Synthesis and Pharmacological Identification of Neutral Histamine H₁-Receptor Antagonists

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In the present study we searched for neutral antagonists for the human histamine H₁-receptor (H₁R) by screening newly synthesized ligands that are structurally related to H₁R agonists for their affinity using radioligand displacement studies and by assessing their functional activity via performing a NF-κB driven reporter-gene assay that allows for the detection of both agonistic and inverse agonistic responses. Starting from the endogenous agonist for the H₁R, histamine, we synthesized and tested various analogues and ultimately identified several compounds with partial inverse agonistic properties and two neutral H₁-receptor antagonists, namely 2-[2-(4,4-diphenylbutyl)-1*H*-imidazol-4-yl]ethylamine (histabudifen, **18d**) (pK_i = 5.8, α = 0.02) and 2-[2-(5,5-diphenylpentyl)-1*H*-imidazol-4-yl]ethylamine (histapendifen, **18e**) (pK_i = 5.9, α = −0.09).

Introduction

Four human G-protein coupled histamine-receptor subtypes (H₁₋₄) are currently recognized to mediate the various actions of the monoamine histamine.[1−10] Specific activation or blockade of these receptor subtypes has led to a tremendous increase in the knowledge of the roles of histamine in both physiology and pathology. Stimulation of the histamine H₁-receptor (H₁R), which is found throughout the body, causes contraction of smooth muscles in, e.g. the airways and intestine.[11] Moreover, histamine plays a role in allergic conditions that have often been treated successfully with H₁R antagonists.[12]

It is increasingly recognized that G protein-coupled receptors (GPCRs) activate their associated signal transduction pathways not only upon agonist activation but also in the absence of agonists, resulting in constitutive receptor activity. Concomitant with the appreciation of constitutive receptor activity, the phenomenon of inverse agonism has found general acceptance as well.[13−18] Many ligands that were previously thought to act as antagonists actually inhibit the constitutive receptor signaling, indicating their inverse agonistic behavior. A third class of ligands, the neutral antagonists, although interacting with the receptor, fail to modulate receptor activity. Inverse agonists display negative intrinsic activity (α) between −1 and 0 and are relatively common, whereas neutral antagonists, ligands without intrinsic activity (α = 0), are quite rare but known for several GPCRs, including histamine H₂- and H₃-receptors.[19−21] The actual therapeutic importance of constitutive GPCR activity has not been clarified yet,

but various human diseases have been ascribed to constitutive receptor activity induced by mutations in genes encoding GPCRs.[22−26] It is obvious that for these genetic disorders inverse agonists are essential for silencing the mutant GPCRs, as neutral antagonists would be of no use.

We have recently shown the constitutive activity of the wild-type human H₁R and we have identified well-known therapeutics such as cetirizine (Zyrtec), epinastine (Flurinol), loratidine (Claritin) as inverse agonists, which led us to reconsider their mechanism of action.[27,28] No data on the physiological relevance of constitutive H₁R activity are currently available. Constitutive receptor activity can be achieved by high receptor expression-levels, but also by other means, i.e., removal of sodium ions[29] and coexpression of their cognate Gα proteins[30−33] or specific G$\beta\gamma$ subunit combinations.[28,34] Moreover, receptor mutation can result in constitutively active (CAM) or inactive (CIM) mutant receptors.[35−38] Therefore, constitutive H₁R activity may contribute to pathophysiological conditions where either H₁R up-regulation occurs, such as in patients with allergic rhinitis,[39] or Gα_q protein levels are found to be elevated, such as in the guinea pig nasal mucosa in a model of nasal hyperresponsiveness.[40] Although an inverse H₁R agonist would suppress any apparent constitutive H₁R activity, long-term exposure of cells expressing constitutively active GPCRs to inverse agonists may result in receptor up-regulation.[19,41−44] This may have clinical significance, since an increased sensitivity, development of tolerance, and recurrence have been attributed to long-term treatment with for instance inverse H₂R agonists.[45,46] However, so far H₁R up-regulation or sensitization has not been reported upon prolonged inverse agonist treatment.

Clearly, the development of a neutral H₁R antagonist would give a valuable pharmacological tool to study the
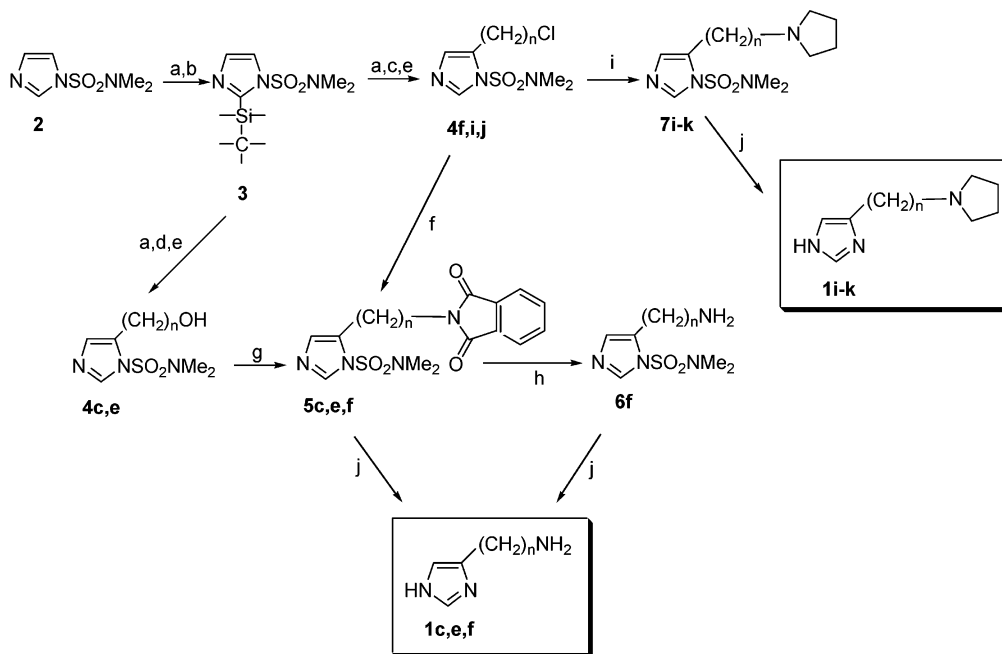
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Scheme 1. Synthesis of Compounds **1c**,**e**,**f**,**i**−**k**

Reagents: (a) *n*-BuLi, THF, −70 °C; (b) *tert*-butyldimethylsilyl chloride; (c) 1-chloro-ω-iodoalkane; (d) 2-(ω-bromoalkyloxy)tetrahydropyran; (e) HCl, rt; (f) isoindole-1,3-dione, Na_2CO_3, DMF, 90 °C; (g) triphenylphosphine, isoindole-1,3-dione, diisopropylazodicarboxylate, THF; (h) hydrazine monohydrate, EtOH, reflux; (i) pyrrolidine, rt; (j) 30% HBr, reflux.

potential physiological role of constitutive H₁R activity which, to date, has been hampered by the lack of neutral H₁R antagonists. In the present study we report the synthesis of a variety of histamine homologues, their binding affinities, and their intrinsic H₁R activity using a reporter-gene assay.

Chemistry

The target aminoalkylimidazoles **1c**,**e**−**k** were prepared by lithiation of a suitable 1,2-diprotected imidazole **3**[47] and subsequent treatment with 1-chloro-ω-iodoalkanes or 2-(ω-bromoalkyloxy)tetrahydropyrans to give compounds **4c**,**e** and **4f**,**i**,**j** (Scheme 1). The ω-hydroxy group of **4c**,**e** was converted into a phthaloyl group via a Mitsunobu reaction[48] followed by hydrolysis of the phthalimide group to give the amines **1c**,**e**. Alternatively, the ω-chloro group of **4f**,**i**,**j** was converted either into a pyrrolidine via reaction with pyrrolidine to give compounds **7i**−**k** or via a Gabriel synthesis[49] into compound **6f**. Deprotection yielded compounds **1f**,**i**−**k**.

For aminoalkylimidazoles **1g**,**h** the synthetic pathway shown in Scheme 2 was adopted. The key step in this synthesis is the conversion of aldehyde **10** into 4-tosyloxazoline **11** using TosMIC followed by treatment with ammonia in methanol under pressure[50] to give amide **12**. Treatment of amide **12** with [*I*,*I*-bis(trifluoroacetoxy)iodo]benzene (PIFA)[51] gave compound **1g**, whereas reduction with LiAlH₄ afforded compound **1h**.

The aminoalkylpyridines **13a**−**g** were prepared according to Scheme 3. Lithiation of methylpyridines **14a**,**d**,**f**[52] and coupling with 1,8-dibromooctane or 2-(ω-bromoalkyloxy)tetrahydropyrans yielded compounds **15a**−**g**. A Gabriel synthesis or a Mitsunobu reaction gave compounds **16a**−**g**. The free amino group was obtained by hydrazinolysis resulting in compounds **13a**−**g**.
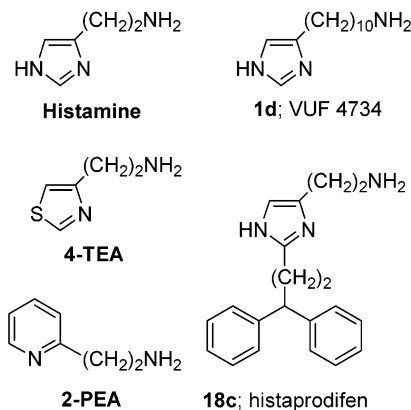

Figure 1. Structures of 2-[1*H*-imidazol-4-yl]ethylamine (histamine), 2-(thiazol-4-yl)ethanamine (4-TEA), 2-(pyridine-2-yl)-ethyl-1-amine (2-PEA), VUF 4734 (**1**), and histaprodifen.

Pharmacological Results and Discussion

The compound 10-(1*H*-imidazol-4-yl)decylamine (**1d**; VUF 4734, Figure 1) has been reported to exhibit the highest affinity for the guinea pig H₁R among a series of higher histamine analogues[53] and subsequently proved to be a partial inverse agonist for the human H₁R (see Table 1). Therefore, we have prepared a series of ω-(1*H*-imidazol-4-yl)alkylamines (**1c**,**e**−**h**) and investigated the effect of the length of the alkyl spacer of the histamine analogues on the human H₁R binding affinity and intrinsic activity. As shown in Table 1, the H₁R binding affinity for the higher homologues of histamine (**1a**−**h**) increases with the elongation of the side chain, whereas for the potency a maximum is reached for compound **1e**, bearing an 11-carbon-atom side chain. The intrinsic activity of the compounds decreases as the length of the side chain increases, meaning that the compounds are turning from partial inverse agonists to full inverse agonists.

