl (6-heteroaryl-7-phenyl-2,3-dihydro-1*H*-pyrrolizin-5-yl)-2-oxoacetate (**5a–d**) and the mixture stirred for 30 min at 60 °C. After addition of potassium hydroxide the mixture was kept at 140 °C for 2 h. When nitrogen evolution ceased the solution was poured onto crushed ice and then acidified with phosphorous acid to a pH value of 3 to 4. The precipitated 6-heteroaryl-7-phenyl-2,3-dihydro-1*H*-pyrrolizin-5-yl-acetic acid was collected by filtration and dried *in vacuo*.

*6-(5-Chloro-thiophen-2-yl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl-acetic acid (**6a**)*

Following method E, precipitated crude acid recrystallized from diethyl ether to give colourless crystals in 56.3% yield.

$C_{21}H_{20}ClNO_2S$, MW = 385.91, mp: 164 °C.

IR (KBr): 1/λ = 2920, 1706, 1599, 1441, 1417, 1250, 1222, 1056, 802, 694.– ^{1}H NMR ($CDCl_3$): δ = 7.24–7.12 (m, 5H, arom.); 6.80–6.62 (AB, 3.8 Hz, thien.), 3.73 (s, 2H, $-CH_2N-$); 3.67 (s, 2H, $-CH_2$-C=O); 2.83 (s, 2H, $-CH_2-$); 1.28 (s, 6H, $C(CH_3)_2$).

*2,2-Dimethyl-7-phenyl-6-(thiophen-3-yl)-2,3-dihydro-1H-pyrrolizin-5-yl-acetic acid (**6b**)*

Following method E, precipitated crude acid purified by CC on a short column (SiO_2, diethyl ether) and recrystallization from diisopropyl ether gave pale pink crystals in 43.5% yield.

$C_{21}H_{21}NO_2S$, MW = 351.46, mp: 157.2 °C.

IR (KBr): 1/λ = 2950, 1700, 1598, 1447, 1412, 1307, 1270, 1224, 790, 685.– ^{1}H NMR ($CDCl_3$): δ = 7.26–7.08 (m, 7H, arom. ABX-thien.), 6.83 (dd, 1H, thien.); 3.76 (s, 2H, $-CH_2$-N-); 3.64 (s, 2H, $-CH_2$-CO-); 2.85 (s, 2H, $-CH_2-$); 1,30 (0.6 Hz, $C(CH_3)_2$).

*6-(Furan-2-yl)-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl-acetic acids (**6c**)*

Method E, precipitated crude acid (80% yield) purified by CC on a short column (SiO_2, diethyl ether) gave a white solid in 27.5% yield.

$C_{21}H_{21}NO_3$, MW = 335.4, mp: 164 °C.

IR (KBr): 1/λ = 3425, 2950, 1702, 1600, 1446, 1289, 1174, 1008, 758, 697.– ^{1}H NMR ($CDCl_3$): δ = 7.37–7.10 (m, 6H, arom. + =C*H*-O-); 6.37 (dd, 1H, *J* = 1.9); 6.13 (d', 1H, *J* = 3.4); 3.75 (s, 2H,); 3.73 (s, 2H,); 2.81 (s, 2H, d); 1.28 (s, 6H, $C(CH_3)_2$).

*6-(Benzo[b]furan-2-yl)-2,2-dimethyl-7-phenyl-2,3-dihydro-1H-pyrrolizin-5-yl-acetic acid (**6d**)*

Following method E, precipitated crude acid purified by CC on a short column of SiO_2, with diethyl ether eluent gave a white solid in 43.5% yield.

$C_{25}H_{23}NO_3$, MW = 385.46, mp: 153.6 °C.

IR (KBr): 1/λ = 2950, 1707, 1600, 1451, 1416, 1251, 1217, 1163, 746, 696.– ^{1}H NMR ($CDCl_3$): δ = 7.48–7.15 (m, 9H, arom. benzofuran); 6.43 (d, 1H, *J* = 0.6 Hz, ArC*H*=C-); 3.89 (s, 2H, $-CH_2$-N-); 3.75 (s, 2H, $-CH_2$-C=O); 2.81 (s, 2H, C-CH_2-C); 1.29 (s, 6H, $-C(CH_3)_2$).

Pharmacology

Materials

All the following solutions and drugs used were of analytical grade: Salts for buffer solutions, solvents (Merck, Darmstadt, Germany); calcium ionophore A 23187 (free acid), Percoll® (colloidal PVP coated silica), trypan blue solution 0.4% (Sigma-Chemie, Deisenhofen, Germany); nordihydroguajaretic acid (NDGA: Aldrich-Chemie, Steinheim, Germany); dimethyl sulfoxide (DMSO: Fluka-Chemie, Buchs, Switzerland); water obtained by reverse osmosis; thromboxane synthase inhibitor (*E/Z*)-7-phenyl-1-(3-pyridyl)-6-heptenoic acid[20]; prostaglandin E_2 and leukotriene B_4 quantification: enzyme linked immunosorbent assay kit [ELISA] (Cayman Chemical Company, Ann Arbor, USA).

