MedChemComm

RESEARCH ARTICLE

View Article Online
View Journal



Cite this: DOI: 10.1039/c8md00527c

# Design, synthesis, and *in vitro* and *in vivo* characterization of 1-{4-[4-(substituted)piperazin-1-yl]butyl}guanidines and their piperidine analogues as histamine $H_3$ receptor antagonists†‡

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Received 18th October 2018,
Accepted 29th November 2018

DOI: 10.1039/c8md00527c

rsc.li/medchemcomm

Previously, we have shown that 1-substituted-[4-(7-phenoxyheptylpiperazin-1-yl)butyl]guanidine with electron withdrawing substituents at position 4 in the benzyl moiety exhibits high *in vitro* affinities toward the guinea pig jejunal histamine $H_3$ receptor with $pA_2$ ranging from 8.49 to 8.43. Here, we present data on the impact of replacement of the piperazine scaffold by the piperidine ring (compounds **2a** and **2b**), moving benzyl- and 4-trifluoromethylbenzyl substituents from position 1 to 3 of the guanidine moiety (compounds **2c** and **2d**), which decreases the guanidine basicity (compound **2e**), and the influence of individual synthons (compounds **2f–h**), present in the lead compounds **1b** and **1c**, on the antagonistic activity against the histamine $H_3$ receptor. Additionally, the most active compounds **1a**, **1c**, and **1d** were evaluated for their affinity to the rat histamine $H_3$ receptor and the human histamine $H_3$ and $H_4$ receptors. It was also shown that compounds **1a**, **1c** and **1d**, given parenterally for five days, reduced the food intake of rats and did not influence the brain histamine or noradrenaline concentrations; however, significantly reduced serotonin and dopamine concentrations were found in rats administered with compounds **1a** and **1c**, respectively.



## 1. Introduction

Histamine is a biogenic amine implicated in a wide range of physiological processes through the activation of four G-protein coupled receptors ($H_1$, $H_2$, $H_3$, and $H_4$).[1] The postsynaptic $H_1$- and $H_2$-receptors are mainly involved in the control of allergic response and gastric acid, respectively, while the $H_4$-receptor seems to be implicated in the inflammatory process.[2] Histamine $H_3$ receptors are located on histaminergic or non-histaminergic neurons, respectively acting as autoreceptors or heteroreceptors, controlling the release and synthesis of histamine[3,4] and of multiple neurotransmitters such as acetylcholine,[5] norepinephrine[6] and dopamine.[7] These data suggested that histamine $H_3$ receptor antagonists could affect a number of behaviors and be useful in the treatment of cognitive deficits associated with a variety of disease states including Alzheimer's disease (AD),[8] attention deficit hyperactivity disorder (ADHD),[9] schizophrenia,[10] and obesity.[11]

The first generation of active histamine $H_3$ receptor ligands was based on a structurally modified imidazole ring.[12] Unfortunately, further pharmacological investigations have proved that the imidazole derivatives show poor brain penetration, CYP450 inhibition, drug–drug interactions, liver toxicity, *etc.*[13,14] For these reasons, and after the successful cloning of the human histamine $H_3$ receptor by Lovenberg,[15] efforts have been directed toward the discovery of histamine $H_3$ receptor antagonists without an imidazole moiety as these compounds may offer improvements in binding affinity, CNS penetration and reduced potential for cytochrome $P_{450}$ enzyme inhibition.[16,17]

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† This article is dedicated to Professor Henk Timmerman on the occasion of his 80th birthday.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c8md00527c

Over the past fifteen years, the imidazole moiety has been successfully replaced with pyrrolidine, piperidine, piperazine and other basic tertiary amines.[18,19]

Although one of the first known histamine $H_3$ receptor antagonists was a guanidine derivative, impromidine, which was found to be a potent agonist of the $H_2$ receptor, only a small number of compounds containing this structure element are described. However, it appears that the guanidine derivatives, both imidazole, *i.e.* 1-cyclohexylmethyl-3-[3-(1*H*-imidazol-4-yl)propyl]guanidinine[20] (Chart 1), and non-imidazole, *i.e.* compound JB 98064 (Chart 1),[21] are potent and selective histamine $H_3$ receptor antagonists.

Based on data reported in patent applications and chemistry papers, many efforts have been made to discover a number of histamine $H_3$ receptor ligands for treatment of cognitive diseases/sleep disorders (*e.g.*, Alzheimer, depression, anxiety, schizophrenia, and narcolepsy).[22–24] Several of these compounds have entered advanced clinical phases.[25,26] In addition to the search for histamine $H_3$ receptor antagonists/inverse agonists for treatment of neurodegenerative diseases, efforts have been made to discover histamine $H_3$ receptor ligands for treatment of metabolic diseases (*e.g.* obesity and dyslipidemia).[27] Based on current knowledge of the involvement of histamine in the regulation of body weight, pharmacological manipulation of the histaminergic system seems to be an obvious target for anti-obesity drugs.[28]

Previously, we have reported the synthesis and preliminary pharmacological *in vitro* characterization of a series of potent histamine $H_3$ receptor non-imidazole antagonists belonging to the class of 1-substituted-1-{ω-[4-(ω-phenoxyalkyl)piperazin-1-yl]alkyl}guanidines.[29]

The prominent members of this family are compounds carrying electron-withdrawing substituents in position 1 of the guanidine moiety or a chlorine atom at position 4 in the benzene ring, **1a** (**ADS-1024**; $pA_2$ = 8.43), **1b** (**ADS-1020**; $pA_2$ =

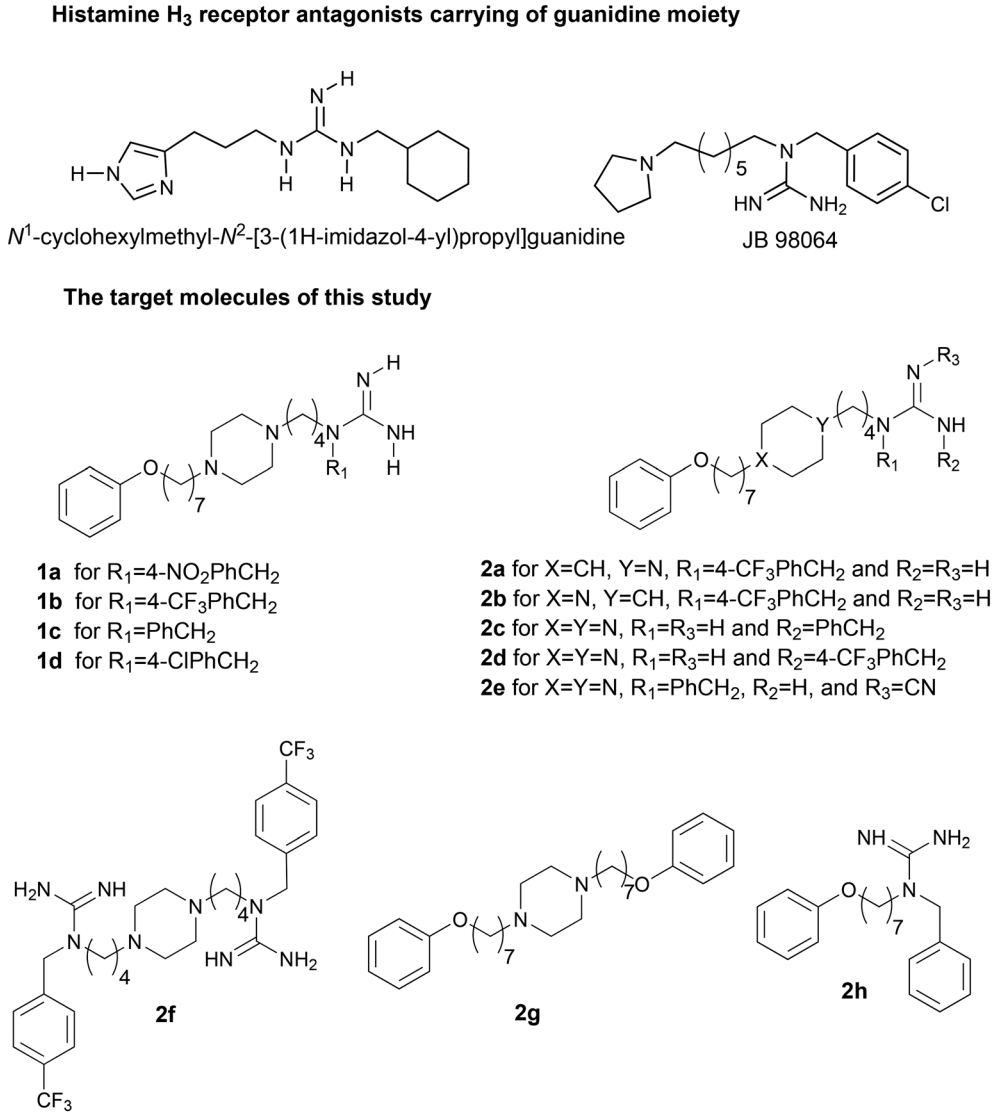


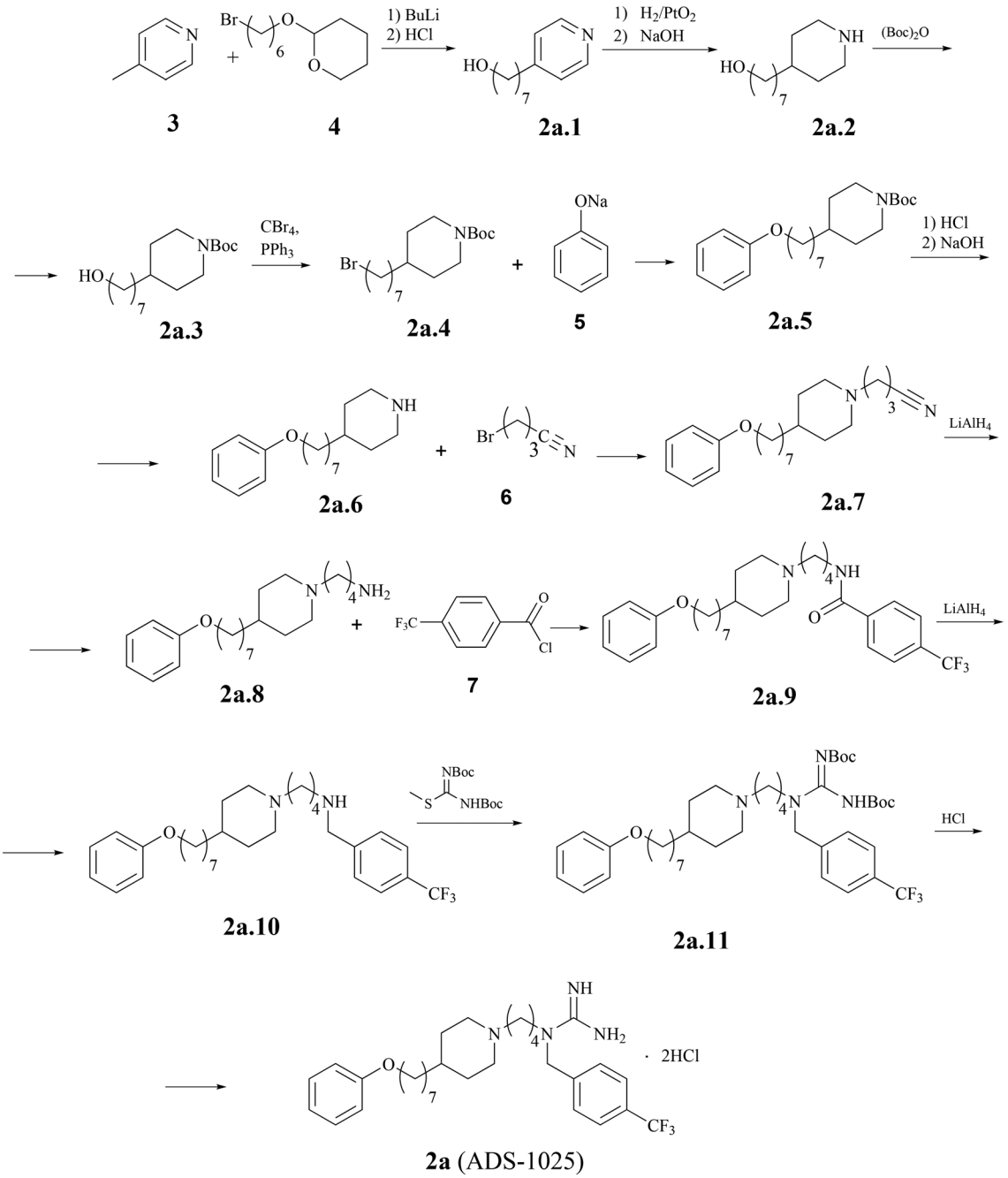

**Chart 1** Example structures of histamine $H_3$ receptor antagonists carrying the guanidine moiety and target molecules of this study.

8.49), **1d**, (**ADS-1022**; $pA_2$ = 7.80), respectively, and an unsubstituted benzyl residue **1c** (**ADS-1017**; $pA_2$ = 8.21) ((Chart 1) for reference **thioperamide** ($pA_2$ = 8.72)). These compounds were also routinely screened for potency towards the histamine $H_1$ receptor and were found to possess weak but competitive $H_1$ antagonistic activity ($pA_2$ = 6.65, $pA_2$ = 6.46, $pA_2$ = 6.62, and $pA_2$ = 6.70, respectively) for this site in the functional assay on isolated tissue preparations of the guinea-pig ileum.

