Fragment Based Design of New H_4 Receptor−Ligands with Anti-inflammatory Properties in Vivo

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Using a previously reported flexible alignment model we have designed, synthesized, and evaluated a series of compounds at the human histamine H_4 receptor (H_4R) from which 2-(4-methyl-piperazin-1-yl)-quinoxaline (**3**) was identified as a new lead structure for H_4R ligands. Exploration of the structure−activity relationship (SAR) of this scaffold led to the identification of 6,7-dichloro 3-(4-methylpiperazin-1-yl)quinoxalin-2(1*H*)-one (VUF 10214, **57**) and 2-benzyl-3-(4-methyl-piperazin-1-yl)quinoxaline (VUF 10148, **20**) as potent H_4R ligands with nanomolar affinities. In vivo studies in the rat reveal that compound **57** has significant anti-inflammatory properties in the carrageenan-induced paw-edema model.

Introduction

Histamine is an endogenous amine that exerts a wide array of physiological activities by a group of four G-protein coupled receptors (GPCR's). The histamine H_1 receptor (H_1R) mediates inflammation and vasodilatation, and H_1R antagonists have long been used for the treatment of allergic disorders such as hay fever.[1] The H_2R is well-known for its regulation of gastric acid secretion and H_2R antagonists have been successfully used for the treatment of gastric ulcers.[2] The H_3R is being studied intensively and has been shown to play an important role in several cognitive processes as well as in the regulation of food intake and the regulation of sleep and wakefulness. Currently H_3R antagonists are being evaluated for their clinical efficacy in ADHD[a], dementia and narcolepsy.[3]

The H_4R is the most recently discovered histamine receptor subtype. The DNA sequence was discovered in the human genome by several groups, and subsequent pharmacological characterization identified it as a novel histamine receptor.[4–8] Although the sequence identity with the H_1R and H_2R is relatively low, the H_4R protein has a considerable sequence identity with the H_3R receptor (31% overall, 54% in the transmembrane region).[9] Nevertheless, the H_4R has a distinct pharmacological profile.[9] The H_4R plays a role in immunological and inflammatory processes and is predominantly expressed on hematopoetic and immune cells such as eosinophils, mast cells, and macrophages as well as in peripheral tissues such as spleen, thymus, and bone marrow.[10] Currently, the H_4R is considered a promising target for the treatment of various chronic inflammatory diseases such as inflammatory bowel disease, asthma, and rheumatoid arthritis.[11–13] Quite recently, its role has also been postulated in the proliferation of colon carcinoma cells, in the modulation of angiogenesis, and in mediating pruritis.[14,15]

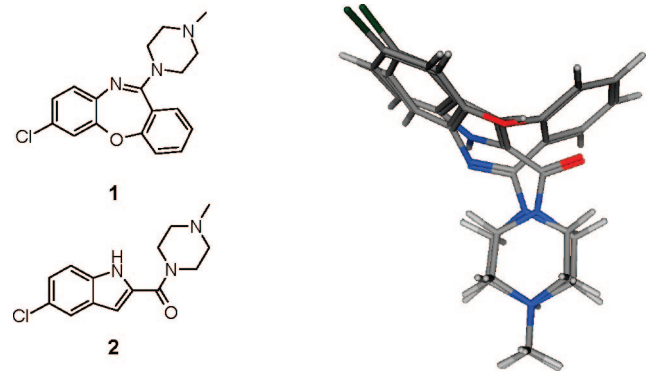

Figure 1. Previously reported H_4R ligands **1** and **2**.

Previously we reported the discovery of VUF6884 (**1**, Figure 1), a tricyclic clozapine analogue with high affinity for the H_4R (pK_i 7.6, full agonist $\alpha = 1$), and saturation binding analysis demonstrated that this ligand bound to the orthosteric binding site of the H_4R.[16]

We also showed that the H_4R antagonist JNJ7777120 (**2**)[17,18] (pK_i 7.8, antagonist $\alpha = 0$) displaces **1** from its H_4R binding site. On the basis of these findings and on the structural similarities between **1** and **2**, we proposed that they had overlapping binding modes and created a model for the future design of H_4R ligands (Figure 1). In this paper, we will discuss the design and synthesis, structure–activity relationship, and pharmacological evaluation of a new class of potent H_4R ligands, which was discovered on the basis of the flexible alignment model of **1** and **2**.[16]

Chemistry

Compounds **3–7** (Table 1) were prepared in one step from their commercially available chloro precursors by substitution with *N*-methylpiperazine under microwave irradiation. Starting from 2,4-dichloroquinazoline (**8**), quinazoline fragment **9** was synthesized according to patent literature (Scheme 1).[19] Starting from phenylenediamine (**10**) and 4,5-dichlorophenylenediamine (**11**) (Scheme 2), intermediate quinoxalinones **12**−**17** were prepared as described in literature,[20–22] except for intermediate

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[a] Abbreviations: ADHD, attention deficit hyperactivity disorder; SEM, standard error of the mean; PEI, pectin esterase inhibitor; DIPEA, diisopropylethylamine, PMB, *para*-methoxybenzylamine.

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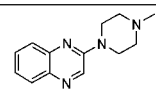
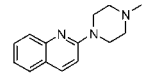
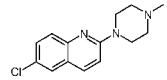
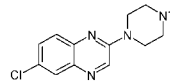
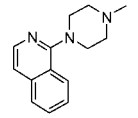
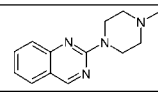
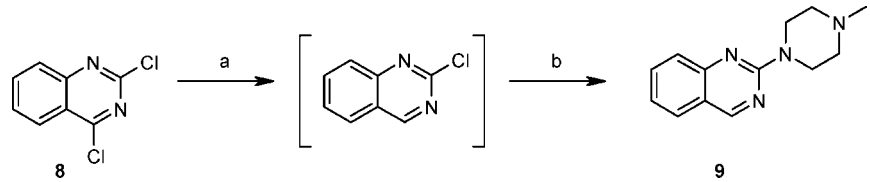
Table 1. Structures and Affinity of Several Heterocyclic Fragments for the H_4R

Nr	Structure	pK_i (μM) ± SEM[a]	Ligand Efficiency[b]
3		6.05 ± 0.07	0.488
4		5.16 ± 0.06	0.416
5		6.23 ± 0.04	0.475
6		7.04 ± 0.01	0.536
7		4.69 ± 0.07	0.378
9		5.12 ± 0.06	0.413

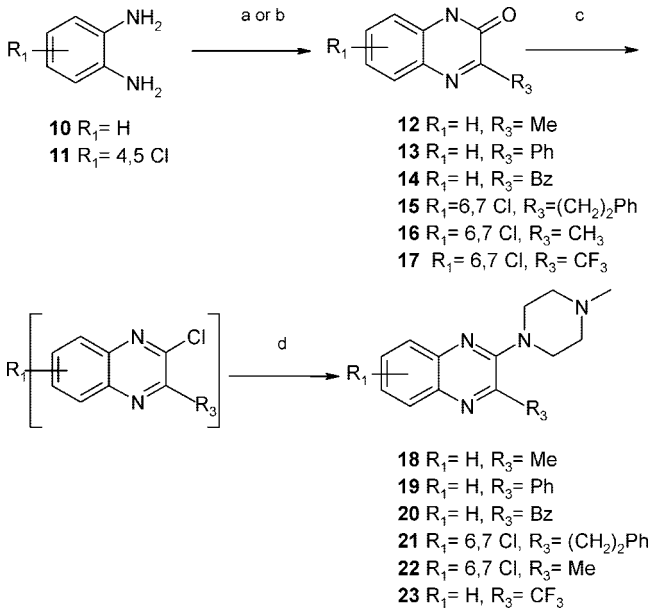
[a] Measured by displacement of [^{3}H]histamine binding using membranes of HEK cells transiently expressing the human H_4R. pK_i's are calculated from at least three independent measurements as the mean ± SEM. [b] Ligand efficiency (Δg) is calculated as the binding energy per non-hydrogen atom ($\Delta g = \Delta G/N_{\text{non-hydrogen atoms}}$ with $\Delta G = -RT \ln K_i$).

Scheme 1[a]



[a] Reagents and conditions: (a) brine with 9% NH_4OH, Zn, DCM, reflux; (b) *N*-methylpiperazine, mw, 140°C.

