Structure–Activity Relationships of Quinoxaline-Based 5-HT₃A and 5-HT₃AB Receptor-Selective Ligands

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Until recently, discriminating between homomeric 5-HT₃A and heteromeric 5-HT₃AB receptors was only possible with ligands that bind in the receptor pore. This study describes the first series of ligands that can discriminate between these receptor types at the level of the orthosteric binding site. During a recent fragment screen, 2-chloro-3-(4-methylpiperazin-1-yl)quinoxaline (VUF10166) was identified as a ligand that displays an 83-fold difference in [³H]granisetron binding affinity between 5-HT₃A and 5-HT₃AB receptors. Fragment hit exploration, initiated from VUF10166 and 3-(4-methylpiperazin-1-yl)-quinoxaline-2-ol, resulted in a series of compounds with higher affinity at either 5-HT₃A or 5-HT₃AB receptors. These ligands reveal that a single atom is sufficient to change the selectivity profile of a compound. At the extremes of the new compounds were 2-amino-3-(4-methylpiperazin-1-yl)quinoxaline, which showed 11-fold selectivity for the 5-HT₃A receptor, and 2-(4-methylpiperazin-1-yl)quinoxaline, which showed an 8.3-fold selectivity for the 5-HT₃AB receptor. These compounds represent novel molecular tools for studying 5-HT₃ receptor subtypes and could help elucidate their physiological roles.

Introduction

5-HT₃ receptors are ligand-gated ion channels that are responsible for fast synaptic neurotransmission in the central (CNS) and peripheral nervous systems (PNS). They are involved in physiological functions as diverse as the vomiting reflex, pain processing, reward, cognition, and anxiety, and modulate the release of neurotransmitters such as acetylcholine, cholecystokinin, dopamine, GABA, glutamate, and serotonin itself.[1] To be distinguished by differences in their 5-HT concentration–response curves (increased EC₅₀ values and shallower Hill slopes), increased single channel conductance (5-HT₃A = sub-pS; 5-HT₃AB = 16-30 pS), increased rate of desensitization, decreased relative Ca²⁺ permeability, and different current–voltage relationships (5-HT₃A is inwardly rectifying, 5-HT₃AB is linear).[1b,5] Pharmacologically distinguishing 5-HT₃A from 5-HT₃AB receptors has historically required the use of compounds that bind in the pore, such as bilobalide, ginkgolide, and picrotoxinin.[6] In contrast, competitive ligands usually have very similar affinities at 5-HT₃A and 5-HT₃AB receptors. Recently, however, a quinoxaline compound (VUF10166) was identified that showed differences in both its binding affinity and functional properties at 5-HT₃A and 5-HT₃AB receptors (Figure 1).[7] Detailed studies of VUF10166 showed that these differences may stem from a second, allosteric site, only found in the 5-HT₃AB receptor, the occupation of which may increase the rate of ligand dissociation from the adjacent orthosteric site.

The actions of a range of quinoxalines have also been previously studied at both 5-HT₃A and native receptors and re-
revealed that these compounds can be relatively potent (sub-micromolar affinities) as antagonists, agonists, and partial agonists, with potential as novel therapeutics.\[8\] There is particular interest, for example, in developing quinoxalines which are impermeable to the blood–brain barrier that would target peripheral 5-HT\textsubscript{3} receptors.\[8a\] None of these studies, however, have evaluated ligand affinities at specific 5-HT\textsubscript{3} receptor subtypes. In this manuscript, we report the synthesis and binding affinities of a series of quinoxalines and demonstrate subtle differences in structure–activity relationships (SAR) for the 5-HT\textsubscript{3}A and 5-HT\textsubscript{3}AB receptor subtypes using competition binding on recombinantly expressed receptors in HEK293 cells.

**Results and Discussion**

**Chemistry**

The pharmacophore features of lead compound 1 and VUF10166, and the effects of these features on 5-HT\textsubscript{3}A and 5-HT\textsubscript{3}AB receptor affinities, was explored by screening a series of compounds that contain the quinoxaline scaffold (Figure 1b). Intermediates 4–5 were synthesized via a two-step ring formation between 2-amino aniline 2 or 3 and the appropriate 2-oxo carboxylic acids (Scheme 1). After conversion into the corresponding 2-chloroquinoxalines with phosphorylchloride, subsequent nucleophilic aromatic substitution with N-methylpiperazine under microwave conditions gave compounds 6 and 7 in moderate to good yields.

Starting from commercially available chloro-quinoxalines 8 and 9, different synthetic routes were followed to synthesize compounds 10 and 11 (Scheme 2). Compound 10 was synthesized through two subsequent nucleophilic aromatic substitution reactions. First, the amine moiety was introduced by reacting compound 8 with ammonia in ethanol. Subsequently, the N-methylpiperazine group was introduced. Both reactions were performed under microwave conditions. Compound 11 was created in a similar manner, although conventional heating was used for this synthesis.

Commercially available quinoxaline-2,3(1H,4H)-dione (12) was treated with phosphorous pentabromide to form 2,3-dibromoquinoxaline (13), which was then allowed to react with N-methylpiperazine in toluene at reflux to yield compound 14 (Scheme 3). For compounds 16, 18, 19, and 21, 2,3-dichloroquinoxaline (8) or 2-chloroquinoxaline (15) were reacted with the corresponding amines using various solvents and temperatures to yield 16, 17, 19, and 20 in good yields. Boc-protected intermediates 17 and 20 were subsequently deprotected with a 4 M solution of hydrochloric acid in dioxane to give compounds 18 and 21 (Scheme 4). The regioselective synthesis of compound 22 was described earlier by our group.\[9\] Here, we used this compound as a precursor in the synthesis of compound 23 (Scheme 5).
Biochemical evaluation and SAR studies

**SAR of quinoxaline compounds for the 5-HT₃A receptors**

Target compounds were evaluated using competition binding with the 5-HT₃-specific ligand [³H]granisetron; the results are summarized in Table 1. SAR data in this table are presented with a focus on different substitution patterns at the R₁, R₂, and R₃ positions of the quinoxaline core scaffold. We found that several quinoxaline compounds show clear differences in their binding affinities at the two receptor subtypes, and the subtype preference differs within the series.