Throughout the study, we choose to retain the length of the linkers connecting the phenoxy and guanidine residues with the piperazine and piperidine rings (the alkyl chain contains seven and four methylene groups, respectively), which is optimal according to our previous results.[29]

In continuation of our search for new highly active and selective non-imidazole histamine $H_3$ receptor antagonists, the first part of this study aimed to clarify whether both nitrogen atoms in the piperazine ring, present in the parent compounds **1a** and **1b**, are necessary to maintain a high activity or whether only one of them (and which one) is absolutely necessary to retain the activity. Therefore, two piperidine analogues, **2a** (**ADS-1025**) and **2b** (**ADS-1026**) (Chart 1), of the lead compound **1b** were synthesized and pharmacologically evaluated (functionally on the *in vitro* test




**Scheme 1** Synthesis of 1-{4-[4-(7-phenoxyheptyl)piperidin-1-yl]butyl}-1-[4-(trifluoromethyl)benzyl]guanidine dihydrochloride – **2a** (**ADS-1025**).

system using guinea pig jejunum preparations).[30] Furthermore, in the piperazine series, the influence of moving the benzyl- and 4-trifluoromethylbenzyl substituents from position 1 to 3 of the guanidine moiety (compounds **2c** (**ADS-1029**) and **2d** (**ADS-1028**) (Chart 1)), and introducing a nitrile group into the $N^2$ guanidine residue (compound **2e** (**ADS-1027**) (Chart 1)) on the histamine $H_3$ receptor antagonistic activity was studied. Finally, three compounds *i.e.* 1,4-bis{4-[1-(4-trifluoromethylbenzyl)guandin-1-yl]but-1-yl}piperazine **2f** (**ADS-1030**), 1,4-bis(phenoxyheptyl)-piperazine **2g** (**ADS-1031**) and 1-benzyl-1-(7-phenoxyhept-1-yl)guanidine **2h** (**ADS-1032**) (Chart 1) were synthesized in order to explain which part of the parent compound **1b** plays the key role in blocking the histamine $H_3$ receptor.

Furthermore, all the newly synthesized compounds were also tested for $H_1$ antagonistic effects *in vitro*, following standard methods, using the guinea pig ileum.[31]

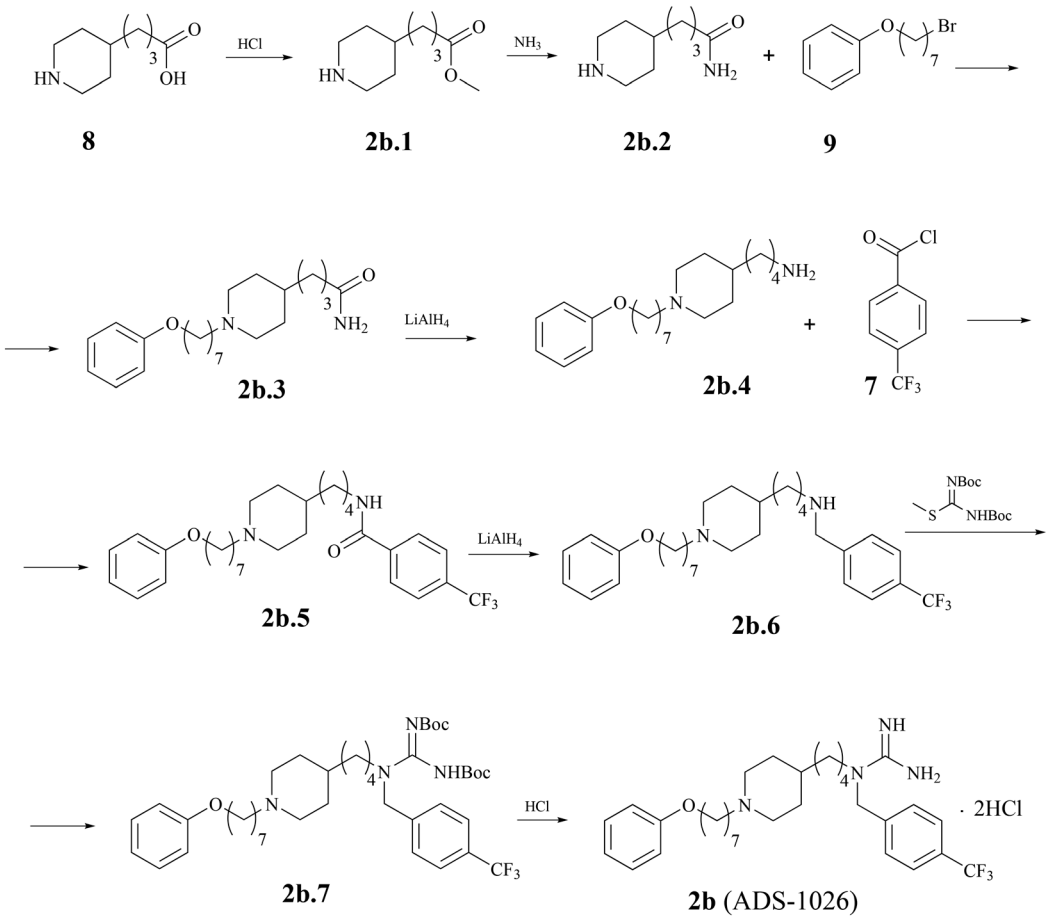
Additionally, the affinity of compounds **1a**, **1c**, and **1d** – the most active compounds that we have so far synthesized in this series – was estimated to the recombinant rat histamine $H_3$ receptor ($rH_3R$) transiently expressed in HEK-293T cells and human histamine $H_3$ and $H_4$ receptors ($hH_3R$, $hH_4R$) on cell membrane preparations from HEK293 with stable expression of the human histamine $H_3$ receptor and from CHO cells stably expressing the human histamine $H_4$ receptor (PerkinElmer, Waltham, MA), respectively. Furthermore, these derivatives were subjected to *in vivo* evaluation of their impact on feeding behavior and brain neurotransmitter systems after repeated peripheral administration to rats. Post-mortem analyses of the rat brain tissues were also carried out to determine the activities of MAO-A, MAO-B, and HNMT.

## 2. Results and discussion

### 2.1. Chemistry

The general synthetic procedures used in this study are illustrated in Schemes 1–7.

1-{4-[4-(7-Phenoxyheptyl)piperidin-1-yl]butyl}-1-[4-(trifluoromethyl)benzyl]guanidine dihydrochloride **2a** (Scheme 1) was prepared by an eleven-step synthesis including deprotonation of the 4-methyl group in 4-methylpyridine **3**, followed by alkylation with 2-(6-bromohexyloxy)tetrahydro-2*H*-pyran **4** to 7-(pyridin-4-yl)heptan-1-ol hydrochloride **2a.1**, hydrogenation with a catalytic amount of platinum dioxide in anhydrous ethanol to 7-(piperidin-4-yl)heptan-1-ol **2a.2**, reaction with di-*tert*-butyl dicarbonate to 7-[1-(*tert*-butoxycarbonyl)piperidin-4-yl]heptan-1-ol **2a.3**, replacement of the hydroxyl group by bromine with tetrabromomethane in the presence of triphenylphosphine to 1-(*tert*-butoxycarbonyl)-




**Scheme 2** Synthesis of 1-{4-[1-(7-phenoxyheptyl)piperidin-4-yl]butyl}-1-[4-(trifluoromethyl)-benzyl]guanidine dihydrochloride **2b** (**ADS-1026**).

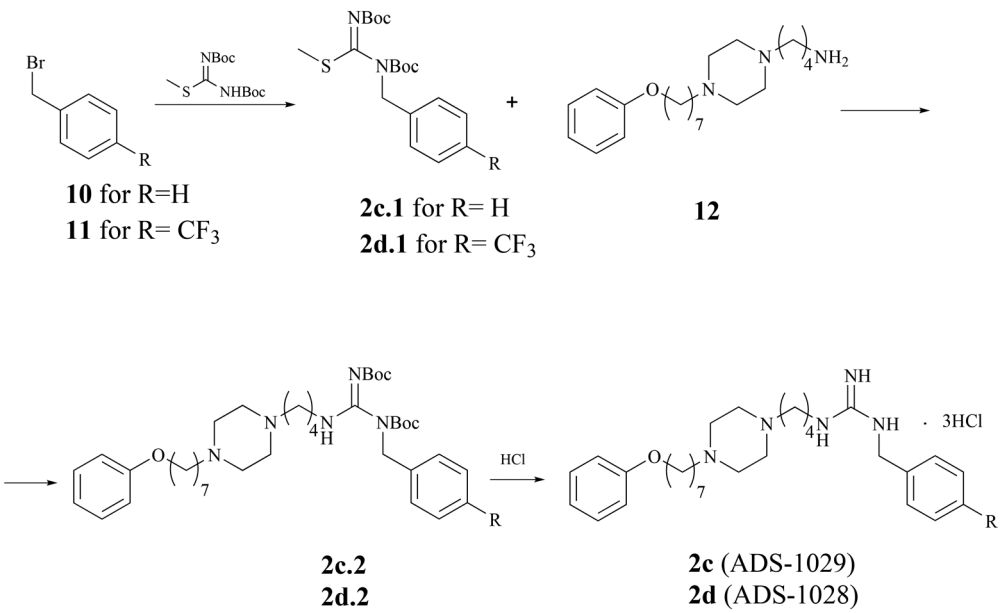
 

4-(7-bromoheptyl)piperidine **2a.4**, etherification with sodium phenoxide **5** in anhydrous ethanol to 1-(*tert*-butoxycarbonyl)-4-(7-phenoxyheptyl)piperidine **2a.5**, removal of the *N*-Boc protecting group in the presence of hydrochloric acid in dioxane to 4-(7-phenoxyheptyl)piperidine **2a.6**, *N*-alkylation with 4-bromobutyronitrile **6** in the presence of potassium carbonate in acetonitrile to 4-[4-(7-phenoxyheptyl)piperidin-1-yl]butanenitrile **2a.7**, reduction with $LiAlH_4$ in dry ethyl ether to 4-[4-(7-phenoxyheptyl)piperidin-1-yl]butan-1-amine **2a.8**, *N*-acylation with 4-(trifluoromethyl)benzoyl chloride **7** in the presence of triethylamine to *N*-{4-[4-(7-phenoxyheptyl)-piperidin-1-yl]butyl}-4-(trifluoromethyl) benzamide **2a.9**, reduction with $LiAlH_4$ in dry ethyl ether to 4-[4-(7-phenoxyheptyl)piperidin-1-yl]-*N*-[4-(trifluoromethyl)benzyl]-butan-1-amine **2a.10**, guanylation with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in the presence of triethylamine and 10% excess of mercury(II) chloride to 2,3-di(*tert*-butoxycarbonyl)-1-{4-[4-(7-phenoxyheptyl)piperidin-1-yl]-butyl}-1-[4-(trifluoromethyl)benzyl]guanidine **2a.11**, and finally acid deprotection of the product from the guanylation to 1-{4-[4-(7-phenoxyheptyl)piperidin-1-yl]butyl}-1-[4-(trifluoromethyl)benzyl]guanidine dihydrochloride **2a**.

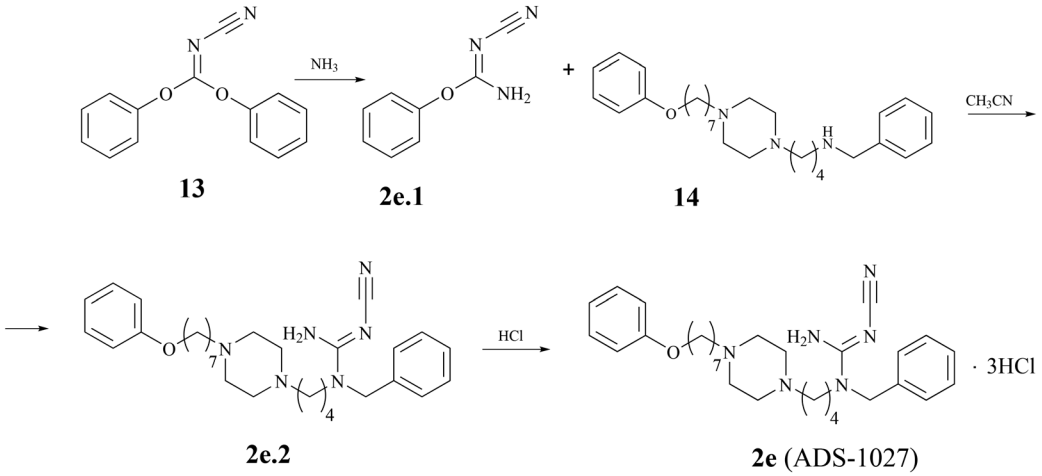
1-{4-[1-(7-Phenoxyheptyl)piperidin-4-yl]butyl}-1-[4-(trifluoromethyl)benzyl]guanidine dihydrochloride **2b** (Scheme 2) was prepared by eight-step synthesis including conversion of 4-piperidine butyric acid hydrochloride **8** to methyl 4-(piperidin-4-yl)butanoate **2b.1**, formation of 4-(piperidin-4-yl)butanamide **2b.2** by the reaction of the ester **2b.1** with aqueous ammonium hydroxide, *N*-alkylation of **2b.2** with 7-phenoxyheptyl bromide to 4-[1-(7-phenoxyheptyl)-piperidin-4-yl]butanamide **2b.3**, reduction with $LiAlH_4$ in dry ethyl ether to 4-[1-(7-phenoxyheptyl)piperidin-4-yl]butan-1-amine **2b.4**, *N*-acetylation with 4-(trifluoromethyl)benzoyl chloride **7** in the presence of triethylamine to *N*-{4-[1-(7-phenoxyheptyl)piperidin-4-yl]butyl}-4-(trifluoromethyl)-benzamide **2b.5**, reduction with $LiAlH_4$ in dry ethyl ether to 4-[1-(7-phenoxyheptyl)piperidin-4-yl]-*N*-[4-(trifluoromethyl)-benzyl]butan-1-amine **2b.6**, guanylation with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in the presence of triethylamine and 10% excess of mercury(II) chloride to 2,3-di(*tert*-butoxycarbonyl)-1-[4-[1-(7-phenoxyheptyl)piperidin-4-yl]-butyl]-1-[4-(trifluoromethyl)benzyl]-guanidine **2b.7**, and finally acid deprotection of the product from the guanylation to 1-{4-[1-(7-phenoxyheptyl)piperidin-4-yl]butyl}-1-[4-(trifluoromethyl)benzyl]-guanidine dihydrochloride **2b**.