Scheme 2[a]



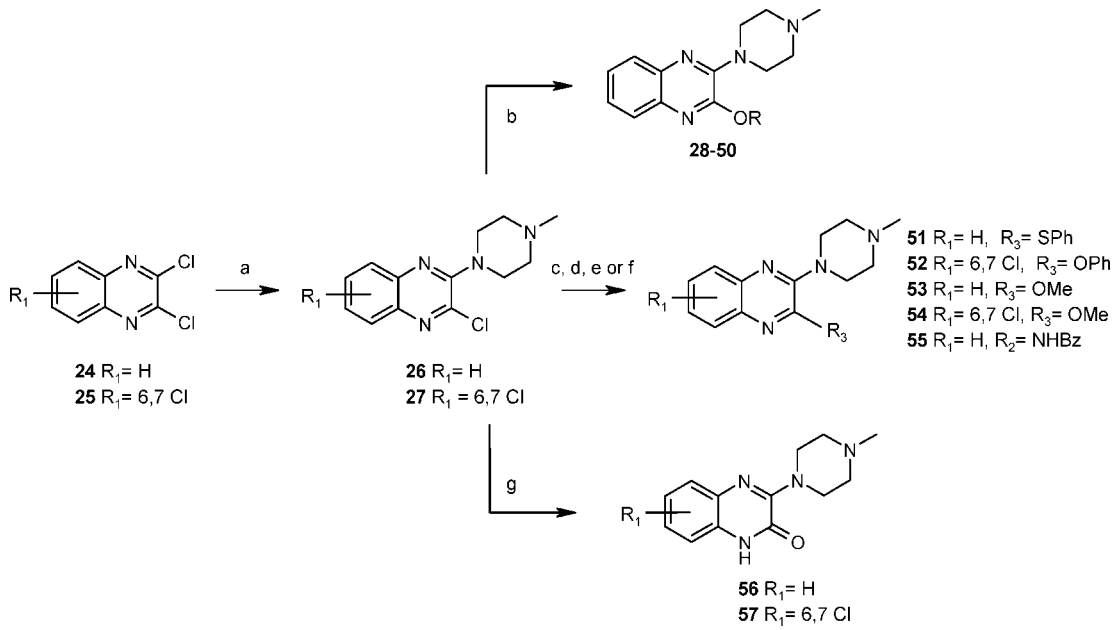
[a] Reagents and conditions: (a) R_2COCO_2H, EtOH, reflux; (b) ethyloxobutyric acid, r.t. (for compound **15**); (c) $POCl_3$, reflux; (d) *N*-methylpiperazine, mw, 140°C.

15, which was synthesized from 4,5-dichlorophenylenediamine (**11**) and ethyl 2-oxo-4-phenylbutyrate. The different quinoxaline-2-ones were converted to their corresponding chlorides with neat phosphorus oxychloride. After removal of excess reagent and no further work up, microwave-assisted heating was used to couple the crude chlorides with neat *N*-methylpiperazine to yield quinoxalines **18**−**23** (Scheme 2).

Commercially available 2,3-dichloroquinoxaline (**24**) and 2,3,6,7-tetrachloroquinoxaline (**25**) were reacted with *N*-methylpiperazine in the presence of diisopropylethylamine in tetrahydrofuran under microwave irradiation to give key intermediates **26** and **27** in high yields (Scheme 3). Unsubstituted quinoxaline **26** was then added to solutions of alcohols and sodium hydride in dimethylformamide to give compounds **28–50** (Table 2).[23] Thioether **51** and phenolether **52** were synthesized by the same procedure from intermediates **26** and **27**, respectively. Methoxy analogues **53** and **54** were synthesized from their corresponding precursors by refluxing in methanol in the presence of sodium methoxide. Benzylamine **55** was synthesized from intermediate **26** by microwave-assisted heating in neat benzylamine. Compound **56** could either be prepared by hydrolysis of methoxy analogue **53** with aqueous sodium hydroxide or by substitution of chloride **26** under microwave-assisted heating with sodium hydroxide in tetrahydrofuran. A similar microwave procedure was used for the synthesis of quinoxalinone **57** from **27**.

Monosubstitution of the 6 position (the 6 position mentioned in the text corresponds to the numbering in Figure 2) of the quinoxaline scaffold was also explored. For this, 2-amino-4-chloroaniline (**58**) was converted to 6-chloro-quinoxalin-2,3(1*H*,4*H*)-dione (**59**) and subsequently to its chlorinated product 2,3,6-trichloroquinoxaline (**60**), as has been described

Scheme 3[a]



[a] Reagents and conditions: (a) *N*-methylpiperazine, DIPEA, THF, mw, 160°C; (b) NaH, ROH, DMF, r.t.; (c) NaH, thiophenol, DMF, r.t.; (d) NaH, phenol, DMF, r.t.; (e) NaMeO, MeOH, reflux; (f) benzylamine, mw, 160°C; (g) aq NaOH, THF, or dioxane, mw, 120°C.

in literature (Scheme 4).[24] From this precursor, 2,6-dichloro-3-methoxy quinoxaline (**61**) was synthesized according to a modified procedure from literature.[24] The original procedure mentioned above failed to give **61** in sufficiently high purity. After multiple attempts, purities around 75% were found by NMR due to formation of two chloro-substituted regioisomers. However, three subsequent crystallizations of this impure material yielded desired product (**61**) in very high purity (>95% by NMR). Subsequent substitution of **61** with *N*-methylpiperazine resulted in methoxy analogue **62**. Attempts to convert **62** to target compound **63** under acidic conditions failed as these conditions led to regeneration of precursor (**59**). However, demethylation of **62** under basic conditions, using sodium hydroxide in water, proceeded more mildly and gives **63** in good yields. Although compound **61** has been reported in literature, no evidence has been given that can confirm its proposed structure; in this particular synthesis, two regioisomers are formed of which the structures cannot be assigned unambiguously by NMR.[24] Absolute proof with regard to the isolated regioisomers of the target compound quinoxalinone **63**, as synthesized following this scheme, could also not be obtained using standard analytical characterization. Because this information is relevant for the SAR, we decided to obtain definite proof of the structure of compound **63** by a fully regioselective synthesis (Scheme 5).

2,5-Dichloro-nitrobenzene (**64**) was substituted with *p*-methoxybenzylamine (PMB), yielding benzylamine **65**, which was subsequently hydrogenated in THF with Raney nickel and hydrogen to give aniline **66**. Ring closure of **66** to *N*-protected quinoxaline **67** was done by heating in neat diethyloxalate. Compound **67** was chlorinated with $POCl_3$ in the presence of Hünigs base and DMF to yield **68**. Analogous to a procedure described in literature, removal of the PMB group from chloroquinoxaline **68** was achieved with concentrated sulfuric acid to give the desired monochloro-substituted quinoxalinone (**69**).[25] Quinoxaline **63** was finally obtained regioselectively through this route by substitution of the 2-chloro position of **69** with *N*-methylpiperazine. A comparison of the melting point, NMR, and LCMS data showed that the compounds obtained with both synthetic routes were indeed identical. Using the same procedure for the preparation of **63** from **69**, quinoxalines **70** and **71** were obtained with *N*-methylhomopiperazine and boc-protected 3-aminomethylpyrrolidine, respectively. Removal of the boc group with HCl/dioxane finally gave compound **72** as the hydrochloric salt.

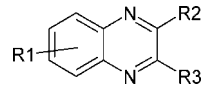
When **68** was directly substituted with *N*-methylpiperazine, PMB-protected compound **73** was obtained.

Finally, to enable comparison of the SAR of the methylpiperazine moiety with other series of H_4R ligands, the *N*-ethylpiperazine substituted compound **74** was prepared by the same method as had been used for **20** (Table 1) using intermediate **14** and *N*-ethylpiperazine.

Results and Discussion

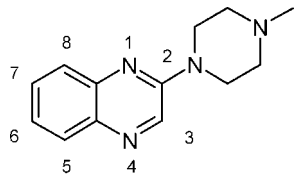
To test our model, we synthesized a series of compounds in which two-ring heterocyclic scaffolds would be directly connected to a *N*-methylpiperazine group. Relatively small compounds such as quinoxaline **3** would be able to fit in the H_4R binding site in such a way that the heterocyclic ring could occupy the same space as the chloro-substituted phenyl ring of **1** and the indole ring of **2** while the *N*-methylpiperazine group would be able to adopt a similar conformation as in **1** and **2** (Figure 3A). Furthermore, because the tricyclic scaffold of **1** (e.g., clozapine) and its analogues are known to be very promiscuous GPCR ligands, it was also considered necessary to move away from this particular scaffold. Therefore, the hybrid scaffolds based on the structures of **1** and **2** were considered a good starting point for further exploration of the H_4R binding site and evaluation of our flexible alignment model. Previous SAR studies of a number of H_4R ligands showed that modification of the *N*-methylpiperazine moiety is very detrimental for H_4R affinity.[16,17] We therefore initially did not alter this moiety in the newly designed compounds. In the initial series, we varied the number and the position of the heterocyclic nitrogen atoms as well as the position of the *N*-methylpiperazine moiety (Table 1). 6-Chloro-substituted compounds **5** and **6** were immediately included in our initial compounds because SAR data from the