First, the SAR of the series will be described for the 5-HT₃A receptor subtype. The alcohol moiety at the R₂ position implies that compound 1 can adopt two different tautomeric states. It

| Compd | R¹ | R² | R³ | pKᵢ (A) | n  | pKᵢ (AB) | n  | Fold diff.ᵃ |
|-------|----|----|----|--------|----|----------|----|------------|
| 1     | H  | Me | OH | 8.93 ± 0.21 | 11 | 9.36 ± 0.06 | 11 | +2.7       |
| 28    | H  | H  | OMe| 9.18 ± 0.16  | 6  | 8.34 ± 0.10 | 7  | −7.1       |
| 29    | H  | H  | O  | 9.00 ± 0.22  | 7  | 8.24 ± 0.10 | 7  | −5.8       |
| 30    | H  | H  | O  | 6.27 ± 0.20  | 4  | 6.71 ± 0.30 | 4  | +2.8       |
| 31    | H  | H  | O  | 7.06 ± 0.07  | 2  | 6.77 ± 0.39 | 2  | −2.0       |
| 32    | H  | H  | O  | 6.89 ± 0.12  | 4  | 7.12 ± 0.13 | 4  | +1.7       |
| 10    | H  | H  | NH₂| 8.53 ± 0.11  | 5  | 7.51 ± 0.32 | 5  | −11        |
| 26    | H  | H  | Me | 8.59 ± 0.19  | 5  | 8.42 ± 0.09 | 5  | −1.5       |
| 6     | H  | H  | Et | 9.20 ± 0.12  | 5  | 8.84 ± 0.46 | 8  | −2.3       |
| 27    | H  | H  | CF₃| 7.35 ± 0.15  | 3  | 7.42 ± 0.39 | 3  | +1.2       |
| 14    | H  | H  | Br | 9.31 ± 0.16  | 6  | 8.85 ± 0.16 | 7  | −2.9       |
| VUF10166 | H  | H  | Cl | 9.82 ± 0.26  | 7  | 7.90 ± 0.49 | 6  | −83        |
| 16    | H  | H  | Cl | 9.11 ± 0.26  | 3  | 8.34 ± 0.23 | 3  | −5.9       |
| 18    | H  | H  | Cl | 6.69 ± 0.16  | 3  | 6.67 ± 0.02 | 3  | −1.0       |
| 23    | H  | 6-Cl| Cl | 8.95 ± 0.18  | 7  | 7.85 ± 0.08 | 14 | −13        |
| 11    | H  | 6,7-Cl| Cl | 8.09 ± 0.34  | 6  | 7.48 ± 0.16 | 6  | −4.1       |

ᵃ Fold difference calculated as |pKᵢ (A) - pKᵢ (AB)|.

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seems that the tautomeric form in which the aromatic nitrogen atom represents a hydrogen bond donor is not involved in binding, as the conversion of the R1 alcohol functionality of 1 into a methoxy (compound 28) or ethoxy (compound 29) group results in compounds with comparable affinities. However, larger ether analogues are not favorable for binding, as observed for compounds 30–32 in which the cyclohexyl, phenyl, and benzyl ether derivatives have ~100-fold lower affinities. When the hydroxy group of 1 at R2 was changed to a different polar moiety (e.g., an amine moiety, as in compound 10), the high affinity was maintained. A decreased affinity was observed for compound 26, which incorporates a methyl group (which is electron-donating) at the R2 position, relative to VUF10166. Addition of an electron-withdrawing CF3 group (compound 27) results in an even larger decrease in 5-HT3A receptor affinity. Compounds that have chlorine or bromine atoms at this position have sub-nanomolar affinities (VUF10166 and 14), indicating that the SAR in this position is very subtle, and an interplay between inductive and resonance effects cannot be ruled out.

For R2 = Cl (VUF10166), different basic moieties were introduced. A small drop in affinity results from replacing R1 = N-methylpiperazine (VUF10166) with R1 = N-methylhomopiperazine (16), but a ~1000-fold drop in affinity is observed for R1 = N-methylpyrrolidin-3-amime (18). As the methylpiperazine moiety leads to the most potent compounds at 5-HT3A receptors, this basic group was used in the R1 position when exploring the effects of different chlorine substitution patterns at the R2 position. Addition of a 6-Cl at R1 (compound 23) causes a ~10-fold drop in affinity, and a second chlorine atom at position R1 (6,7-Cl, 11) results in another ~10-fold decrease. Again, VUF10166 (R3 = H) shows the highest affinity for the 5-HT3A receptor. For compounds with R2 = OH (1), a similar trend is observed. Affinity at the 5-HT3A receptor is highest for R3 = H (1) and decreases significantly for both compound 22 (R3 = 6-Cl) and 34 (R3 = 6,7-Cl), which both have a pKi in the mid-nanomolar range.

The same modifications to R1 and R2 were made for the most simple 2-N-methylpiperazine quinoxaline compound of the series (R2 = H, 24), which has a pKi of 8.21. Addition of chlorine atoms at position R1 (33, 7) results in compounds with similar affinity at the 5-HT3A receptor. Finally, replacement of the N-methylpiperazine group of compound 24 with an N-methylhomopiperazine group (19) has no effect on 5-HT3A receptor affinity, but for R1 = N-methylpyrrolidin-3-amime (21), a small decrease in affinity is observed. This is different to what is observed for R2 = Cl, where basic moieties other than the N-methylpiperazine group resulted in more pronounced differences in affinity (e.g., compare 19 and 21 with 16 and 18, respectively).

