Design and Synthesis of Novel 2-Amino-5-hydroxyindole Derivatives That Inhibit Human 5-Lipoxygenase

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Compounds that inhibit 5-lipoxygenase (5-LO), the key enzyme in the biosynthesis of leukotrienes (LTs), possess potential for the treatment of inflammatory and allergic diseases as well as of atherosclerosis and cancer. Here we present the design and the synthesis of a series of novel 2-amino-5-hydroxyindoles that potently inhibit isolated human recombinant 5-LO as well as 5-LO in polymorphonuclear leukocytes, exemplified by ethyl 2-[(3-chlorophenyl)amino]-5-hydroxy-1*H*-indole-3-carboxylate (**3n**, IC_{50} value $\cong$ 300 nM). Introduction of an aryl/arylethylamino group or 4-arylpiperazin-1-yl residues into position 2 of the 5-hydroxyindoles was essential for biological activity. Whereas the 4-arylpiperazin-1-yl derivatives were more potent in cell-free assays as compared to intact cell test systems, aryl/arylethylamino derivatives inhibited 5-LO activity in intact cells and cell-free assays almost equally well. On the basis of their 5-LO inhibitory properties, these novel 2-amino-5-hydroxyindoles represent potential candidates for the pharmacological intervention with LT-associated diseases.

Introduction

5-Lipoxygenase (5-LO) is the key enzyme in the conversion of arachidonic acid (AA) to the bioactive leukotrienes (LTs) (for review, see ref 1). LTs cause constriction and mucus secretion in the lung, increase vascular permeability, and are potent vasoconstrictors of coronary arteries, but also function as proinflammatory mediators by acting as chemotactic and chemokinetic agents toward granulocytes.[2] On the basis of these properties, LTs are regarded as powerful mediators, playing crucial roles in asthma and inflammatory reactions, in vascular diseases including atherosclerosis,[3] myocardial infarction, and stroke,[4] as well as in cell proliferation and survival particularly of prostate and pancreatic cancer cells.[5] In search of pharmacological strategies that intervene with LTs, a huge number of different types of low molecular weight inhibitors that potently suppress LT synthesis (i.e. redox-, iron ligand-, and nonredox-type 5-LO inhibitors) have been developed within the past 20 years.[1] However, despite the large number of therapeutic indications and the strong need for efficient and safe drugs that target the 5-LO pathway, no 5-LO inhibitor is available on the market for the therapy of human subjects today.[6] Hence, the development of novel appropriate compounds lacking the disadvantages of former 5-LO inhibitors is an important challenge. Such compounds may provide substantial therapeutic benefit for the pharmacological treatment of inflammatory and allergic disorders, cardiovascular diseases, and cancer.

Here we present the design and synthesis of novel 2-amino-5-hydroxyindoles that proved to be potent inhibitors of human 5-LO in cell-free assays as well as in intact cells. These compounds exhibit no typical structural properties or characteristics of classical 5-LO inhibitors such as iron ligand-type inhibitors, redox-active inhibitors, or nonredox-type inhibitors and have not been reported before to interfere with LT formation. Our data suggest a therapeutic potential for these novel 2-amino-5-hydroxyindoles in the treatment of diseases related to LTs and other 5-LO-derived lipid mediators.

Results and Discussion

Chemistry. For the synthesis of 5-hydroxyindoles with possible bioactivity, the Nenitzescu reaction[7] is a suitable approach. We prepared the title compounds by reacting 1,4-benzoquinone (**1**) with primary-secondary and primary-tertiary ketene aminals **2a**−**m** (Scheme 1). In all cases, stirring of the ketene aminals **2** with 1.2 equiv of **1** in ethanol at room temperature gave the desired 2-amino-5-hydroxyindoles **3a**−**m** in moderate to good yields (28−82%). The primary 2-amino-5-hydroxyindoles **4a**−**c** (Table 1) were synthesized as previously reported.[8] The preparation of the ketene aminals of type **2** was first described by Meyer et al.[9] Accordingly, ethyl 3-ethoxy-3-aminoacrylate (**5**) was treated with an appropriate primary or secondary amine **6** in ethanol under reflux. Following this or a slightly modified methodology, the preparation of the ketene aminals **2a**−**k** has been already described by different authors.[10−16] We prepared these compounds as well as the enediamines **2l**,**m** and **13** according to the method of Meyer et al.[9] The hitherto unknown ketene aminals **2n**,**o** were not available by refluxing the ketene *N*,*O*-acetal **5** with the aromatic amines 3-chloroaniline (**6n**) and 3-aminopyridine (**6o**), respectively, in different solvents (e.g. ethanol, *n*-propanol, toluene), presumably due to the insufficient nucleophilicity of the aromatic amines in this reaction. Surprisingly, by adding 1.2 equiv of 1,4-benzoquinone (**1**) to the reaction mixture, the resulting Nenitzescu reaction gave rise to the expected 2-ethoxyindole **3p**,[17] but also to the indoles **3n**,**o**. In a previous paper,[8] a plausible mechanism for this unexpected result has already been provided and the interested reader is referred to that report. To