Table 2. SAR of Quinoxalines Substituted at the R3 Position



no.	R1	R2	R3	pK_i ± SEM[a]
		histamine		7.92 ± 0.07
		thioperamide		7.20 ± 0.06
3	H	*N*-methylpiperazine	H	6.05 ± 0.07
18	H	*N*-methylpiperazine	CH_3	6.70 ± 0.02
19	H	*N*-methylpiperazine	C_6H_5	4.99 ± 0.06
20	H	*N*-methylpiperazine	$CH_2C_6H_5$	7.40 ± 0.04
51	H	*N*-methylpiperazine	SPh	6.44 ± 0.02
55	H	*N*-methylpiperazine	$NHCH_2Ph$	5.13 ± 0.04
28	H	*N*-methylpiperazine	OPh	6.49 ± 0.02
29	H	*N*-methylpiperazine	OCH_2Ph	6.53 ± 0.05
30	H	*N*-methylpiperazine	$O(CH_2)_2Ph$	6.32 ± 0.03
31	H	*N*-methylpiperazine	$O(CH_2)_4Ph$	5.53 ± 0.15
32	H	*N*-methylpiperazine	OCH_2-2-Pyridyl	6.33 ± 0.05
33	H	*N*-methylpiperazine	OCH_2-3-Pyridyl	5.53 ± 0.03
34	H	*N*-methylpiperazine	OCH_2-4-Pyridyl	5.36 ± 0.11
35	H	*N*-methylpiperazine	OCH_2-(4-OCH_3−Ph)	6.15 ± 0.40
36	H	*N*-methylpiperazine	OCH_2-(4-CH_3−Ph)	5.86 ± 0.16
37	H	*N*-methylpiperazine	OCH_2-(4-Cl-Ph)	5.64 ± 0.05
38	H	*N*-methylpiperazine	OCH_2-(3-Cl-Ph)	6.57 ± 0.05
39	H	*N*-methylpiperazine	O(3-pyridyl)	5.89 ± 0.03
40	H	*N*-methylpiperazine	O(4-Cl-Ph)	5.63 ± 0.06
41	H	*N*-methylpiperazine	O(3,4-Cl-Ph)	5.77 ± 0.03
42	H	*N*-methylpiperazine	O(4-F-Ph)	5.80 ± 0.04
43	H	*N*-methylpiperazine	O(3-CH_3−Ph)	6.24 ± 0.08
44	H	*N*-methylpiperazine	O(4-CH_3−Ph)	5.66 ± 0.08
45	H	*N*-methylpiperazine	O-(4-OCH_3−Ph)	5.63 ± 0.20
46	H	*N*-methylpiperazine	O-(3-*N,N*-dimethylaniline)	5.81 ± 0.02
47	H	*N*-methylpiperazine	O-cyclohexyl	4.88 ± 0.03
48	H	*N*-methylpiperazine	$OCH_2CH(CH_3)_2$	5.24 ± 0.03
49	H	*N*-methylpiperazine	OCH_2CH_3	6.64 ± 0.08
50	H	*N*-methylpiperazine	$O(CH_2)_3$-*N,N*-dimethylamine	5.40 ± 0.19
52	H	*N*-methylpiperazine	OCH_3	6.47 ± 0.02
56	H	*N*-methylpiperazine	OH	7.21 ± 0.03
62	6-Cl	*N*-methylpiperazine	OCH_3	7.58 ± 0.04
63	6-Cl	*N*-methylpiperazine	OH	7.93 ± 0.05
57	6,7-Cl	*N*-methylpiperazine	OH	8.25 ± 0.07
21	6,7-Cl	*N*-methylpiperazine	$(CH_2)_2Ph$	5.40 ± 0.04
22	6,7-Cl	*N*-methylpiperazine	CH_3	7.20 ± 0.09
52	6,7-Cl	*N*-methylpiperazine	OPh	5.93 ± 0.11
54	6,7-Cl	*N*-methylpiperazine	OCH_3	7.24 ± 0.03
23	H	*N*-methylpiperazine	CF_3	5.60 ± 0.02
26	H	*N*-methylpiperazine	Cl	6.64 ± 0.03
73		structure as depicted in Scheme 5		6.32 ± 0.06
70	6-Cl	*N*-methylhomopiperazine	OH	6.01 ± 0.07
72	6-Cl	aminomethylpyrrolidine	OH	5.83 ± 0.08
74	H	*N*-ethylpiperazine	$CH_2C_6H_5$	5.57 ± 0.01

[a] Measured by displacement of [^{3}H]histamine binding using membranes of HEK cells transiently expressing the human H_4R. pK_i's are calculated from at least three independent measurements as the mean ± SEM.



3

Figure 2. 2-(4-Methylpiperazin-1-yl)quinoxaline (3) as a new lead compound for H_4R ligands.

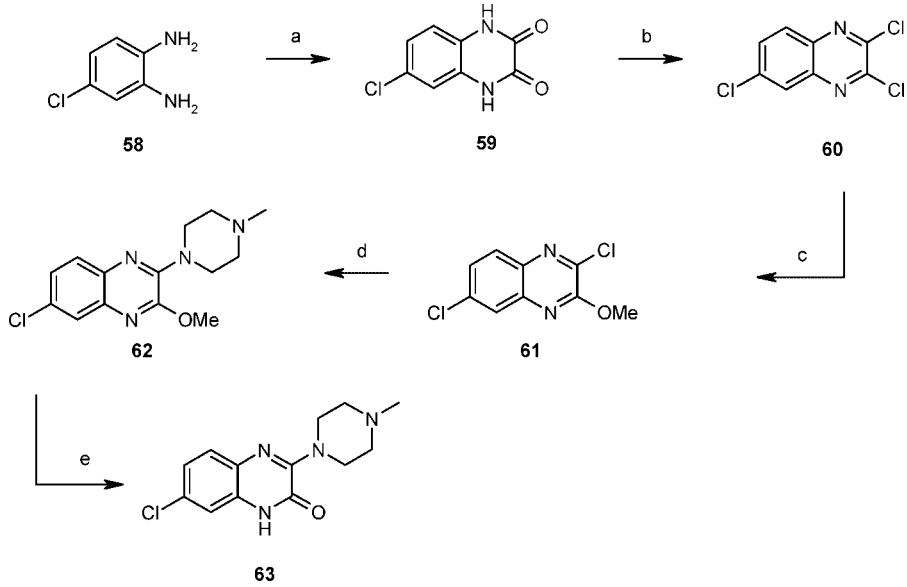
reported series of H_4R ligands indicated that the introduction of a chlorine atom on the all-carbon six-membered ring of several heterocyclic scaffolds could be beneficial for H_4R affinity.[16–18]

After the synthesis and pharmacological evaluation of the compounds in Table 1, we identified 2-(4-methylpiperazin-1-yl)quinoxaline (**3**) as a new lead compound for the development of H_4R ligands. Fragment **3**, together with its chloro analogue **6** has the highest ligand efficiency and thus can be considered the most promising for further optimization.[26]

The introduction of a second nitrogen at the four position of quinoline **4** enhanced the affinity 5-fold, as can be seen in quinoxaline **3**. The introduction of a second nitrogen atom at the three position gave quinazoline **9**, which showed slightly decreased H_4R binding properties. As had been proposed before, the introduction of a chlorine atom at the 6-position was beneficial and resulted in an increase in H_4R affinity of approximately 10-fold in both the quinoline (compare **4** and **5**) and quinoxaline (compare **3** and **6**) scaffolds. Isoquinoline **7** was inactive at the H_4R because the methylpiperazine moiety is most likely positioned unfavorably relative to the heterocyclic ring.

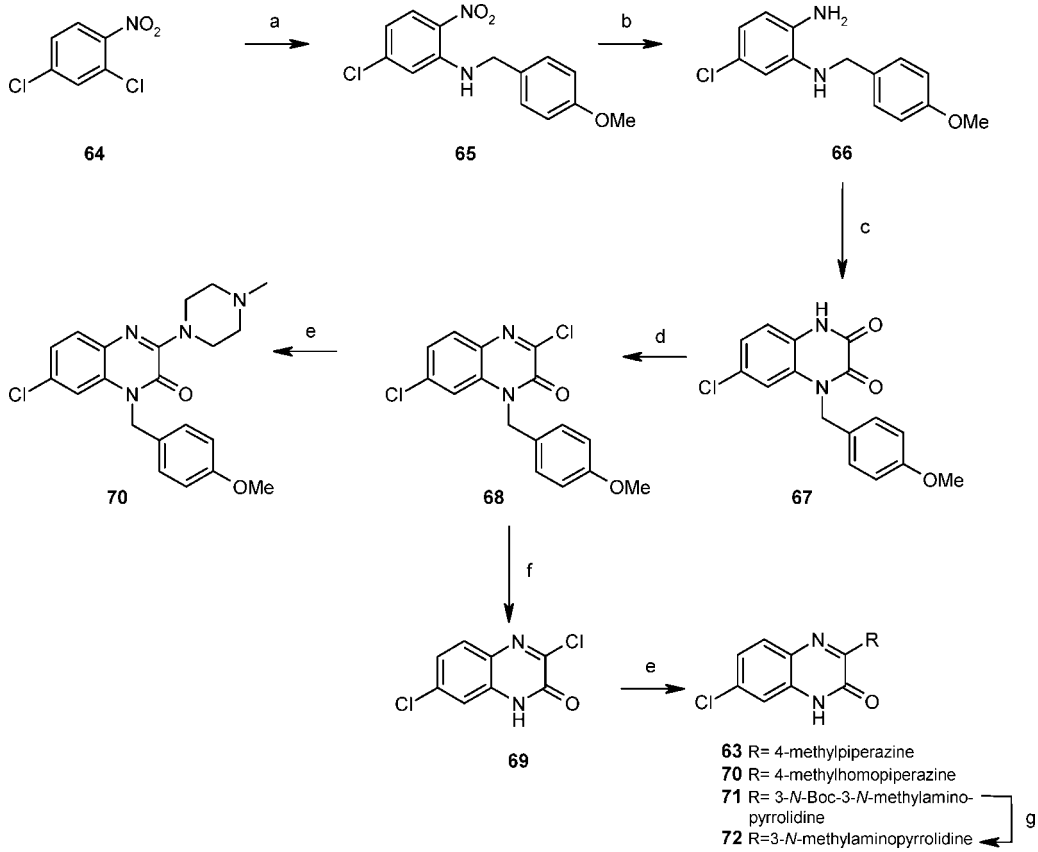
The alignment model suggested that substitution of quinoxaline **3** with an additional aromatic ring system could occupy the aromatic pocket, which is also occupied by one of the

Scheme 4[a]



[a] Reagents and conditions: (a) diethyloxalate, reflux; (b) $POCl_3$, reflux; (c) NaOMe, MeOH, 50°C; (d) *N*-methylpiperazine, DIPEA, THF, mw; (e) 5% NaOH, 70°C.