Scheme 2. Synthesis of Compounds **1g**,**h**
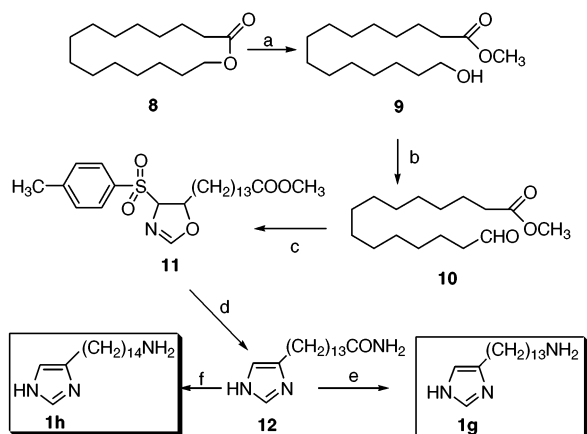
Reagents: (a) MeOH, *p*-toluenesulfonic acid monohydrate, reflux; (b) Swern oxidation; (c) TosMIC, NaCN, EtOH abs; (d) NH₃/MeOH, 90–110 °C, 10 atm; (e) PIFA, CH₃CN/H₂O (1/1); (f) LiAlH₄, THF.

Scheme 3. Synthesis of Compounds **13a–g**
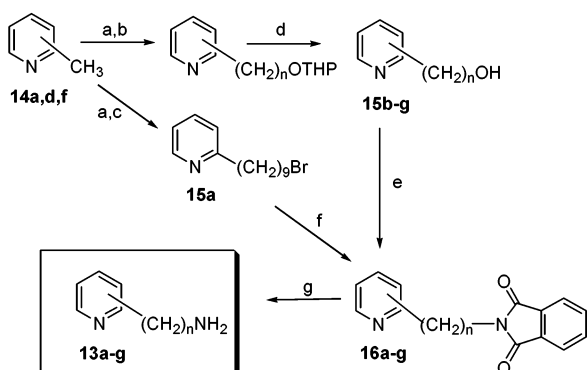
Reagents: (a) *n*-BuLi (ortho-isomers) *or* LDA (meta- and para-isomers), THF, −50 °C; (b) 2-(ω-bromoalkyloxy)tetrahydropyran, −50 °C; (c) 1,8-dibromooctane, −50 °C; (d) HCl, rt; (e) triphenylphosphine, isoindole-1,3-dione, diisopropylazodicarboxylate, THF; (f) isoindole-1,3-dione, Na₂CO₃, DMF, 90 °C; (g) hydrazine monohydrate, EtOH, reflux.

In an attempt to obtain compounds with a higher H$_1$R affinity, the amino group of some of the higher homologues of histamine was exchanged for a pyrrolidine moiety (**1i**–**k**), by analogy with the classical H$_1$R antagonists for which it is known that the substitution of the primary amino function by a pyrrolidine ring results in an increase in H$_1$R affinity of an order of magnitude or more.[12] This exchange results in an increased binding affinity of one log unit only in the case of compound **1i**, whereas in the case of longer side chains (**1j** and **1k**) the binding affinities are unchanged or reduced. Moreover, there is no significant difference among the binding affinities of the three compounds bearing a pyrrolidine ring at the end of the side chain (**1i**–**k**). No definite relation between the length of the side chain and the potency can be observed. Opposite to observations made for the series of compounds bearing a primary amino function (**1a**–**h**), in this series the intrinsic activity increases as the length of the side chain increases, meaning that the compounds are turning from full inverse agonists to partial inverse agonists. However, no neutral antagonist were identified in this series.
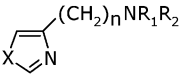
H$_1$R specific agonists have been developed by replacement of the imidazole ring for a pyridine ring, which

does not influence the capability of the resulting compound to bind to the H$_1$R, provided that the aminoethyl chain is placed in the 2-position of the pyridine ring,[12,54] as in 2-(pyridine-2-yl)ethanamine (2-PEA, Figure 1). On this basis we decided to investigate the importance of the imidazole ring in the binding affinity and intrinsic H$_1$R activity of the higher homologues of histamine. Various aminoalkylpyridines have been synthesized in which a long side chain has been attached in the 2- (**13a**–**c**), 3- (**13d**,**e**), and 4-position (**13f**,**g**) of the pyridine ring (Table 2). For the 2-isomers (**13a**–**c**), an increase in the H$_1$R binding affinity with the elongation of the side chain can be observed, similarly to what happens for the aminoalkylimidazoles with a primary amino function (compounds **1c**, **1d**, and **1f**), and the pK_i values are comparable to those of the aminoalkylimidazoles. Although **13b** and especially **13c** appear to be lacking intrinsic H$_1$R activity, these compounds exhibited non-H$_1$R-mediated effects at high concentrations that interfered with an accurate estimation of their intrinsic H$_1$R activity in our cell based assay (data not shown). Interestingly, the 3- (**13d**,**e**) and 4-isomers (**13f**,**g**) are still active on the receptor, although no significant differences are observed for the binding affinities with elongation of the side chain or shifting the side chain from the meta to the para position. All the aminoalkylpyridines are (partial) inverse H$_1$R agonists, with the exception of 2-PEA (Table 2), but there is no apparent relation between the length or the position of the side chain and the efficacy of the compound.

Substitution of the 2-position of histamine has proven to be a successful strategy to obtain H$_1$R-selective agonists,[55] such as the full agonist histaprodifen (see Figure 1 and Table 3).[56] As shown in Table 3 the monosubstituted compounds **17a** and **17b** are agonists with comparable binding affinities and potencies. Interestingly, compound **17a** displays an intrinsic activity (α) that is greater than that of histamine itself (Table 3, Figure 3C). The disubstituted compound (**17c**) is a partial agonist, displaying a reduced binding affinity and potency, suggesting that the accommodation in the ligand-binding pocket of the H$_1$R[57] is hindered due to steric effects of the chlorine atom in the para-position.
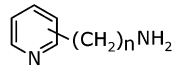
Replacement of the imidazole ring of histamine for a thiazole moiety leads to 2-(thiazol-4-yl)ethanamine (4-TEA,[12] Figure 1), with a very low affinity for the receptor (Table 1). Subsequent substitution of the 2-position of 4-TEA with a (substituted) phenyl ring or a (substituted) phenylmethyl residue was achieved by compounds **19a**–**d**, all displaying a similar (and low) binding affinity for the receptor.

Recently, Elz et al. synthesized a series of compounds constituting a new class of highly active H$_1$R agonists that combine a histamine moiety linked at the 2-position with an ω,ω-diphenylalkyl substituent (**18a**–**e**; histaprodifens, Figure 1) and examined them for H$_1$-histaminergic properties on the guinea pig ileum preparation.[58] In this study the affinities, potencies, and efficacies of these compounds, and other analogues,[56,58,59] on the cloned human H$_1$R were determined. The diphenylmethyl substituent present in the histaprodifen series is a common feature of various clinically used H$_1$R antagonists,[12] like cetirizine, and is believed to confer

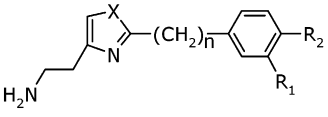**Table 1.** Chemical Structures and Pharmacological Properties of the Aminoalkylimidazoles **1a**−**k** for the Human H$_1$ Receptor

compd	name or code	X	n	R$_1$	R$_2$	pK_i^a	pIC$_{50}^a$	α^a
	histamine	NH	2	H	H	4.2 ± 0.1	6.8 ± 0.1^b	1.00
	4-TEA	S	2	H	H	<4	−^{c}	−^{d}
1a	VUF 4732	NH	6	H	H	3.3 ± 0.4	−^{e}	−^{e}
1b	VUF 4733	NH	8	H	H	4.4 ± 0.2	5.5 ± 0.8	−0.54 ± 0.13
1c	VUF 5695	NH	9	H	H	4.7 ± 0.2	5.7 ± 0.2	−0.72 ± 0.03
1d	VUF 4734	NH	10	H	H	5.3 ± 0.1	6.3 ± 0.2	−0.54 ± 0.06
1e	VUF 5696	NH	11	H	H	5.6 ± 0.1	6.6 ± 0.1	−0.61 ± 0.04
1f	VUF 5671	NH	12	H	H	6.0 ± 0.1	6.0 ± 0.2	−0.62 ± 0.09
1 g	VUF 5697	NH	13	H	H	6.4 ± 0.1	5.9 ± 0.2	−0.84 ± 0.06
1h	VUF 5673	NH	14	H	H	6.6 ± 0.1	5.7 ± 0.1	−0.90 ± 0.11
1i	VUF 5669	NH	8	−(CH$_2$)$_4$−		5.4 ± 0.1	6.2 ± 0.1	−0.99 ± 0.05
1j	VUF 5670	NH	10	−(CH$_2$)$_4$−		5.4 ± 0.2	6.4 ± 0.3	−0.61 ± 0.08
1k	VUF 5672	NH	12	−(CH$_2$)$_4$−		5.7 ± 0.1	5.4 ± 0.1	−0.46 ± 0.33
	mepyramine					8.7 ± 0.1	7.9 ± 0.1	−1.00
	tripelennamine					8.0 ± 0.1	7.4 ± 0.1	−0.84 ± 0.02

^{a} The values are expressed as means ± SEM of separate experiments, each performed in triplicate. $N > 3$. ^{b} pEC$_{50}$ value. ^{c} Could not be estimated. ^{d} Tested up to 100 μM; no effects up to 10 μM. ^{e} Low affinity for the receptor; not tested for the efficacy.

