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"Methylene Bridge" to 5-HT_3 Receptor Antagonists: Conformationally-Constrained Phenylguanidines

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ABSTRACT

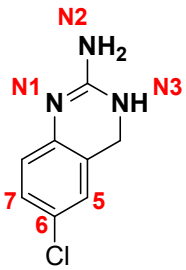
Arylguanidines, depending upon their aromatic substitution pattern, display varying actions at 5-HT_3 receptors (e.g., partial agonist, agonist, superagonist). Here, we demonstrate that conformational constraint of these agents as dihydroquinazolines (such as A6CDQ; **1**) results in their conversion to 5-HT_3 receptor antagonists. We examined the SAR of **1**. Replacement/removal of any of the guanidinium nitrogen atoms of **1** resulted in decreased affinity. All three nitrogen atoms of **1** are necessary for optimal binding affinity at 5-HT_3 receptors. Introduction of substituents as small as an N2-methyl group abolishes affinity. The results are consistent with homology modeling/docking studies and binding data from site-directed mutagenesis studies. Introducing a "methylene bridge" to the arylguanidine structure, regardless of its functional activity, results in a 5-HT_3 receptor antagonist.

KEYWORDS: Dihydroquinazolines, functional activities, binding affinities, site-directed mutagenesis, SAR, 3D-graphic models

INTRODUCTION

Despite their wide distribution both in the peripheral and central nervous systems, the role of ligand-gated ion channel 5-HT_3 receptors as therapeutic targets appears to be limited and underexplored. Currently, only the antiemetic properties of 5-HT_3 receptor antagonists have earned them the status of clinically used agents in the chemotherapeutic treatment of cancer.[1] However, recent literature reports might refresh the outlook on the possible therapeutic role of 5-HT_3 receptor ligands and open new avenues for further investigations. In addition to their postulated involvement in cognition, pain, depression, anxiety, alcohol abuse, schizophrenia, and inflammation,[1, 2] 5-HT_3 receptors have been shown to play a role in neuroprotection.[3] The latter might be due to a modulatory role of 5-HT_3 receptors in neurotransmission and/or signaling pathways of other endogenous ligands (i.e., acetylcholine, dopamine, GABA and glutamate)[4] implicating the 5-HT_3 receptor as a beneficial target in the treatment of Alzheimer's disease, multiple sclerosis, and stroke.[3] Recently, antagonists of 5-HT_3 receptors were also indicated as a potential pharmacological therapy for the treatment of opioid-induced pruritus.[5]

Our laboratory recently identified a novel class of 5-HT_3 receptor antagonists.[6] An example of this class is 2-amino-6-chloro-3,4-dihydroquinazoline (A6CDQ; **1**). Little is known about the structure-activity relationships (SAR) of **1**.



A6CDQ (**1**)

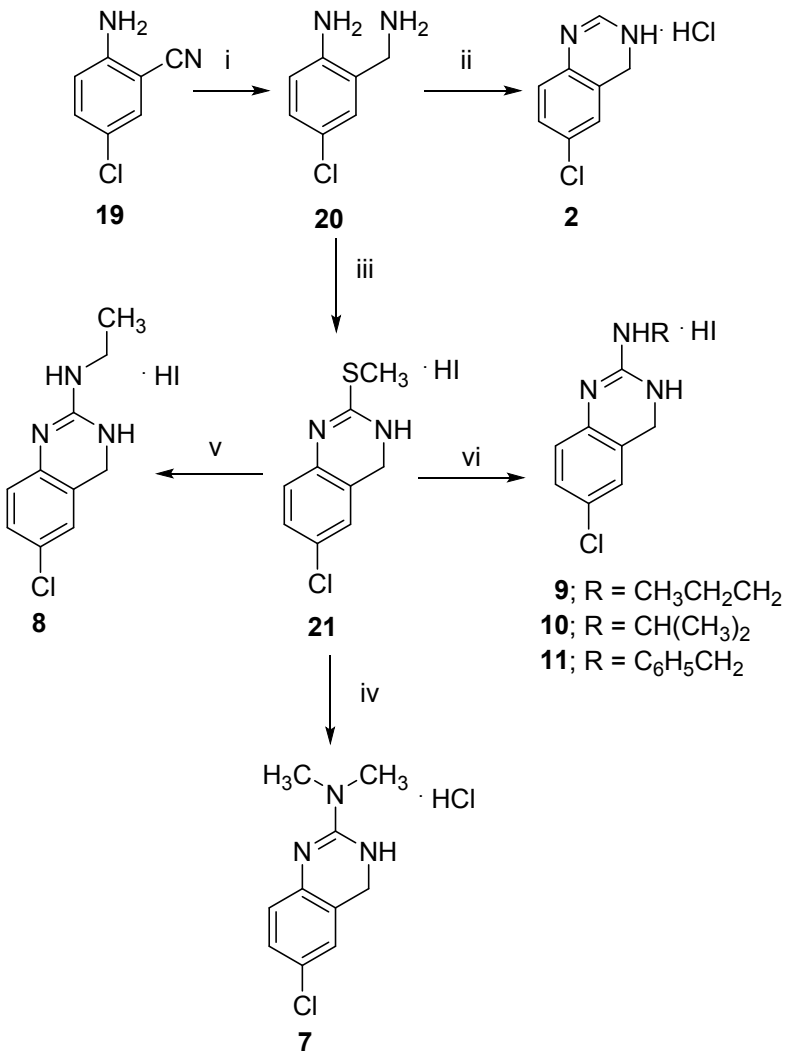
The aim of the studies reported here was several-fold: 1) to determine the necessity of the N1, N2 and N3 nitrogen atoms of A6CDQ for binding at h5-HT_3A receptors; 2) to examine the role of primary amine (i.e., N2) substituents of A6CDQ on binding at h5-HT_3A receptors; 3) to investigate the structural