Scheme 5[a]



[a] Reagents and conditions: (a) *p*-methoxybenzylamine, 1-propanol, reflux; (b) Raney Nickel/H_2, THF, r.t.; (c) diethyloxalate, reflux; (d) $POCl_3$, DIPEA, toluene, reflux; (e) NHR_1R_2, DIPEA, EtOAC, microwave, 120°C; (f) H_2SO_4, r.t.; (g) 2 M HCl in Et_2O, r.t.

aromatic rings of **1** when it binds to the H_4R (Figure 3B).[16] Analogues of quinoxaline **3** could be readily synthesized with a variety of substituents at the 3-position. To probe the aromatic pocket, we therefore initially synthesized the methyl (**18**), phenyl (**19**), and benzyl (**20**) analogues of quinoxaline **3**. The methyl analogue showed an almost 10-fold increase in H_4R affinity compared to **3**, whereas phenyl substitution in **19** is not well tolerated and leads to a drop in H_4R affinity. Interestingly, benzyl-substituted analogue **20** bound to the H_4R with high affinity (33 nM), suggesting that an additional interaction with an aromatic pocket had indeed been found. Therefore, the investigation was extended by introducing various substituents as well as heteroatoms such as oxygen, nitrogen, and sulfur at the 3-position (Table 2).

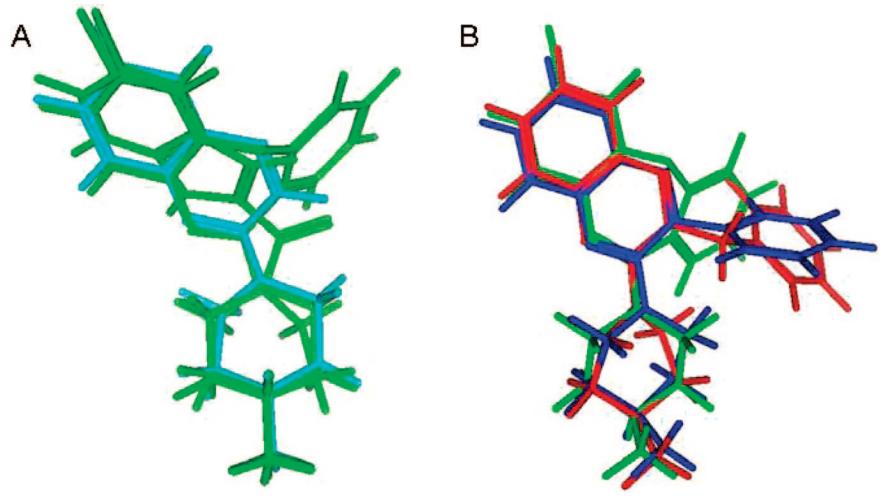


Figure 3. (A) Superposition of **1** and **2** (both in green) with quinoxaline **3** (in light blue) can be seen. Quinoxaline **3** is able to occupy the shared aromatic pockets of **1** and **2**, while all three *N*-methylpiperazine moieties are in the same position. (B) Quinoxalines substituted with a phenyl (compound **19** in blue) and benzyl (compound **20** in red) moiety at the 3-position and their superposition with **1**.

The introduction of oxygen (**28**) or sulfur (**51**) in the benzyl moiety of **20** was detrimental for H_4R affinity, but both compounds bind with higher affinity than quinoxaline **3**, suggesting that a large number of substituents at the 3-position indeed enhance H_4R affinity.

Benzylamine analogue **55** did not improve binding, whereas the benzyl alcohol substituted analogue **29** showed about a 5-fold enhancement of affinity. The introduction of extra carbon atoms in the alkoxy spacer of **29** showed that two carbons (compare **29** and **30**) were tolerated but four carbons (compare **29** and **31**) were detrimental for H_4R binding. Because compound **29** had quite reasonable H_4R affinity, we investigated whether the properties of the aromatic ring of the benzyl alcohol moiety could be optimized for H_4R interaction. 2-Pyridyl analogue **32** had similar affinity to **29**, whereas a nitrogen in the 3- or 4-position of the pyridine ring (compounds **33** and **34**) decreased the H_4R affinity. Analogues **35**−**37** with $-OCH_3$, $-CH_3$, or chlorine substituents did not show improved H_4R affinity.

Parallel to the synthesis of the benzyl alcohols, we synthesized a series of pyridinol and phenol-substituted compounds based on **28** in which we also introduced a number of different substituents. In these series (compounds **39**−**46**), none of the synthesized compounds showed an affinity that was higher than **28**.

Several quinoxalines were synthesized with aliphatic ethers that enhanced the binding affinity of **3** only slightly when substituted with an ethoxy (**49**) or methoxy (**52**) group. However, when the size of these substituents was increased to isobutoxy (**48**) or cyclohexyloxy (**47**), the affinity dropped up to 10-fold for compound **47**. The introduction of a flexible aliphatic side chain with a basic amine (compound **50**) at the 3-position of quinoxaline **3** was not tolerated.

More interesting, quinoxalinone **56** had an affinity almost comparable to benzylquinoxaline **20**. It was anticipated that the introduction of a chlorine atom on the heterocyclic core would improve the H_4R affinity after having optimized the R_2 position as had been the case in other series.[16,17] By this rationale, compounds **62** and **63** were synthesized. The 6-chloro substituent gave an enhancement in H_4R afiinity of 14- and 5-fold in compounds **62** and **63**, respectively. We also synthesized 6,7-dichloro-substituted quinoxaline **57** and found that it was even more potent, showing an H_4R affinity of 6 nM.

To see if the introduction of two chlorine atoms on the quinoxaline heterocycle would give a linear SAR, we synthesized phenethyl-, methyl-, phenoxy- and methoxy analogues **21**, **22**, **52**, and **54**. As can be seen in Table 2, two chlorine substituents increased the affinity of the methoxy (**52**) and methyl (**22**) substituted quinoxalines but not of the phenylethyl (**21**) and phenoxy (**22**) substituted quinoxalines. In the last two compounds, this modulation appeared to even be quite detrimental for H_4R affinity. During the course of these investigations, a series of substituted quinoxalinones was reported by Johnson and Johnson scientists in patent literature as new and very potent H_4R antagonists. Although only a small series of compounds is reported, quinoxalines **63** and **57** are also reported as H_4R antagonists with nanomolar affinities (31 and 32 nM, respectively).[27]

The SAR of the methylpiperazine group was studied to a limited extent because even minor changes such as the replacement of the methyl group by an ethyl group (compare **20** and **74**) is very detrimental for H_4R affinity. The sensitivity to chemical modulation of the *N*-methylpiperazine group was seen before in benzimidazole, indole, and dibenzo-oxazepine series that were reported as H_4R ligands.[17,16,28] However, patent literature reports that in a series of aminopyrimidines and benzofuropyrimidines, the *N*-methylpiperazine moiety can be successfully replaced with 3-aminomethylpyrollidine and several other bioisosteres.[29,30] In the quinoxaline series, attempts to find a suitable bioisostere were unsuccesfull because homopiperazine **70** and aminomethylpyrollidine **72** were found to have affinities that were about 100-fold lower than *N*-methylpiperazine **63**.

For further pharmacological characterization, quinoxalines **20** and **57** were selected from Table 2. The affinity for the other histaminergic receptors was determined in order to assess the selectivity of these ligands for the H_4R over the other histamine receptor subtypes (Table 3). Although compound **57** showed limited inhibition of radioligand binding to the other histaminergic receptors, at a concentration of 10 μM, compound **20** effectively displaces [^{3}H]mepyramine binding to the H_1R. Compound **20** was designed with a working model that made use of compound **1** (Figure 3), which had been demonstrated to have a high affinity ($pK_i = 8.11 \pm 0.10$) for the H_1R.[16] Therefore, we were prompted to determine the pK_i at the H_1R, which was found to be 6.13 ± 0.1 and showed that **20** also possessed some affinity for the H_1R. As was already pointed

Table 3. Pharmacological Evaluation of Selected Quinoxalines at the Four Human Histaminergic Receptor Subtypes

compound	H_1[a] (% inhibition)	H_2 (% inhibition)	H_3 (% inhibition)	H_4[b] (pK_i ± SEM)
20	86[c]	62	26	7.40 ± 0.04
57	7	42	64	8.25 ± 0.07

[a] % Inhibition of radioligand binding to the human H_1−H_3 receptors was determined in duplo at a concentration of 10 μM of ligand. [b] Values are taken from Table 2. [c] The pK_i of **20** at the human H_1R was determined to be 6.13 ± 0.1 (n = 3).