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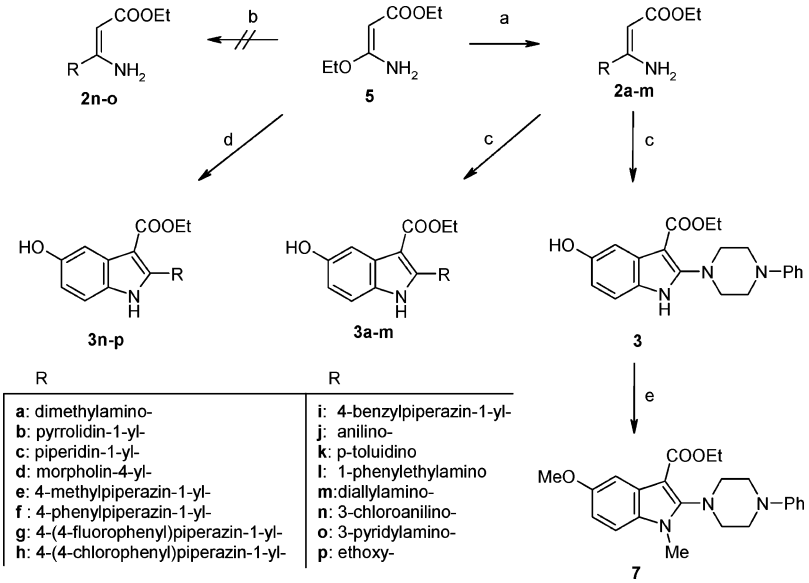
Scheme 1. Synthesis of 2-Amino-5-hydroxyindoles (**3**) via the Nenitzescu Reaction[a]



[a] Reagents and conditions: (a) amine (**6a**−**m**), EtOH, reflux, 48 h; (b) amine (**6n**,**o**), toluene, reflux; (c) 1,4-benzoquinone (**1**), EtOH, rt, 50 min; (e) NaH, dimethyl sulfate, DMF, reflux, 1 h.

abolish the reducing properties of the type **3** 5-hydroxyindoles, the bioactive compound **3f** was doubly methylated by refluxing with NaH and dimethyl sulfate resulting in **7f** that requires oxidative demethylation in order to act as an antioxidant (for all reactions, see Scheme 1). To avoid the formation of quinoneimines by metabolic oxidation, we further synthesized the 5-unsubstituted indoles **9a** and **9b** by a modification of Bergmann's methodology[18] (Scheme 2). Next, the ester moiety of **3l** was replaced by a nitrile group. Although a direct conversion of **3l** to **11** is laborious, this compound was easily accessible by reaction of (*E*) (*RS*) 3-amino-3-[(1-phenylethyl)-amino]acrylonitrile (**10**)[19] with 1,4-benzoquinone (**1**) at room temperature in ethanol, giving (*RS*) 5-hydroxy-2-[(1-phenyl)-ethylamino]-1*H*-indole-3-carbonitrile (**11**) in good yields (Scheme 3).

To test the necessity of the ethyl ester moiety, we replaced the ethyl by a methyl group. The methyl ester **14** can be synthesized by stirring *p*-benzoquinone (**1**) with the ketene aminal **13** in methanol at room temperature. **13** is available by treating methyl 3-amino-3-methoxyacrylate (**12**)[20] with (*RS*)-1-phenylethylamine (**6l**) in boiling methanol (Scheme 4).