Apparatus and Analytical Systems

For Centrifugation: Suprafuge 22, coolable (Heraeus Instruments, Hanau, Germany).– Microscopy: Olympus CHS microscope (Olympus Optical Co., Weilheim, Germany) for cell counting and investigation of cell viability.– The incubation procedures were performed in a rocking water bath (GFL®, Burgwedel, Germany).– For ultra-sound treatment of cells: Sonorex RK 100 (Bandelin, Berlin, Germany).– Scales: Sartorius Analytical Scale, type 1702 (Sartorius, Ismaning, Germany) and Mettler PC 180 scale (Mettler, Grüssen, Switzerland).– For the evaluation: Emax™ precision microplate reader, detection wavelength 405nm and SOFTmax® For Windows™ Version 2.31 User's Manual (Molecular Devices Corporation, Gräfelfing, Germany) for analysis of the dose response curves.

Subjects

Human whole blood from four healthy donors (male or female, 20 mL each) was removed by venopuncture and collected in heparin-NH_4-monovettes [15 IE/mL] (Sarstedt, Nümbrecht, Germany) on the day of the experiment. The freshly drawn blood was obtained from the DRK (German Red Cross).

Preparations

Isolation and purification of human platelets and PMNLs was performed as previously described[17]. The final platelet pellet was resuspended in EDTA-PBS-solution (3 mM EDTA disodium, 133 mM sodium chloride, 2.6 mM potassium chloride, 5.4 mM disodium hydrogen phosphate, 1 mM sodium dihydrogen phosphate, and 1.4 mM potassium dihydrogen phosphate) and adjusted to 0.3×10^8 cells/mL and corresponding the PMNLs were adjusted to 0.125×10^7 cells/mL in IKP buffer (137 mM sodium chloride, 2.7 mM potassium chloride, 2 mM potassium dihydrogen phosphate, 5 mM disodium hydrogen phosphate, and 5.55 mM α-D-glucose).

Incubation procedures

5-Lipoxygenase assay

Aliquots (800 µL) of human PMNL suspension (0.125×10^7 cells/mL) were preincubated in 16 mL polypropylene tubes (Nalgene, Rochester, NY, USA) in the absence (control) or presence of various concentrations of test compound (10 µL DMSO solution) at 37 °C in a vibrating water bath for 10 min. After 5 min, 200 µL calcium chloride solution (10 mM in 0.8% sodium chloride solution) was added. The cells were stimulated at $t = 0$ by adding 10 µM of calcium ionophore solution A 23187 (10 µL in DMSO) and incubation was continued for 1.5 min at 37 °C. Leukotriene formation was terminated by placing the samples in an ultra-sound ice-cold water bath under the simultaneous addition of 2 mL NDGA solution 0.02 mM (an oxygen scavenger) and 1 mL double-distilled water. After centrifugation at 46,000 $\times g$ for 12 min at 4 °C the sample supernatants were removed and immediately stored at –20 °C in polypropylene snap-caps (Eppendorf, Hamburg, Germany).

Cyclooxygenase-1 assay

The assay was carried out under the conditions described for the 5-lipoxygenase assay (see above) except for the application of 800 µL human platelet suspension (0.3×10^8 cells/mL) containing 1 µM thromboxane synthase inhibitor (*E/Z*)-7-phenyl-1-(3-pyridyl)-6-heptenoic acid[20] (10 µL in DMSO) instead of the PMNL suspension and a stimulation time of 45 s instead of 1.5 min after addition of calcium ionophore A 23187.

ELISA

The samples for PGE_2 and LTB_4 determination were diluted in the standard diluent of the assay (EIA buffer preparation) and were examined at a final dilution of 1:200. Each determination was run by an eight-point standard curve in duplicate.

The PGE_2 kits were coated with goat anti-mouse IgG monoclonal antibody and the detection limit (80% B/B_0) of the assay was 29 pg/mL for PGE_2.

The LTB_4 kits were coated with mouse anti-rabbit IgG monoclonal antibody and the detection limit (80% B/B_0) of the assay was 7 pg/mL for LTB_4.

Statistical analysis

The test compounds were tested in solutions of 10^{-5} to 10^{-9} M. The selectivity of the test compounds was evaluated by calculation of the percentage inhibition of PGE_2 and LTB_4 production respectively. Potency estimates are presented as IC_{50} values (inhibitor concentration, which reduce mediator synthesis by 50%). The results were expressed as mean ± SEM.

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☆ Dedicated to Professor Dr. W. Wiegrebe, Regensburg, on the occasion of his 65th birthday.

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