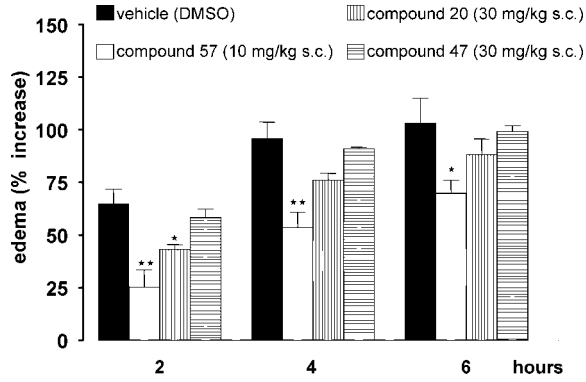


Figure 4. Anti-inflammatory effect of compounds **20** and **57** and inactive control **47** on paw edema induced by subplantar injection of carrageenan (1% in CMC) in rats. Data are expressed as mean ± SEM n = 6 rats per group. Comparisons between multiple groups were made by using one-way analysis of variance (ANOVA), followed by Dunnett's test. *P < 0.05 compared with vehicle-treated animals (Student t test for grouped data).

out earlier, it can be envisioned that dual action H_1/H_4 ligands might have great potential in the treatment of several histamine-mediated allergic and inflammatory disorders.[31]

Compound **20** and **57** were selected to study the anti-inflammatory effect on the new series of H_4R ligands in vivo. After 2 h both compounds, respectively, coadministered at 10 mg/kg and 30 mg/kg with carrageenan, caused a significant inhibition of carrageenan-induced edema when compared to the vehicle (Figure 4). The effect of **57** was still found to be significant 6 h after administration. Compound **47** (pK_i = 4.88 ± 0.03), which was selected for this study as an inactive control, displayed no significant inhibition of the carrageenan-induced edema.

Conclusion

Albeit limited by the available GPCR protein structural information and the complications when applying biophysical screening approaches to these targets, the growing of a micromolar affinity fragment into nanomolar ligands can be considered a fragment based design approach that has been successfully used in many GPCR projects before.[2,33] In this work, we have described the discovery of a series of new histamine H_4R ligands starting from an in silico flexible alignment model of the known H_4R agonist **1** and antagonist **2**. It was used to design a series of small heterocyclic fragments as H_4 receptor–ligands, of which 2-(4-methylpiperazin-1-yl)quinoxaline (**3**) binds to the H_4R with a K_i in the submicromolar range. The growing of fragments into more druglike compounds by introducing a variety of substituents onto this new scaffold was guided by the in silico model, thereby strengthening the rational design. This approach led to a series of potent H_4R ligands with affinities in the low nanomolar range. Two of these compounds, 6,7-dichloro-3-(4-methylpiperazin-1-yl)quinoxalin-2(1*H*)-one (**57**) and 2-benzyl-3-(4-methyl-piperazin-1-yl)quinoxaline (**20**) were evaluated in vivo and displayed significant anti-inflammatory activity in the carrageenan induced paw edema model in rats.

Experimental Section

In Vivo Pharmacology. Carrageenan-Induced Edema Model. Animals. Male Wistar rats (180–200 g; Harlan-Italy, Milan) were housed under controlled standard conditions (23 °C temperature, 12 h light/dark cycle, and 65% humidity). Food and water were provided ad libitum. The experiments received the approval of the local Animal Ethics Committee of the University of Parma, Italy.

Induction of Acute Inflammation. Inflammation was induced in fasted rats by subplantar injection of carrageenan (0.1 mL of 1% suspension in carboxymethycellulose) into the left hind paw. As previously described, carrageenan-induced edema was measured with a plethysmometer (Basile, Comerio, Italy) immediately prior to the injection of carrageenan and thereafter at 2, 4, and 6 h.[33] Edema was expressed for each animal as % increase in paw volume after carrageenan injection relative to the preinjection value, considered as 100.

Compound Administration. Equivalent volumes (0.1 mL per 100 g) of the test compounds or vehicle were administered subcutaneously (s.c.) in separate groups of rats immediately prior to carrageenan injection. Before use solutions of the histamine H_4 receptor–ligands in DMSO were freshly prepared.

Data Analysis. Data are expressed as mean ± SEM. A value of P < 0.05 was considered statistically significant. The software package Prism GraphPad 3.0 (GraphPad Software Inc., San Diego, CA) was used to process data.

In Vitro Pharmacology. Radioligand Displacement Studies at the Human H_1, H_2, and H_3 Receptors. Single-point radioligand displacement studies at the human H_1, H_2, and H_3 receptors were preformed by Cerep (Le Bois l'Evêque, France) at a concentration of 10 μM of ligand. These measurements were performed in duplo. The pK_i at the H_1R was determined according to a procedure described in literature.[34]

Radioligand Displacement Studies at the Human H_4 Receptor. Cell Culture and Transfection. HEK 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/mL penicillin, and 50 μg/mL streptomycin in 5% CO_2 humidified atmosphere at 37 °C. Approximately 4 million cells were seeded in a 10 cm dish and cultured overnight before transfection. For transfection of each dish of cells, the transfection mixture was prepared in 1 mL serum-free DMEM and contained 5 μg of human H_4R receptor plasmid and 15 μL of 1 mg/mL 25 kDa linear polyethyleneimine (Polyscience, Inc., USA). The mixture was incubated for 10–15 min at room temperature before it was added into the monoloyer cell culture loaded with 5 mL of fresh cell culture medium. Two days after transfection, the cells were washed with PBS containing 1 mM EDTA, collected as pellet by centrifuging, and stored at –20 °C until use.

[^{3}H] Histamine Binding Assay. For the radioligand binding study, pellets of transfected cells were homogenized in H_4R binding buffer (100 mM Tris-HCl, pH 7.4). The saturation binding assay was performed using different concentrations of [^{3}H]histamine (Perkin-Elmer Life Science, Inc., USA), while nonspecific binding was determined by incubation in the presence of 3–10 μM of compound **2** in a total assay volume of 200 μL. For the displacement binding assay, the membranes were typically incubated with 10^{-4}−10^{-11} M of ligands (stock concentration was 10 mM 1 DMSO) in the presence of [^{3}H]histamine in a total volume of 200 μL. The reaction mixtures were incubated for 1 h at room temperature (22 °C), and harvested on 96-well glass fiber C plates that were pretreated with 0.3% 750 kDa PEI. The binding assay data were analyzed using Prism 4.0 (Graphpad Software Inc., USA).

Flexible Alignment. The alignment model was created with Molecular Operating Environment 2006.08 (MOE) from Chemical Computing Group (Montreal, Canada). The compounds in Figure 3 were aligned with the flexible alignment module of MOE using

default parameters and similarity term "partial charge" added with a weight factor of 1. The alignment with highest mutual similarity score F ($F = 32.1926$, $S = 159.991$, $U = 127.7983$) was selected and refined further using the "refine existing alignment" option with an energy cutoff of 7.0 kcal/mol.

General Remarks. Carrageenan was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals and reagents were obtained from commercial suppliers and were used without further purification. Yields given are isolated yields unless mentioned otherwise. Flash column chromatography was typically carried out on an Argonaut Flashmaster II flash chromatography system, using prepacked Isolute Flash Si II columns with the UV detector operating at 254 nm. All melting points are uncorrected and were measured on an Optimelt automated melting point system from Stanford Research Systems. All ^{1}H NMR and ^{13}C NMR spectra were measured on a Brüker 200 or Brüker 400.

Analytical HPLC-MS analyses were conducted using a Shimadzu LC-8A preparative liquid chromatograph pump system with a Shimadzu SPD-10AV UV–vis detector with the MS detection performed with a Shimadzu LCMS-2010 liquid chromatograph–mass spectrometer. The buffer mentioned under conditions I and II is a 0.4% (w/v) NH_4CO_3 solution in water, adjusted to pH 8.0 with NH_4OH. The analyses were performed using the following two conditions:

Condition I: an Xbridge (C18)5μm column (100 mm × 4.6 mm) with the following two solvents: solvent A, 90% MeCN−10%; solvent B, 90% water−10% buffer; flow rate = 2.0 mL/min; start: 5% A, linear gradient to 90% A in 10 min, then 10 min at 90% A, then 10 min at 5% A. Total run time: 30 min.