### Affinity differences at 5-HT3A and 5-HT3AB receptors

The affinity of compound 1 is slightly higher (2.7-fold) for 5-HT3AB receptors than for 5-HT3A receptors. Methoxy and ethoxy analogues 28 and 29 both show a 10-fold decrease in affinity at 5-HT3AB receptors relative to compound 1; in contrast, these modifications do not result in a change in affinity at the 5-HT3A receptor. The larger ether analogues 30–32 have pKs values of ~7 for the 5-HT3AB receptor, which are similar to

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**Table 1. (Continued)**

| Compd | R1 | R2 | R3 | pKi (A) | n | pKi (AB) | n | Fold diff. |
|-------|----|----|----|---------|---|----------|---|------------|
| 22    | OH | 7-Cl | 7.50 ± 0.13 | 3 | 8.14 ± 0.21 | 3 | +4.3 |
| 34    | OH | 6,7-Cl | 7.30 ± 0.39 | 6 | 7.14 ± 0.26 | 9 | −1.4 |
| 24    | H | H | 8.21 ± 0.26 | 5 | 9.13 ± 0.30 | 5 | +8.3 |
| 33    | H | 6-Cl | 7.79 ± 0.20 | 3 | 6.99 ± 0.31 | 3 | −6.3 |
| 7     | H | 6,7-Cl | 8.41 ± 0.29 | 6 | 8.07 ± 0.27 | 5 | −2.2 |
| 19    | H | H | 8.09 ± 0.20 | 5 | 7.48 ± 0.13 | 5 | −4.0 |
| 21    | H | H | 7.49 ± 0.08 | 6 | 7.11 ± 0.15 | 5 | −2.4 |

[a] +/− refer to an increase or decrease at 5-HT3AB relative to 5-HT3A.
their affinities for the homomeric receptor. Replacement of the alcohol moiety with an amine moiety (compound 10) resulted in a large decrease in affinity (−70-fold) for 5-HT₃AB receptors but had little effect on the affinity for 5-HT₃A receptors. Similar affinities are observed at both the 5-HT₃A and 5-HT₃AB receptors for compounds that have methyl and ethyl substituents in the R² position (i.e., 26 and 6, respectively), as well as for trifluoromethyl derivative 27. For the halogen-substituted compounds, a different trend is observed. For R² = Br, the pKᵢ for 5-HT₃AB receptors is close to 9, which is similar to that for 5-HT₃A receptors, but the affinity of VUF10166 (R² = Cl) is significantly decreased at 5-HT₃AB receptors, resulting in a −100-fold difference relative to 5-HT₃A receptors. The effect of replacing R¹ = N-methylpiperazine (VUF10166) for R¹ = N-methylhomopiperazine while R² = Cl (16) is negligible at 5-HT₃AB receptors, which is again different to what is observed at 5-HT₃A receptors. For R¹ = N-methylpyrrolidin-3-amine (18), a >10-fold decrease in affinity for 5-HT₃AB receptors is observed. 5-HT₃AB receptor affinities for compounds with a chlorine atom at position R² (VUF10166, 23, and 11) do not change substantially when increasing numbers of chlorine atoms are added at the R² position, although compound 11 has the lowest 5-HT₃AB receptor affinity of this subset. This is different to what is observed for the 5-HT₃A receptor affinities of these compounds, where addition of chlorine at R² resulted in a large decrease in affinity. When R² = OH, compound 1 (R² = H) had the highest 5-HT₃AB receptor affinity, compound 22 (R¹ = 6-Cl) showed a −10-fold decrease in affinity, and a further −10-fold decrease in affinity was observed for compound 34 (R¹ = 6,7-Cl). 5-HT₃A receptor affinities shown by 22 and 34 are similar. When R² = H, the highest 5-HT₃A receptor affinity was observed for R¹ = H (24), but a −100-fold drop in affinity was observed for R¹ = 6-Cl (33) and only a −10-fold drop for R¹ = 6,7-Cl (7). The effect on 5-HT₃AB receptor affinity when replacing R¹ = N-methylpiperazine (24) for R¹ = N-methylhomopiperazine (19), while R² = H, is a −45-fold decrease in affinity. For compound 21, a similar lowering in 5-HT₃AB receptor affinity is observed. This is in contrast to what is observed for 5-HT₃A receptors and can primarily be attributed to the sub-nanomolar affinity of compound 24 at 5-HT₃AB receptors, which is almost 10-fold higher than its 5-HT₃A receptor affinity.

Table 1 shows that compound 24 shows the highest selectivity for 5-HT₃AB over 5-HT₃A receptors (−10-fold), and VUF10166 has the highest selectivity for 5-HT₃A over 5-HT₃AB receptors (−100-fold). The difference between these two compounds is solely the atom at position R², R² = H for compound 24 and R² = CI for VUF10166. When the hydrogen atom is replaced with a chlorine atom, the 5-HT₃A receptor affinity increases −40-fold, while the affinity for 5-HT₃AB receptors decreases −20-fold. Both of these compounds comprise the N-methylpiperazine moiety, which is the preferred basic group for selectivity. For 5-HT₃A receptor affinity, R² = Cl (VUF10166) is superior, with Br (14), Et (6), OMe (28), and OEt (29) having similar lower affinities. For the 5-HT₃AB receptor, an alcohol moiety at position R² (as observed for compound 1) is preferred, but a hydrogen atom at R² also results in high affinity (compound 24). At 5-HT₃AB receptors, R² = Br and the smaller alkyl (26, 6) and ether analogues (28 and 29) also have high affinities, whereas incorporation of larger ether groups at R² results in decreased affinity. However, for R² = Cl (VUF10166) and NH₂ (10), only 5-HT₃AB receptor affinity is decreased, resulting in 100- and 10-fold selectivity for 5-HT₃A over 5-HT₃AB receptors, respectively. Different substitution patterns at the R² position also caused marked changes. For example, when R² = Cl, replacement of R² = H (VUF10166) with a chlorine atom results in a −10-fold (R¹ = 6-Cl, 23) or −100-fold (R¹ = 6,7-Cl, 11) decrease in affinity for the 5-HT₃A receptor, but this replacement does not have a large effect on 5-HT₃AB receptor affinity. When R² = H, the affinity for 5-HT₃A receptors does not show a large difference upon addition of chlorine atoms to the R² position, but the 5-HT₃AB receptor affinity changes significantly. It can be concluded that, in either case, the greatest 5-HT₃ receptor subtype selectivity is achieved for R¹ = N-methylpiperazine.