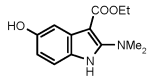
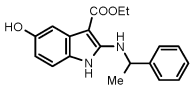
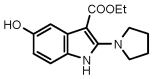
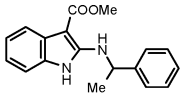
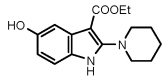
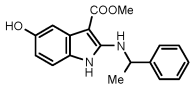
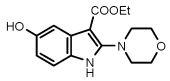
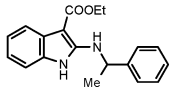
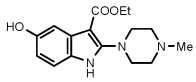
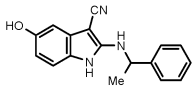
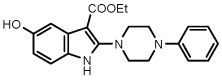
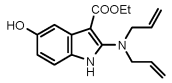
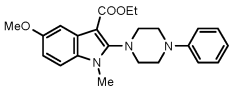
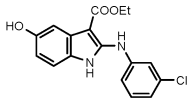
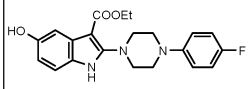
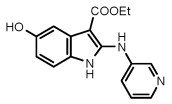
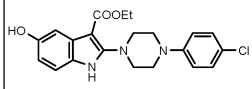
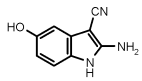
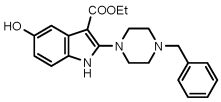
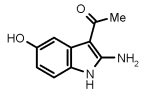
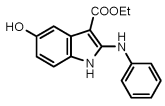
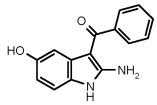
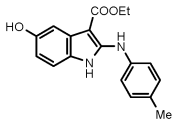
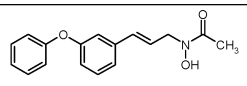
Evaluation of 5-LO Activity and Structure−**Activity Relationships.** In an initial screening round, several 2-amino-5-hydroxyindoles were tested at a concentration of 10 μM for their ability to inhibit 5-LO in human PMNL. The PMNL were stimulated with 2.5 μM Ca^{2+} ionophore A23187 that evokes most pronounced 5-LO product synthesis in these cells.[1] Under these conditions, the Ca^{2+}-ionophore A23187 causes liberation of AA as substrate for 5-LO from cellular phospholipids by $cPLA_2$,[21] due to an elevation of the intracellular Ca^{2+} concentration. Hence, reduced levels of 5-LO products may result not necessarily from inhibition of the 5-LO enzyme, but could also be due to suppression of AA release via inhibition of $cPLA_2$. Thus, to exclude effects on AA supply from endogenous sources, selected compounds were assayed in Ca^{2+}-ionophore A23187-challenged PMNL supplemented with exogenous AA (20 μM), thereby circumventing $cPLA_2$ activity. Finally, to assess direct suppressive effects on 5-LO activity, the test compounds (10 μM, each) were screened for inhibition of crude enzymatic activity of 5-LO in a cell-free assay using S100 of lysates of *E. coli* expressing human recombinant 5-LO that were incubated with 20 μM AA.[22] BWA4C, a highly potent and well-recognized iron ligand-type 5-LO inhibitor,[1,23] closely related to the most far developed 5-LO inhibitor zileuton,[6,24] was used as reference drug in both assays.

As shown in Table 1, as long as the substituent in position 2 is a primary amine (**4a**−**c**), no inhibition of 5-LO was recorded in Ca^{2+}-ionophore A23187-challenged PMNL, regardless of the substituent in position 3 (COOEt (not shown), CN, COMe, COPh) of the indole. Also, increasing the lipophilicity of the 2-amino group by incorporation of the nitrogen into a pyrolidin-1-yl, piperidin-1-yl, 4-methylpiperidin-1-yl, or morpholin-1-yl moiety (**3a**−**e**), the compounds remained inactive at concentrations up to 30 μM. However, when the 4-methyl residue in the piperazinyl ring of **3e** was replaced by a phenyl (**3f**,**g**) or benzyl (**3i**) moiety, the compounds slightly suppressed 5-LO product synthesis in PMNL (15 to 22% inhibition at 10 μM). Notably, the 4-(4-chlorophenyl)piperazin-1-yl derivative **3h** (10 μM) (but not the 4-(4-fluorophenyl)piperazin-1-yl derivative **3g**) was quite potent and blocked 5-LO product synthesis by 84% (Table 1). In contrast, for inhibition of crude 5-LO activity in S100, all 4-arylpiperazin-1-yl derivatives (**3f**−**i**) were much more efficient (61 to 81% inhibition). Although the reason for this is not clear, low intracellular availability due to high polarity and thus low cell-permeability (positive charge of the basic piperazine ring) is conceivable.