Condition II: Xbridge (C18)5μm column (100 mm × 4.6 mm) with the following two solvents: solvent A, MeCN with 0.1% formic acid; solvent B, water with 0.1% formic acid; flow rate = 2.0 mL/min; start: 5% A, linear gradient to 90% A in 10 min, then 5 min at 90% A, then 5 min at 5% A. Total run time: 20 min. Compound purities under both conditions were calculated as the percentage peak area of the analyzed compound by UV detection at 254 nm.

Analytical HPLC-MS analyses for condition III were carried out on Agilent 1100 Series HPLC-MS system including an Agilent G1315B DAD. Condition III: XBridge (C18)3.5μm column (2.1 mm × 50 mm) with the following two solvents: solvent A, a 5 mM solution of NH_4HCO_3 in water set to pH 9.0 using 19 mM NH_3; solvent B, 100% MeCN. The MS detection was performed with an Agilent G1956B LC/MSD SL using a multimode ion source. Flow rate = 1.2 mL/min; start: 95% A, linear gradient to 5% A in 1.25 min, then 0.75 min at 5% A, followed by 1.0 min 95% A, 5% B. Total run time: 3 min.

Synthetic Methods. 2-(4-Methylpiperazin-1-yl)quinoxaline (3). 2-Chloroquinoxaline (309 mg, 1.88 mmol) and *N*-methylpiperazine (2.0 mL) were added to a microwave tube and heated at 160 °C for 5 min. The resulting mixture was diluted with water and extracted with EtOAc. Drying over Na_2SO_4 and evaporation of the solvent yielded 402 mg (1.76 mmol, 94%) of a brown solid. Mp 108.6–110.9 °C; ^{1}H NMR ($CDCl_3$): δ (ppm) 8.56 (s, 1H), 7.85 (dd, $J = 8.2$ Hz, $J = 1.3$ Hz, 1H), 7.66 (dd, $J = 8.4$ Hz, $J = 1.3$ Hz, 1H), 7.59–7.50 (m, 1H), 7.41–7.24 (m, 1H), 3.79 (t, $J = 5.1$ Hz, 4H), 2.54 (t, $J = 5.1$ Hz, 4H), 2.34 (s, 3H); ^{13}C NMR ($CDCl_3$): δ (ppm) 152.09, 141.46, 136.66, 135.55, 129.92, 128.50, 126.32, 124.60, 54.61, 46.07, 44.48.

2-(4-Methylpiperazin-1-yl)quinazoline (9). A solution of 2,4-dichloroquinazoline (1.2 g, 6.03 mmol) in DCM (24 mL) covered with brine (24 mL) containing 9% NH_4OH was treated with powdered zinc (1.2 g), and the resulting biphasic mixture was then refluxed for 3.5 h. After cooling to r.t., it was filtered through celite and the organic layer was removed under reduced pressure. The residue was diluted with EtOAc and washed with 1 N HCl solution, after which it was dried and concentrated to dryness. Without purification, *N*-methylpiperazine (4.0 mL) was added, causing a crystalline solid to separate almost immediately. The product was diluted with ethyl acetate and washed with $NaHCO_3$ and water. Drying over Na_2SO_4 and rotary evaporation yielded the crude title compound. Purification over SiO_2 yielded 180 mg (0.79 mmol, 13%) of a light-orange solid; mp 74.4–76.0 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 8.81 (s, 1H), 7.52–7.40 (m, 3H), 7.04–6.96 (m, 1H), 3.85 (t, $J = 5.0$ Hz, 4H), 2.34 (t, $J = 5.0$ Hz, 4H), 2.17 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 161.05, 158.86, 151.98, 133.68, 127.06, 125.41, 122.13, 119.32, 54.78, 46.01, 43.69; MS (ESI) m/z 229 $(M + H)^+$.

2-Benzyl-3-(4-methylpiperazin-1-yl)quinoxaline (20). 3-Benzylquinoxalin-2(1*H*)-one (**14**) (998 mg, 4.2 mmol) and $POCl_3$ (6.0 mL) were added to a round-bottom flask and heated at reflux. After 5 h, the mixture was allowed to cool to r.t. and was poured onto crushed ice. Concentrated ammonia was then used to adjust the mixture to pH 6, and the obtained suspension was left to stir vigorously overnight at r.t. After filtration of the suspension and washing of the obtained solids with water, the product was dried in vacuo yielding 1.06 g (41.6 mmol, 99%) of 2-benzyl-3-chloroquinoxaline as a white solid. This was used directly in the next step without further purification. A microwave tube charged with 2-benzyl-3-chloroquinoxaline (400 mg, 1.57 mmol) and *N*-methylpiperazine (3.0 mL) was heated at 120 °C for 5 min. Then water (25 mL) was added and the aqueous layer was extracted with EtOAc. The combined organic extracts were dried with brine and Na_2SO_4. Evaporation of the organic solvents and subsequent purification over SiO_2 (EtOAc 90%, Et_3N 5%, MeOH 5%) yielded 446 mg (1.40 mmol, 89%) of a yellow solid; mp 89.0–90.0 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.82 (dd, $J = 1.4$ Hz, $J = 8.3$ Hz, 1H), 7.62–7.46 (m, 2H), 7.34–7.14 (m, 5H), 4.35 (s, 2H), 3.32 (t, $J = 4.8$ Hz, 4H), 2.57 (t, $J = 4.8$ Hz), 2.35 (s, 3H). ^{13}C NMR (DMSO-d_6) δ (ppm): 155.89, 151.33, 140,04, 138.80, 138.24, 128.97, 128.63, 128.29, 128.16, 127.18, 126.69, 126.31, 54.75, 49.59, 45.98, 40.71; MS (ESI) m/z 319 $(M + H)^+$.

2-Chloro-3-(4-methylpiperazin-1-yl)quinoxaline (26). To a solution of 2,3-dichloroquinoxaline (6.60 g, 33.2 mmol) in toluene (45 mL) was added triethylamine (4.6 mL, 33.0 mmol) and the solution was heated at reflux. *N*-methylpiperazine (3.7 mL, 33.4 mmol) was added and heating was continued for 2 h. The resulting mixture was diluted with saturated $NaHCO_3$ solution. The organic layer was separated, and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. Purification of the residue over SiO_2 (90% EtOAc, 5% Et_3N, 5% MeOH) (EtOAc) yielded 7.70 g (29.3 mmol, 88%) of a yellow solid; mp 67.4–68.6 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.87–7.78 (m, 2H), 7.65–7.47 (m, 2H), 3.60 (t, $J = 4.9$ Hz, 4H), 2.63 (t, $J = 4.9$ Hz, 4H), 2.37 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 152.38, 141.46, 139.99, 138.03, 129.94, 127.54, 127.13, 126.87, 54.55, 48.77, 45.96. MS (ESI) m/z 263 $(M + H)^+$.

2,6,7-Trichloro-3-(4-methylpiperazin-1-yl)quinoxaline (27). A microwave tube charged with 2,3,6,7-tetrachloro-quinoxaline (500 mg, 1.87 mmol), *N*-methylpiperazine (0.21 mL, 1.89 mmol), DIPEA (0.37 mL, 2.12), and THF (3.0 mL) was heated at 140 °C. After 5 min, the reaction mixture was diluted with water and extracted with EtOAc. The combined organic layers were washed with brine and dried over Na_2SO_4. The extracts were concentrated and filtered over a short column of SiO_2 (EtOAc as eluent) Evaporation of the solvent yielded 523 mg (1.58 mmol, 84%) of the title compound. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.90 (s, 1H), 7.87 (s, 1H), 3.61 (t, $J = 4.8$ Hz, 1H), 2.62 (t, $J = 4.8$ Hz, 1H), 2.36 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 152.66, 142.30, 138.93, 136.46, 134.28, 130.89, 128.05, 127.42, 54.22, 48.60, 45.89.

2-(4-Methylpiperazin-1-yl)-3-phenoxyquinoxaline (28). To a round-bottom flask were added 2-chloro-3-(4-methylpiperazin-1-yl)quinoxaline (**26**) (1.0 g, 3.81 mmol), phenol (0.43 g, 4.57 mmol), DMF (3.0 mL), and NaOH (0.23 g, 5.75 mmol). The resulting suspension was stirred at r.t. for 2 h. After completion, the mixture was diluted with water and the aqueous layer was extracted with EtOAc. The combined organic extracts were thoroughly washed with water and brine and then dried over Na_2SO_4. Evaporation of the solvent gave an oil that was purified over SiO_2 (90% EtOAc, 5% Et_3N, 5% MeOH) yielding 0.89 g (2.78 mmol, 73%) of a yellow solid; mp 96.8–97.6 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.73 (dd, $J = 8.1$ Hz, $J = 1.2$ Hz, 1H), 7.56–7.20 (m, 8H), 3.82 (t, $J = 4.9$ Hz,

4H), 2.61 (t, $J = 4.9$ Hz, 4H), 2.36 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 152.61, 149.95, 147.02, 138.95, 135.79, 129.37, 127.18, 126.49, 126.00, 125.62, 125.03, 121.60, 54.88, 47.67, 46.04; MS (ESI) m/z 321 $(M + H)^+$.