Table 2. Chemical Structures and Pharmacological Properties of the Aminoalkylpyridines **13a**−**g** for the Human H$_1$ Receptor

compd	name or code	isomer	n	pK_i^a	pIC$_{50}^a$	α^a
	PEA	ortho	2	3.8 ± 0.1	5.9 ± 0.1^b	−1.11 ± 0.21
13a	VUF 5680	ortho	9	4.5 ± 0.1	5.1 ± 0.2	−0.92 ± 0.08
13b	VUF 5674	ortho	10	4.9 ± 0.1	−^{c}	−^{d}
13c	VUF 5677	ortho	12	6.0 ± 0.1	−^{c}	−^{d}
13d	VUF 5675	meta	10	5.1 ± 0.1	5.6 ± 0.1	−0.76 ± 0.01
13e	VUF 5678	meta	12	5.4 ± 0.1	5.9 ± 0.1	−0.78 ± 0.13
13f	VUF 5676	para	10	5.3 ± 0.1	5.6 ± 0.2	−1.00 ± 0.06^e
13g	VUF 5679	para	12	5.4 ± 0.1	6.4 ± 0.2	−0.67 ± 0.05

^{a} The values are expressed as means ± SEM of separate experiments, each performed in triplicate. $N > 3$. ^{b} pEC$_{50}$ value. ^{c} Could not be estimated. ^{d} Tested up to 10 μM; no effects up to 1 μM. ^{e} Estimated (see Figure 3B).

Table 3. Chemical Structures and Pharmacological Properties of Compounds **17a**−**e** and **19a**−**d** for the Human H$_1$ Receptor

compd	name or code	n	X	R$_1$	R$_2$	pK_i^a	pEC$_{50}^a$	α^a
17a	VUF 5963	0	NH	Cl	H	5.2 ± 0.1	5.7 ± 0.1	1.28 ± 0.06
17b		0	NH	CF$_3$	H	5.4 ± 0.1	6.0 ± 0.1	0.91 ± 0.08
17c	VUF 8811	0	NH	Cl	Cl	4.7 ± 0.1	4.3 ± 0.1	0.50 ± 0.07
17d	FUB 113	4	NH	H	H	<4	−^{b}	−^{b}
17e	FUB 114	5	NH	H	H	<4	−^{b}	−^{b}
19a		0	S	H	H	4.5 ± 0.1	−^{c}	−^{d}
19b		0	S	CF$_3$	H	4.9 ± 0.1	−^{c}	−^{d}
19c		1	S	H	H	4.2 ± 0.2	−^{c}	−^{d}
19d		1	S	Cl	H	4.5 ± 0.1	−^{c}	−^{d}

^{a} The values are expressed as means ± SEM of separate experiments, each performed in triplicate. $N > 3$. ^{b} Low affinity for the receptor; not tested for the efficacy. ^{c} Could not be estimated. ^{d} Tested up to 100 μM; no effects up to 10 μM.

high receptor affinity on these antihistamines. We investigated the influence of this substituent by testing several substituted histaprodifens and substituted thiazoles.[60,61]

Compounds **18a**−**i** have interesting structure−activity relationships, as shown in Table 4. The elongation of the spacer results in an increased H$_1$R binding affinity (compounds **18b**−**e**) together with a reduction of the intrinsic activity from full agonism (**18c**) to neutral antagonism (**18d** and **18e**; see also Figure 4).

Considering the binding data, the replacement of a phenyl ring with a cyclohexyl ring (**18f**) leads to a reduced affinity for the receptor, and removal of one phenyl ring (**17d,e**) led to a dramatic decrease in binding affinity. However, substitution of one of the phenyl rings in the para-position either with an electron-withdrawing substituent (**18g**) or an electron-releasing substituent (**18h**) does not affect the affinity, suggesting that the electron density on that phenyl ring as well as substitution on the para position are not important for the interaction with the receptor. This striking observa-
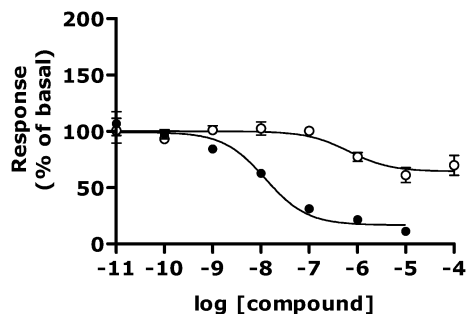
Figure 2. The effects of the full inverse H_1 agonist mepyramine (●) and the partial inverse H_1 agonist VUF 4734 (○) on the basal signaling of the H_1R, as measured by the bioluminescent reporter gene assay (see also Table 1). Data are normalized to the basal signaling observed in the assay (set to 100%).

tion is in contrast with the well-known structure–activity relationships for the classical antihistamines[12,62] and with data for agonists.[63] Those studies indicated in fact that para-substitution of the "cis" ring of classical H_1R antagonists with a small lipophilic group (i.e. CH_3, Cl) is favorable and confers the compounds an increased affinity on the receptor. The aromatic character of this ring seems to be indispensable for H_1R activity. In contrast, the second aromatic "trans" ring can be replaced by nonaromatic lipophilic groups (e.g. cyclohexyl) without drastic effects on H_1R-blocking activity.[62] Unlike the known effects for classical H_1R antagonists, the replacement of the primary amino function with a pyrrolidine ring in compound **18i** does not influence its binding affinity, similarly to what was observed with the series of aminoalkylimidazoles (compounds **1j** and **1k**).

Previously, we have shown the human histamine H_1-receptor to be constitutively active both by measuring the inositolphosphate levels in transiently transfected COS-7 cells and by measuring the luciferase activity in a reporter-gene assay we set up in which a firefly luciferase reporter-gene is under the transcriptional control of five NF-κB enhancer elements. However, in general, the physiological relevance of inverse agonists needs to be verified in a more physiological environment before the biological effect of antagonists can be primarily ascribed to negative intrinsic activity.[14,15] Therefore, we investigated the effects of (partial) inverse agonists and the identified neutral antagonist for the human histamine H_1-receptors endogenously expressed by HeLa cells.

Long-term exposure of cells expressing constitutively active GPCRs to inverse agonists may result in receptor up-regulation.[19,41–44] Also, sensitization of receptors by inverse agonist treatment has been shown in cell lines stably expressing receptors at relatively low density[64] as well as in cell lines in which receptor expression is inducible.[65] Although inverse agonist induced receptor up-regulation may have clinical significance,[45,46] no data on the physiological relevance of constitutive H_1R activity are currently available, in part due to the absence of available neutral H_1R antagonists. So far a receptor up-regulation or sensitization has not been reported for the H_1R upon prolonged inverse agonist treatment. We assessed the long-term effects of prolonged inverse agonist treatment of HeLa cells endogenously expressing the H_1R at low expression levels ($B_{max} = 55$ fmol/
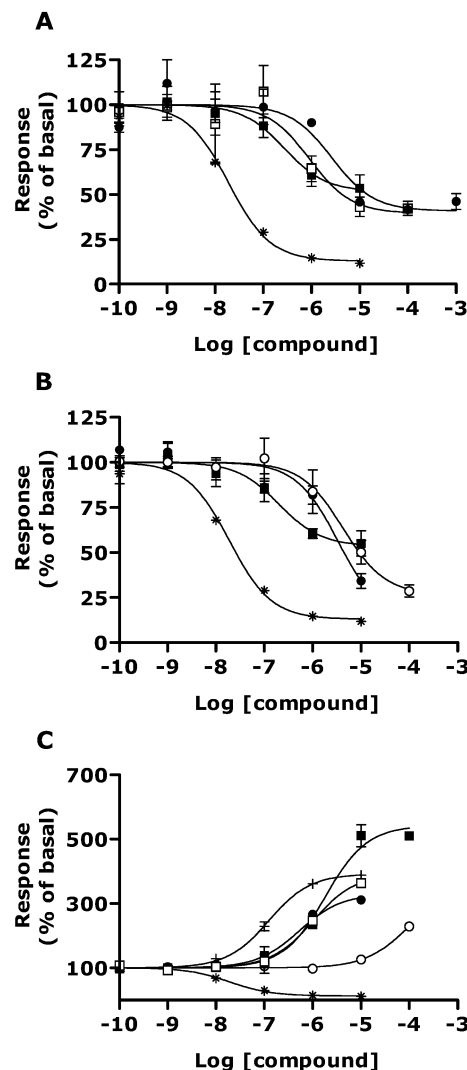

Figure 3. The effects of several selected compounds on the basal signaling of the H_1R, as measured by the bioluminescent reporter gene assay. (A) Dose–response curves of the partial inverse H_1 agonists VUF 5695 (●), VUF 5696 (■), and VUF 5673 (□) (see also Table 1). (B) Dose–response curves of the inverse H_1 agonists VUF 5675 (○), VUF 5676 (●), and VUF 5679 (■) (see also Table 2). (C) Dose–response curves of the H_1R agonists histamine (+), histaprodifen (●), 2-(3-trifluoromethylphenyl)histamine (□), and VUF 5963 (■) (see also Table 3 and Table 4). Representative dose–response curves of the full inverse H_1 agonist mepyramine (∗)[27,28] are shown (A, B, and C). Data are normalized to the basal signaling observed in the assay (set to 100%).

mg of protein; Figure 5A) on the H_1R expression levels and H_1R signaling properties. The procedure of measuring the inverse-agonist-induced up-regulation of H_1Rs is greatly dependent on the dissociation rates of the inverse agonists, as the preparation needs to be free of the inverse agonist used to pretreat the cells, to determine either the expression level of the H_1R or its signaling properties. Whereas tripelennamine is one of the few antihistamines that dissociates rather quickly from the receptor and allows for the measurement of H_1R up-regulation and the increase in E_{max} of histamine, many other antihistamines, such as mepyramine and cetirizine, dissociate more slowly from the H_1R.[66] As seen in Figure 5, a 24 h treatment of HeLa cells with the inverse H_1R agonist tripelennamine[28] results in a 2-fold increase of the total H_1R-expression level (Figure

Table 4. Chemical Structures and Pharmacological Properties of Compounds **18a**−**i** and **20a**,**b** for the Human H₁ Receptor

compd	name or code	n	R_1	R_2	R_3	$pK_i{}^a$	$pIC_{50}{}^a$	α^a
	histamine		H	H		4.2 ± 0.1	6.8 ± 0.1^b	1.00
18a		0	H	H	phenyl	<4	$-^c$	$-^c$
18b		1	H	H	phenyl	5.1 ± 0.1	$-^d$	$-^e$
18c	histaprodifen	2	H	H	phenyl	5.7 ± 0.1	6.4 ± 0.1^b	0.69 ± 0.06
18d	histabudifen	3	H	H	phenyl	5.8 ± 0.1	$-^d$	0.02 ± 0.03
18e	histapendifen	4	H	H	phenyl	5.9 ± 0.1	$-^d$	-0.09 ± 0.18
18f		2	H	H	cyclohexyl	5.0 ± 0.1	$-^d$	$-^f$
18g		2	H	H	p-Br-phenyl	5.6 ± 0.1	$-^d$	$-^f$
18h		2	H	H	p-CH₃-phenyl	5.5 ± 0.1	$-^d$	$-^f$
18i		2	\(-(CH_2)_4-\)		phenyl	5.6 ± 0.1	$-^d$	$-^e$