1-Benzyl-3-{4-[4-(7-phenoxyheptyl)piperazin-1-yl]butyl}-guanidine trihydrochloride **2c** and 1-[4-[4-(7-phenoxyheptyl)-piperazin-1-yl]butyl]-3-[4-(trifluoromethyl)benzyl]guanidine trihydrochloride **2d** were prepared as outlined in Scheme 3. Treatment of the appropriate benzyl bromide **10** and **11** with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in the presence of 60% sodium hydride oil dispersion in DMF afforded 1,3-di(*tert*-butoxycarbonyl)-1-benzyl-2-methyl-2-thiopseudourea **2c.1** and 1,3-di(*tert*-butoxycarbonyl)-1-[4-(trifluoromethyl)benzyl]-2-methyl-2-thiopseudourea **2d.1**, respectively. Compounds **2c.1** and **2d.1** were reacted with 4-[4-(7-phenoxyheptyl)piperazin-1-yl]butan-1-amine **12** in a mixture of THF–water to give compounds 2,3-di(*tert*-butyloxycarbonyl)-3-benzyl-1-{4-[4-(7-phenoxyheptyl)piperazin-1-yl]butyl}guanidine **2c.2** and 2,3-di(*tert*-butyloxycarbonyl)-1-{4-[4-(7-phenoxyheptyl)-piperazin-1-yl]butyl}-3-[4-(trifluoromethyl)-benzyl]guanidine **2d.2**, respectively. Finally, compounds **2c.2** and **2d.2** were treated with HCl in dioxane to cleave the Boc moieties respectively, affording compounds **2c** and **2d** in quantitative yields.

The synthesis of 1-benzyl-3-cyano-1-{4-[4-(7-phenoxyheptyl)-piperazin-1-yl]but-1-yl}guanidine trihydrochloride **2e** was

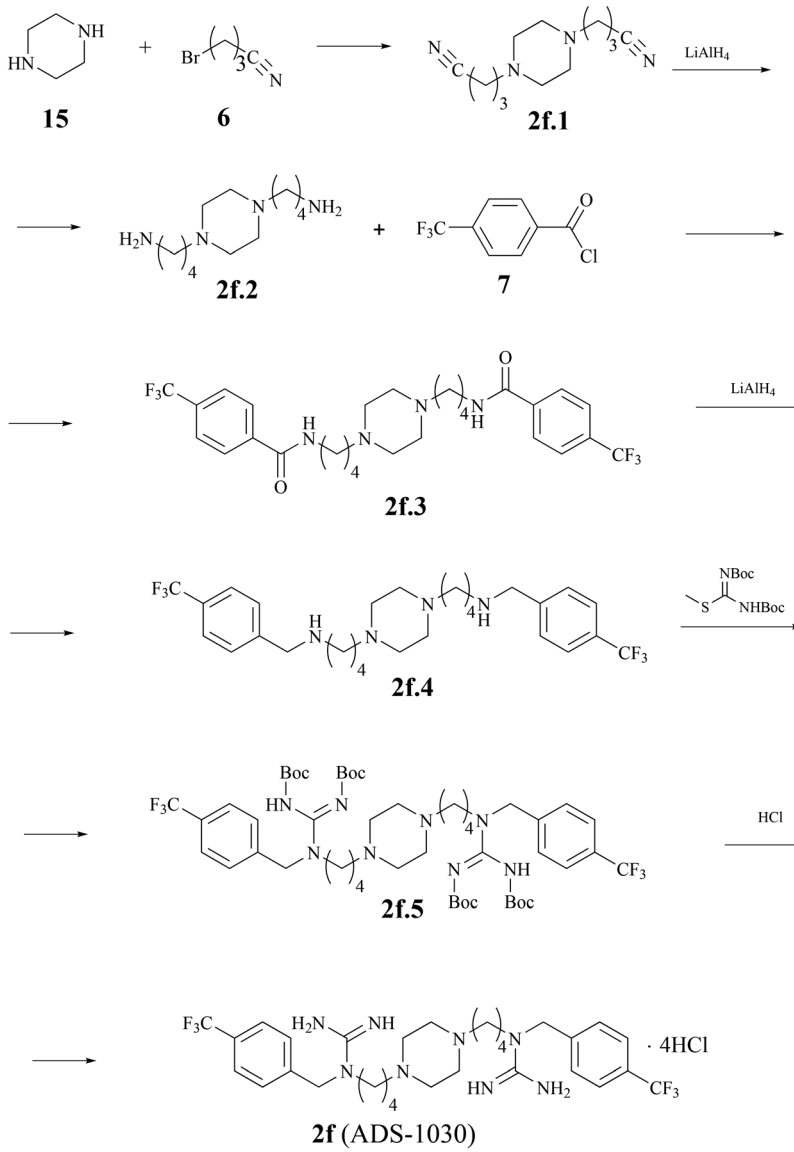



**Scheme 3** Synthesis of 1-benzyl-3-{4-[4-(7-phenoxyheptyl)piperazin-1-yl]butyl}guanidine trihydrochloride **2c** (**ADS-1029**) and 1-[4-[4-(7-phenoxyheptyl)piperazin-1-yl]butyl]-3-[4-(trifluoromethyl)-benzyl]guanidine trihydrochloride **2d** (**ADS-1028**).




13 2e.1 14 2e.2 2e (ADS-1027)

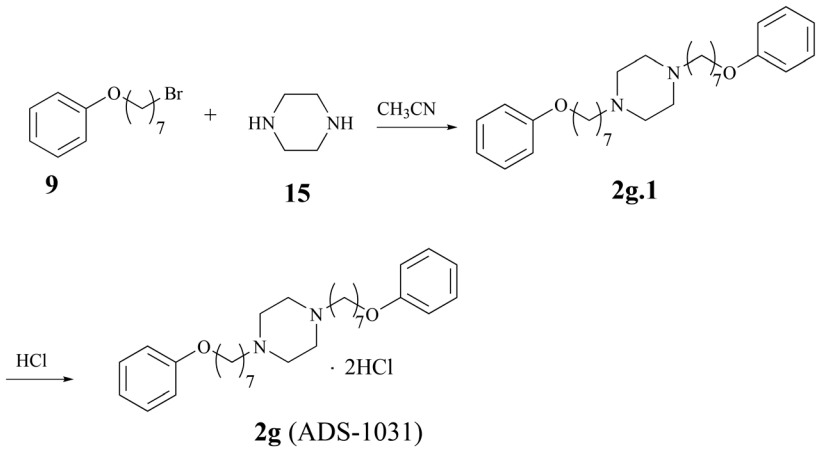
**Scheme 4** Synthesis of 1-benzyl-3-cyano-1-{4-[4-(7-phenoxyheptyl)piperazin-1-yl]but-1-yl}guanidine trihydrochloride **2e** (**ADS-1027**).




15 6 2f.1 2f.2 7 2f.3 2f.4 2f.5 2f (ADS-1030)

**Scheme 5** Synthesis of 1,4-bis{4-[1-(4-trifluoromethylbenzyl)guandin-1-yl]but-1-yl}piperazine tetrahydrochloride **2f** (**ADS-1030**).



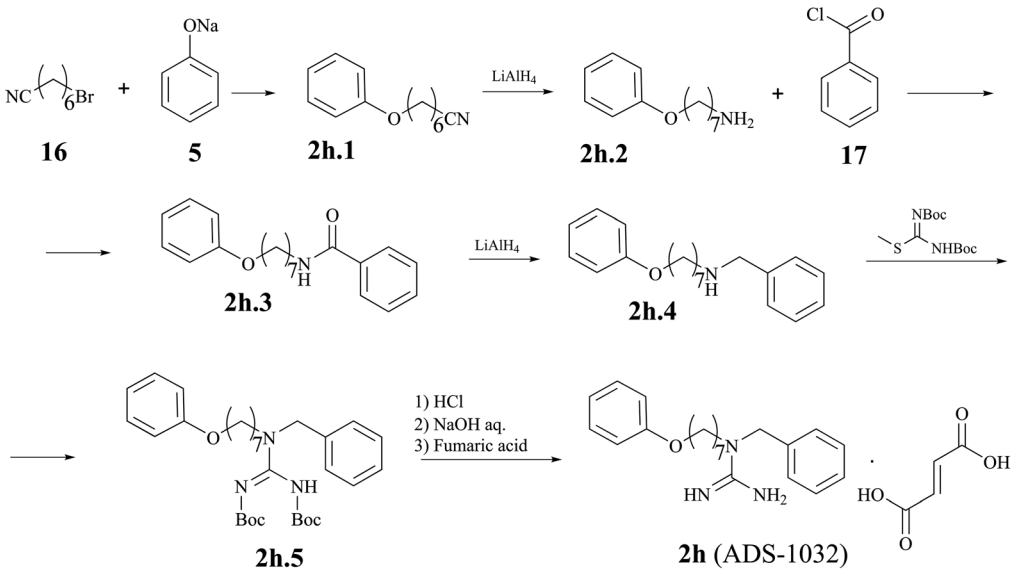

**Scheme 6** Synthesis of 1,4-bis(7-phenoxyheptyl)piperazine dihydrochloride **2g** (**ADS-1031**).

carried out as outlined in Scheme 4. Treatment of *N*-cyanodiphenylimidocarbonate **13** with a 10% ammonia solution in methanol afforded 1-cyano-2-phenylisourea **2e.1**. The reaction of *N*-benzyl-4-[4-(7-phenoxyheptyl)piperazin-1-yl]butan-1-amine **14** with guanylating reagent **2e.1** in acetonitrile yielded **2e.2** which was converted into trihydrochloride salt **2e** in 16% hydrogen chloride–methanol solution.

1,4-Bis{4-[1-(4-trifluoromethylbenzyl)guandin-1-yl]but-1-yl}-piperazine tetrahydrochloride **2f** (Scheme 5) was prepared by six-step synthesis including bis-*N*-alkylation of piperazine **15** with 4-bromobutyronitrile **6** in the presence of potassium carbonate in acetonitrile to 1,4-bis(4-nitrilobutyl)piperazine **2f.1**, reduction with $LiAlH_4$ in dry ethyl ether to 1,4-bis(4-aminobutyl)piperazine **2f.2**, bis-*N*-acylation with 4-(trifluoromethyl)benzoyl chloride **7** in the presence of triethylamine to 1,4-bis[*N*-(4-trifluoromethylbenzoil)aminobut-4-yl]piperazine **2f.3**, reduction with $LiAlH_4$ in dry ethyl ether to 1,4-bis[*N*-(4-trifluoromethylbenzyl)aminobut-4-yl]piperazine **2f.4**, guanylation with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in the presence of triethylamine and 10% excess of mercury(II) chloride to 1,4-bis{4-[1-(4-trifluoromethylbenzyl)-2,3-di(*tert*-butyloxycarbonyl)guandin-1-yl]but-1-yl}piperazine **2f.5**, and finally acid deprotection of the product from the guanylation to 1,4-bis{4-[1-(4-trifluoromethylbenzyl)guandin-1-yl]but-1-yl}piperazine tetrahydrochloride **2f.**

1,4-Bis(7-phenoxyheptyl)piperazine dihydrochloride **2g** (Scheme 6) was obtained by condensing 2 moles of 7-phenoxyheptylbromide **9** with piperazine **15** in acetonitrile with the presence of $K_2CO_3$. The free base was treated with a 4 M solution of hydrogen chloride–dioxane and dihydrochloride was precipitated with dry methanol.

1-Benzyl-1-(7-phenoxyhept-1-yl)guanidine fumarate **2h** (Scheme 7) was prepared by six-step synthesis including etherification of sodium phenoxide **5** with 7-bromoheptanenitrile **16** to 7-phenoxyheptanenitrile **2h.1**, reduction




**Scheme 7** Synthesis of 1-benzyl-1-(7-phenoxyhept-1-yl)guanidine fumarate **2h** (**ADS-1032**).

with $LiAlH_4$ in dry ethyl ether to 7-phenoxyheptan-1-amine **2h.2**, *N*-acylation with benzoyl chloride **17** in the presence of triethylamine to *N*-(7-phenoxyheptyl)benzamide **2h.3**, reduction with $LiAlH_4$ in dry ethyl ether to *N*-benzyl-7-phenoxyheptan-1-amine **2h.4**, guanylation with 1,3-bis(*tert*-butoxycarbonyl)-2-methyl-2-thiopseudourea in the presence of triethylamine and 10% excess of mercury(II) chloride to 1-benzyl-1-(7-phenoxyhept-1-yl)-2,3-di(*tert*-butyloxycarbonyl)-guanidine **2h.5**, and finally acid deprotection of the product from the guanylation to 1-benzyl-1-(7-phenoxyhept-1-yl)guanidine fumarate **2h**.

Detailed synthetic procedure and analytical data for compounds **2a.1–2a.11**; **2b.1–2b.7**; **2c.1**, **2c.2**; **2d.1**, **2d.2**; **2e.1**, **2e.2**; **2f.1–2f.5**, and **2h.1–2h.5** are shown in Appendix A (subsection 1): ESI.‡

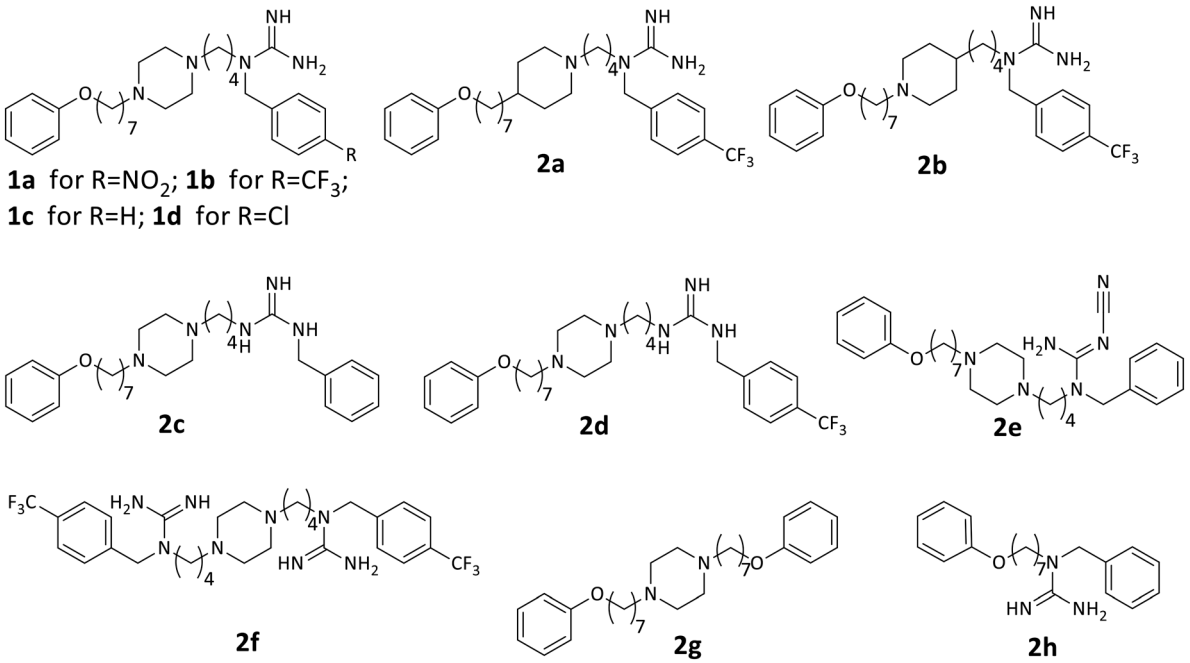
## 2.2. Pharmacology

### 2.2.1. *In vitro* pharmacological studies

*2.2.1.1. Histamine $H_3$ receptor antagonistic activity of compounds **1a–d** and **2a–h**.* The compounds were tested *in vitro* as histamine $H_3$ receptor antagonists against histamine $H_3$ receptor agonist-induced inhibition of the electrically evoked contraction of the guinea-pig jejunum.[30] The potencies of the newly synthesized compounds **2a–e** and **2f–h** are reported in Table 1 including the previously described data for compounds **1a–d** (ref. 29) (Table 1). Derivatives **2a–h** show weak to pronounced antagonist activity against the histamine $H_3$ receptor.