2-Ethoxy-3-(4-methylpiperazin-1-yl)quinoxaline (49). To 2-chloro-3-(4-methylpiperazin-1-yl)quinoxaline (**26**) (355 mg, 1.28 mmol) in ethanol (5.0 mL) was added NaOH (54 mg, 1.35 mmol, in 1.0 mL water) and the obtained solution was heated at reflux for 2 h. After cooling to room temperature, the solution was added to a separatory funnel containing EtOAc and water and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with water and brine and dried over $MgSO_4$. Purification over SiO_2 (EtOAc 90%, MeOH 5%, Et_3N 5%) yielded 214 mg (0.79 mmol, 61%) of the title compound as a yellow oil. As a side-product, 30 mg (0.12 mmol, 9%) of compound **56** was also isolated. Analytical data for **49**: ^{1}H NMR ($CDCl_3$) δ (ppm): 7.70–7.62 (m, 2H), 7.44–7.30 (m, 2H), 4.54 (q, $J = 7.1$ Hz, 2H), 3.73 (t, $J = 4.9$ Hz, 4H), 2.57 (t, $J = 5.0$ Hz, 4H), 2.34 (s, 3H), 1.46 (t, $J = 7.0$ Hz, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 150.34, 146.87, 138.04, 136.36, 126.13, 126.01, 125.81, 125.38, 62.25, 54.81, 47.46, 46.01, 14.23; MS (ESI) m/z 273 $(M + H)^+$.

2-Methoxy-3-(4-methylpiperazin-1-yl)quinoxaline (52). 2-Chloro-3-(4-methylpiperazin-1-yl)quinoxaline (**26**) (1.0 g, 3.81 mmol) and a 5.4 M solution of NaOMe in MeOH (0.9 mL, 4.86 mmol) were dissolved in MeOH (20 mL) and heated at reflux. After 3 h, the obtained mixture was cooled to r.t. and diluted with EtOAc (60 mL). The organic layer was then washed with water and brine and dried over Na_2SO_4. Evaporation of the organics yielded 932 mg (3.61 mmol, 95%) of a light-brown solid; mp 74.0–76.3 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.71–7.65 (m, 2H), 7.45–7.33 (m, 2H), 4.09 (s, 3H), 3.71 (t, $J = 4.9$ Hz, 4H), 2.57 (t, $J = 5.0$ Hz, 4H), 2.34 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 150.77, 146.95, 138.14, 136.36, 126.30, 126.07, 125.85, 125.51, 54.87, 53.52, 47.50, 46.05; MS (ESI) m/z 259 $(M + H)^+$.

3-(4-Methylpiperazin-1-yl)quinoxalin-2(1*H*)-one (56). Method 1: Synthesis from 2-Chloro-3-(4-methylpiperazin-1-yl)quinoxaline. A microwave tube containing 2-chloro-3-(4-methylpiperazin-1-yl)quinoxaline (**26**) (150 mg, 0.57 mmol), NaOH (60 mg, 1.5 mmol), THF (2.0 mL), and water (0.5 mL) was heated at 120 °C for 20 min. After completion, the solution was partitioned between EtOAc and water and the aqueous layer was extracted with EtOAc. After drying over Na_2SO_4, the solvent was evaporated and the residue was purified over SiO_2 (EtOAc 90%, Et_3N 5%, MeOH 5%) yielding 100 mg (0.41 mmol, 72%) of a white solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 11.29 (bs, 1H), 7.53–7.48 (m, 1H), 7.24–7.08 (m, 3H), 4.03 (t, $J = 4.9$ Hz, 4H), 2.58 (t, $J = 5.0$– Hz, 4H), 2.34 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 153.41, 150.74, 133.05, 128.32, 125.75, 124.91, 124.02, 114.21, 54.95, 46.50, 45.99; MS (ESI) m/z 245 $(M + H)^+$.

Method 2: Synthesis from 2-Methoxy-3-(4-methylpiperazin-1-yl)quinoxaline. 2-Methoxy-3-(4-methylpiperazin-1-yl)-quinoxaline (**52**) (100 mg, 0.39 mmol) and 5% NaOH in water (5.0 mL) were heated at reflux for 20 h. After cooling to room temperature, the pH was adjusted to 7.0 with diluted HCl. The aqueous solution was extracted with DCM, and the combined extracts were washed with brine. Drying over Na_2SO_4 and evaporation of the solvent yielded 84 mg (0.34 mmol, 88%) of a white solid. An analytical sample was recrystallized from EtOAc to yield white needles; mp 206.4–207.8 °C.

6,7-Dichloro-3-(4-methylpiperazin-1-yl)quinoxalin-2(1*H*)-one (57). To a microwave tube were added 2,6,7-trichloro-3-(4-methylpiperazin-1-yl)-quinoxaline (**26**) (212 mg, 0.64 mmol), dioxane (3.0 mL), NaOH (52 mg, 1.30 mmol), and water (1.0 mL). The mixture was heated in the microwave for 5 min at 120 °C and diluted with water. The obtained suspension was thoroughly extracted with DCM, and the organic layers were dried with Na_2SO_4. The residue that was obtained after evaporation of the solvent was triturated with EtOAc to yield 114 mg (0.36 mmol, 57%) of an off-white solid; mp 262.4–263.6 °C. ^{1}H NMR (DMSO-d_6) δ (ppm): 7,52 (s, 1H), 7.26 (s, 1H), 3.92 (m, 4H), 2.41 (t, $J = 4.9$ Hz, 4H), 2.19 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 151.51, 150.96, 132.27, 128.87, 125.20, 125.20, 124.43, 114.87, 54.32, 45.70, 45.42; MS (ESI) 313 m/z $(M + H)^+$.

2,6-Dichloro-3-methoxyquinoxaline (61). A 5.4 M solution of NaOMe in methanol (2.9 mL, 15.7 mmol) was diluted with methanol (36 mL) and added over a 6 h period to a slurry of 2,3,6-trichloroquinoxaline (3.0 g, 12.8 mmol) in methanol (36 mL) at 50 °C. After addition, the obtained solution was refluxed for 16 h and the solvent was evaporated. The residue was taken up in $CHCl_3$ (100 mL) and washed with water and brine. After drying over Na_2SO_4, the organic layer was removed and the residue was purified over SiO_2 (toluene) to yield a white solid. After three crystallizations, 369 mg (1.61 mmol, 13%) of the title compound was obtained as white needles (purity >95% by NMR); mp 110.9–111.4 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.86–7.82 (m, 2H), 7.51 (dd, $J = 8.8$ Hz, $J = 2.2$ Hz, 1H), 4.14 (s, 3H).

6-Chloro-3-methoxy-2-(4-methylpiperazin-1-yl)quinoxaline (62). To a microwave tube were added 2,6-dichloro-3-methoxy-quinoxaline (**61**) (200 mg, 0.87 mmol), *N*-methylpiperazine (0.2 mL, 1.80 mmol), and THF (3.0 mL). After heating the solution for 5 min at 140 °C, the obtained suspension was diluted with EtOAc (30 mL) and washed with saturated $NaHCO_3$, water, and brine. Drying with Na_2SO_4 and evaporation of the organic layer yielded an off-white solid. Purification over SiO_2 (EtOAc 90%, MeOH 5%, Et_3N 5%) yielded 168 mg (0.57 mmol, 66%) of a white-yellow solid; mp 99.9–101.6 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.66 (d, $J = 2.3$ Hz, 1H), 7.59 (d, $J = 8.7$ Hz, 1H), 7.34 (dd, $J = 8.8$ Hz, $J = 2.4$ Hz, 1H), 4.01 (s, 3H), 3.71 (t, $J = 5.0$ Hz, 4H), 2.56 (t, $J = 5.0$ Hz, 4H), 2.34 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 151.24, 146.83, 136.80, 136.73, 130.49, 127.02, 126.85, 125.09, 54.85, 53.72, 47.42, 46.03; MS (ESI) m/z 293 $(M + H)^+$.

6-Chloro-3-(4-methylpiperazin-1-yl)quinoxalin-2(1*H*)-one (63). Method 1: Synthesis from 6-Chloro-3-methoxy-3-(4-methylpiperazin-1-yl)quinoxaline (62). 6-Chloro-3-methoxy-3-(4-methylpiperazin-1-yl)quinoxaline (**62**) (135 mg, 0.46 mmol) and 5% aqueous NaOH (10 mL) were heated at 70 °C. After 4 h, the clear solution was neutralized (pH 7) with diluted HCl and extracted with DCM. The combined extracts were then washed with brine. After drying over Na_2SO_4 and evaporation of the organics, the residue was purified over SiO_2 (EtOAc 90%, MeOH 5%, Et_3N 5%) to yield 35 mg (0.13 mmol, 28%) of the title compound as a white solid; mp 228.5–230.0 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 11.23 (bs, 1H), 7.40 (d, $J = 8.5$ Hz, 1H), 7.14 (dd, $J = 8.6$ Hz, $J = 2.2$ Hz, 1H), 7.03 (d, $J = 2.2$ Hz, 1H), 4.03 (t, $J = 4.8$ Hz, 4H), 2.58 (t, $J = 5.0$ Hz, 4H), 2.35 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 153.32, 150.36, 131.74, 129.94, 128.91, 126.74, 124.40, 113.91, 54.89, 46.47, 45.93; MS (ESI) m/z 279 $(M + H)^+$.