In addition to the 2-[(4-aryl)piperazin-1-yl]-indole-3-carboxylates, also introduction of secondary phenyl- or phenylethylamine residues into position 2 led to potent 5-LO inhibitors exemplified by **3j**, **3k**, **3l**, **3n**, and **14** that blocked 5-LO activity at 10 μM in intact PMNL as well as in cell-free assays in the range of 65 to 97% (Table 1). BWA4C (1 μM) suppressed 5-LO activity by 93%. The respective (basic) 2-(pyridin-3-ylamino)-indole derivative (**3o**) was significantly less potent. Of interest, the only bioactive nonaromatic compound was the 2-diallylamino substituted indole **3m**, which has similar lipophilic properties as compared to the aromatic compounds. Together, introduction of a neutral, lipophilic (aromatic) system into the 2-amino group or into the 2-piperazin-1-yl moiety of 5-hydroxyindole-3-carboxylates is required for inhibition of 5-LO activity.

One pharmacological strategy for inhibition of 5-LO activity is the application of lipophilic reducing agents including

Table 1. Inhibition of 5-LO Activity in Intact PMNL and in a Cell-Free Assay[a]

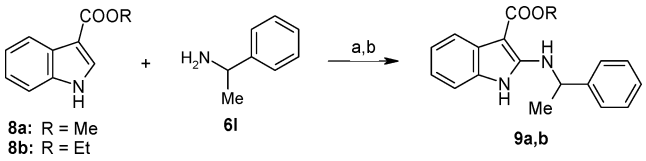
A	B	C	D	A	B	C	D
3a		0	n.d.	**3l**		94±3	72±4
3b		0	n.d.	**9a**		48±2	42±9
3c		0	n.d.	**14**		77±6	94±4
3d		0	n.d.	**9b**		53±4	51±2
3e		0	n.d.	**11**		85±1	62±4
3f		22±2	79±2	**3m**		71±5	65±2
7f		14±8	36±7	**3n**		90±3	97±2
3g		22±7	81±1	**3o**		19±14	24±10
3h		84±4	69±14	**4a**		0	n.d.
3i		15±8	61±1	**4b**		0	n.d.
3j		65±9	80±7	**4c**		0	n.d.
3k		76±5	77±4	**BWA4C**		93±2	86±4

[a] Values (percentage of inhibition of 5-LO activity by test compounds at 10 μM; BWA4C was used at 1 μM) are expressed as mean ± SE., $n = 3-4$; n.d. = not determined. A: compound; B: structure; C: % inhibition of 5-LO at 10 μM (intact PMNL); D: % inhibition of 5-LO at 10 μM (cell-free).

nordihydroguaretic acid, caffeic acid, flavonoids, or coumarins that act by reducing the active-site iron of 5-LO.[25,26] It was shown that 5-hydroxyindoles possess antioxidative properties[27−29] but on the other hand may also act as pro-oxidants, depending on the assay conditions.[27] Interestingly, compounds that are essentially devoid of any antioxidative features, such as **7f**, the 2-fold methylated analogue of **3f** as well as the deshydroxylated analogues of **3l** and **14**, namely **9b** or **9a**, respectively, still significantly inhibited 5-LO, although somewhat less potent as compared to the parental compounds (Table 1). Therefore, the antioxidative potential may in part contribute, but is not fully responsible for the 5-LO inhibitory effects of the 2-amino-5-hydroxyindoles.