Recently, we described a novel 1-{4-[4-(7-phenoxyheptyl)-piperazin-1-yl]butyl}-1-[4-(substituted)benzyl]guanidine series[29]

**Table 1** Histamine $H_3$- and $H_1$-receptor antagonistic potency in the *in vitro* test system in the Guinea pig jejunum and radioligand binding results on human histamine $H_3$ and $H_4$ receptors for compounds **1a–d** and **2a–h**




| Cpd. | gp p$A_2$ (sem) $H_3$ | *N* (*caviae*) | gp p$A_2$ (sem) $H_1$ | *N* (*caviae*) | r$H_3$R $pK_i$ ± sem | h$H_3$R p$K_i$ ± sem | h$H_4$R p$K_i$ ± sem | h$H_3$R selectivity ($H_4/H_3$) |
|---|---|---|---|---|---|---|---|---|
| **1a** (ref. 29) | **8.43** (0.11) | 10 (3) | **6.65** (0.08) | 9 (*3*) | 7.8 ± 0.1 | 6.84 ± 0.02 | 5.92 ± 0.01 | 8.3 |
| **1b** (ref. 29) | **8.49** (0.05) | 13 (4) | **6.46** (0.06) | 9 (*3*) | | | | |
| **1c** (ref. 29) | **8.21** (0.05) | 12 (4) | **6.70** (0.10) | 12 (*4*) | 7.9 ± 0.1 | 6.80 ± 0.04 | 5.49 ± 0.07 | 20.9 |
| **1d** (ref. 29) | **7.80** (0.1) | 9 (3) | **6.62** (0.15) | 15 (*5*) | 7.6 ± 0.1 | 6.56 ± 0.03 | 5.97 ± 0.03 | 3.9 |
| **2a** | **7.90** (0.05) | 12 (4) | **7.19** (0.05) | 8 (*2*) | | | | |
| **2b** | **8.35** (0.08) | 12 (4) | **6.90** (0.06) | 12 (*4*) | | | | |
| **2c** | **8.10** (0.06) | 12 (4) | **7.10** (0.11) | 8 (*2*) | | | | |
| **2d** | **7.97** (0.02) | 9 (3) | **7.18** (0.01) | 8 (*2*) | | | | |
| **2e** | **7.30** (0.08) | 12 (4) | **6.97** (0.16) | 12 (3) | | | | |
| **2f** | **7.99** (0.08) | 12 (4) | **7.22** (0.10) | 12 (*3*) | | | | |
| **2g** | **5.78** (0.13) | 9 (3) | **NA** | | | | | |
| **2h** | **5.78** (0.03) | 9 (3) | **6.80** (0.25) | 8 (*2*) | | | | |
| **Thioperamide** | **8.98** (0.08) | 16 (5) | | | 7.9 ± 0.1 | 6.75 ± 0.11 | 7.34 ± 2.55 | 0.3 |
| **Pyrilamine** | | | **9.18** (0.05) | 20 (5) | | | | |

sem: standard error of the mean; *N*: number of different animal preparations; *caviae*: number of animals. gp: guinea pig; r: rat; h: human.

with various substituents at position 4 in the benzyl moiety. The introduction of strong electron-withdrawing substituents led to compounds **1a** ($pA_2$ = 8.43) and **1b** ($pA_2$ = 8.49) showing the highest potency for that series. Slightly lower potency was shown by derivative **1c** ($pA_2$ = 8.21) bearing an unsubstituted benzyl moiety. Additionally, all the compounds (**1a–1d**) showed weak, but competitive $H_1$-antagonistic activity, with $pA_2$ ranging from 6.46 to 6.70.

With the aim of optimizing the structure of the lead compound **1b**, we wanted to clarify whether both nitrogen atoms in the piperazine ring, present in the parent compound **1b**, are necessary to maintain a high activity or whether only one of them (and which one) is absolutely necessary to retain the activity. Therefore, two piperidine analogues, **2a** – where the butyl-[4-(trifluoromethyl)benzyl]guanidine residue was directly bonded to the piperidine nitrogen, and **2b** – where the 7-phenoxyheptyl moiety was attached to the piperidine nitrogen, were synthesized and pharmacologically evaluated. On comparing the potency of **2a** ($pA_2$ = 7.90) and **2b** ($pA_2$ = 8.35) with that of the parent compound **1b** ($pA_2$ = 8.49; Table 1), it was shown that the piperidine analogues possessed slightly lower antagonistic activity than the lead compound **1b** containing the piperazine scaffold, but derivative **2b** possessed higher potency to the $H_1$ receptor ($pA_2$ = 6.90) than the parent compound **1b** ($pA_2$ = 6.46). Taking into consideration the obtained results, we chose to retain the 1-butyl-4-(7-phenoxyheptyl)piperazine moiety as an optimal fragment for further structural modification. Therefore, the influence of moving the benzyl- and 4-trifluoromethylbenzyl substituents from $N^1$ to the $N^3$ position of the guanidine moiety in compounds **2c** and **2d** (Table 1) on the antagonistic activity against the histamine $H_3$ receptor was investigated. As seen in the case of the piperidine analogues, both 1,3-disubstituted guanidines (**2c** and **2d**) were less active ($pA_2$ = 8.10 and $pA_2$ = 7.97) than the corresponding 1,1-disubstituted guanidines **1c** and **1b** ($pA_2$ = 8.21 and $pA_2$ = 8.49, respectively). Slightly higher potency was demonstrated by derivative **2c** with an unsubstituted benzyl ring than its 4-trifluoromethylbenzyl analogue **2d**. Additionally, in order to decrease the guanidine basicity, a nitrile group was introduced into the $N^2$ nitrogen of derivative **1c** which resulted in compound **2e** showing the lowest potency ($pA_2$ = 7.3) within the series of compounds **2a–e** (Table 1). Because of the lower potency of **2e** compared to **1c**, we might conclude that the decreasing basicity of the $N^2$ nitrogen has a negative effect on blocking the histamine $H_3$ receptor.

Finally, in order to explain which synthon of the parent compounds **1b** and **1c** plays the key role in blocking the

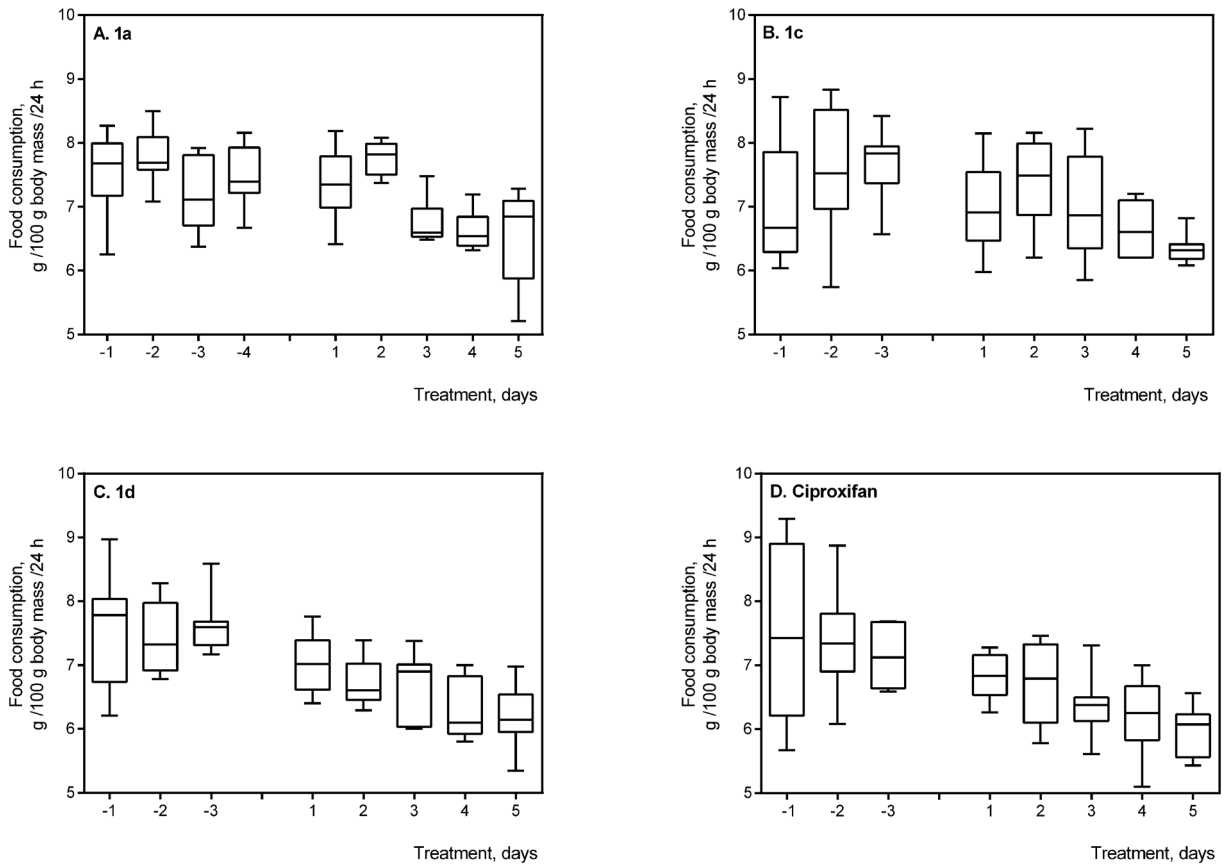



**Fig. 1** Daily food consumption during pre-test (three days) and five days of treatment with the newly synthesized histamine $H_3$ receptor antagonists **1a**, **1c**, and **1d**, and ciproxifan. Ciproxifan, a histamine $H_3$-receptor antagonist/inverse agonist, was used as a reference compound. The median (the line in the middle of the box) and the range of values (whiskers) are given for eight rats.

histamine $H_3$ receptor, three compounds were synthesized and pharmacologically evaluated *in vitro* (Table 1): 1,4-bis{4-[1-(4-trifluoromethylbenzyl)guandin-1-yl]but-1-yl}piperazine tetrahydrochloride **2f**, 1,4-bis(7-phenoxyheptyl)piperazine **2g**, and 1-benzyl-1-(7-phenoxyhept-1-yl)guanidine **2h**. Bisguanidine derivative **2f** showed potency ($pA_2$ = 7.99; Table 1) on the same level as compound **2d** ($pA_2$ = 7.97; Table 1). In the case of bis(7-phenoxyheptyl) derivative **2g**, a drastic reduction of affinity ($pA_2$ = 5.78; Table 1) was observed. Removal of the 1-butylpiperazine fragment from derivative **1c** resulted in compound **2h**, with only a weak activity ($pA_2$ = 5.78; Table 1) against the $H_3$ receptor. Based on the obtained results, it is seen that the trifluoromethylbenzylguandinbutyl moiety plays the key role in blocking the histamine $H_3$ receptor in the piperazine series.

All the graphs of antagonism by **2a–h** and thioperamide and the inhibitory effect of *R*-(−)-α-methyl-histamine (RAMH) on the electrically induced contraction of guinea-pig ileum strips are shown in Appendix B (subsection 2.2.1; Fig. 1): ESI.‡

*2.2.1.2. $H_1$ antagonistic activity of compounds **2a–h**.* Due to the fact that the most active previously obtained compounds (**1a–d**) showed weak, competitive $H_1$-antagonistic activity, all the newly synthesized compounds (**2a–h**) were also tested for $H_1$ antagonistic effects *in vitro*, following standard methods, using the guinea pig ileum.[31] The highest potency to the $H_1$ receptor was seen for compounds **2a**, **2c**, **2d** and **2f** ($pA_2$ = 7.19, $pA_2$ = 7.10, $pA_2$ = 7.18, and $pA_2$ = 7.22, respectively; Table 1). A similar potency for the histamine $H_1$ receptors and histamine $H_3$ receptors was observed for compound **2e** ($H_1pA_2$ = 6.97, $H_3pA_2$ = 7.30) (Table 1). In the case of compound **2h** (where the butylpiperazine fragment was removed) the antagonistic activity against the $H_1$ receptor was higher ($pA_2$ = 6.8) than that against the histamine $H_3$ receptor ($pA_2$ = 5.78; Table 1). No activity for the $H_1$ receptor was observed for derivative **2g** only, but a drastic reduction of potency was observed for the histamine $H_3$ receptor ($pA_2$ = 5.78), compared to the parent compound **1c** ($pA_2$ = 8.21). All the graphs of antagonism by **2a–h** and pyrilamine and the inhibitory effect of histamine on the contraction of guinea-pig ileum strips are shown in Appendix B (subsection 2.2.2; Fig. S2): ESI.‡

**2.2.2. Histamine $H_3$ and $H_4$ receptor affinity.** Taking into consideration the potency of the newly synthesized compounds **2a–h**, the parent compounds **1a**, **1c**, and **1d** demonstrated significantly higher potency. For this reason, derivatives **1a**, **1c**, and **1d** were evaluated for their affinity to the recombinant rat histamine $H_3$ receptor ($rH_3R$) and human histamine $H_3$ and $H_4$ receptors ($hH_3R$ and $hH_4R$).