5-HT₃ receptor binding sites

Orthosteric binding sites in 5-HT₃AB receptors could theoretically exist at A₊ + A₋, A₊ + B₋, B₊ + A₋, and B₊ + B₋ interfaces, but the majority of 5-HT₃ receptor-competitive ligands only bind to an A₊ + A₋ interface. There is evidence, however, that at least one of the quinoxaline compounds studied here (VUF10166) binds to both an A₊ + A₋ and an A₊ + B₋ interface; binding to the A₊ + B₋ interface may decrease the affinity of ligands binding to the A₊ + A₋ site by allosterically increasing the rate of ligand dissociation. Other quinoxalines may similarly bind to both sites; thus, to identify potential interactions, we constructed models of the two interfaces.

Homology models were based on a tropisetron-bound AChBP crystal structure (PDB code: 2WNC) as no quinoxaline-bound Cys-loop receptor structure has been solved to date, and tropisetron is the closest structurally related compound to those described here (Figure 3). Tropisetron is an antagonist at the 5-HT₁ receptor but can act as an agonist at some nACh receptors, thus, it is an ideal choice from the available structures as quinoxalines can act as both agonists and antagonists at 5-HT₁ receptors, though they were not evaluated in this study. As with all homology models, caution must be applied in data interpretation, especially now that recent electron microscopy images of the nACh receptor have shown that the difference between the structure of unbound and agonist-bound binding site sites is less than that observed in AChBP.

Nevertheless, it is likely that our compounds adopt a broadly similar orientation to tropisetron; therefore, the models serve as means of identifying residues that could potentially be responsible for the differences in affinities of the quinoxaline ligands at 5-HT₃A and 5-HT₃AB receptors. As discussed below, several of the identified residues are known to interact with a range of 5-HT₃ receptor ligands (Figures 2 and 3). Some of these are the same in both A₊ + A₋ and A₊ + B₋ binding sites and are unlikely to be responsible for differences in affinity, while others are different and may provide possible explanations for the varied ligand affinities at the two receptor subtypes.
Studies of the 5-HT3A receptor have identified an aromatic binding cavity formed by residues Trp90 (loop D), Trp183 (loop B) and Tyr234 (loop C), mutagenesis of which effects both 5-HT activation and the binding of 5-HT3 receptor competitive antagonists. [13] Our homology models predict that both A+\textsubscript{A}/C0 and A+\textsubscript{B}/C0 binding sites contain these residues, providing an aromatic environment to accommodate the positively charged moiety that is a well-known pharmacophore feature of 5-HT3 ligands. [14] Another pharmacophore feature, a hydrogen bond acceptor (HBA), is observed in both models as an interaction between the carbonyl oxygen atom of tropisetron and water from a water network that has been observed in AChBP crystal structures; water molecules in this network are also stabilized by interactions with the backbone of the protein and the side chain of Tyr234. In both binding sites, the positively charged moieties of tropisetron are also stabilized by ionic interactions with Glu129 (loop A), and by a hydrogen bonding interaction between the protonated nitrogen atom and the carbonyl backbone of Trp183. Mutagenesis studies have shown that both Glu129 and Trp183 are essential for 5-HT function and granisetron binding. [13]

Because the principle faces of both A+\textsubscript{A} and A+\textsubscript{B} binding sites are identical, we must look toward the A/C0 and B/C0 Interfaces for differences between the two binding sites. Of the differing residues, A Ile71/B Phe71, A Arg92/B Gln92 and A Gln151/B Glu151 are closest to tropisetron, and might also be expected to interact with the structurally related quinoxaline ligands described here. A Arg92 mutations to Ala and Leu had no effect on granisetron binding affinity, suggesting the residue at this location does not affect ligand binding. [13] Conversely substitution of A Arg92 changed the affinities of several 5-HT3 ligands, including 5-HT and granisetron, and we have previously speculated that a cation–π interaction could exist between A Arg92 and the aromatic parts of (iso)quinolines and quinazolines, as it does with granisetron. [10, 15] As this type of interaction would be absent in an A+\textsubscript{B} site (as Gln is a neutral residue), quinoxalines might adopt quite a distinct orientation in this binding pocket. In support of this speculation, we have previously shown that introduction of a Cys substitution at this location has no effect on 5-HT or granisetron, but eliminates the allosteric effects of VUF10166 in heteromeric receptors. [7] Similarly, the change in charge at location 151 (A Gln151/B Glu151) could have a significant effect on ligand binding properties. Although the effect of this residue has not yet been studied, mutation of the closely located B Tyr153 residue also eliminates the allosteric effects of VUF10166, showing that this region influences binding at the B interface. [7] For VUF10166, the effects of these B-substitutions are known to alter both the binding properties and the functional response, but for the other quinoxalines studied here, it has yet to be determined whether the differing binding affinities also translate into functional changes.

Conclusions

In summary, most quinoxaline compounds examined here show no difference in their affinities at 5-HT\textsubscript{A} and 5-HT\textsubscript{AB} receptors, and we suggest that these compounds may only bind to the A+\textsubscript{A} binding site that is found in both receptor types,
consistent with all other 5-HT₃ receptor-competitive ligands.¹⁰,¹⁶ Some, however, show significant differences and thus may bind to the A + B interface as has previously been shown for VUF10166.¹⁰ These novel ligands could be valuable in both experimental and computer-aided drug design, with potential for the development of novel therapeutic agents.