To determine the exact potency of those compounds that showed significant inhibition of 5-LO activity at 10 μM, selected (potent) compounds (**3f**, **3h**, **3j**, **3k**, **3l**, **3m**, **3n**, **11**, and **9b**)

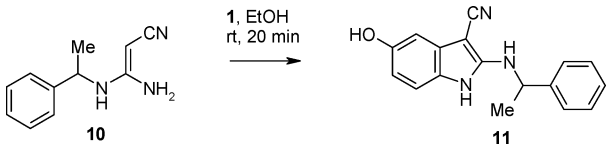
Scheme 2. Synthesis of (*RS*) Methyl and (*RS*) Ethyl 2-[(1-Phenylethyl)amino]-1*H*-indole-3-carboxylate (**9a**,**b**)[a]



8a: R = Me
8b: R = Et
6l
9a,b

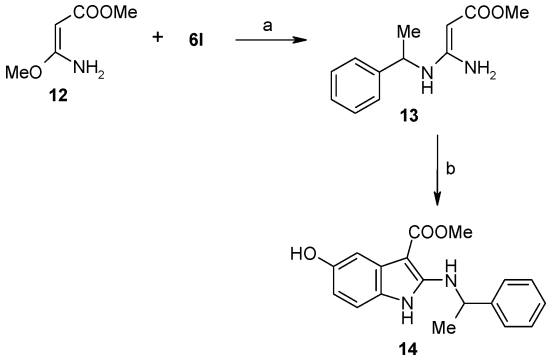
[a] Reagents and conditions: (a) NCS, *N*,*N*′-dimethylpiperazine, CH_2Cl_2, 0 °C, 2 h, (b) trichloroacetic acid, (*RS*)-phenylethylamine (**6l**), rt, 2 h.

Scheme 3. Synthesis of (*RS*) 5-Hydroxy-2-[(1-phenylethyl)Amino]-1*H*-indole-3-carbonitrile (**11**)



10
11

Scheme 4. Synthesis of (*RS*) Methyl 5-Hydroxy-2-[(1-phenylethyl)amino]-1*H*-indole-3-carboxylate (**14**)[a]



12
13
14

[a] Reagents and conditions: (a) MeOH, reflux, 48 h; (b) 1,4-benzoquinone (**1**), MeOH, rt, 20 min.

Table 2. Determination of IC_{50} Values of Selected Compounds

	5-lipoxygenase inhibition (IC_{50} values in μM)	
compound	intact PMNL	cell-free assay
3f	>20[a]	3.1
3h	5[a]/5.8[b]	2.3
3j	7.3[a]	2.3
3k	5.2[a]	5
3l	1.2[a]/4.6[b]	3
9b	9.7[a]/ 5.9[b]	9.8
3m	7.2[a]	7.1
3n	2.4[a]	0.3
11	2.8[a]/4.1[b]	10

Intact PMNL were stimulated with ionophore[a] or ionophore plus 20 μM AA[b].

were further investigated in concentration−response studies. First, the compounds were analyzed for inhibition of 5-LO product synthesis in intact PMNL, challenged with Ca^{2+}-ionophore A23187. All compounds concentration-dependently inhibited 5-LO product synthesis, and as shown in Table 2, the IC_{50} values were determined in the range of 1.2 to 9.7 μM. In agreement with previous reports,[23,30] the IC_{50} value for the reference drug BWA4C was 0.06 μM. As mentioned above, inhibition of Ca^{2+} ionophore A23187-induced cellular 5-LO product synthesis could be related also to reduced supply of endogenous AA. However, selected compounds out of the 4-aryl-piperazin-1-yl series (**3h**) and the phenylethyl-amino series (**3l**, **9b,** and **11**) still blocked 5-LO product synthesis efficiently, when PMNL were stimulated with Ca^{2+}-ionophore A23187 plus 20 μM exogenous AA, thus circumventing $cPLA_2$ activity (Table 2). Increasing the lipophilicity by introduction of *p*-methyl (**3k**)- or *m*-chloro (**3n**) moieties into the phenyl of **3j**, further enhanced the potency. Among all compounds, the phenylethyl-2-amino substituted indole-3-carboxylate (**3l**) was most efficient. Replacement of the ethyl 3-carboxylate of **3l** by a methyl ester in **14** did not markedly affect the efficacy. Also, exchanging the ethyl 3-carboxylate substituent of **3l** by a nitrile leading to **11** was almost not detrimental, implying no absolute requirement of a carboxylate moiety in position 3. Compounds with a free 3-carboxylic moiety were synthetically not accessible and thus not tested. Notably, most (potent) 5-LO inhibitors of different classes virtually do not possess a carboxylic moiety,[1,6] although for many NSAIDs and inhibitors of the 5-LO-activating protein (FLAP) a free carboxylic group is essential for high efficacy.