ᵃ The values are expressed as means ± SEM of separate experiments, each performed in triplicate. $N > 3$. *ᵇ* pEC₅₀ value. *ᶜ* Low affinity for the receptor; not tested for the efficacy. *ᵈ* Could not be estimated. *ᵉ* Tested up to 100 μM; no effects up to 10 μM. *ᶠ* Tested up to 10 μM; no effects up to 1 μM.
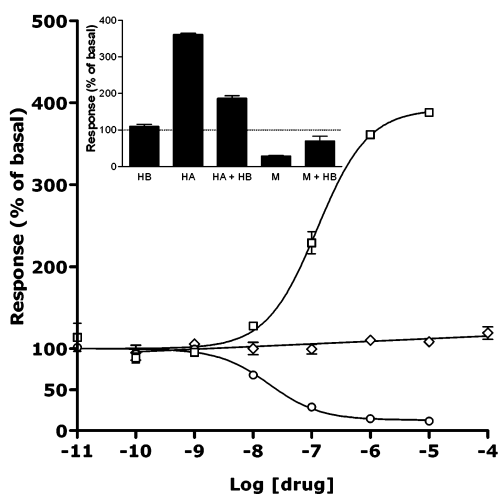

Figure 4. Histabudifen as a neutral H₁R antagonist. The effects of the endogenous agonist histamine (□), the full inverse H₁ agonist mepyramine (○), and histabudifen (◇) on the modulation of basal H₁R signaling, as measured by the bioluminescent reporter gene assay (see also Table 4). Data are normalized to the basal signaling observed in the assay (set to 100%). (Inset) Effects of histabudifen (10 μM, HB), histamine (1 μM, HA), and mepyramine (0.1 μM, M) on H₁R-mediated signaling, and the inhibition of histamine- and mepyramine-induced H₁R-mediated responses by histabudifen.

5A) that is concomitant with a dose-dependent increase in the E_{max}, but not in the EC₅₀ value of histamine-induced inositolphosphates formation (Figure 5B,C). Although similar effects were observed when mepyramine was used to preincubate the cells, the EC₅₀ of histamine after such pretreatment of the cells was rightward shifted (data not shown), most likely due to the kinetics of mepyramine binding. The maximum enhancement of H₁R signaling observed upon inverse H₁-agonist treatment is a 2-fold increase. These data are clearly consistent with the notion of constitutive H₁R activity and suggests that the inverse H₁R agonist tripelennamine may act to stabilize the H₁R in the cell membrane. In contrast to the effects observed with pretreatment of the cells with the inverse agonist tripelennamine, pretreatment of the cells with 0.63 mM histamine (10 times its K_i value[28]) completely prevented histamine-stimulated inositolphosphates formation (Figure 5C).

Pretreatment of HeLa cells with 100 μM of the partial inverse H₁R agonist VUF 4734 (**1d**), which corresponds to 20 times its K_i value, did not result in a significant increase of the E_{max} of histamine. The E_{max} of histamine after pretreatment with VUF 4734 was 146 ± 26% of that of control cells. Pretreatment of HeLa cells with 16 μM of the identified neutral H₁-antagonist histabudifen (**18d**), corresponding to 10 times its K_i value, also did not result in a significant increase in the E_{max} of histamine compared to control cells. VUF 4734 and histabudifen could not be tested at higher concentrations due to non-H₁R-mediated effects in our cell-based assay.

Conclusions

In the present study we reported the synthesis of a variety of histamine homologues and testing of their binding affinities and intrinsic H₁R activities. A NF-κB driven reporter-gene assay allowing testing of H₁R ligands for a dynamic range of intrinsic activities has been used.[28] Moreover, we show an up-regulation of endogenously expressed H₁R and an increased effectiveness of histamine to induce the formation of inositol phosphates upon inverse H₁R agonist treatment, underscoring the potential benefit of neutral antagonists.

Novel ligands for the histamine H₁-receptor were synthesized and tested for their affinity and intrinsic activity for the human histamine H₁-receptor, and several known H₁-receptor ligands were screened in the same system. Most of the compounds presented in this research are (partial) inverse agonists. Especially aminoalkylimidazoles **1a**−**k** and aminoalkylpyridines **13a**,**d**−**g** are structurally diverse from classical histamine H₁-antagonists, as they possess a structure similar to histamine and H₁-agonists; therefore, they constitute a novel class of inverse agonist ligands for this receptor. The substitution of the primary amino function in the aminoalkylimidazole series with a pyrrolidine ring led to the expected increase of affinity for the receptor only in the case of compound **1i**, possibly due to a different binding site for the other ligands compared to classical H₁-antagonists.[62,67] Previously we have indeed proposed that different H₁R agonists binding pockets could exist.[57] The position of the nitrogen in the aminoalkyl-pyridines series does not seem to be important, since
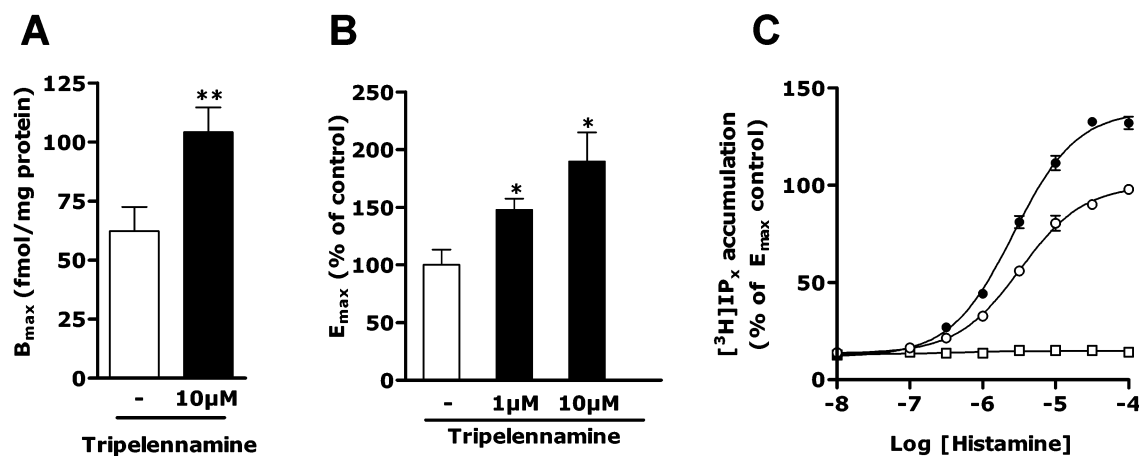
Figure 5. Effects of a 24 h pretreatment with the inverse H_1 agonist tripelennamine on H_1R expression and H_1R-mediated signaling of HeLa cells endogenously expressing H_1Rs. (A) Upregulation of endogenously expressed H_1Rs after a 24 h pretreatment of the cells with 10 μM tripelennamine. (B) Increase in the E_{max} of histamine-induced inositolphosphates accumulation upon pretreatment of the cells with either 1 or 10 μM tripelennamine for 24 h. (C) Effect of tripelennamine (1 μM, 24 h, ●) or histamine (0.63 mM, 24 h, □) pretreatment of HeLa cells on the E_{max} and pEC_{50} of histamine-induced inositolphosphates accumulation in comparison to control cells (○).

all the compounds bind to the receptor. The elongation of the side chain from the classical aminoethyl chain turns the compounds into (partial) inverse agonists, analogous to the aminoalkylimidazoles series, with the exception of compound **13b**, for which we currently have no explanation available.

It has proven difficult to identify neutral H_1-receptor antagonists by modification of the agonist histamine, a strategy previously successfully used to identify neutral H_2- and H_3-receptor antagonists.[19,20] Nonetheless, two histaprodifen-like compounds (**18d** and **18e**), which exhibit hybrid structures composed of the endogenous agonist histamine combined with the classical H_1R antagonist pharmacophore, have been ultimately identified as neutral H_1-receptor antagonists. Consequently, the modification of agonist structures has again proven a successful strategy to obtain neutral histamine receptor antagonists. The benzhydryl group in compounds **18d** and **18e** seems to be important for their neutral H_1R antagonism. Although at this stage it is not possible to draw definite conclusions on the structural requirements necessary for neutral antagonism on the histamine H_1-receptor, the structure–activity relationships for neutral H_1R antagonists seem to differ from the structure–activity relationships of inverse H_1R agonists. In contrast to the effects of pretreatment with inverse H_1-agonist, pretreatment with weak partial inverse agonists or neutral antagonists for the H_1-receptor had no effect on the histamine-induced H_1-receptor-mediated responses. These compounds will therefore be useful pharmacological tools and may serve as a starting point for the development of high-affinity neutral H_1R antagonists, the availability of which will allow the assessment of the importance of constitutive H_1R activity in vivo as well as the requirement of inverse H_1R agonistic properties for the therapeutic value of antihistamines.

Experimental Section

Chemistry. General Procedures. Melting points were measured on an Electrothermal IA 9200 apparatus. ^{1}H NMR spectra were recorded on a Bruker AC-200 (200 MHz) spectrometer. Chemical shifts are given in ppm downfield from tetramethylsilane as internal standard. Elemental analyses were performed by the Department of Microanalysis, Groningen University, Groningen, The Netherlands. Chromatography was performed on J. T.Baker silica gel for flash chromatography. THF was freshly distilled from $LiAlH_4$, and DMF was dried on molecular sieves. All reactions were performed under an atmosphere of dry nitrogen.