**Experimental Section**

**Chemistry:** Chemicals and solvents were purchased from Sigma–Aldrich and used as received. Unless indicated otherwise, all reactions were carried out under an inert atmosphere of dry N₂. TLC analyses were performed with Merck F254 alumina silica plates using UV visualization or staining. Column purifications were carried out automatically using the Biotage equipment. All HRMS spectra were recorded on Bruker microTOF mass spectrometer using ESI in positive ion mode.¹³ H NMR spectra were recorded on a Bruker 250 (250 MHz) or a Bruker 500 (500 MHz) spectrometer. Purification of compounds was performed using flash chromatography. Purities were calculated as the percentage peak area of the major peak in the H NMR spectra. NOESY experiments were used in conjunction with the Bruker 500 MHz spectrometer to confirm the structure of the compound. Purification of compounds was performed using flash chromatography. Purities were calculated as the percentage peak area of the major peak in the H NMR spectra. NOESY experiments were used in conjunction with the Bruker 500 MHz spectrometer to confirm the structure of the compound.

**3-Ethyl-3,4-dihydroxyquinolin-2(1H)-one (4):** Benzene-1,2-diamine (2) (1.07 g, 28.4 mmol) and 2-oxobutanoic acid (2.90 g, 28.4 mmol) were dissolved in 50 mL CH₂Cl₂, and the resulting solution was stirred overnight at room temperature. The precipitate was collected via filtration over a Büchner funnel. Purification was carried out using flash chromatography. Purities were calculated as the percentage peak area of the major peak in the H NMR spectra. NOESY experiments were used in conjunction with the Bruker 500 MHz spectrometer to confirm the structure of the compound.

**6,7-Dichloro-3,4-dihydroxyquinolin-2(1H)-one (5):** 4,5-dichloro-benzene-1,2-diamine (3) (842 mg, 4.76 mmol) and 2-oxoacetic acid (715 mg, 4.83 mmol) were dissolved in CH₂Cl₂ (50 mL) and stirred at room temperature for 30 min. The mixture was concentrated under reduced pressure, H₂O was added, and the resulting mixture was extracted with EtOAc. The combined organic phases were dried over MgSO₄ and concentrated under reduced pressure to yield 133 mg of 5 (0.62 mmol, 13%) as a dark-brown solid:¹² H NMR (250 MHz, DMSO) δ = 8.19 (s, 1 H), 8.04 (s, 1 H), 7.45 ppm (s, 1 H).

**2-Ethyl-3-(4-methylpiperazin-1-yl)quinoline (6):** A solution of 4 (1.64 g, 9.39 mmol) in phosphoryl trichloride (100 mL) was stirred at 100 °C for 1 h. The reaction mixture was then concentrated under reduced pressure, H₂O was added to the remaining solid, and the mixture was extracted with CH₂Cl₂. The organic layers were combined, dried over Na₂SO₄, and concentrated under reduced pressure to yield 1.59 g (8.24 mmol, 88%) of 2-chloro-3-ethyl-4-methylpiperazin-1-ylquinoline as a dark-pink solid:¹³ H NMR (250 MHz, CDCl₃) δ = 8.10–8.03 (m, 1 H), 8.02–7.95 (m, 1 H), 7.79–7.67 (m, 2 H), 3.17 (q, J = 7.5 Hz, 2 H), 1.44 ppm (t, J = 7.5 Hz, 3 H). Next, 2-chloro-3-ethyl-4-methylpiperazin-1-ylquinoline (555 mg, 2.88 mmol) was dissolved in N-methylpyrerpiprazine (2 mL), and the resulting solution was heated at 120 °C for 15 min using microwave (mw) radiation. After cooling to room temperature, excess N-methylpiperazin-1-ylpyrerpiprazine was removed under reduced pressure, and the product was purified over SiO₂ (EtOAc/Et₂N, 96:4, v/v) to yield 566 mg of 6 (2.21 mmol, 77%) as a yellow solid:¹³ H NMR (250 MHz, CDCl₃) δ = 7.95–7.87 (m, 1 H), 7.85–7.78 (m, 1 H), 7.61–7.45 (m, 2 H), 3.43–3.31 (m, 4 H), 2.97 g (J = 7.4 Hz, 2 H), 2.68–2.58 (m, 4 H), 2.38 (s, 3 H), 1.41 ppm (t, J = 7.4 Hz, 3 H);¹⁵ C NMR (126 MHz, CDCl₃) δ = 155.74, 154.15, 139.92, 138.88, 128.68, 128.01, 127.27, 126.57, 55.04, 49.67, 46.24, 27.70, 12.64 ppm; LCMS: tᵣ = 2.82 min, purity 95%, [M + H]⁺ 257.00; HRMS m/z: [M + H]⁺ calcd for C₁₃H₁₈N₂; 257.1761, found: 257.1763.