*2.2.2.1. Histamine $H_3$ receptor affinity studies.* The affinity of the aforementioned compounds was evaluated by measuring the displacement curve of [$^3$H]-N$^\alpha$-methylhistamine on the rat ($rH_3R$) histamine $H_3$ receptor in HEK-293T cell membranes as described by Bongers.[32]

*2.2.2.1.1. Competitive binding of rat histamine $H_3$ receptor ligands.* Saturation of the rat histamine $H_3$ receptor – membranes expressing $rH_3R$ were incubated with different concentrations of [$^3$H]-N$^\alpha$-MH (0–20 nM) in the absence or presence of unlabeled thioperamide (10 μM) to determine the total and non-specific binding, respectively, for 2 h at 25 °C. The reaction was terminated by rapid filtration on GF/C 96 well plates and the bound radioligand was measured by scintillometry. Specific binding was defined as the difference between the total and non-specific binding conditions. A representative graph of the saturation of the rat histamine $H_3$ receptor can be found in Appendix B (subsection 2.2.3; Fig. S3): ESI.‡

Analysis of the [$^3$H]-N$^\alpha$-MH saturation binding yielded on the rat histamine $H_3$ receptor a $K_D$ value of 2.72 ± 0.34 nM and a $B_{max}$ value of 2715 ± 445 fmol mg$^{-1}$ protein and on the human histamine $H_3$ receptor a $K_D$ value of 0.9 ± 0.08 nM and a $B_{max}$ value of 632 ± 52 fmol mg$^{-1}$ protein.

Competitive radioligand binding – the affinity of **1a**, **1c**, **1d**, histamine, and thioperamide – the reference compound, was determined by measuring the displacement curves of [$^3$H]-N$^\alpha$-methylhistamine binding on the rat histamine $H_3$ receptor expressed in HEK-293T membranes. Derivatives **1c** and **1a** possess a high nanomolar affinity for the rat histamine $H_3$ receptor with a $pK_i$ value of 7.9 ± 0.1 and 7.8 ± 0.1, respectively. Slightly lower affinity is seen for compound **1d** with a $pK_i$ value of 7.6 ± 0.1 in comparison with the $pK_i$ of thioperamide ($pKi$ value = 7.9 ± 0.1). All the investigated compounds show slightly higher affinity than histamine ($pK_i$ = 7.3 ± 0.1). A representative graph of the competitive binding of histamine $H_3$ receptor ligands on the rat histamine $H_3$ receptor can be found in Appendix B (subsection 2.2.2.1; Fig. S4): ESI.‡

*2.2.2.2. Competitive binding of human $H_3$ and $H_4$ receptor ligands.* Previously, compounds **1a**, **1c**, and **1d**, and thioperamide – the reference compound – were investigated for human histamine $H_3$ and $H_4$ receptor *in vitro* binding studies.[33] [$^3$H]N$^\alpha$-methylhistamine was used as the radioligand in binding assays to test the affinity to the human recombinant histamine $H_3$ receptor stably expressed in HEK293 cells[34] (the experimentally determined radioligand $K_D$ value of 0.34 nM was used). [$^3$H]histamine was used as the radioligand in binding assays to test the affinity on membrane preparations from CHO cells, expressing the recombinant human histamine $H_4$ receptor[35] (the experimentally determined radioligand $K_D$ value of 9.70 nM was used). Pharmacological results are presented in Table 1.

Based on preliminary functional pharmacological studies, subsection 2.2.1.1, the radioligand binding studies confirmed the affinity of the investigated derivatives **1a**, **1c**, and **1d** with the human histamine $H_3$ receptor (Table 1). All the investigated compounds showed good affinities for the human histamine $H_3$ receptor in a $pK_i$ range of 6.84–6.56 in comparison with the $pK_i$ of thioperamide ($K_i$ value 6.75). The competitive radioligand binding assay on $hH_4R$ revealed weak selectivity towards the human histamine $H_3$ receptor for derivatives **1a** and **1d** carrying a substituent at position 4 in the *N*-benzylguanidine moiety. Significant selectivity towards the

histamine $H_3$ receptor was shown for unsubstituted *N*-benzylguanidine derivative **1c** (Table 1). This compound also showed higher affinity to the human histamine $H_3$ receptor with a $pK_i$ value of 6.80 compared with thioperamide ($pK_i$ value = 6.75).

**2.2.3. Verification of *in vivo* activity for compounds 1a, 1c and 1d.** Furthermore, derivatives **1a**, **1c**, and **1d** were subjected to preliminary *in vivo* evaluation of their impact on brain neurotransmitter systems. This assessment concerned:

- The effects of the compounds on the feeding behavior of rats after their repeated peripheral administration.

- The influence on the cerebral amine neurotransmitter concentrations, as well as the activity of monoamine oxidases A and B and histamine *N*-methyltransferase. The latter was accomplished by *postmortem* analyses of the brain tissue of the treated rats.

Derivatives **1a**, **1c** and **1d** were chosen for the aforementioned studies and not one of the latest compounds, as for this class of non-imidazole antagonists so far no proof of *in vivo* activity has been reported. As such, we have chosen the best compounds available for the *in vivo* studies.

In accordance with the earlier reported experimental data,[36–38] it was assumed that if a histamine $H_3$ receptor antagonist subcutaneously injected into rats crossed the blood–brain barrier (BBB), it would affect the animal food consumption. To evaluate the central effects of peripheral administration of **1a**, **1c**, and **1d** to rats, the feeding behavior of rats was monitored after each drug administration.

As presented in Fig. 2, treatment of rats with either of the four histamine $H_3$ receptor antagonists **1a** (3 mg $kg^{-1}$ s.c.), **1c** (3 mg $kg^{-1}$ s.c.) and **1d** (3 mg $kg^{-1}$ s.c.) and ciproxifan (3 mg $kg^{-1}$ s.c. – based on the literature data[39] the dose 3 mg $kg^{-1}$ was selected to evaluate the central effects of peripheral administration of derivatives **1a**, **1c**, and **1d** to rats on their feeding behavior) over a period of five days evoked a statistically significant reduction in the amount of consumed food by rats compared to the pre-treatment period.

Based on *in vitro* and *in vivo* studies in the literature,[40] ciproxifan was shown as a pure competitive antagonist of the histamine $H_3$ receptor. Therefore, ciproxifan was used for the *in vivo* studies, instead of thioperamide because the *in vivo* potency of thioperamide is rather low compared with it's *in vitro* potency, which can result in restricted drug bioavailability, particularly its brain penetration.

Male Wistar or Lewis rats were used to verify the *in vivo* efficacy of the selected histamine $H_3$ receptor ligands. Four days before the experiment, the rats were placed individually in metabolic cages (Tecniplast Gazzada, Italy) with free access to feed and water. After a one-day adaptation period to the new conditions, food consumptions were monitored daily, between 8:00–8:30 a.m., for the next three days and the means related to the pretreatment were calculated. Each of the tested compounds was given in a dose of 3 mg $kg^{-1}$ per day, subcutaneously, for five days. Ciproxifan was employed as the reference. Every morning, the consumed food was recorded. Fig. 1 illustrates a decrease in the daily food intake upon the repeated drug administration.

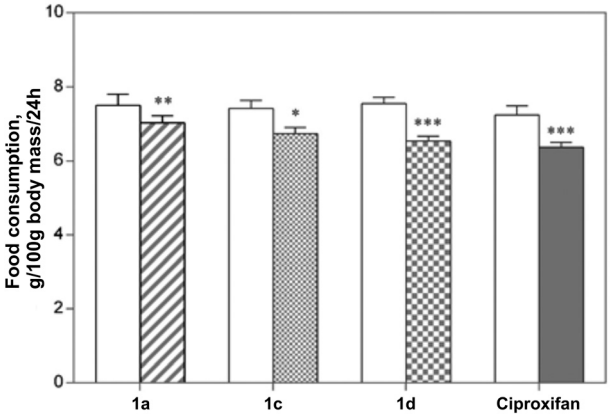



**Fig. 2** The effect of the newly synthesized histamine $H_3$ receptor antagonists **1a** (s.c. 3 mg $kg^{-1}$ daily for 5 days), **1c** (s.c. 3 mg $kg^{-1}$ daily for 5 days), and **1d** (s.c. 3 mg kg daily for 5 days) on food consumption. Ciproxifan (s.c. 3 mg $kg^{-1}$ daily for 5 days), a histamine $H_3$-receptor antagonist/inverse agonist, was used as a reference compound. The values are mean ± sem. All white bars correspond respectively to food consumed during the 3-day pre-test, whereas bars with patterns correspond to food consumed during the 5-day treatment with appropriate drugs. Paired *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs.* before treatment.

In Fig. 2 the effect of five-day treatment *versus* pretreatment is presented as related to the consumed food. It can be inferred from these data that all the tested histamine $H_3$ receptor ligands have entered the brain for they significantly modified the feeding pattern, with compound **1d** exhibiting a potency similar to that of ciproxifan.

A statistically significant decline in food consumption was noted in rats treated with all the compounds examined (Fig. 1 and 2); **1d**, being the most effective, caused a total of 13.5% reduction in food consumption compared with the results recorded before administration of the drug (7.54 ± 0.17 *vs.* 6.52 ± 0.13 g per 100 g of body weight) (Fig. 2, $p < 0.001$). This effect was most noticeable on the 5th day (Fig. 1) and was similar to that caused by ciproxifan (Fig. 1 and 2). **1a** decreased the pellet consumption to a lesser extent by 10%, *i.e.* 7.42 ± 0.22 *vs.* 6.73 ± 0.17 g per 100 g of body weight, $P = 0.0205$ (Fig. 2). The lowest reduction in food consumption was noted for compound **1c**.

*2.2.3.1. Post-mortem biochemical analysis of the brain tissues of **1a**, **1c**, and **1d** treated rats.* To find out whether there are any post-treatment alterations in the cerebral amine neurotransmitter systems, after the last record was taken in metabolic cages the rats were sacrificed and their brains were collected to measure the amine concentrations by RIA or ELISA (DIAsource ImmunoAssays S.A., Nivelles, Belgium), as well as the histamine *N*-methyltransferase and monoamine oxidase (MAO) activities by radioisotopic assays.

None of the tested compounds influenced the histamine or noradrenaline concentrations in the rat cortex. However, cortical concentrations of serotonin and dopamine were significantly lower in rats administered with compounds **1a** and **1c**, respectively, when compared to the concentrations measured in ciproxifan-treated rats. The serotonin concentration

was roughly one third and the dopamine concentration two-thirds of those obtained in ciproxifan-treated rats. A representative graph of cerebral amine neurotransmitter concentration in rats subchronically treated with the newly synthesized **1a**, **1c**, and **1d** histamine $H_3$ receptor antagonists and the reference compound ciproxifan can be found in Appendix B (subsection 2.2.3.1; Fig. S5): ESI.‡

The activities of the brain catabolic enzymes have not been affected by the tested histamine $H_3$ receptor ligands. Clearly, the MAO enzymes do not contribute to the changes in serotonin or dopamine concentrations resulting from the treatment with **1a**, **1c**, and **1d**. The effect of subchronic administration of **1a**, **1c** and **1d** (s.c. 3 mg $kg^{-1}$ daily for 5 days) and ciproxifan (s.c. 3 mg $kg^{-1}$ daily for 5 days) on cerebral MAO and HNMT activities can be found in Appendix B (subsection 2.2.3.1; Table S1): ESI.‡

# Conclusions

Of both series (**1a–d** and **2a–h**), the 1-substituted-1-{4-[4-(7-phenoxyalkyl)piperazin-1-yl]butyl}guanidine derivatives **1a** ($pA_2$ = 8.43) and **1b** ($pA_2$ = 8.49) carrying electron-withdrawing substituents at position 4 in the benzene ring demonstrated the highest *in vitro* potency as histamine $H_3$ receptor antagonists. For **1a** and **1b**, the *in vitro* potency was found to slightly decrease when the piperazine scaffold was replaced by a piperidine ring (**2a** – $pA_2$ = 7.90 and **2b** – $pA_2$ = 8.35). In the piperazine series, transfer of the benzyl- and 4-trifluoromethylbenzyl substituents from the $N^1$ to the $N^3$ position of the guanidine moiety resulted in further decreases in potency (**2c** – $pA_2$ = 8.10 and **2d** – $pA_2$ = 7.97). Decreasing the guanidine basicity following the introduction of a nitrile substituent into the $N^2$ nitrogen derivative **1c** resulted in compound **2e**, showing the lowest potency ($pA_2$ = 7.3) within the series of compounds **2a–e**. In the series of compounds **2f–h**, where the synthons: phenoxyheptyl-, trifluoromethylbenzylguandinbutyl-, and 1-piperazinynylobutyl- were removed from the structure of the parent compound **1b**, only bisguanidine derivative **2f** showed a similar potency ($pA_2$ = 7.99) to **2d** ($pA_2$ = 7.97). Compounds **2g** and **2h** showed weak potency ($pA_2$ = 5.78, $pA_2$ = 5.78, respectively) for the histamine $H_3$ receptor. All the newly obtained compounds **2a–h** ($pA_2$ from 6.90 to 7.22) exhibited higher potency for the $H_1$ receptor than the lead compounds **1a–d** ($pA_2$ from 5.85 to 6.7). Competitive radioligand binding studies on the rat histamine $H_3$ receptors for compounds **1a**, **1c** and **1d** showed an affinity ($pK_i$ from 7.9 to 7.6, respectively) on a comparable level to the reference compound – thioperamide ($pK_i$ = 7.9), confirming the high potency shown by the tested compounds in the functional assay in the guinea pig jejunum. Additionally, competitive radioligand binding studies on the human histamine $H_3$ receptor also showed good affinity for these compounds with a $pK_i$ value ranging from 6.84 to 6.56 while a $pK_i$ value of 6.75 was found for thioperamide. The competitive radioligand binding assay on the $H_4$ receptor revealed weak selectivity towards the human histamine $H_3$ receptor for compounds **1a** and **1d**. The highest histamine $H_3$ receptor selectivity was seen for compound **1c**; ratio $K_iH_4/K_iH_3$ = 20.9.

Compounds **1a**, **1c** and **1d**, given parenterally for five days, reduced the food intake of rats. Of the tested compounds, the best turned out to be **1d** which had a similar potency to ciproxifan in terms of the food consumption decrease; a somewhat lower effect was found for **1c** and a lower one for **1a**. Post-mortem biochemical analysis of the brain tissues of **1a**, **1c**, and **1d** treated rats indicates that none of the tested compounds influenced the brain histamine or noradrenaline concentrations. However, significantly reduced serotonin and dopamine concentrations were found in the rats administered with compounds **1a** and **1c**, respectively.