The 1-chloro-ω-iodoalkanes were prepared by refluxing the corresponding 1,ω-dichloroalkanes with 0.33 equiv of sodium iodide in acetone and purification by distillation.[68]

The 2-(ω-bromo-alkyloxy)tetrahydropyrans were prepared from the corresponding ω-bromoalkan-1-ols.[69,70]

5-(9-Hydroxynonyl)imidazole-1-sulfonic Acid Dimethylamide (4c). Imidazole-1-sulfonic acid dimethylamide **2**[53] (6.86 g, 0.039 mol) was dissolved in THF (150 mL) and cooled to -70 °C. n-Butyllithium in hexane (29.25 mL, 0.047 mol) was added dropwise at -65 °C. After 15 min, a solution of $tert$-butyldimethylsilyl chloride (7.07 g, 0.047 mol) in THF was added at -65 °C and the solution was allowed to warm to room temperature and stirred for an additional 30 min. The mixture was cooled to -70 °C again, and n-butyllithium in hexane (24.5 mL, 0.039 mol) was added dropwise. After 30 min, a solution of 2-(9-bromononyloxy)tetrahydropyran (10.00 g, 0.033 mol) in THF was added gradually and the mixture was allowed to (slowly) warm to room temperature (rt) overnight. The reaction mixture was poured into water and the THF was removed under reduced pressure. The product was extracted with dichloromethane, washed with water, dried ($MgSO_4$), and concentrated in vacuo. HCl (37%) was added to the crude product till pH = 1, and the mixture stirred for 1 h and was then diluted with water, basified with K_2CO_3, and extracted with dichloromethane; the organic layers were combined, dried ($MgSO_4$), and concentrated in vacuo. The product was purified by flash chromatography with ethyl acetate as eluent to afford 1.09 g (10%) of white crystals. ^{1}H NMR ($CDCl_3$): δ 1.20 (m, 10H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 2.60 (t, 2H, imidazole-5-CH_2), 2.78 (s, 6H, N(CH_3)$_2$), 3.53 (t, 2H, CH_2OH), 6.70 (s, 1H, imidazole-4H), 7.78 (s, 1H, imidazole-2H).

5-(11-Hydroxyundecyl)imidazole-1-sulfonic Acid Dimethylamide (4e). 2-($tert$-Butyl-dimethylsilanyl)imidazole-1-sulfonic acid dimethylamide **3**[47] (9.23 g, 0.032 mol) was dissolved in THF (120 mL) and cooled to -70 °C. n-Butyllithium in hexane (20 mL, 0.032 mol) was added dropwise at -65 °C. After 30 min, a solution of (11-bromoundecyloxy)-tetrahydropyran (10.63 g, 0.032 mol) in THF was added dropwise and the mixture was allowed to (slowly) warm to room temperature overnight. The solution was acidified with HCl (2 M) and stirred for 1 h, then the solvents were removed in vacuo. The residue was diluted with water, basified with

K₂CO₃, extracted with dichloromethane, dried (Na₂SO₄), and concentrated in vacuo. The crude product was purified by flash chromatography switching gradually the eluent from dichloromethane/ethyl acetate (8/2) into ethyl acetate to afford 1.60 g of product (mixed with 25% of **2**) which was used without further purification.

5-(12-Chlorododecyl)imidazole-1-sulfonic Acid Dimethylamide (4f). Using the same procedure as for **4c** with 4.46 g (0.026 mol) of **2** and 5.61 g (0.017 mol) of 1-chloro-12-iodododecane gave 2.69 g of product (mixed with 45% of 5-(*tert*-butyldimethylsilanyl)imidazole-1-sulfonic acid dimethylamide) which was used without further purification.

5-(8-Chlorooctyl)imidazole-1-sulfonic Acid Dimethylamide (4i). Using the same approach as for **4e** with 9.23 g (0.032 mol) of **3** and 8.70 g (0.032 mol) of 1-chloro-8-iodooctane gave 9.59 g (93%) of brown oil, which did not need any further purification by flash chromatography. ¹H NMR (CDCl₃): δ 1.25 (m, 8H, central CH_2's), 1.65 (m, 4H, 2 × CH_2), 2.63 (t, 2H, imidazole-5-CH_2), 2.78 (s, 6H, N(CH_3)₂), 3.43 (t, 2H, CH_2Cl), 6.73 (s, 1H, imidazole-4H), 7.73 (s, 1H, imidazole-2H).

5-(10-Chlorodecyl)imidazole-1-sulfonic Acid Dimethylamide (4j). Using the same procedure as for **4c** with 7.96 g (0.046 mol) of **2** and 13.05 g (0.043 mol) of 1-chloro-10-iodododecane gave 5.32 g of product (mixed with 60% of 5-(*tert*-butyldimethylsilanyl)imidazole-1-sulfonic acid dimethylamide) which was used for the next step without further purification.

5-[9-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)nonyl]imidazole-1-sulfonic Acid Dimethylamide (5c). A mixture of **4c** (1.09 g, 0.0035 mol), triphenylphosphine (0.91 g, 0.0035 mol), and isoindole-1,3-dione (0.51 g, 0.0035 mol) in dry THF (5 mL) was stirred vigorously and placed in an ice/water bath. Diisopropyl azodicarboxylate (0.698 g, 0.00345 mol) in dry THF (5 mL) was added dropwise and stirred at room temperature overnight. The solvent was removed in vacuo and the residue was purified by flash chromatography with ethyl acetate as eluent to afford 0.96 g (63%) of beige solid. ¹H NMR (CDCl₃): δ 1.28 (m, 10H, central CH_2's), 1.65 (m, 4H, 2 × CH_2), 2.67 (t, 2H, imidazole-5-CH_2), 2.83 (s, 6H, N(CH_3)₂), 3.62 (t, 2H, CH_2-isoindole-1,3-dione), 6.78 (s, 1H, imidazole-4H), 7.50−7.78 (m, 5H, isoindole-1,3-dione-H + imidazole-2H).

5-[11-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)undecyl]imidazole-1-sulfonic Acid Dimethylamide (5e). The same procedure as for **5c** starting from 1.60 g (± 0.0046 mol) of **4e** was used. The residue was purified by flash chromatography using ethyl acetate/dichloromethane as eluent to afford 2.11 g of product that was used for the next step without further purification.

5-[12-(1,3-Dioxo-1,3-dihydroisoindol-2-yl)dodecyl]imidazole-1-sulfonic Acid Dimethylamide (5f). Compound **4f** (1.54 g, ±0.0025 mol) was mixed with isoindole-1,3-dione (1.22 g, 0.0083 mol), Na₂CO₃ (0.87 g, 0.0083 mol), and NaI (0.075 g, 0.50 mmol) in dry DMF (40 mL) and heated at 90 °C. After 20 h the solvent was evaporated under reduced pressure, and the residue was diluted with water and extracted with CHCl₃. The combined organic extract were washed with water, dried (Na₂SO₄), and concentrated in vacuo to give **5f** as a brown solid (2.07 g) which was used without further purification.

5-(12-Aminododecyl)imidazole-1-sulfonic Acid Dimethylamide (6f). Compound **5f** (2.07 g) was dissolved in warm ethanol (60 mL), hydrazine monohydrate (0.50 g, 0.01 mol) was added, and the solution was refluxed for 3 h, cooled to room temperature, and filtered. The filtrate was concentrated in vacuo and the residue was diluted with NaOH (1 M) and then extracted with CHCl₃. The organic extracts were washed with NaOH (0.5 M), dried (Na₂SO₄), and concentrated in vacuo. The crude product was purified by flash chromatography switching gradually the eluent from ethyl acetate into methanol/triethylamine (9/1) (R_f = 0.5); 340 mg (38%) of a yellow solid was isolated. ¹H NMR (CDCl₃): δ 1.25 (m, 16H, central CH_2's + NH_2), 1.65 (m, 6H, 3 × CH_2), 2.65 (m, 4H, imidazole-5-CH_2 + CH_2NH₂), 2.83 (s, 6H, N(CH_3)₂), 6.75 (s, 1H, imidazole-4H), 7.78 (s, 1H, imidazole-2H).

5-(8-Pyrrolidin-1-yloctyl)imidazole-1-sulfonic Acid Dimethylamide (7i). Compound **4i** (1.5 g, 0.0047 mol) was

stirred at room temperature with pyrrolidine (4.37 g, 0.062 mol). After 48 h the unreacted pyrrolidine was removed under reduced pressure and the residue was diluted with water, basified with K₂CO₃, extracted with dichloromethane, dried (Na₂SO₄), and concentrated in vacuo to afford 1.30 g (78%) of brown oil. ¹H NMR (CDCl₃): δ 1.25 (m, 10H, central CH_2's), 1.75 (m, 6H, 3 × CH_2), 2.35 (m, 6H, 2 × CH_2 + CH_2-pyrrolidine), 2.65 (t, 2H, imidazole-5-CH_2), 2.83 (s, 6H, N(CH_3)₂), 6.75 (s, 1H, imidazole-4H), 7.78 (s, 1H, imidazole-2H).

5-(10-Pyrrolidin-1-yldecyl)imidazole-1-sulfonic Acid Dimethylamide (7j). The same procedure as for **7i** with 4.43 g (±0.0029 mol) of **4j** was used. The product was purified by flash chromatography switching gradually the eluent from ethyl acetate into methanol/triethylamine (9/1); 760 mg (67%) of a brown oil was obtained. ¹H NMR (CDCl₃): δ 1.25 (m, 14H, central CH_2's), 1.75 (m, 6H, 3 × CH_2), 2.50 (m, 6H, 2 × CH_2 + CH_2-pyrrolidine), 2.65 (t, 2H, imidazole-5-CH_2), 2.83 (s, 6H, N(CH_3)₂), 6.85 (s, 1H, imidazole-4H), 7.90 (s, 1H, imidazole-2H).

5-(12-Pyrrolidin-1-yldodecyl)imidazole-1-sulfonic Acid Dimethylamide (7k). Using the same procedure as for **7j** starting with **4f** (1.00 g, ±0.0016 mol) gave 500 mg (74%) of a brown oil. ¹H NMR (CDCl₃): δ 1.25 (m, 16H, central CH_2's), 1.75 (m, 8H, 4 × CH_2), 2.45 (m, 6H, 2 × CH_2 + CH_2-pyrrolidine), 2.70 (t, 2H, imidazole-5-CH_2), 2.83 (s, 6H, N(CH_3)₂), 6.80 (s, 1H, imidazole-4H), 7.80 (s, 1H, imidazole-2H).

14-[4-(Toluene-4-sulfonyl)-4,5-dihydrooxazol-5-yl]tetradecanoic Acid Methyl Ester (11). To a stirred suspension of tosyl methyl isocyanide (TosMIC) (4.98 g, 0.026 mol) and 15-oxo-pentadecanoic acid methyl ester **10** [71] (6.99 g, 0.026 mol) in absolute ethanol (80 mL) was added finely powdered sodium cyanide (0.12 g, 0.0026 mol). The yellow-orange suspension was stirred for 30 min, filtered, and washed with ethanol/ether. The light yellow solid was immediately used for the next step. ¹H NMR (CDCl₃): δ 1.30 (m, 18H, central CH_2's), 1.60 (m, 6H, 3 × CH_2), 2.25 (t, 2H, CH_2COOCH₃), 2.46 (s, 3H, p-CH_3), 3.65 (s, 3H, CH_3OCO), 4.76 (d, 1H, oxazoline-4H), 5.05 (q, 1H, oxazoline-5H), 6.95 (s, 1H, oxazoline-2H), 7.35 (d, 2H, 2,6-phenyl-H), 7.80 (d, 2H, 3,5-phenyl-H).