Method 2: Synthesis from 3,7-Dichloroquinoxalin-2(1*H*)-one (69). *N*-Methylpiperazine (1.0 mL), 3,7-dichloro-quinoxalin-2(1*H*)-one (**69**) (300 mg, 1.40 mmol), and EtOAc (2.0 mL) were added to a microwave tube and were heated at 120 °C for 10 min. After cooling to r.t., the mixture was diluted with EtOAc and washed with water and brine. Drying of the organic phase over Na_2SO_4 and evaporation of the solvent gave the crude product that was recrystallized from EtOAc to yield 227 mg (0.81 mmol, 58%) of off-white crystals; mp 230.0–232.0 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 11.01 (bs, 1H), 7.36 (d, $J = 8.6$ Hz, 1H), 7.10 (dd, $J = 8.6$ Hz, $J = 2.2$ Hz, 1H), 7.03 (d, $J = 2.1$ Hz, 1H), 3.98 (t, $J = 4.9$ Hz, 4H), 2.52 (t, $J = 5.0$ Hz, 4H), 2.30 (s, 3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 153.26, 150.38, 131.75, 129.91, 128.91, 126.73, 124.38, 113.88, 54.90, 46.49, 45.94; MS (ESI) m/z 279 $(M + H)^+$.

5-Chloro-*N*-(4-methoxybenzyl)-2-nitroaniline (65). A solution of 3,4-dichloro-nitrobenzene (37.0 g, 0.19 mol) and 4-methoxybenzylamine (50 mL, 0.38 mol) in *n*-propanol (200 mL) was heated at reflux for 16 h, after which the solvent was removed under reduced pressure. The residue was taken up in EtOAc and washed with water and brine. Drying of the organic phase over Na_2SO_4 and removal of the solvent yielded a yellow solid that was recrystallized from abs EtOH to yield 42.6 g (0.15 mol, 76%) of yellow crystals; mp 70.0–72.0 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 8.33 (br s, 1H), 8.11 (d, $J = 9.1$ Hz, 1H), 7.27–7.23 (m, 2H), 6.89 (d,

J = 8.7 Hz, 2H), 6.82 (d, J = 2.1 Hz, 1H), 6.61 (dd, J = 9.1 Hz, J = 2.1 Hz, 1H), 4.41 (d, J = 5.4 Hz, 2H), 3.80 (s, 3H).

5-Chloro-*N*′-(4-methoxybenzyl)benzene-1,2-diamine (65). To a solution of **64** (8.7 g, 29.7 mmol) in THF (150 mL) in a round-bottom flask was added Raney nickel (2.0 g of a 50% suspension in water) and the resulting mixture was stirred vigorously. Hydrogen gas was added with a balloon until TLC indicated complete conversion of the starting material (16 h). The catalyst was removed by filtration, and the reaction mixture was evaporated to dryness, yielding 7.74 g (29.5 mmol, 98%) of a dark-brown solid that was used in the next step without further purification. ^{1}H NMR (DMSO-d_6) δ (ppm): 7.27 (d, J = 8.7 Hz, 2H), 6.89 (d, J = 8.7 Hz, 2H), 6.50 (d, J = 8.2 Hz, 1H), 6.37 (dd, J = 8.1 Hz, J = 2.2 Hz, 1H), 6.26 (d, J = 2.3 Hz, 1H), 5.33 (m, 1H), 4.71 (s, 2H), 4.21 (d, J = 6.1 Hz, 2H), 3.73 (s, 3H).

7-Chloro-1-(4-methoxybenzyl)quinoxaline-2,3(1*H*,4*H*)-dione (67). Crude **66** (7.7 g, 0.029 mol) and diethyloxalate (16.0 mL, 154 mmol) were heated at 140 °C for 16 h. After completion, the reaction mixture was carefully cooled to r.t. upon which the desired product crystallized from the reaction flask. The product was filtered off and washed with cold EtOH to yield 8.35 g (26.4 mmol, 91%) of the title compound as white crystals; mp 236.0–238.0 °C. ^{1}H NMR (DMSO-d_6): δ (ppm): 12.18 (br s, 1H), 7.28–7.13 (m, 5H), 6.88 (d, J = 8.7 Hz, 2H), 5.30 (s, 2H), 3.71 (s, 3H). ^{13}C NMR (DMSO-d_6) δ (ppm): 158.25, 155.39, 153.17, 127.87, 127.27, 126.96, 126.58, 124.84, 123.06, 116.77, 114.89, 113.84, 54.80, 44.74.

3,7-Dichloro-1-*N*-(4-methoxybenzyl)quinoxalin-2-one (68). Compound **67** (6.46 g, 20.4 mmol) and DIPEA (6.8 mL, 39.0 mmol) were dissolved in DMF (5.0 mL), after which $POCl_3$ (6.0 mL, 64.4 mmol) was carefully added. The resulting mixture was heated at 95 °C for 60 min and carefully poured onto crushed ice. After vigorous stirring, the obtained suspension was extracted with DCM, and the combined organic extracts were washed with water and brine and dried over Na_2SO_4. The solvent was reduced to about 20 mL and the solution was filtered over a pad of silica using DCM with 5% of methanol to elute the compound. Evaporation of the collected filtrate yielded 5.15 g (15.4 mmol, 75%) of the title compound as a white solid; mp 182.0–183.0 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.80 (d, J = 8.6 Hz, 1H), 7.59 (d, J = 2.0 Hz, 1H), 7.43 (dd, J = 8.6 Hz, J = 2.0 Hz, 1H), 7.27 (d, J = 8.6 Hz, 2H), 6.89 (d, J = 8.6 Hz, 2H), 5.45 (s, 2H), 3.71 (s, 3H).

3,6-Dichloroquinoxalin-2(1*H*)-one (69). Compound **68** (1.0 g, 2.98 mmol) was dissolved in concentrated H_2SO_4 (6.0 mL) and stirred at room temperature. After 20 min, the deep-red reaction mixture was carefully poured onto a small amount of crushed ice and neutralized with 15 N sodium hydroxide solution while cooling at 0 °C. A cloudy suspension was obtained that was extracted thoroughly with *n*-butanol. The combined organic layers were washed with a small amount of brine and dried over Na_2SO_4. Removal of the solvent yielded 640 mg (100%) of the crude title compound as a light-yellow solid. ^{1}H NMR (DMSO-d_6) δ (ppm): 7.73 (d, J = 8.6 Hz, 1H), 7.39–7.30 (m, 2H); ^{13}C NMR (DMSO-d_6) δ (ppm): 150.80, 149.27, 134.57, 133.15, 129.40, 129.29, 123.58, 114.67.

7-Chloro-3-(3-(methylamino)pyrrolidin-1-yl)quinoxalin-2(1*H*)-one Hydrochloride (72). To compound **69** (100 mg, 0.47 mmol) in a microwave tube were added Boc-aminomethylpyrrolidine (103 mg, 0.51 mmol), DIPEA (0.9 mL, 0.51 mmol), and EtOAc (2.0 mL), and the resulting suspension was heated at 120 °C for 10 min. After cooling to room temperature, the mixture was diluted with EtOAc and washed with water and brine. Drying of the organic phase over Na_2SO_4 and rotary evaporation gave the crude product that was purified over SiO_2 (EtOAC:hexanes, 1:4) to yield 124 mg (64%) of the Boc-protected intermediate (**72**) as a light-yellow solid. This compound was then stirred in 2 M HCl ether (2.0 mL) and dioxane (2.0 mL) until removal of the Boc group was completed (LCMS). Evaporation of the solvents yielded the hydrochloric salt as a yellow-white solid. ^{1}H NMR (400 MHz, 341 K, D_2O) δ (ppm): 7.85 (d, J = 8.8 Hz, 1H), 7.62 (dd, J = 8.8 Hz, J = 2.2 Hz, 1H), 7.58 (d, J = 2.1 Hz, 1H), 4.92 (dd, J = 14.1 Hz, J = 6.8 Hz, 1H), 4.79 (dd, J = 14.2 Hz, J = 5.0 Hz, 1H), 4.59–4.54 (m, 1H), 4.50–4.41 (m, 2H), 3.18 (s, 3H), 3.00–2.92 (m, 1H), 2.75–2.66 (m, 1H). ^{13}C NMR (D_2O) δ (ppm): 153.49, 147.74, 132.71, 128.45, 126.48, 125.11, 121.49, 116.65, 59.01, 54.58, 51.10, 33.14, 28.02; MS (ESI) m/z 279 $(M + H)^+$.

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Supporting Information Available: LCMS purity data for compounds **5**, **7**, **9**, **18**−**23**, **26**, **28**−**57**, **62**, **63**, **70**, and **72**−**74** and synthetic procedures for compounds **4**−**7**, **18**, **19**, **21**−**32**, **29**−**48**, **50**−**52**, **54**, **55**, **70**, **73**,and **74**. This material is available free of charge via the Internet at http://pubs.acs.org.

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