By conducting detailed inhibition studies of 5-LO activity in cell-free assays, the IC_{50} values were determined in the range of 0.3 to 10 μM (Table 2). The IC_{50} value for the reference drug BWA4C was determined at 0.15 μM. Compound **3n** (IC_{50} = 0.3 μM) is clearly the most potent representative, being about 8-fold more efficient in cell-free assays as compared to intact cells. Similarly, **3f** hardly reduced 5-LO activity in intact cells (IC_{50} > 20 μM, compare Table 1 and 2) but strongly suppressed enzymatic activity under cell-free conditions, and also **3j** was about 3-fold more potent for crude 5-LO. Together, regardless of these different efficacies between intact cells and cell-free assays, the IC_{50} values are in a close range and structure−activity relationships are obvious.

With respect to their molecular mode of action, 5-LO inhibitors can be categorized into redox-active compounds, iron-chelators, and nonredox-type inhibitors. Comparison of the 2-amino-5-hydroxyindoles with the diverse structures of known 5-LO inhibitors does not indicate any relations, and analysis of the overall structural features does not allow a defined classification into any of the established categories of 5-LO inhibitors. For example, no iron-chelating properties or substrate (AA)-competitive features of 2-amino-5-hydroxyindoles are immediately apparent from the chemical structures. As stated above, the reducing properties of the 5-hydroxyindole moiety may be one possible explanation for 5-LO inhibition. However, the O- and N-methylated derivative **7f** as well as the deshydroxy-analogues **9b** and **9a** of the potent parental compounds **3l** and **14**, respectively that essentially lack reducing features, still blocked the activity of the crude 5-LO enzyme in vitro, though some potency was lost. Apparently, antioxidant properties or the free 5-hydroxy moiety as potential pharmacophoric group is not an absolute prerequisite for efficacy, but nevertheless contributes to potent 5-LO inhibition. Besides the antioxidant functionality, the 5-hydroxy group may also increase the binding toward the 5-LO enzyme.

In summary, we designed and synthesized novel 2-amino-5-hydroxyindoles that block the biosynthesis of the bioactive LTs. The lead structures **3n** and **3l** represent potent 5-LO inhibitors with IC_{50} values (0.3 to 2.4 μM) being in the range of the potency of the most successfully developed 5-LO inhibitor zileuton (IC_{50} values 0.5 to 1 μM in intact cells[24,31]) that was available on the market in the US since 2000 until recently for the treatment of asthma.

Conclusions

Due to the frequently reported lack of benefit of 5-LO inhibitors in the clinic, the value of such drugs and the role of 5-LO products as key players in inflammatory and allergic

diseases have been doubted. However, experiments with 5-LO knock-out mice and successful therapy of humans with LT receptor antagonists (i.e. montelukast, pranlukast, and zafirlukast) clearly proof for the involvement of LTs in the respective pathophysiology,[2] giving rise to the pharmaceutical industry to launch inhibitor screening programs in order to identify and develop novel and potent 5-LO inhibitors. Here we have presented the design and synthesis of novel 2-amino-5-hydroxyindoles that proved to be potent inhibitors of 5-LO in crude 5-LO enzyme assays as well as in intact cells. Importantly, no structurally related compounds have been reported thus far as 5-LO inhibitors and no clear classification of the 2-amino-5-hydroxyindoles to any of the well recognized traditional categories of 5-LO inhibitors can be made. Unfortunately, the 3D structure of 5-LO has not been resolved yet, and neither the substrate-binding site nor any binding site of well-known 5-LO inhibitor could be determined thus far. At the moment, the precise molecular mode of action of the 2-amino-5-hydroxyindoles for 5-LO inhibition remains elusive. Nevertheless, there are obvious structure−activity relationships for the 2-amino-5-hydroxyindole derivatives. While the nature of the substituent in position 3 could be varied, neutral, preferably aromatic, or at least highly lipophilic substituents of the 2-amino group appear to be essential for bioactivity. Of interest, for both the 4-aryl-piperazin-1-yl- and the phenylethyl-amino derivatives, those containing chloro-substituted phenyl residues (**3h** and **3n**) were the most potent representatives, respectively. Collectively, on the basis of their potent 5-LO inhibitory properties, the 2-amino-5-hydroxyindoles may possess potential for the treatment of inflammatory and allergic disorders, select cardiovascular diseases, and various types of cancer. Future analysis using inflammatory animal models remain to allow a judgment of (a) possible benefit(s) of these compounds to treat LT-related diseases in humans.