14-(1H-Imidazol-4-yl)tetradecanoic Acid Amide (12). In a resealable pressure tube, a solution of **11** in a saturated solution of ammonia in dry methanol was heated between 120 and 140 °C. After 48 h the reaction mixture was cooled to rt and the solvent was removed in vacuo. The residue was triturated with dichloromethane (150 mL) and filtered. A total of 4.41 g (58%) of a beige solid was collected. ¹H NMR (DMSO): δ 1.25 (m, 18H, central CH_2's), 1.50 (m, 4H, 3 × CH_2), 2.05 (t, 2H, CH_2CONH₂), 2.50 (t, 2H, imidazole-4(5)-CH_2), 6.70 (s, 1H, imidazole-4(5)H), 7.65 (s, 1H, imidazole-2H).

9-(1H-Imidazol-4-yl)nonylamine Dioxalate (1c). Compound **5c** (0.96 g, 0.0022 mol) was dissolved in 30% HBr (12 mL) and heated under reflux. After 16 h the mixture was cooled and concentrated in vacuo, and the residue was diluted with water, washed with diethyl ether, basified with K₂CO₃, and extracted with CHCl₃/EtOH (9/1). The organic layers were combined, dried (MgSO₄), and concentrated in vacuo to afford 0.30 g (65%) of brown solid.

The free base was converted into the dioxalate salt, using the following procedure: the free base was dissolved in ethyl acetate with a few drops of methanol and then added dropwise with a solution of 2 equiv of oxalic acid dihydrate in ethyl acetate/methanol. The precipitate that formed was collected by filtration to afford 0.42 g (77%) of a light yellow powder. Mp: 126−128 °C. ¹H NMR (D₂O): δ 1.27 (m, 10H, central CH_2's), 1.62 (m, 4H, 2 × CH_2), 2.67 (t, 2H, imidazole-4(5)-CH_2), 2.94 (t, 2H, CH_2NH₂), 7.14 (s, 1H, imidazole-4(5)H), 8.50 (s, 1H, imidazole-2H). Anal. (C₁₆H₂₇N₃O₈) C, H, N.

11-(1H-Imidazol-4-yl)undecylamine (1e). Using the same procedure as for **1c** starting with 2.11 g (±0.0040 mol) of **5e** gave 0.78 g (82%) of light yellow powder. Mp: 87.5−88.5 °C. ¹H NMR (CDCl₃): δ 1.27 (m, 16H, central CH_2's + NH_2), 1.50 (m, 4H, 2 × CH_2), 2.60 (m, 4H, imidazole-4(5)-CH_2 + CH_2NH₂), 6.70 (s, 1H, imidazole-4(5)H), 7.50 (s, 1H, imidazole-2H). Anal. (C₁₄H₂₇N₃) C, H, N.

12-(1*H*-Imidazol-4-yl)dodecylamine (1f). The same procedure as for **1c** with 0.34 g (0.95 mmol) of **6f** was used. After basification the water was removed in vacuo and the remaining solid was washed with 10 mL portions of 2-propanol. The filtrate was concentrated under reduced pressure and washed with fresh 2-propanol. The filtrates were concentrated in vacuo to give 0.16 g (67%) of a beige powder. Mp: 150−152 °C. ^{1}H NMR (CDCl$_3$): δ 1.20 (m, 16H, central CH_2's + NH_2), 1.55 (m, 6H, 3 × CH_2), 2.60 (m, 4H, imidazole-4(5)-CH_2 + CH_2NH$_2$), 6.70 (s, 1H, imidazole-4(5)H), 7.50 (s, 1H, imidazole-2H). Anal. (C$_{15}$H$_{29}$N$_3$) C, H, N.

13-(1*H*-Imidazol-4-yl)tridecylamine Dioxalate (1g). PIFA[51] (1.72 g, 0.0040 mol) was dissolved in 6 mL of acetonitrile, and 6 mL of distilled water was added. To this solution was added **12** (1.17 g, 0.0040 mol), and the suspension was stirred at room temperature. After 20 h the reaction mixture was diluted with water (75 mL) and HCl (37%, 8 mL), stirred for another 30 min, and washed with diethyl ether. The combined ether layers were extracted with HCl (10%, 30 mL). The aqueous layers were combined, basified with K$_2$CO$_3$, extracted with chloroform/ethanol (10%), dried (MgSO$_4$), and concentrated in vacuo. The residue was purified by flash chromatography with methanol and then methanol/triethylamine (9/1) to afford 240 mg (23%) of amine. The free base was converted into a dioxalate; 335 mg (83%) of a white powder was obtained. Mp: 118−120 °C. ^{1}H NMR (D$_2$O): δ 1.27 (m, 18H, central CH_2's), 1.62 (m, 4H, 2 × CH_2), 2.67 (t, 2H, imidazole-4(5)-CH_2), 2.94 (t, 2H, CH_2NH$_2$), 7.14 (s, 1H, imidazole-4(5)H), 8.50 (s, 1H, imidazole-2H). Anal. (C$_{20}$H$_{35}$N$_3$O$_8$) C, H, N.

14-(1*H*-Imidazol-4-yl)tetradecylamine Dioxalate (1h). A suspension of **12** (2.00 g, 0.068 mol) in THF (100 mL) was added, with efficient stirring, to a suspension of LiAlH$_4$ (1.30 g, 0.034 mol) in THF (30 mL). The reaction mixture was stirred for 1 h at rt, and then heated under reflux. After 20 h the suspension was cooled to rt and water (1.5 mL) followed by NaOH 10% aqueous solution (1.5 mL) was added dropwise, and the mixture was refluxed. After 30 min the reaction mixture was cooled to room temperature, filtered, and concentrated in vacuo to afford 1.66 g (88%) of a yellow solid that was converted into the dioxalate salt. The yellow precipitate collected by filtration was washed (under stirring) with methanol (10 mL) and filtered again to afford 1.12 g (42%) of a light-yellow powder. Mp: 122−126 °C. ^{1}H NMR (D$_2$O): δ 1.27 (m, 20H, central CH_2's), 1.62 (m, 4H, 2 × CH_2), 2.67 (t, 2H, imidazole-4(5)-CH_2), 2.94 (t, 2H, CH_2NH$_2$), 7.14 (s, 1H, imidazole-4(5)H), 8.50 (s, 1H, imidazole-2H). Anal. (C$_{21}$H$_{37}$N$_3$O$_8$) C, H, N.

4-(8-Pyrrolidin-1-yloctyl)-1*H*-imidazole Dioxalate (1i). Using the same procedure as for **1c** with **7i** (1.25 g, 0.0035 mol) gave 800 mg (92%) of product. ^{1}H NMR (CDCl$_3$): δ 1.25 (m, 8H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 1.75 (m, 4H, 2 × CH_2), 2.45 (m, 8H, imidazole-4(5)-CH_2 + CH_2-pyrrolidine + 2 × CH_2), 6.70 (s, 1H, imidazole-4(5)H), 7.45 (s, 1H, imidazole-2H). The free base was converted into the dioxalate salt and crystallized from methanol/diethyl ether to afford 150 mg (11%) of light yellow solid. Anal. (C$_{19}$H$_{31}$N$_3$O$_8$) C, H, N.

4-(10-Pyrrolidin-1-yldecyl)-1*H*-imidazole (1j). Using the same procedure as for **1c** with **7j** (0.76 g, 0.0020 mol), followed by the treatment of the free base with activated charcoal, gave 480 mg (87%) of a brown waxy compound. ^{1}H NMR (CDCl$_3$): δ 1.20 (m, 12H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 1.70 (m, 4H, 2 × CH_2), 2.45 (m, 8H, imidazole-4(5)-CH_2 + CH_2-pyrrolidine + 2 × CH_2), 6.70 (s, 1H, imidazole-4(5)H), 7.45 (s, 1H, imidazole-2H). Anal. (C$_{17}$H$_{31}$N$_3$) C, H, N.

4-(12-Pyrrolidin-1-yldodecyl)-1*H*-imidazole (1k). Using the same procedure as for **1j** gave 210 mg (57%) of a waxy yellow solid. ^{1}H NMR (CDCl$_3$): δ 1.20 (m, 16H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 1.70 (m, 4H, 2 × CH_2), 2.45 (m, 8H, imidazole-4(5)-CH_2 + CH_2-pyrrolidine + 2 × CH_2), 6.70 (s, 1H, imidazole-4(5)H), 7.45 (s, 1H, imidazole-2H). Anal. (C$_{19}$H$_{35}$N$_3$) C, H, N.

2-(9-Bromononyl)pyridine (15a). 2-Methylpyridine **14a** (0.54 g, 0.0058 mol) in THF (5 mL) was added dropwise to a solution of *n*-butyllithium (3.65 mL, 0.0058 mol) in 5 mL of THF at −50 °C over 10 min, then the cooling bath was removed and the temperature was allowed to rise to −20 °C. The cold dark red solution was added dropwise to a cold solution (−50 °C) of dibromooctane (2.44 g, 0.0090 mol) in THF (5 mL) and the mixture was allowed to warm to room temperature and stirred for an additional 0.5 h. HCl (37%) was added till pH = 1 and stirred for 1.5 h, then the solvent was removed in vacuo. The residue was washed with diethyl ether, basified with K$_2$CO$_3$, extracted with dichloromethane, dried (MgSO$_4$), and evaporated in vacuo. The residue (0.82 g) was used directly for the next step.