features of A6CDQ underlying its 5-HT_3 receptor antagonist action; and 4) to understand how A6CDQ and its analogs interact/bind with 5-HT_3A receptors at the atomic level.

CHEMISTRY

To understand the role of the nitrogen atoms N1, N2, and N3 and their contribution to the binding affinity at h5-HT_3A receptors of the parent **1** we synthesized analogs **2**-**5** by removing the N2 atom (i.e., **2**) or replacing the N1 and/or N3 atoms by sp^3 hybridized carbon atoms (i.e., **3**-**5**). The parent compound **1** was resynthesized according to a previously reported procedure by us.[6] Des-amino quinazoline analog **2** was obtained in a two-step reaction (Scheme 1). First, the nitrile group of commercially available 2-amino-5-chlorobenzonitrile (**19**) was reduced with $LiAlH_4$[7] to the corresponding diamine **20**, followed by cyclization with HCO_2H in the presence of a catalytic amount of H_2SO_4 to afford the free base of **2** in 38% yield.

Scheme 1. [a]Synthetic route for preparation of compounds **2** and **7-11**.

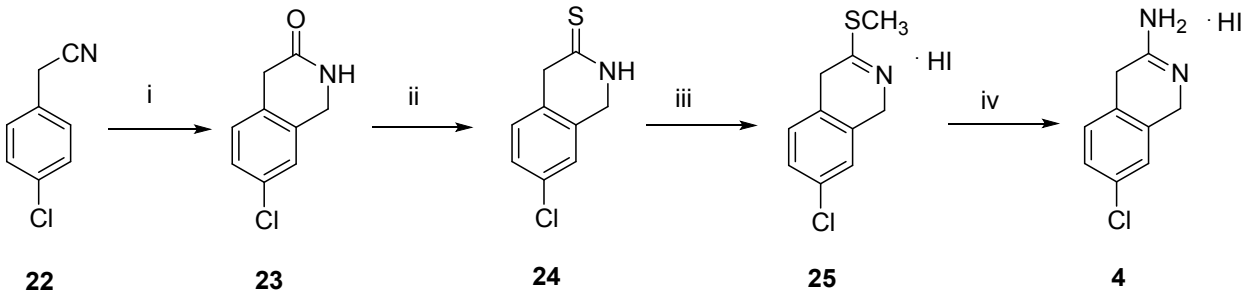


[a]**Reagents and conditions:** (i) $LiAlH_4$/THF, reflux, 2 h; (ii) (a) HCO_2H, H_2SO_4, reflux, 18 h; (b) HCl/EtOAc, room temperature; (iii) (a) CS_2, KOH, room temperature, 2 h, reflux, 18 h; (b) CH_3I, EtOH, reflux, 2 h; (iv) (a) $(CH_3)_2NH$ (40% wt in H_2O), EtOH, reflux, 18 h; (b) HCl/EtOH, room temperature, overnight; (v) $CH_3CH_2NH_2$ (70% wt in H_2O), EtOH, reflux, overnight; (vi) RNH_2, MeCN, reflux, 4-8 h.

The *des*-N3 quinoline analog **3** was obtained from known 6-chloro-3,4-dihydroquinoline-2(1*H*)-thione[8] via an amination reaction[9] with ammonium hydroxide and $HgCl_2$ as Lewis acid in a THF/DMF (5:1) mixture.