Experimental Section

General Methods. Chromatography was carried out with 230−400 mesh silica gel (Merck, Darmstadt, Germany). The MPLC-apparatus consisted of the following segments: MPLC-pump, BÜCHI (type B688); fraction collector, LKB (type 2111 Multirac and SuperFracTM); columns: Büchi, 15 × 460, 26 × 460, 36 × 460, 49 × 460 mm. Melting points (mp): Büchi melting point apparatus, uncorrected. Mass spectra: Finnigan MAT TSQ 70 instrument. ^{1}H NMR spectra: Bruker AM 360 spectrometer at 360 MHz. Spectra were measured in d_6-DMSO using TMS as internal standard. Elemental analyses were performed by the Organic Chemistry Department of the Friedrich-Alexander University Erlangen-Nuernberg; Apparatus: Carlo Erba (model 1108) and Heraeus (CHN− Rapid). Infrared spectra were recorded on the FTIR spectrometers Perkin-Elmer (type1740) and Jasco (Type 410).

General Procedure for the Preparation of the New Ketene Aminals 2j−m. 2.0 g (12.6 mmol) of ethyl 3-amino-3-ethoxyacrylate (**5**) was dissolved in 20 mL ethanol. After addition of 12.6 mmol of amine **6j−m**, the resulting mixture was refluxed for 48 h. Evaporation of the solvent resulted in a syrupy residue, which was purified via flash chromatography. If necessary, crystallization of the compounds was performed in solvents.

General Procedure for the Preparation of the 2-Amino-5-hydroxyindoles 3a−m, 11, and 14. 1.30 g (12 mmol) of 1,4-benzoquinone (**1**) was added slowly to a solution of ketene aminal **2**, **10**, respectively (10 mmol), in 40 mL of ethanol. In case of ketene aminal **13** we used methanol as solvent. After the reaction was stirred at room temperature for 20 min, the solvent was removed under reduced pressure to give a black residue, which was purified by MPLC or flash chromatography on silica gel. If necessary, crystallization of the compounds was performed in solvents.

General Procedure for the Preparation of the 2-Amino-5-hydroxyindole-3-carboxylates 3n,o. 1590 mg (10 mmol) of ethyl 3-amino-3-ethoxyacrylate (**5**) was dissolved in 40 mL of ethanol. After addition of 10 mmol of the amine **6n**, **6o**, respectively, the resulting mixture was stirred at room temperature for 30 min. 1.30 g (12 mmol) of 1,4-benzoquinone (**1**) was added over a period of 5 min. After additional 20 min of stirring, the solvent was evaporated, resulting in a dark tarry resin, which was purified by means of MPLC on silica gel and crystallized in the media.

Ethyl 5-Methoxy-1-methyl-2-(4-phenylpiperazin-1-yl)-1*H*-indole-3-carboxylate (7f). A mixture of 500 mg (1.37 mmol) of **3f**, ∼3 mmol of NaH, and 5 mL of DMF was stirred at room temperature, until the hydrogen development stopped completely. After addition of 360 mg (2.85 mmol) dimethyl sulfate, the reaction mixture was refluxed for 1 h. 20 mL of a saturated solution of ammonia in ethanol was added, and after the reaction was stirred at room temperature for additional 20 min, the solvent was evaporated under reduced pressure. The residue was purified via MPLC.