The high potency and affinity for $H_3$ receptors and the *in vivo* activity warrant further study on compounds **1a** and **1c**.

# 3. Experimental section

**General methods**

All melting points (mp) were measured in open capillaries on electrothermal apparatus and are uncorrected. All $^1$H NMR and $^{13}$H NMR spectra were recorded on Varian Mercury VX 300 MHz and Bruker Avance III 600 MHz ($^1$H NMR spectra were run at 600 MHz, while $^{13}$C NMR spectra were run at 150.95 MHz) spectrometers in deuterated dimethyl sulfoxide (DMSO-$d_6$), chloroform-d and deuterium oxide. Chemical shifts are expressed in ppm downfield from internal TMS as a reference. $^1$H NMR data are reported in the order: multiplicity (br, broad; s, singlet; d, doublet; t, triplet; q, quartet; qt, quintet; m, multiplet; *, exchangeable by deuterium oxide), number of protons, and approximate coupling constant in Hertz. Elemental analysis (C, H, and N) for all compounds was conducted on a Perkin Elmer Series II CHNS/O Analyzer 2400 and results are within ±0.4% of the theoretical values. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 plates (Merck) and visualized using a UV Lamp (254 nm) and cerium molybdate stain. Flash column chromatography was performed on silica gel 60 Å 50 mm (J. T. Baker B. V.) and Normasil 60 silica gel 40–63 μm (VWR Chemicals), employing the eluent indicated by TLC.

2-(6-Bromohexyloxy)tetrahydro-2*H*-pyran, di-*tert*-butyl dicarbonate, tetrabromomethane, 4-bromobutyronitrile, 4 M solution hydrogen chloride in dioxane, 4-(trifluoromethyl)-benzoyl chloride, 4-piperidine butyric acid hydrochloride, benzyl bromide, 4-(trifluoromethyl)benzyl bromide, and piperazine were purchased from commercial suppliers (Aldrich, TCI, Fluorochem, Fluka) and used without further purification.

*N*-Benzyl-4-(4-(7-phenoxyheptyl)piperazin-1-yl)butan-1-amine was prepared according to Staszewski and Walczyński.[29]

### 3.1. Chemistry

**General procedure for the preparation of guanidines 2a, 2b, 2c, 2d, 2f and 2h.** To a solution of the appropriate 2,3-

bis(*tert*-butoxycarbonyl)guanidine (**2a.11**, **2b.7**, **2c.2**, **2d.2**, **2f.5** and **2h.5**) (1 eq.) in chloroform, a 4 M solution of hydrogen chloride–dioxane (20 eq.) was added dropwise. The reaction was stirred overnight at room temperature and then the solvent was removed under vacuum. The crude product was evaporated twice with chloroform and twice with EtOAc, then recrystallized from dry ethanol or methanol to yield the pure product. In the case of compound **2h**, the following procedure was used: the crude product was evaporated twice from chloroform and twice from EtOAc then evaporated, dissolved in methanol, alkalized by 5% sol. of sodium hydroxide, extracted with DCM and dried over $Na_2SO_4$. The crude product was dissolved in methanol and 1 eq. fumaric acid was added. After 30 minutes the solvent was evaporated, then the product was recrystallized from dry 2-propanol to yield the pure product.

***2a*** – *1-{4-[4-(7-phenoxyheptyl)piperidin-1-yl]butyl}-1-(4-(trifluoromethyl)-benzyl)guanidine dihydrochloride.* $C_{31}H_{45}F_3N_4O{\cdot}2HCl$. *M* = 619.65. White solid. 89.1% yield. mp: 159.6–161.5 °C. $^1$H NMR (600 MHz, $D_2O$) $\delta$ ppm 7.82–7.81 (m, $2H^{arom.}$, $(CH)_2CCF_3$), 7.52–7.51 (m, $2H^{arom.}$, $(CHCH)_2CCF_3$), 7.44–7.41 (m, $2H^{phenoxy.}$, $C(CHCH)_2CH$), 7.10–7.07 (m, $3H^{phenoxy.}$, $C(CHCH)_2CH$), 4.73 (s, 2H, $CH_2Ph$), 4.15–4.13 (t, 2H, $OCH_2$, *J* = 6.52 Hz), 3.52–3.47 (m, 4H: $2H^{alif.}$ $CH_2CH_2NC(N)$, $2H^{piperidine}$ $CH(CH_2CH_2)_2N$), 3.06–3.04 (m, 2H, $CH_2N^{piperidine}$), 2.88–2.84 (m, $2H^{piperidine}$, $CH(CH_2CH_2)_2N$), 1.99–1.97 (m, $2H^{piperidine}$, $CH(CH_2CH_2)_2N$), 1.83–1.78 (qt, 2H, $OCH_2CH_2$), 1.71–1.70 (m, 4H, $NCH_2CH_2CH_2CH_2N$), 1.57–1.55 (m, $1H^{piperidine}$, $CH(CH_2CH_2)_2N$), 1.51–1.46 (m, 2H, $OCH_2CH_2CH_2$), 1.43–1.30 (m, $10H^{alif.}$: $CHCH_2$, $CH_2CH_2CH_2$). $^{13}$C NMR (600 MHz, *DMSO*) $\delta$ ppm 158.54 ($1C^{quat./phenoxy.}$, CO), 156.55 ($1C^{quat.}$, C═NH), 140.79 ($1C^{quat./arom.}$, $CCH_2$), 129.33 ($2C^{phenoxy.}$, $C(CHCH)_2CH$), 128.35, 128.14, 127.93, 127.72 ($1C^{arom}$, $CCF_3$), 127.36 ($2C^{arom.}$, $(CHCH)_2CCF_3$), 126.81, 125.01, 123.20, 121.40 (1C, $CF_3$), 125.43, 125.41 ($2C^{arom.}$, $(CH)_2CCF_3$), 120.27 ($1C^{phenoxy.}$, $C(CHCH)_2CH$), 114.29 ($2C^{phenoxy.}$, $C(CHCH)_2CH$), 67.15 (1C, $PhOCH_2$), 55.32 (1C, $CH_2N^{piperidine}$), 51.71 ($1C^{piperidine}$, $CH(CH_2CH_2)_2N$), 51.70 ($1C^{piperidine}$, $CH(CH_2CH_2)_2N$), 50.29 (1C, $NCH_2Ph$), 47.86 (1C, $CH_2CH_2NC(N)$), 35.19 (1C, $O(CH_2)_3CH_2$), 32.83 ($1C^{piperidine}$, $CH(CH_2CH_2)_2N$), 28.94 ($1C^{piperidine}$, $CH(CH_2CH_2)_2N$), 28.67 (1C, $CH_2CH_2CH_2$), 28.57 (3C: $1C^{piperidine}$, $CH(CH_2CH_2)_2N$, $2C^{alif.}$, $CH_2CH_2CH_2$), 25.69 (1C, $O(CH_2)_2CH_2$), 25.38 (1C, $CH_2CH_2CH_2$), 24.05 (1C, $CH_2CH_2NC(N)$), 20.02 (1C, $CH_2CH_2CH_2$). Anal. calcd: C, 60.09; H, 7.65; N, 9.04. Found: C, 59.69; H, 8.05; N, 9.06.

***2b*** – *1-{4-[1-(7-phenoxyheptyl)piperidin-4-yl]butyl}-1-[4-(trifluoro methyl)-benzyl]guanidine dihydrochloride.* $C_{31}H_{45}F_3N_4O{\cdot}2HCl{\cdot}0.5H_2O$. *M* = 628.66. White solid. 96.6% yield. mp: 88.2–90.2 °C. $^1$H NMR (600 MHz, $D_2O$) $\delta$ ppm 7.81–7.79 (m, $2H^{arom.}$, $(CHCH)_2CCF_3$), 7.53–7.52 (m, $2H^{arom.}$, $(CHCH)_2CCF_3$), 7.43–7.40 (m, $2H^{phenoxy.}$, $C(CHCH)_2CH$), 7.09–7.05 (m, $3H^{phenoxy.}$, $C(CHCH)_2CH$), 4.78 (s, 2H, $CH_2Ph$), 4.12–4.09 (t, 2H, $OCH_2$, *J* = 6.46 Hz), 3.52–3.50 (m, $2H^{Piperidine}$, $CH(CH_2CH_2)_2N$), 3.46–3.44 (m, 2H, $CH_2CH_2NC(N)$), 3.07–3.04 (m, 2H, $CH_2N^{piperidine}$), 2.82–2.78 (m, $2H^{Piperidine}$, $CH(CH_2CH_2)_2N$), 1.91–1.88 (m, $2H^{Piperidine}$, $CH(CH_2CH_2)_2N$), 1.83–1.79 (qt, 2H, $OCH_2CH_2$), 1.75–1.70 (m, 2H, $CH_2CH_2N^{piperidine}$), 1.66–1.61 (m, 2H, $CH_2CH_2NC(N)$), 1.52–1.26 (m, 13H: $3H^{Piperidine}$, $10H^{alif.}$, $CHCH_2$, $CH_2CH_2CH_2$). $^{13}$C NMR (600 MHz, *DMSO*) $\delta$ ppm 158.36 ($1C^{quat./phenoxy.}$, CO), 156.79 ($1C^{quat.}$, C═NH), 139.63 ($1C^{quat./arom.}$, $CCH_2$), 129.97 ($2C^{phenoxy.}$, $C(CHCH)_2CH$), 129.77, 129.55, 129.34, 129.11 ($1C^{arom}$, $CCF_3$), 127.43 ($2C^{arom.}$, $(CHCH)_2CCF_3$), 126.98, 125.18, 123.38, 121.75 (1C, $CF_3$), 125.92, 125.89 ($2C^{arom.}$, $(CH)_2CCF_3$), 121.75 ($1C^{phenoxy.}$, $C(CHCH)_2CH$), 115.03 ($2C^{phenoxy.}$, $C(CHCH)_2CH$), 68.55 (1C, $PhOCH_2$), 57.07 (1C, $CH_2N^{piperidine}$), 52.94 ($2C^{piperidine}$, $CH(CH_2CH_2)_2N$), 51.81 (1C, $CH_2Ph$), 49.56 (1C, $CH_2CH_2NC(N)$), 34.43 (1C, $CH_2CH_2CH_2$), 32.74 (1C, $CH_2CH_2CH_2$), 29.20 ($2C^{piperidine}$, $CH(CH_2CH_2)_2N$), 28.34 ($1C^{piperidine}$, $CH(CH_2CH_2)_2N$), 27.86 (1C, $CH_2CH_2CH_2$), 26.61 (1C, $CH_2CH_2CH_2$), 25.74 (1C, $CH_2CH_2CH_2$), 24.94 (1C, $CH_2CH_2CH_2$), 23.27 (1C, $CH_2CH_2CH_2$), 22.79 (1C, $CH_2CH_2CH_2$). Anal. calcd: C, 59.23; H, 7.70; N, 8.91. Found: C, 59.47; H, 7.87; N, 8.89.

***2c*** – *1-benzyl-3-{4-[4-(7-phenoxyheptyl)piperazin-1-yl]butyl}-guanidine trihydrochloride.* $C_{29}H_{45}N_5O{\cdot}3HCl{\cdot}0.5H_2O$. *M* = 598.10. White solid. 95.3% yield. mp: 218.6–220.6 °C with decomposition. $^1$H NMR (600 MHz, $D_2O$) $\delta$ ppm 7.52–7.50 (m, $2H^{arom.}$), 7.46–7.42 (m, 5H: $2H^{phenoxy.}$, $C(CHCH)_2CH$, $3H^{arom.}$, $C(CHCH)_2CH$), 7.12–7.07 (m, $3H^{phenoxy.}$, $C(CHCH)_2CH$), 4.52 (s, 2H, $CH_2Ph$), 4.15–4.13 (t, 2H, $OCH_2$, *J* = 6.48 Hz), 3.70 (br, $8H^{piperazine}$), 3.34–3.31 (m, 6H, $CH_2N^{piperazine}$, $CH_2CH_2NC(N)$), 1.84–1.76 (m, 6H, $CH_2CH_2CH_2$), 1.72–1.67 (qt, 2H, $CH_2CH_2CH_2$), 1.53–1.46 (m, 6H, $CH_2CH_2CH_2$). $^{13}$C NMR (600 MHz, *DMSO*) $\delta$ ppm 158.54 ($1C^{quat./phenoxy.}$, CO), 155.86 ($1C^{quat.}$, C═NH), 137.24 ($1C^{quat./arom.}$, $CCH_2$), 129.35 ($2C^{phenoxy.}$, $C(CHCH)_2CH$), 128.41 ($2C^{arom.}$, $C(CHCH)_2CH$), 127.28 ($1C^{arom.}$, $C(CHCH)_2CH$), 127.06 ($2C^{arom.}$, $C(CHCH)_2CH$), 120.25 ($1C^{phenoxy.}$, $C(CHCH)_2CH$), 114.32 ($2C^{phenoxy.}$, $C(CHCH)_2CH$), 67.14 (1C, $PhOCH_2$), 55.52 (1C, $CH_2N^{piperazine}$), 55.12 (1C, $CH_2N^{piperazine}$), 47.83 ($4C^{piperazine}$), 43.89 (1C, $NCH_2Ph$), 40.15 (1C, $CH_2CH_2NC(N)$), 28.47 (1C, $CH_2CH_2CH_2$), 28.09 (1C, $CH_2CH_2CH_2$), 25.81 (1C, $CH_2CH_2CH_2$), 25.53 (1C, $CH_2CH_2CH_2$), 25.19 (1C, $CH_2CH_2CH_2$), 22.79 (1C, $CH_2CH_2CH_2$), 20.15 (1C, $CH_2CH_2CH_2$). Anal. calcd: C, 58.24; H, 8.26; N, 11.71. Found: C, 58.58; H, 8.61; N, 11.63.