10-Pyridin-2-yldecan-1-ol (15b). Compound **14a** (5.63 g, 0.061 mol) in THF (30 mL) was added dropwise to a solution of *n*-butyllithium (37.8 mL, 0.061 mol) in 50 mL of THF at −50 °C over 10 min, then the cooling bath was removed and the temperature allowed to rise to −20 °C. The red solution was cooled again to −50 °C and 16.77 g (0.055 mol) of 2-(9-bromononyloxy)tetrahydropyran in THF (30 mL) was added dropwise, then the mixture was allowed to (slowly) warm to room temperature overnight. The reaction mixture was poured into water and the THF was removed under reduced pressure. The residue was acidified with HCl (37%) and stirred for 30 min, washed with diethyl ether, basified with K$_2$CO$_3$, extracted with dichloromethane, dried (MgSO$_4$), and evaporated in vacuo. The product was purified by flash chromatography switching gradually the eluent from dichloromethane/ethyl acetate (9/1) into ethyl acetate; 4.49 g (35%) of a white waxy compound was isolated. ^{1}H NMR (CDCl$_3$): δ 1.15 (m, 12H, central CH_2's), 1.50 (m, 4H, 2 × CH_2), 2.60 (t, 2H, pyridine-2-CH_2), 2.98 (br s*, 1H, OH), 3.45 (t, 2H, CH_2OH), 6.90 (m, 2H, pyridine-3H + pyridine-5H), 7.38 (m, 1H, pyridine-4H), 8.30 (d, 1H, pyridine-6H).

12-Pyridin-2-yldodecan-1-ol (15c). The same procedure as for **15b** with 12.35 g (0.037 mol) of 2-(11-bromoundecyloxy)tetrahydropyran was used. After the basification the product precipitated and was filtered and washed with water to afford 5.67 g (58%) of a beige powder. ^{1}H NMR (CDCl$_3$): δ 1.23 (m, 14H, central CH_2's), 1.60 (m, 6H, 3 × CH_2), 2.75 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.05 (m, 2H, pyridine-3H + pyridine-5H), 7.55 (m, 1H, pyridine-4H), 8.45 (d, 1H, pyridine-6H).

10-Pyridin-3-yldecan-1-ol (15d). A solution of 2.89 g (0.031 mol) of 3-methylpyridine **14d** in THF (20 mL) was added dropwise at −20 °C to a freshly prepared solution of lithium diisopropylamide (19.38 mL, 0.031 mol of *n*-butyllithium and 3.74 g, 0.037 mol of diisopropylamine) in 70 mL of THF. The cooling bath was removed and the temperature allowed to rise to 10 °C. After stirring for 30 min at this temperature the reaction mixture was cooled to −40 °C and 8.00 g (0.026 mol) of 2-(9-bromononyloxy)tetrahydropyran in THF (30 mL) was added dropwise. The cooling bath was removed and the mixture was allowed to (slowly) warm to room temperature overnight. The reaction mixture was poured into water and the THF was removed under reduced pressure. The crude product was acidified with HCl (37%), stirred for 30 min, washed with diethyl ether (3 × 50 mL), basified with K$_2$CO$_3$, extracted with dichloromethane, dried (MgSO$_4$), and evaporated in vacuo, to afford 5.50 g (90%) of a brown waxy compound. ^{1}H NMR (CDCl$_3$): δ 1.25 (m, 12H, central CH_2's), 1.52 (m, 4H, 2 × CH_2), 2.58 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.15 (m, 1H, pyridine-5H), 7.45 (d, 1H, pyridine-4H), 8.38 (m, 2H, pyridine-2H + pyridine-6H).

12-Pyridin-3-yldodecan-1-ol (15e). Using the same procedure as for **15d** with 1.78 g (0.019 mol) of **14d** and 5.36 g (0.016 mol) of 2-(11-bromoundecyloxy)tetrahydropyran gave 2.56 g (61%) of a brown waxy compound. ^{1}H NMR (CDCl$_3$): δ 1.25 (m, 16H, central CH_2's), 1.55 (m, 6H, 3 × CH_2), 2.55 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.15 (m, 1H, pyridine-5H), 7.45 (d, 1H, pyridine-4H), 8.38 (m, 2H, pyridine-2H + pyridine-6H).

10-Pyridin-4-yldecan-1-ol (15f). The same procedure as for the preparation of **15d** was employed, using 3.02 g (0.032 mol) of 4-methylpyridine **14f**, with the exception that **14f**

solution was added at −40 °C and the reaction mixture was stirred for 30 min at 20 °C. Upon addition of HCl (37%) a white precipitate formed, which was filtered. The solid was dissolved in water, basified with K_2CO_3, and filtered to afford 4.89 g (77%) of a yellow solid. ¹H NMR (CDCl₃): δ 1.25 (m, 12H, central CH_2's), 1.55 (m, 4H, 2 × CH_2), 1.85 (br s, 1H, OH), 2.55 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.10 (d, 2H, pyridine-3H + pyridine-5H), 8.45 (m, 2H, pyridine-2H + pyridine-6H).

12-Pyridin-4-yldodecan-1-ol (15g). Using the same procedure as for **15f** with 1.78 g (0.019 mol) of **14f** gave 3.67 g (87%) of a brown oil. ¹H NMR (CDCl₃): δ 1.25 (m, 14H, central CH_2's), 1.55 (m, 6H, 3 × CH_2), 1.75 (br s, 1H, OH), 2.55 (t, 2H, pyridine-2-CH_2), 3.60 (t, 2H, CH_2OH), 7.10 (d, 2H, pyridine-3H + pyridine-5H), 8.45 (m, 2H, pyridine-2H + pyridine-6H).

2-(10-Pyridin-2-yl-decyl)isoindole-1,3-dione (16b). The same procedure as for **5c** with 4.49 g (0.019 mol) of **15b** was used. After removal of the solvent the residue was dissolved in ethyl acetate and extracted with HCl (1 M). The aqueous layer was washed once with ethyl acetate, basified with K_2CO_3, extracted with CHCl₃, dried (MgSO₄), and evaporated to afford 6.25 g (90%) of white powder. ¹H NMR (CDCl₃): δ 1.23 (m, 12H, central CH_2's), 1.65 (m, 4H, 2 × CH_2), 2.75 (t, 2H, pyridine-2-CH_2), 3.62 (t, 2H, CH_2-N), 7.05 (m, 2H, pyridine-3H + pyridine-5H), 7.50 (m, 1H, pyridine-4H), 7.65 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.45 (d, 1H, pyridine-6H).

2-(12-Pyridin-2-yldodecyl)isoindole-1,3-dione (16c). The same procedure as for **16b** with 5.67 g (0.022 mol) of **15c** was used. During the extraction with HCl (1 M) the hydrochloric salt of the compound crystallized and was separated by filtration. The salt was dissolved in water, basified with K_2CO_3, extracted with CHCl₃, dried (MgSO₄), and concentrated in vacuo to afford 3.19 g (38%) of white powder. ¹H NMR (CDCl₃): δ 1.23 (m, 14H, central CH_2's), 1.60 (m, 6H, 3 × CH_2), 2.75 (t, 2H, pyridine-2-CH_2), 3.65 (t, 2H, CH_2-N), 7.05 (m, 2H, pyridine-3H + pyridine-5H), 7.55 (m, 1H, pyridine-4H), 7.68 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.45 (d, 1H, pyridine-6H).

2-(10-Pyridin-3-yldecyl)isoindole-1,3-dione (16d). Using the same procedure as for **16b** with 5.50 g (0.023 mol) of **15d** gave 7.57 g (90%) of brown oil. ¹H NMR (CDCl₃): δ 1.23 (m, 12H, central CH_2's), 1.55 (m, 4H, 2 × CH_2), 2.55 (t, 2H, pyridine-2-CH_2), 3.62 (t, 2H, CH_2-isoindole-1,3-dione), 7.15 (m, 2H, pyridine-3H + pyridine-5H), 7.15 (m, 1H, pyridine-5H), 7.45 (d, 1H, pyridine-4H), 7.65 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.38 (m, 2H, pyridine-2H + pyridine-6H).

2-(10-Pyridin-4-yldecyl)isoindole-1,3-dione (16f). Using the same procedure as for **16b** with 4.89 g (0.021 mol) of **15f** gave 6.42 g (82%) of a yellow solid. ¹H NMR (CDCl₃): δ 1.23 (m, 12H, central CH_2's), 1.65 (m, 4H, 2 × CH_2), 2.55 (t, 2H, pyridine-2-CH_2), 3.62 (t, 2H, CH_2-isoindole-1,3-dione), 7.05 (d, 2H, pyridine-3H + pyridine-5H), 7.45 (d, 1H, pyridine-4H), 7.65 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.45 (m, 2H, pyridine-2H + pyridine-6H).

2-(12-Pyridin-4-yldodecyl)isoindole-1,3-dione (16g). Using the same procedure as for **16b** with 3.67 g (0.014 mol) of **15g** gave 4.37 g (79%) of brown oil. ¹H NMR (CDCl₃): δ 1.23 (m, 14H, central CH_2's), 1.55 (m, 6H, 3 × CH_2), 2.55 (t, 2H, pyridine-2-CH_2), 3.62 (t, 2H, CH_2-isoindole-1,3-dione), 7.05 (d, 2H, pyridine-3H + pyridine-5H), 7.65 (m, 2H, isoindole-1,3-dione), 7.80 (m, 2H, isoindole-1,3-dione), 8.45 (m, 2H, pyridine-2H + pyridine-6H).

9-Pyridin-2-ylnonylamine Dihydrochloride (13a). The same procedure as for **5f** with 0.82 g of crude **15a** was used. The residue obtained after extraction was purified by flash chromatography using ethyl acetate as eluent. The product fractions were hydrazinolysed as described for **6f** to give 0.12 g (59%) of free base.

The free base was converted into the dihydrochloride, using the following procedure: the free base was mixed with 2 equiv of HCl (37%) and stirred for 30 min, then the water was removed in vacuo to afford 0.15 g (95%) of a brown waxy compound. ¹H NMR (D₂O): δ 1.25 (m, 10H, central CH_2's), 1.70 (m, 6H, 2 × CH_2 + NH_2), 3.00 (m, 4H, pyridine-2-CH_2 + CH_2NH₂), 7.85 (m, 2H, pyridine-3H + pyridine-5H), 8.43 (m, 1H, pyridine-4H), 8.55 (d, 1H, pyridine-6H). Anal. (C₁₄H₂₆Cl₂N₂) C, H, N.

10-Pyridin-2-yldecylamine Dihydrochloride (13b). Using the same procedure as for **6f** with 6.25 g (0.017 mol) of **16b** gave 2.99 g (74%) of free base. The free base was converted into the dihydrochloride, decolorized with activated charcoal, and crystallized from ethanol/ethyl acetate to afford 2.18 g (56%) of white crystals. Mp: 143.5−146.5 °C. ¹H NMR (D₂O): δ 1.25 (m, 14H, central CH_2's + NH_2), 1.70 (m, 4H, 2 × CH_2), 3.00 (m, 4H, pyridine-2-CH_2 + CH_2NH₂), 7.85 (m, 2H, pyridine-3H + pyridine-5H), 8.43 (m, 1H, pyridine-4H), 8.55 (d, 1H, pyridine-6H). Anal.(C₁₅H₂₈Cl₂N₂) C, H, N.