**6,7-Dichloro-2-(4-methylpiperazin-1-yl)quinoline (7):** A solution of 5 (133 mg, 0.62 mmol) in phosphoryl trichloride (50 mL) was stirred at 100 °C for 1 h. The reaction mixture was concentrated under reduced pressure, H₂O was added to the remaining solid, and the mixture was extracted with CH₂Cl₂. The organic layers were combined, dried over Na₂SO₄, and concentrated under reduced pressure to yield 66 mg (28.0 mmol, 46%) of 2,6,7-trichloroquinoline:¹³ H NMR (250 MHz, CDCl₃) δ = 8.77 (s, 1 H), 8.25 (s, 1 H), 8.15 ppm (s, 1 H). Then, 2,6,7-trichloroquinoline (66 mg, 0.28 mmol) was dissolved in EtOAc (2 mL), N-methylpiperazin-1-ylpyrerpiprazine (0.1 mL, 0.90 mmol) was added, and the resulting solution was heated at 160 °C for 1 h using microwave radiation. After cooling to room temperature, EtOAc and excess N-methylpiperazinpyrerpiprazine were removed under reduced pressure, and the product was purified over SiO₂ (EtOAc/Et₂N, 96:4, v/v) to yield 36 mg of 7 (0.12 mmol, 43%) as a light-brown solid:¹³ H NMR (250 MHz, CDCl₃) δ = 8.55 (s, 1 H), 7.96 (s, 1 H), 7.77 (s, 1 H), 3.85–3.79 (m, 4 H), 2.50–2.47 (m, 4 H), 2.37 ppm (s, 3 H);¹⁵ C NMR (126 MHz, CDCl₃) δ = 152.87, 142.50, 139.15, 138.88, 136.67, 134.50, 131.10, 128.26, 127.62, 54.00, 48.80, 46.07 ppm; LCMS: tᵣ = 2.39 min, purity > 99%, [M + H]⁺ 296.90; HRMS m/z: [M + H]⁺ calcd for C₂₁H₁₉N₂Cl₂; 297.0668, found: 297.0662.

**3-(4-Methylpiperazin-1-yl)quinolin-2-amine (8):** 2-(4-Methylpiperazin-1-yl)quinoline (5) (1.09 g, 5.68 mmol) was dissolved in CH₂Cl₂ (50 mL) and heated at 120 °C for 1 h using microwave radiation. After cooling to room temperature, EtOAc and excess N-methylpiperazinpyrerpiprazine were removed under reduced pressure, and the product was purified over SiO₂ (EtOAc/Et₂N, 96:4, v/v) to yield 36 mg of 8 (0.12 mmol, 43%) as a white solid:¹³ H NMR (250 MHz, CDCl₃) δ = 9.28 (s, 1 H), 7.74 (s, 1 H), 7.49 ppm (s, 1 H).

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7.60 (d, J = 7.8 Hz, 1H), 7.50–7.33 (m, 2H), 4.98 (s, 2H), 3.56–3.27 (m, 4H), 2.76–2.51 (m, 4H), 2.46–2.29 ppm (m, 3H); 13C NMR (126 MHz, CDCl 3) δ = 148.39, 147.69, 138.94, 137.41, 127.34, 127.26, 125.25, 125.08, 55.07, 48.54, 46.24 ppm; LCMS: tR = 2.25 min, purity > 99%, [M + H]+ 244.00; HRMS m/z: [M + H]+ calc for C10H9N3O: 244.1557, found: 244.1552.

2,6,7-Trichloro-3-(4-methylpyperazin-1-yl)quinoxaline (11): 2,6,7-Tetrachloroquinoxaline (9) (1.25 g, 4.58 mmol) was dissolved in THF (50 mL). N-methylpyperazine (0.58 mL, 4.58 mmol) and triethylamine (0.65 mL, 4.66 mmol) were added, and the mixture was stirred at 80 °C for 90 min. The resulting mixture was stirred with Et2O, H2O, and 1N NaOH solution of HCl, and the mixture was stirred vigorously for 30 min. The mixture was extracted with Et2O, washed with 1N NaOH and 1N HCl solution, and dried over MgSO4. The crude was purified over SiO2 (EtOAc/n-heptanes, 1:2, v/v) to give 0.62 g (77.5%) as a yellow solid: 1H NMR (250 MHz, CDCl 3) δ = 7.99 (s, 1H), 7.92 (s, 1H), 3.68–3.59 (m, 3H, 2H), 2.70–2.64 ppm (m, 2H, 1H, 4H), 2.42 (s, 3H), 2.10 ppm (dt, J = 7.0, 2.0 Hz), 3.86–3.81 (m, 2H, 2H), 2.90–2.83 (m, 2H, 2H), 2.70–2.64 (m, 2H, 1H). 13C NMR (126 MHz, CDCl 3) δ = 148.39, 147.69, 138.94, 137.41, 127.34, 127.26, 125.25, 125.08, 55.07, 48.54, 46.24 ppm; LCMS: tR = 2.69 min, purity > 99%, [M + H]+ 244.00; HRMS m/z: [M + H]+ calc for C10H9N3O: 244.1557, found: 244.1552.

2,3-Dibromoquinoxaline (13) (2.96 g, 18.3 mmol) and pentabromophosphorane (17.06 g, 39.6 mmol) were dissolved in toluene (200 mL) and heated at 160 °C for 3 h. After cooling to room temperature, H2O was added, and the mixture was stirred at 90 °C for 3 h. The mixture was directly used in the synthesis of 18.

2-Chloro-3-(4-methyl-1,4-diazepan-1-yl)quinoxaline (16) to give 1.01 g (3.65 mmol, 73%): 1H NMR (250 MHz, CDCl 3) δ = 7.90 (s, 1H), 7.86–7.77 ppm (m, 2H). 13C NMR (126 MHz, CDCl 3) δ = 147.42, 137.60, 136.72, 136.02, 131.08, 132.79, 132.59, 54.70, 49.48, 46.18 ppm; LCMS: tR = 2.90 min, purity > 99%, [M + H]+ 263.05; HRMS m/z: [M + H]+ calc for C10H9N3O: 263.1058, found: 263.1055.