***2d*** – *1-{4-[4-(7-phenoxyheptyl)piperazin-1-yl]butyl}-3-[4-(trifluoromethyl)-benzyl]guanidine trihydrochloride.* $C_{30}H_{44}F_3N_5O{\cdot}3HCl$. *M* = 657.10. Light yellow solid. 90.9% yield. mp: 227.4–229.4 °C with decomposition. $^1$H NMR (600 MHz, $D_2O$) $\delta$ ppm 7.79–7.78 (m, $2H^{arom.}$), 7.57–7.55 (m, $2H^{arom.}$), 7.44–7.41 (m, $2H^{phenoxy.}$, $C(CHCH)_2CH$), 7.11–7.08 (m, $1H^{phenoxy.}$, $C(CHCH)_2CH$), 7.06–7.05 (m, $2H^{phenoxy.}$, $C(CHCH)_2CH$), 4.59 (s, 2H, $CH_2Ph$), 4.11–4.09 (t, 2H, $OCH_2$, *J* = 6.50 Hz), 3.72 (br, $8H^{piperazine}$), 3.36–3.31 (m, 6H: $CH_2N^{piperazine}$, $CH_2CH_2NC(N)$), 1.85–1.76 (m, 6H, $CH_2CH_2CH_2$), 1.73–1.68 (qt, 2H, $CH_2CH_2CH_2$), 1.50–1.44 (m, 6H, $CH_2CH_2CH_2$). $^{13}$C NMR (600 MHz, *DMSO*) $\delta$ ppm 158.52 ($1C^{quat./phenoxy.}$, CO), 155.94 ($1C^{quat.}$, C═NH), 142.28 ($1C^{quat./arom.}$, $CCH_2$), 129.31($2C^{phenoxy.}$, $C(CHCH)_2CH$), 128.15, 127.94, 127.73, 127.52 ($1C^{arom.}$, $CCF_3$), 127.70 ($2C^{arom.}$, $(CHCH)_2CCF_3$), 126.83, 125.03, 123.23, 121.42 (1C, $CF_3$), 125.22, 125.20 ($2C^{arom.}$, $(CH)_2CCF_3$), 120.21 ($1C^{phenoxy.}$, $C(CHCH)_2CH$), 114.29 ($2C^{phenoxy.}$, $C(CHCH)_2CH$), 67.11(1C, $PhOCH_2$), 55.48 (1C, $CH_2N^{piperazine}$), 55.06 (1C, $CH_2N^{piperazine}$), 47.78 ($4C^{pip.}$), 43.36 (1C, $NCH_2Ph$), 40.16 (1C, $CH_2CH_2NC(N)$), 28.44 (1C, $CH_2CH_2CH_2$), 28.06 (1C, $CH_2CH_2CH_2$), 25.79 (1C,

$CH_2CH_2CH_2$), 25.52 (1C, $CH_2CH_2CH_2$), 25.16 (1C, $CH_2CH_2CH_2$), 22.72 (1C, $CH_2CH_2CH_2$), 20.10 (1C, $CH_2CH_2CH_2$). Anal. calcd: C, 54.84; H, 7.21; N, 10.66. Found: C, 54.91; H, 7.37; N, 10.61.

*2f – 1,4-bis{4-[1-(4-trifluoromethylbenzyl)guandin-1-yl]but-1-yl}piperazine tetrahydrochloride.* $C_{30}H_{42}F_6N_8 \cdot 4HCl \cdot 1.5H_2O$. $M$ = 801.58. White solid. 91.60% yield. mp: 261.2–263.4 °C with decomposition. $^1$H NMR (600 MHz, $D_2O$) $\delta$ ppm 7.82–7.81 (m, 4H$^{arom.}$), 7.52–7.51 (m, 4H$^{arom.}$), 4.80 (s, 4H, $CH_2Ph$), 3.69–3.68 (m, 8H$^{piperazine}$), 3.54–3.53 (m, 4H, $CH_2CH_2N$), 3.31–3.29 (m, 4H, $CH_2CH_2N$), 1.79–1.78 (m, 8H, $CH_2CH_2CH_2$). $^{13}$C NMR (600 MHz, $D_2O$) $\delta$ ppm 156.67 (2C$^{quat.}$, C═NH), 139.04 (2C$^{quat./arom.}$, $CCH_2$), 129.68, 129.47, 129.26, 129.05 (2C$^{arom.}$, $CCF_3$), 126.93 (4C$^{arom.}$, $(CHCH)_2CCF_3$), 126.75, 124.96, 123.16, 121.36 (2C, $CF_3$), 125.86, 125.84 (4C$^{arom.}$, $(CH)_2CCF_3$), 56.24 (2C, $CH_2CH_2N$), 56.23 (2C, $CH_2CH_2N$), 51.46 (2C, $CH_2PhCF_3$), 48.59, 48.56 (4C$^{piperazine}$), 23.51 (2C, $CH_2CH_2CH_2$), 20.63 (2C, $CH_2CH_2CH_2$). Anal. calcd: C, 44.95; H, 6.16; N, 13.98. Found: C, 45.28; H, 6.34; N, 13.65.

*2h – 1-benzyl-1-(7-phenoxyhept-1-yl)guanidine fumarate.* $C_{21}H_{29}N_3O \cdot C_4H_4O_4$. $M$ = 455.54. White solid. 40.6% yield. mp: 103.4–105.0 °C. $^1$H NMR (600 MHz, $CD_3OD$) $\delta$ ppm 7.40–7.38 (m, 2H$^{arom.}$, $C(CHCH)_2CH$), 7.33–7.31 (m, 1H$^{arom.}$, $C(CHCH)_2CH$), 7.26–7.22 (m, 4H: 2H$^{phenoxy.}$, $C(CHCH)_2CH$), 2H$^{arom.}$, $C(CHCH)_2CH$, 6.90–6.87 (m, 3H$^{phenoxy.}$, $C(CHCH)_2CH$), 6.69 (s, 2H$^{fum.}$, CH), 4.62 (s, 2H, $PhCH_2$), 3.96–3.94 (t, 2H, $OCH_2$, $J$ = 6.37 Hz), 3.34–3.32 (t, 2H, $CH_2CH_2C(N)N$), 1.77–1.72 (qt, 2H, $CH_2CH_2CH_2$), 1.65–1.59 (qt, 2H, $CH_2CH_2CH_2$), 1.49–1.44 (qt, 2H, $CH_2CH_2CH_2$), 1.40–1.30 (m, 4H, $CH_2CH_2CH_2$). $^{13}$C NMR (600 MHz, $CD_3OD$) $\delta$ ppm 171.17 (2C, C═O), 160.55 (1C$^{quat./phenoxy.}$, CO), 158.38 (1C$^{quat.}$, C═N), 136.42 (1C$^{quat./arom.}$), 136.12 (2C, C═C), 130.39, 130.05 (4C$^{arom.}$, $C(CHCH)_2CH$), 129.14 (1C$^{arom.}$, $C(CHCH)_2CH$), 128.06 (2C$^{phenoxy.}$, $C(CHCH)_2CH$), 121.55 (1C$^{phenoxy.}$, $C(CHCH)_2CH$), 115.53 (2C$^{phenoxy.}$, $C(CHCH)_2CH$), 68.78 (1C, $OCH_2$), 52.67 (1C, $PhCH_2$), 49.87 (1C, $CH_2CH_2C(N)N$), 30.25 (1C, $CH_2CH_2CH_2$), 30.04 (1C, $CH_2CH_2CH_2$), 28.07 (1C, $CH_2CH_2CH_2$), 27.39 (1C, $CH_2CH_2CH_2$), 26.99 (1C, $CH_2CH_2CH_2$). Anal. calcd: C, 64.64; H, 7.38; N, 9.05. Found: C, 64.86; H, 7.09; N, 9.12.

**Synthesis of 1-benzyl-3-cyano-1-{4-[4-(7-phenoxyheptyl)-piperazin-1-yl]but-1-yl}guanidine trihydrochloride 2e.** To a solution of 1-benzyl-3-cyano-1-[4-[4-(7-phenoxyheptyl)piperazin-1-yl]butyl]guanidine (**2e.2**) (1 eq.) in methanol, 16% w/w solution of hydrogen chloride–methanol (4 eq.) was added dropwise. The reaction was stirred for 1 hour at room temperature and then the solvent was removed under vacuum. The crude product was evaporated twice from EtOAc, then recrystallized from dry ethanol to yield the pure product.

*2e – 1-benzyl-3-cyano-1-{4-[4-(7-phenoxyheptyl)piperazin-1-yl]but-1-yl}-guanidine trihydrochloride.* $C_{30}H_{44}N_6O \cdot 3HCl \cdot 1.5H_2O$. $M$ = 641.13. White solid. 64.2% yield. mp: 124.4–126.4 °C. $^1$H NMR (600 MHz, $D_2O$) $\delta$ ppm 7.47–7.44 (m, 2H$^{arom.}$), 7.42–7.39 (m, 1H$^{arom.}$), 7.37–7.35 (2H$^{phenoxy.}$, $C(CHCH)_2CH$), 7.29–7.28 (m, 2H$^{arom}$), 7.04–6.99 (m, 3H$^{phenoxy.}$, $C(CHCH)_2CH$), 4.75 (s, 2H, $CH_2Ph$), 4.07–4.05 (t, 2H, $OCH_2$, $J$ = 6.49 Hz), 3.59–3.51 (m, 10H: 8H$^{pip}$, $CH_2CH_2NC(N)$), 3.25–3.19 (m, 4H: $CH_2N^{piperazine}$), 1.76–1.71 (m, 8H, $CH_2CH_2CH_2$), 1.45–1.38 (m, 6H, $CH_2CH_2CH_2$). $^{13}$C NMR (600 MHz, $D_2O$) $\delta$ ppm 158.19 (1C$^{quat./phenoxy.}$, CO), 155.89 (1C$^{quat.}$, C═NH), 153.94 (1C$^{quat.}$, C≡N), 133.78 (1C$^{quat./arom.}$, $CCH_2$), 129.98 (2C$^{phenoxy.}$, $C(CHCH)_2CH$), 129.26 (1C$^{arom.}$, $C(CHCH)_2CH$), 128.44 (2C$^{arom.}$), 126.88 (2C$^{arom.}$), 121.53 (1C$^{phenoxy.}$, $C(CHCH)_2CH$), 115.07 (2C$^{phenoxy.}$, $C(CHCH)_2CH$), 68.65 (1C, $PhOCH_2$), 57.07 (1C, $CH_2N^{piperazine}$), 56.27 (1C, $CH_2N^{piperazine}$), 52.15 (1C, $NCH_2Ph$), 48.68, 48.63 (5C: 4C$^{piperazine.}$, $CH_2CH_2NC(N)$), 28.22 (1C, $CH_2CH_2CH_2$), 27.81 (1C, $CH_2CH_2CH_2$), 25.49 (1C, $CH_2CH_2CH_2$), 24.92 (1C, $CH_2CH_2CH_2$), 23.39 (1C, $CH_2CH_2CH_2$), 23.26 (1C, $CH_2CH_2CH_2$), 20.72 (1C, $CH_2CH_2CH_2$). Anal. calcd: C, 56.20; H, 7.86; N, 13.11. Found: C, 56.37; H, 8.11; N, 13.05.

**Synthesis of 1,4-bis(7-phenoxyheptyl)piperazine dihydrochloride 2g.** A 4 M solution of hydrogen chloride–dioxane (3 eq.) was added dropwise to a solution of 1,4-bis(7-phenoxyheptyl)piperazine (**2g.1**) (1 eq.) in chloroform. The reaction was stirred overnight at room temperature, then the solvent was removed under vacuum. The crude product was evaporated twice with chloroform and twice with EtOAc, then recrystallized from dry methanol to yield the pure product.

*2g – 1,4-bis(7-phenoxyheptyl)piperazine dihydrochloride.* $C_{30}H_{46}N_2O_2 \cdot 2HCl$. $M$ = 539.64. White solid. 82.6% yield. mp: 244.0–246.0 °C with decomposition. $^1$H NMR (600 MHz, $CD_3OD$) $\delta$ ppm 7.26–7.23 (m, 4H$^{phenoxy.}$, $C(CHCH)_2CH$), 6.91–6.88 (m, 6H$^{phenoxy.}$, $C(CHCH)_2CH$), 3.99–3.97 (t, 4H, $OCH_2$, $J$ = 6.33 Hz), 3.90–3.31 (br, 8H$^{piperazine}$), 3.19 (m, 4H, $CH_2N^{piperazine}$), 1.81–1.77 (qt, 8H, $CH_2CH_2CH_2$), 1.56–1.51 (qt, 4H, $CH_2CH_2CH_2$), 1.49–1.46 (m, 8H, $CH_2CH_2CH_2$). $^{13}$C NMR (600 MHz, $CD_3OD$) $\delta$ ppm 160.54 (2C$^{quat./phenoxy.}$, CO), 130.41 (4C$^{phenoxy.}$, $C(CHCH)_2CH$), 121.57 (2C$^{phenoxy.}$, $C(CHCH)_2CH$), 115.53 (4C$^{phenoxy.}$, $C(CHCH)_2CH$), 68.74 (2C, $OCH_2$), 57.62 (2C$^{piperazine}$), 57.48 (2C,$CH_2N^{piperazine}$), 57.33 (2C$^{piperazine}$), 30.25 (2C, $CH_2CH_2CH_2$), 29.84 (2C, $CH_2CH_2CH_2$), 27.49 (2C, $CH_2CH_2CH_2$), 26.93 (2C, $CH_2CH_2CH_2$), 25.12 (2C, $CH_2CH_2CH_2$). Anal. calcd: C, 66.77; H, 8.97; N, 5.19. Found: C, 66.38; H, 9.35; N, 5.16.

### 3.2. *In vitro* pharmacology

All experiments with animals (guinea pigs and rats) were performed in compliance with the Polish legislation concerning the protection and welfare of animals used for scientific purposes and followed the university guidelines. All animal procedures have been approved by the 9 Local Ethical Committee for Animal Experiments in Lodz, approval no. 60/ŁB681/2013 and 86/ŁB696/2013, respectively. The HEK293T cell line was kindly provided by Prof. Dr Rob Leurs, Vrije Universiteit Amsterdam; the HEK-hH3R cell line was kindly provided by Prof. Dr. Jean-Charles Schwartz, Bioprojet, France; CHO cells stably expressing $hH_4R$ were obtained from PerkinElmer, Waltham, MA, U.S.