12-Pyridin-2-yldodecylamine Dihydrochloride (13c). Using the same procedure as for **13b** with 3.19 g (0.0081 mol) of **16c** gave 1.65 g (66%) of white crystals. Mp: 161−164 °C. ¹H NMR (D₂O): δ 1.25 (m, 16H, central CH_2's), 1.70 (m, 4H, 2 × CH_2), 3.00 (m, 4H, pyridine-2-CH_2 + CH_2NH₂), 7.85 (m, 2H, pyridine-3H + pyridine-5H), 8.48 (m, 1H, pyridine-4H), 8.58 (d, 1H, pyridine-6H). Anal. (C₁₇H₃₂Cl₂N₂) C, H, N.

10-Pyridin-3-yldecylamine Dihydrochloride (13d). The same procedure as for **6f** was employed starting with 7.57 g (0.021 mol) of **16d**. The free base obtained after extraction was converted into the dihydrochloride and crystallized from ethanol/ethyl acetate to afford 1.78 g (28%) of a beige powder. Mp: 93.5−96.5 °C. ¹H NMR (D₂O): δ 1.30 (m, 12H, central CH_2's), 1.68 (m, 4H, 2 × CH_2), 2.82 (t, 2H, pyridine-2-CH_2), 2.95 (t, 2H, CH_2NH₂), 7.95 (m, 1H, pyridine-5H), 8.45 (d, 1H, pyridine-4H), 8.60 (m, 2H, pyridine-2H + pyridine-6H). Anal. (C₁₅H₂₈Cl₂N₂) C, H, N.

12-Pyridin-3-yldodecylamine Dihydrochloride (13e). The same procedure as for **5c** with 2.56 g (0.0098 mol) of **15e** was used. The solvents were removed in vacuo and the remaining oil was hydrazinolysed using the same procedure as for **6f**.

The free base was converted into the dihydrochloride and crystallized from ethanol/ethyl acetate to afford 1.32 g (40%) of a yellow powder. Mp: 110−112 °C. ¹H NMR (D₂O): δ 1.30 (m, 18H, central CH_2's + NH_2), 1.68 (m, 4H, 2 × CH_2), 2.82 (t, 2H, pyridine-2-CH_2), 2.95 (t, 2H, CH_2NH₂), 7.95 (m, 1H, pyridine-5H), 8.45 (d, 1H, pyridine-4H), 8.60 (m, 2H, pyridine-2H + pyridine-6H). Anal. (C₁₇H₃₂Cl₂N₂) C, H, N.

10-Pyridin-4-yldecylamine Dihydrochloride (13f). The same procedure as for **6f** with 6.42 g (0.018 mol) of **16f** was used. The free base was converted into the dihydrochloride, crystallized from ethanol/ethyl acetate, and decolorized with activated charcoal to afford 2.65 g (49%) of light yellow powder. Mp: 143.5−144.5 °C. ¹H NMR (D₂O): δ 1.25 (m, 14H, central CH_2's + NH_2), 1.65 (m, 4H, 2 × CH_2), 2.95 (m, 4H, pyridine-2-CH_2 + CH_2NH₂), 7.85 (d, 2H, pyridine-3H + pyridine-5H), 8.58 (d, 2H, pyridine-2H + pyridine-6H). Anal. (C₁₅H₂₈Cl₂N₂) C, H, N.

12-Pyridin-4-yldodecylamine Dihydrochloride (13g). The same procedure as for **6f** with 4.37 g (0.011 mol) of **16g** was used. The crude product was purified by flash chromatography switching gradually the eluent from ethyl acetate into methanol/triethylamine (9/1) (R_f = 0.4) to afford 1.48 g (50%) of yellow solid. The free base was converted into the dihydrochloride, crystallized from ethanol/ethyl acetate, and decolorized with activated charcoal to afford 1.28 g (68%) of light yellow powder. Mp: 137.5−139.5 °C. ¹H NMR (D₂O): δ 1.25 (m, 18H, central CH_2's + NH_2), 1.65 (m, 4H, 2 × CH_2), 2.95 (m, 4H, pyridine-2-CH_2 + CH_2NH₂), 7.85 (d, 2H, pyridine-3H + pyridine-5H), 8.58 (d, 2H, pyridine-2H + pyridine-6H). Anal. (C₁₇H₃₂Cl₂N₂) C, H, N.

Pharmacology. Materials. pNF-κB-Luc was obtained from Stratagene (La Jolla, CA). Mepyramine (pyrilamine maleate) and tripelennamine hydrochloride were obtained from RBI. ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), histamine dihydrochloride, polyethyleneimine, and Tween 20 were purchased from Sigma Chemical Company. D-Luciferin was obtained

from Duchefa Biochemie BV, glycerol from Riedel-de-Haën, and Triton X-100 from Fluka. Cell culture media, penicillin, and streptomycin were obtained from Life Technologies (Merelbeke, Belgium). Fetal calf serum (FCS) was obtained from Integro B. V. (Dieren, The Netherlands), dialyzed fetal calf serum was obtained from HyClone Laboratories Inc. [³H]Mepyramine (30 Ci/mmol) and myo-[2-³H]inositol (17 Ci/mmol) were from Amersham International.

All other compounds, including VUF 4732 (**1a**), VUF 4733 (**1b**), VUF 4734 (**1d**),[53] VUF 8811 (**17c**), VUF 5963 (**17a**), 2-(pyridine-2-yl)ethyl-1-amine (PEA) dihydrochloride, 2-(thiazol-4-yl)ethanamine (4-TEA), 2-(3-trifluoromethylphenyl)-histamine dihydrogenmaleate (**17b**),[56] histaprodifen dimaleate (**18c**), histabudifen dimaleate (**18d**), histapendifen dimaleate (**18e**), the pyrrolidin analogue of histaprodifen as dioxalate (**18i**),[58] the ring-substituted analogues of histaprodifen dihydromaleate [4-Br-histaprodifen (**18g**) and 4-methyl-histaprodifen (**18h**)],[59] the histaprodifen analogues 2-(diphenylmethyl)histamine dihydrochloride (**18a**) and 2-(diphenylethyl)histamine dihydrochloride (**18b**), 2-(4-phenylbutyl)histamine dihydrogenmaleate (FUB 113, **17d**) and 2-(5-phenylpentyl)histamine dihydrogenmaleate (FUB 114, **17e**), the cyclohexyl analogue of histaprodifen dihydromaleate (**18f**),[55] the 2-(2-phenyl-4-thiazolyl)ethyl-1-amines (**19a,b**), and 2-(2-benzyl-4-thiazolyl)ethyl-1-amines (**19c,d**),[60] were taken from our own stock.

Gifts of mianserine hydrochloride (Organon NV), pcDEF₃ (Dr. J. Langer), and the cDNA encoding the human histamine H₁-receptor (Dr. H. Fukui)[2] are greatly acknowledged.

Cell Culture and Transfection. COS-7 African green monkey kidney cells were maintained at 37 °C in an humidified 5% CO₂/95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin, and 5% (v/v) fetal calf serum (FCS); HeLa cells were maintained in the same manner in medium containing 10% (v/v) FCS. COS-7 cells were transiently transfected using the DEAE-dextran method as previously described.[28] The expression level of the histamine H₁-receptor in transiently transfected COS-7 cells was 3.2 ± 0.4 pmol/mg of protein as determined by radioligand binding assays. The total amount of DNA transfected was maintained constant by addition of either pcDEF₃ or pcDNA₃.

Reporter-Gene Assay. Reporter-gene assays were preformed as previously described.[28] Briefly, after cotransfection with pNFκB-Luc (125 μg/1 × 10⁷ cells) and either pcDEF₃ or pcDEF₃hH₁ (25 μg/1 × 10⁷ cells) cells were seeded in 96 well whiteplates in serum free culture medium and incubated with drugs. After 48 h, cells were assayed for luminescence for 3 s/well in a Victor² (Wallac) 30 mim after aspiration of the medium and the addition of 25 μL/well luciferase assay reagent [0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM MgCl₂, 0.78 μM Na₂H₂P₂O₇, 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton-X-100, and 2.6 μM DTT]. To detect potential nonhuman H₁R-mediated effects of the tested compounds, the compounds were tested in parallel on control cells not expressing the human H₁R.

Histamine H₁-Receptor Binding Studies. HeLa cells or COS-7 cells (harvested 48 h after transfection) for radioligand binding studies were harvested and homogenized in ice-cold H₁-binding buffer (50 mM Na₂/K-phosphate buffer, pH = 7.4) as previously described.[28] HeLa cell membranes were further purified by centrifugation of the cell homogenate for 5 min at $1400g$ and subsequent centrifugation of the supernatant for 20 min at $15\,000g$. For radioligand binding studies, COS-7 cell homogenates or HeLa cell membranes were incubated for 30 min at 30 °C in H₁-binding buffer containing 1 nM [³H]mepyramine. The nonspecific binding was determined in the presence of 1 μM mianserin. Incubations were terminated by rapid dilution and subsequent filtration over Whatman GF/C filters that had been treated with 0.3% polyethyleneimine using ice-cold H₁-binding buffer. The radioactivity retained on the filters was measured by liquid scintillation counting. Binding data were evaluated by a nonlinear, least

squares curve-fitting procedure using Graphpad Prism (GraphPad Software, Inc., San Diego, CA).

[³H]Inositol Phosphate Formation. HeLa cells were seeded in 24-well plates (10⁵ cells/well) and after 24 h cells were washed with PBS, labeled, and pretreated with various concentrations of inverse H₁-agonists in inositol-free culture medium supplemented with 1 μCi/mL myo-[2-³H]inositol for 24 h. Subsequently, the medium was aspirated and cells were incubated with histamine for 1 h at 37 °C in DMEM containing 25 mM Hepes (pH 7.4) and 20 mM LiCl. Incubations were stopped by aspiration of the culture medium and the addition of cold 10 mM formic acid. After 90 min incubation at 4 °C, [³H]inositol phosphates were isolated by anion exchange chromatography and counted by liquid scintillation.

Analytical Methods. Protein levels were determined according to the method of Bradford,[72] using BSA as a standard. All data shown are expressed as mean $\pm$ SEM, and statistical analyses were carried out by Student's t-test. P values < 0.05 were considered to indicate a significant difference.

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