2-Chloroquinoxaline (18) (0.40 g) was dissolved in dioxane (10 mL) and stirred at room temperature. A 4 M solution of HCl in dioxane (20 mL) was added dropwise, and precipitation was observed. The resulting suspension was stirred overnight and subsequently filtered over a Büchner funnel, and the residue was washed with 1.4-dioxan. The residue was then dried under reduced pressure to yield 0.302 mg of 18 as a light-yellow solid (0.68 mmol, 61%). 1H NMR (500 MHz, CDCl 3) δ = 7.87–7.74 ppm (m, 2H), 7.66 (d, J = 8.4, 7.1, 1.4 Hz, 1H), 7.58–7.46 (m, 1H), 4.27–4.10 (m, 3H, 2H), 2.83 (s, 3H), 2.61–2.45 (m, 1H), 2.37–2.23 ppm (m, 1H); 13C NMR (126 MHz, CDCl 3) δ = 142.74, 137.60, 136.72, 136.02, 131.08, 132.79, 132.59, 54.70, 49.48, 46.18 ppm; LCMS: tR = 2.69 min, purity > 99%, [M + H]+ 244.00; HRMS m/z: [M + H]+ calc for C10H9N3O: 244.1560, found: 244.1560.

N-Methyl-(1-quinooxalin-2-yl)pyrroloidin-3-amine (20): 2-Chloroquinoxaline (1.79 g, 10.9 mmol) was dissolved in DMF (50 mL). K2CO3 (1.51 g, 10.9 mmol) and tert-butyl methyl(pyrrolidin-3-yl)carbamate (2.00 g, 10.0 mmol) were added, and the mixture was stirred at 80 °C for 6 h. The mixture was cooled to room temperature, diluted with H2O, and extracted with EtO. The combined organic layers were dried over MgSO4 and concentrated under reduced pressure to give 3.20 g of 20, which was directly used in the synthesis of 21.
constructed by homology modeling using MOE (version 2010.10, Chemical Computing Group, Montreal). The sequence of the human 5-HT3A gene (O05264) was aligned with the sequence of the 5-HT-A gene (P46098) using the “Protein Align” option in MOE (standard settings) and was adjusted manually. The final sequence alignment is shown in Figure 2. The 5-HT-A receptor homology model was selected to serve as the template. Structural waters located in the binding pocket of the original crystal structure (PDB code: 2WNC)\(^{16}\) formed a conserved protein–ligand hydrogen bond interaction network in several other AChBP crystals (e.g., 2BYR, 2PGZ, 2BYS, 2XTY) and were included in the 5-HT-A receptor model. The template backbone, the ligand, and the water molecules were fixed, and ten receptor models were constructed based on the template backbone. During this construction, the ligand and waters of the original co-crystal structure were considered as an additional restraint using the “Environment” option within MOE. The structural quality of the models was checked using the evaluation modules in MOE; protein geometry of receptor atoms was evaluated for bound lengths, bond angles, atom clashes, and contact energies. Ramachandran plots were used to check the Phi and Psi angles of all residues. The best model was selected for further refinement, hydrogen atoms were added, partial atomic charges were calculated, and the protein was minimized around the fixed ligand and static water molecules using the Amber09 force field in MOE.

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\[^{[1]}\] A. J. Walstab, G. Rappold, B. Niesle, *Pharmacol. Ther.* 2010, 128, 146–169; b) A. J. Thompson, S. C. Lumnis, *Curr. Pharm. Des.* 2006, 12, 3615–3630; c) A. J. Thompson, S. C. Lumnis, *Expert Opin. Ther. Targets* 2007, 11, 527–540.

\[^{[2]}\] A. K. Hu, R. W. Peoples, *J. Biol. Chem.* 2008, 283, 6826–6831; b) B. Niesler, J. Kapeller, C. Hammer, G. Rappold, *Pharmacogenomics* 2008, 9, 501–504.

\[^{[3]}\] A. A. Jensen, P. A. Davies, H. Brauner-Osborne, K. Krzywykowski, *Trends Pharmacol. Sci.* 2008, 29, 437–444.

\[^{[4]}\] A. J. Thompson, H. A. Lester, S. C. Lumnis, *Q. Rev. Biophys.* 2010, 43, 449–499.

\[^{[5]}\] a) S. P. Kelley, J. I. Dunlop, E. F. Kirkness, J. J. Lambert, J. A. Peters, *Nature 2003*, 424, 321–324; b) A. E. Dubin, R. Huvar, M. R. D’Andrea, J. Pyati, J. Y. Zhu, K. C. Joy, S. J. Wilson, J. E. Galindo, C. A. Glass, L. Luo, M. R. Jackson, T. W. Lovenberg, M. G. Erlander, *J. Biol. Chem.* 1999, 274, 30799–810.

\[^{[6]}\] A. J. Thompson, R. K. Duke, S. C. Lumnis, *Mol. Pharmacol.* 2011, 80, 183–190; b) D. Das, G. H. Dillon, *Brain Res. Mol. Brain Res.* 2003, 119, 207–12.

\[^{[7]}\] A. J. Thompson, M. H. P. Verheji, I. J. P. de Esch, S. C. R. Lumnis, *J. Pharmacol. Exp. Ther.* 2012, 347, 350–359.

\[^{[8]}\] a) S. Butini, R. Budriesi, M. Hamon, E. Morelli, S. Gemma, M. Brindisi, G. Borrelli, E. Novellino, I. Fiorini, P. Ioan, A. Chiarini, T. Giannini, C. Fracasso, S. Caccia, G. Campiani, *J. Med. Chem.* 2009, 52, 6946–6950; b) G. Campiani, A. Cappelli, V. Nacci, M. Anzini, S. Vomero, M. Hamon, A. Cagnotto, C. Fracasso, C. Uboldi, S. Caccia, S. Consolo, T. Mennini, J. Med. Chem. 1997, 40, 3670–3678; c) R. Mahesh, R. V. Peru...
a) D. L. Beene, G. S. Brandt, W. Zhong, N. M. Zacharias, H. A. Lester, D. A. Dougherty, Biochemistry 2002, 41, 10262–10269; b) D. L. Beene, K. L. Price, H. A. Lester, D. A. Dougherty, S. C. Lummis, J. Neurosci. 2004, 24, 9097–90104; c) K. L. Price, S. C. Lummis, J. Biol. Chem. 2004, 279, 23294–23301; d) K. L. Price, K. S. Bower, A. J. Thompson, H. A. Lester, D. A. Dougherty, S. C. R. Lummis, Biochemistry 2008, 47, 6370–6377; e) A. J. Thompson, M. Lochner, S. C. Lummis, Biophys. J. 2008, 95, 5728–5736; f) A. J. Thompson, K. L. Price, D. C. Reeves, S. L. Chan, P. L. Chau, S. C. Lummis, J. Biol. Chem. 2005, 280, 20476–20482; g) P. Venkataraman, P. Joshi, S. P. Venkatatachalan, M. Muthalaghi, H. S. Parihar, K. S. Kirschbaum, M. K. Schulte, BMC Biochem. 2002, 3, 16; h) D. Yan, M. K. Schulte, K. E. Bloom, M. M. White, J. Biol. Chem. 1999, 274, 5537–5541.