Procedure for the Preparation of 9a,b. The appropriate indole-3-carboxylate **8a**,**b** (1.19 mmol) was dissolved in CH_2Cl_2 (5 mL). After cooling to 0 °C, 75 mg of *N*,*N*′-dimethylpiperazine (0.66 mmol) and 175 mg of *N*-chlorosuccinimide (1.31 mmol) were added, and the reaction mixture was allowed to stand at 0 °C for 2 h, whereupon a solution of 50 mg of trichloroacetic acid (0.3 mmol) and 284 mg of (*RS*)-1-phenylethylamine (2.34 mmol, **6l**) in CH_2Cl_2 (5 mL) was added. After standing for 2 h at room temperature, the reaction mixture was washed with 10% aqueous $NaHCO_3$ solution, with 1 M aqueous hydrochloric acid, and with water. The resulting solution was dried over Na_2SO_4, filtered, and evaporated to give a residue, which was purified by flash chromatography on silica gel (cyclohexane/ethyl acetate 7:3).

(*E*/*Z*) (*RS*) Methyl 3-Amino-3-[(1-phenylethyl)amino]acrylate (13). 1.65 g (12.6 mmol) of methyl 3-amino-3-methoxyacrylate (**12**) was dissolved in 20 mL of methanol. After addition of 1.53 g (12.6 mmol) of (*RS*)-1-phenylethylamine (**6l**), the resulting mixture was heated at reflux for 48 h. Evaporation of the solvent resulted in a syrupy residue, which was purified via flash chromatography.

Cells. Human PMNL were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation at 4000*g* for 20 min at 20 °C for preparation of leukocyte concentrates. PMNL were promptly isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously.[32] PMNL (7.5×10^6 cells/mL; purity >96−97%) were finally resuspended in phosphate-buffered saline pH 7.4 (PBS) plus 1 mg/mL glucose.

Determination of 5-Lipoxygenase Product Formation in Intact Cells. For assays of intact cells, 10^7 freshly isolated PMNL were finally resuspended in 1 mL of PBS plus 1 mg/mL glucose and 1 mM $CaCl_2$. After preincubation with the indicated compounds at 37 °C, 5-LO product formation was started by addition of 2.5 μM ionophore A23187 together with or without 20 μM AA, as indicated. After 10 min at 37 °C, the reaction was stopped with 1 mL of methanol, and 30 μL of 1 N HCl, 200 ng prostaglandin B1, and 500 μL of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described.[33] 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells which includes LTB_4 and its all-trans isomers, 5(*S*),12(*S*)-dihydroxy-6,-10-*trans*-8,14-cis-eicosatetraenoic acid (5(*S*),12(*S*)-DiHETE), and 5(*S*)-hydro(pero)xy-6-*trans*-8,11,14-cis-eicosatetraenoic acid (5-H(p)ETE). Cysteinyl LTs (LTC_4, D_4, and E_4) were not detected and oxidation products of LTB_4 were not determined. IC_{50} values are expressed as mean ± SE., $n = 3-4$.

Expression and Determination of Enzymatic Activity of 5-LO from *E. coli*. Expression of 5-LO was performed in *E. coli* JM 109 cells, transfected with pT3−5LO, and purification of 5-LO was performed as described previously.[34] In brief, cells were lysed by incubation in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/mL), 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysozyme (500 μg/mL), homogenized by sonication (3 × 15 s), and centrifuged at 19000*g* for 15 min.

Proteins including 5-LO were precipitated with 50% saturated ammonium sulfate during stirring on ice for 60 min. The precipitate was collected by centrifugation at 16000*g* for 25 min and the pellet was resuspended in 20 mL of PBS containing 1 mM EDTA and 1 mM PMSF. After centrifugation at 100000*g* for 70 min at 4 °C, the pellet was removed and the supernatant (S100) was used for 5-LO activity assays. ATP (1 mM) and the test compounds or vehicle (DMSO, <0.3% vol/vol) were added to the S100, and after 5−10 min at 4 °C, samples were prewarmed for 30 s at 37 °C. To start 5-LO product formation, 2 mM $CaCl_2$ and 20 μM AA at the indicated concentrations were added. The reaction was stopped after 10 min at 37 °C by addition of 1 mL of ice-cold methanol, and the formed metabolites were analyzed by HPLC as described for intact cells.

Statistics. All values are reported as mean ± SE. Statistical comparisons were determined using the Student's *t*-test for correlated samples (inhibitor versus control), and significance was accepted at $p < 0.05$.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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