**3.2.1. Histamine $H_3$ receptor antagonistic activity of compounds 2a–h.** In the first step, all the newly synthesized compounds were tested for histamine $H_3$ receptor antagonistic effects *in vitro*, following standard methods, using an electrically-contracting guinea pig jejunum.[30]

Male guinea pigs weighing 300–400 g were sacrificed and a portion of the small intestine, 20–50 cm proximal to the ileocaecal valve (jejunum), was removed and placed in Krebs buffer (composition (mM) NaCl 118; KCl 5.6; $MgSO_4$ 1.18; $CaCl_2$ 2.5; $NaH_2PO_4$ 1.28; $NaHCO_3$ 25; glucose 5.5 and indomethacin ($1 \times 10^{-6}$ mol $L^{-1}$)). Whole jejunum segments (2 cm) were prepared and mounted between two platinum electrodes (4 mm apart) in 20 mL Krebs buffer, continuously gassed with 95% $O_2$ : 5% $CO_2$ and maintained at 37 °C. Contractions were recorded isotonically under 1.0 g tension with a Hugo Sachs Hebel-Messvorsatz (Tl-2)/HF-modem (Hugo Sachs Elektronik, Hugstetten, Germany) connected to a pen recorder. The equilibration was performed for one hour with washings every 10 minutes. The muscle segments were then stimulated at a maximum between 15 and 20 volts, continuously at a frequency of 0.1 Hz for a duration of 0.5 ms, with rectangular-wave electrical pulses, delivered by a Grass Stimulator S-88 (Grass Instruments Co., Quincy, USA). After 30 min of stimulation and five minutes before adding (*R*)-α-methylhistamine, pyrilamine ($1 \times 10^{-5}$ mol $L^{-1}$ concentration in an organ bath) was added, and then cumulative concentration–response curves (half-log increments) of (*R*)-α-methylhistamine, a histamine $H_3$ receptor agonist, were recorded until no further change in response was found. Five minutes before adding the tested compounds, pyrilamine ($1 \times 10^{-5}$ mol $L^{-1}$ concentration in an organ bath) was added, and after 20 minutes cumulative concentration–response curves (half-log increments) of (*R*)-α-methylhistamine, an histamine $H_3$ receptor agonist, were recorded until no further change in response was found. Statistical analysis was carried out with Student's *t*-test. In all the tests, a $p < 0.05$ was considered statistically significant. The potency of an antagonist is expressed by its $pA_2$ value, calculated from the Schild[31] regression analysis where at least three concentrations were used. The $pA_2$ values were compared with the potency of thioperamide.

**3.2.2. $H_1$ antagonistic activity of compounds 2a–h.** All the final compounds were tested for $H_1$ antagonistic effects *in vitro*, following standard methods, using the guinea pig ileum.[30] The donors were male guinea pigs (300–400 g) as mentioned above. The excised ileum was placed in phosphate buffer at room temperature (pH 7.4) containing (mM) NaCl (136.9), KCl (2.68), and $NaHPO_4$ (7.19). The intraluminal content was flushed and segments of about 2 cm long were cut and mounted for isotonic contractions in water mixed with 20 mL organ baths filled with oxygenated ($O_2$ : $CO_2$ = 95 : 5, v/v) Krebs buffer containing (mM) NaCl (117.5), KCl (5.6), $MgSO_4$ (1.18), $CaCl_2$ (2.5), $NaH_2PO_4$ (1.28), $NaHCO_3$ (25), glucose (5.5) and indomethacin (1 $10^{-6}$ mol $L^{-1}$) at 37 °C under a constant load of 0.5 g. After a 30 min equilibration period with washings every 10 min, a submaximal priming dose of histamine (1 mM) was given and washed out (standard washing procedure: 3 changes of buffer for 30 min). After washing out, the antagonistic activity of the given compounds was measured by recording a concentration–response curve (CRC) for histamine in the presence of the tested compounds which were added 10 min before histamine. This procedure was repeated with higher concentrations of the compounds. The antagonism has a competitive nature causing a parallel shift in the CRC. The $pA_2$-values were calculated according to Arunlakshana and Schild.[31] The $pA_2$ values were compared with the affinity of pyrilamine.

**3.2.3. Antagonist binding to the rat histamine $H_3$ receptor ($rH_3R$), human histamine $H_3$ receptor ($hH_3R$), and human histamine $H_4$ receptor ($hH_4R$)**

*3.2.3.1. Antagonist binding to the rat histamine $H_3$ receptor ($rH_3R$).* The affinities of the most active compounds **1a**, **1c** and **1d** were evaluated by measuring the displacement curve of [$^3$H]-$N^\alpha$-methylhistamine on the rat ($rH_3R$) histamine $H_3$ receptor in HEK-293Tcell membranes as described by Bongers.[32]

### Cell culture and transfection

Human embryonic kidney cells (HEK293T) were cultured in DMEM supplemented with 10% fetal bovine serum and 100 IU $mL^{-1}$ penicillin and 100 μg $mL^{-1}$ streptomycin at 37 °C and 5% $CO_2$. The day prior to transfection, two million cells were seeded in 10 cm dishes. Approximately four million cells were transfected by the polyethyleneimine (PEI) method with 5 μg of cDNA at a ratio of 1 : 4 (DNA : PEI). Briefly, 0.5 μg of pcDNA3-$rH_3R$ and 4.5 μg of empty plasmid (pcDNA3.1) were mixed with 20 μg of 25 kDa linear PEI in 500 μl of 150 mM NaCl and incubated for 30 minutes at 22 °C. Meanwhile, the medium from 10 cm dishes was replaced with fresh culture medium and the transfection mix was subsequently added dropwise to the cells and incubated for 48 hours at 37 °C and 5% $CO_2$.

### Crude membrane extracts

Forty-eight hours after transfection, cells were washed with ice-cold phosphate-buffered saline (PBS) and scrapped and the homogenate was centrifuged for 10 minutes at ~2000*g*, 4 °C. The supernatant was aspirated and cell pellets were resuspended in 1 ml of ice-cold PBS and centrifuged again under the same conditions; the supernatant was aspirated and the membranes were stored at −20 °C until further use.

### [$^3$H]-$N^\alpha$-methylhistamine binding

Frozen cell pellets were dissolved in 50 mM Tris-HCl buffer (pH 7.4), homogenized by sonication (40 Watt Labsonic 1510) for 3 to 5 s and kept on ice until use. Cell homogenates were incubated in the presence of increasing concentrations of [$^3$H]-NαMH (0–20 nM) in 50 mM Tris-HCl binding buffer for saturation binding. Total and non-specific binding was determined in the absence or presence of excess non-labeled thioperamide (10 μM), respectively. For the competitive binding assay, the homogenates were incubated with increasing concentrations of receptor ligands ($10^{-11}$ to $10^{-4}$ M) and ~2.5 nM [$^3$H]-NαMH. All assays were incubated at 25 °C for two hours on a shaking table (600 rpm). The reaction was terminated by rapid filtration into 0.5% polyethyleneimine pre-soaked glass fiber C plates (GF/C Perkin Elmer) followed by three washes with ice-cold Tris-HCl buffer (pH 7.4 at 4 °C). The plates were

dried for one hour at 50 °C and scintillation liquid was added to each well (25 μl). The retained radioactivity was determined by liquid scintillation counting in a Wallac Microbeta (Perking Elmer). Protein determination for $B_{max}$ estimation was performed with a Pierce BCA protein assay kit and measured by spectrophotometry in a Power Wave X340 (Biot-Tek Instruments Inc.).

### Chemicals

Dubelcco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS) trizma base, and polyethyleneimine solution (50%, PEI) were purchased from Sigma Aldrich. Fetal bovine serum (FBS, Bodinco BV, the Netherlands), penicillin/streptomycin (streptomycin 10 000 IU $mL^{-1}$; penicillin and 10 000 μg $mL^{-1}$, Thermo Fischer Scientific), linear 25 kDa polyethyleneimine (PEI, Polysciences, Warrington, PA, U.S.), [$^3$H]-N-α-methylhistamine (specific activity 79.7 Ci $mmol^{-1}$, Perkin Elmer), thioperamide, (Abcam), and histamine (TCI).

*3.2.3.2. Antagonist binding to the human histamine $H_3$ receptor ($hH_3R$).* Competitive radioligand binding to cell membranes of HEK293 cells stably expressing the human histamine $H_3$ receptor was conducted as presented in detail elsewhere.[34] [$^3$H]-$N^{\alpha}$-methylhistamine (1 nM) was incubated with the cell membrane preparation in 50 mM Tris/HCl, 5 mM $MgCl_2$ assay buffer (35 μg of protein in a single assay tube) and serial dilutions of the tested compounds for 1 h at 25 °C with continuous shaking (400 rpm). Total radioligand binding was measured in the presence of 2.5% DMSO and nonspecific binding was assessed by addition of unlabeled (*R*)(−)-α-methylhistamine (20 μM). The bound radioligand was detected after filtration of the incubation mixture through GF/B filters presoaked with 0.5% (m/v) polyethyleneimine. For the calculation of $K_i$ values, the Cheng–Prusoff equation and experimentally determined radioligand $K_D$ value of 0.34 were used.

*3.2.3.3. Antagonist binding to human $hH_4R$.* Histamine $H_4$ receptor affinity was evaluated on membrane preparations from CHO cells, expressing the recombinant human histamine $H_4$ receptor. Experiments were performed as described before.[35] In brief: cell membranes (15 μg protein per sample) were incubated for 1 h at 25 °C with tritiated histamine (10 nM) and serial dilutions of the evaluated compounds in assay buffer (50 mM Tris/HCl and 5 mM EDTA in ultrapure water, pH 7.4). Non-specific binding was determined in the presence of 100 μM un-labeled histamine. The bound radioligand was collected on GF/B filters pretreated with 0.3% (m/v) polyethyleneimine and the captured radioactivity was measured by liquid scintillation counting. Affinity values ($pK_i$) were calculated according to the Cheng–Prusoff equation ($K_D$ for [$^3$H]histamine: 9.7 nM).

### 3.3. Verification of *in vivo* activity for compounds 1a, 1c, and 1d

The histamine $H_3$ receptor antagonistic activity of **1a**, **1c**, and **1d** toward the brain histamine receptors was assessed *in vivo* by the intravital study of the feeding behavior and then by post-mortem analyses of neurotransmitter systems in the brain tissues from the treated rats.

For the first stage, food intakes were measured daily during subchronic drug administration. The histamine $H_3$ receptor antagonists show the ability to inhibit appetite.

Male rats aged between 9 and 10 weeks old were used. In experiments with compound **1a**, Wistar rats were used; the Lewis strain was employed with compounds **1c** and **1d** due to a shortage of Wistar rats of the required age. Although these rat strains differ somewhat in the cerebral amine concentrations and enzyme activities, both responded in the same way to ciproxifan as checked. Metabolic cages (Tecniplast, Italy) were used to measure feed consumption. The cages have a standardized size that allows the animal to move inside freely. Throughout the adaptive period and the experimental one, access to feed and fluid was unlimited. The illumination cycle was stable at 12 h light on, 12 h light off. The rats were individually placed in the cages four days before the onset of treatment to adapt to a new environment and conditions of housing. The consumption and excretion were recorded every morning and the monitoring continued as long as the rats stayed in metabolic cages. The treatment ran for five days from the fifth day.

Subcutaneous injections of either **1a**, **1c**, **1d** or ciproxifan, which was used as a reference (3 mg $kg^{-1}$ per day to $n$ = 8 rats), in the study group and physiological saline (0.2 ml per day to $n$ = 8 rats) in the case of the control group were conducted. Records of consumption were taken as g of feed per 100 g body mass.

**3.3.1. Post-mortem biochemical analyses.** Post-mortem brain analyses included estimation of the amine neurotransmitter concentrations as well as the activities of the degradative enzymes HNMT, MAO-A and MAO-B.

The rats were sacrificed 24 hours after the last injection. The brains were collected, cerebral cortex separated according to the Glowinski and Iversen method,[38] immediately frozen in liquid nitrogen and kept at −80 °C until assayed. The thawed tissue was homogenized in 3 volumes of bi-distilled water and aliquots were further processed to estimate the concentrations of the amines and activities of the enzymes. The amine concentrations: dopamine, noradrenaline, and serotonin were measured by RIA and histamine by Research ELISA kits (DIAsource ImmunoAssays S.A. Louvain-la-Neuve, Belgium). The enzyme activities of histamine *N*-methyltransferase and monoamine oxidase A and B were estimated by radioisotopic assays according to Taylor and Snyder[41] and Fowler and Tipton[42] with modifications by Gómez *et al.*,[43] respectively. For MAO-A, serotonin (5-[2-14C]-hydroxytryptamine binoxalate) was used as a substrate and for MAO-B, β-[ethyl-1-$^{14}$C]-phenylethylamine hydrochloride was used as a substrate; clorgyline (MAO-A) and deprenyl (MAO-B) (fin. conc. $10^{-9}$ M) were used as enzyme inhibitors. The protein was measured by the Lowry method.[44]

## Author contributions

K. Walczyński was responsible for the supervision and development of the whole project.

M. Staszewski performed the chemical syntheses of the newly synthesized compounds and the preliminary pharmacological studies *in vitro*, both on the histamine $H_3$ receptor and histamine $H_1$ receptor, elaborated and described the results.

A. Stasiak performed the extended pharmacological studies *in vivo*, elaborated and described the results.

T. Karcz performed the human histamine $H_3$ receptor and human histamine $H_4$ receptor binding affinity tests, elaborated and described the results.

D. McNaught Flores performed the rat histamine $H_3$ receptor binding affinity test, elaborated and described the results.

W. A. Fogel coordinated the advanced pharmacological studies *in vivo* and interpreted the obtained results.

K. Kieć-Kononowicz coordinated the human histamine $H_3$ receptor and human histamine $H_4$ receptor binding affinity tests and interpreted the obtained results.

R. Leurs coordinated the rat histamine $H_3$ receptor binding affinity test and interpreted the obtained results.

## Conflicts of interest

The authors have declared no conflict of interest.

## Acknowledgements

This study was supported by departmental sources: Medical University of Lodz grant numbers 503/3-016-01/503-31-001; 503/5-087-02/503-01 and COST Action CA 15135.

The authors would like to thank Mr. Mieczyslaw Wośko, president of the pharmaceutical company Polfarmex SA, for providing financial support for the purchase of the necessary reagents for *in vivo* studies and also to thank Prof. H. Stark, from Heinrich-Heine-Universität Düsseldorf, Germany for kindly donating ciproxifan for use as the reference compound for the *in vivo* study.

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