[10] R. L. Papke, J. K. P. Papke, G. M. Rose, Bioorg. Med. Chem. Lett. 2004, 14, 1849–1853.

[11] N. Unwin, Y. J. Fujiyoshi, J. Mol. Biol. 2012, 422, 617–34.

[12] a) A. Asagarasu, T. Matsui, H. Hayashi, S. Tamaoki, Y. Yamauchi, M. Sato, Chem. Pharm. Bull. 2009, 57, 34–42; b) A. Cappelli, M. Anzini, S. Vomero, L. Mennuni, F. Makovec, E. Doucet, M. Hamon, M. C. Menziani, P. G. De Benedetti, G. Giorgi, C. Ghelardini, S. Collina, Bioorg. Med. Chem. 2002, 10, 779–801; c) R. D. Clark, A. B. Miller, J. Berger, D. B. Repke, K. K. Weinhardt, B. A. Kowalczyk, R. M. Eglen, D. W. Bonhaus, C. H. Lee, J. Med. Chem. 1993, 36, 2645–2657; d) S. Evans, A. Galdes, M. Gall, Pharmacol. Biochem. Behav. 1991, 40, 1033–1040; e) M. Hibert, R. Hoffmann, R. Miller, A. Carr, J. Med. Chem. 1990, 33, 1594–1600; f) R. Mahesh, R. V. Perumal, P. V. Pandi, Biol. Pharm. Bull. 2004, 27, 1403–1405.

[13] a) D. L. Beene, G. S. Brandt, W. Zhong, N. M. Zacharias, H. A. Lester, D. A. Dougherty, Biochemistry 2002, 41, 10262–10269; b) D. L. Beene, K. L. Price, H. A. Lester, D. A. Dougherty, S. C. Lummis, J. Neurosci. 2004, 24, 9097–90104; c) K. L. Price, S. C. Lummis, J. Biol. Chem. 2004, 279, 23294–23301; d) K. L. Price, K. S. Bower, A. J. Thompson, H. A. Lester, D. A. Dougherty, S. C. R. Lummis, Biochemistry 2008, 47, 6370–6377; e) A. J. Thompson, M. Lochner, S. C. Lummis, Biophys. J. 2008, 95, 5728–5736; f) A. J. Thompson, K. L. Price, D. C. Reeves, S. L. Chan, P. L. Chau, S. C. Lummis, J. Biol. Chem. 2005, 280, 20476–20482; g) P. Venkataraman, P. Joshi, S. P. Venkatatachalan, M. Muthalaghi, H. S. Parihar, K. S. Kirschbaum, M. K. Schulte, BMC Biochem. 2002, 3, 16; h) D. Yan, M. K. Schulte, K. E. Bloom, M. M. White, J. Biol. Chem. 1999, 274, 5537–5541.

[14] a) A. Asagarasu, T. Matsui, H. Hayashi, S. Tamaoki, Y. Yamauchi, M. Sato, Chem. Pharm. Bull. 2009, 57, 34–42; b) A. Cappelli, M. Anzini, S. Vomero, L. Mennuni, F. Makovec, E. Doucet, M. Hamon, M. C. Menziani, P. G. De Benedetti, G. Giorgi, C. Ghelardini, S. Collina, Bioorg. Med. Chem. 2002, 10, 779–801; c) R. D. Clark, A. B. Miller, J. Berger, D. B. Repke, K. K. Weinhardt, B. A. Kowalczyk, R. M. Eglen, D. W. Bonhaus, C. H. Lee, J. Med. Chem. 1993, 36, 2645–2657; d) S. Evans, A. Galdes, M. Gall, Pharmacol. Biochem. Behav. 1991, 40, 1033–1040; e) M. Hibert, R. Hoffmann, R. Miller, A. Carr, J. Med. Chem. 1990, 33, 1594–1600; f) R. Mahesh, R. V. Perumal, P. V. Pandi, Biol. Pharm. Bull. 2004, 27, 1403–1405.

[15] D. Yan, M. M. White, Mol. Pharmacol. 2005, 68, 365–371.

[16] J. Peters, M. Cooper, M. Livesey, J. Garland, J. Lambert in Ion Channels: From Structure to Function (Eds.: J. N. C. Kew, C. H. Davies), Oxford University Press, 2010, pp. 231–251.

[17] Y. Cheng, W. H. Prusoff, Biochem. Pharmacol. 1973, 22, 3099–3108.

[18] M. H. P. Verheij, A. J. Thompson, J. E. van Mulijwijk-Koezen, S. C. R. Lummis, R. Leurs, I. J. P. de Esch, J. Med. Chem. 2012, 55, 8603–14.

[19] R. E. Hibbs, G. Sulzenbacher, J. Shi, T. T. Talley. S. Conrod, W. R. Kem, P. Taylor, P. Marchot, Y. Bourne, EMBO J. 2009, 28, 3040–3051.

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