The *des*-N1 dihydroisoquinoline, analog **4** was obtained in four steps (Scheme 2). Initially, an acid-catalyzed cyclization reaction of commercially available *p*-chlorophenylacetonitrile (**22**) was carried out with pyrophosphoric acid and paraformaldehyde at 200 °C (oil bath) to afford 7-chloro-1,2,3,4-tetrahydroisoquinolin-3-one (**23**) in 72% yield.[10] Similar to a literature procedure,[11] thionation of **23** with Lawesson's reagent in anhydrous THF gave 7-chloro-1,2,3,4-tetrahydroisoquinolin-3-thione (**24**). Compound **24** was methylated with iodomethane to give the corresponding 7-chloro-3-(methylthio)-1,4-dihydroisoquinoline hydroiodide **(25).** Amination of **25** was achieved by treatment with ammonia to give **4**.

Scheme 2. [a]Synthetic route for compound **4**.



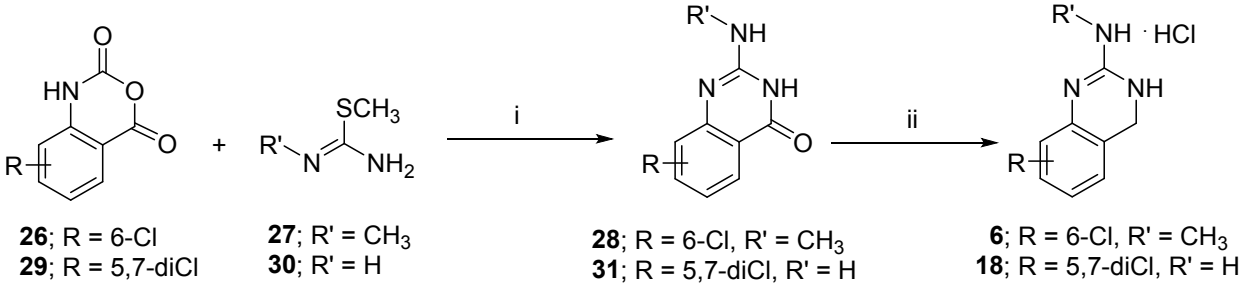
22 **23** **24** **25** **4**

[a]**Reagents and conditions:** (i) Pyrophosphoric acid, paraformaldehyde, 200 °C, 2 h; (ii) Lawesson's reagent, THF, reflux, 18 h; (iii) CH_3I, MeCN, reflux, 2 h; (iv) (a) NH_3, butanol, 200 °C, 1 h.

Compound **5** was obtained in one step by reductive amination of 6-chloro-β-tetralone with $NaCNBH_3$ and NH_4OAc.

The N-methyl analog of **1**, secondary amine **6**, was prepared in a similar manner to **1**, except *N,S*-dimethylthioisourea hydroiodide was used instead of *S*-methylisothiourea sulfate (Scheme 3).

Scheme 3. [a]Synthetic pathway for preparation of compounds **6** and **18**.



[a] **Reagents and conditions:** (i) Na_2CO_3, aq. MeCN (80%), reflux, 24 h; (ii) (a) BH_3·THF, reflux, 6 h; (b) HCl/EtOH, room temperature, 1 h.

The tertiary amine **7** and secondary amines **8-11** were synthesized by amination of **21** with dimethylamine (aq. 40%) and the corresponding primary amines, respectively (Scheme 1). The unsubstituted dihydroquinazoline analog **13**[12] and a positional isomer of **1**, 5-Cl dihydroquinazoline **16**[13] were synthesized according to a literature procedure except that HCl salts were prepared instead of the reported HI and HBr salts, respectively. Analog **18**, the 5,7-diCl analog of **13**, was prepared by a general procedure (Scheme 3) for the preparation of dihydroquinazolines utilized by us in the preparation of **1**.[6]

All target compounds were prepared as water soluble salts and their structures confirmed by ^{1}H NMR, and C, H, N analysis.

RESULTS AND DISCUSSION

Radioligand Binding. Figure 1 shows the h5-HT_3A receptor affinity of A6CDQ (**1**) and the analogs where one (i.e., **2**-**4**) or two (i.e., **5**) of the nitrogen atoms have been removed or replaced by a carbon atom. All three nitrogen atoms of **1** contribute to binding and removal of any one of them (i.e., **2**-**4**) results in a 5- to 10-fold reduction in affinity (Figure 1). Removal of both ring nitrogen atoms or, more accurately, replacement of the ring nitrogen atoms N1 and N3 by sp^3-hybridized carbon atoms, resulted in loss of affinity (i.e., **5**; K_i > 10,000 nM).

Retaining all three nitrogen atoms, N-alkylated analogs of **1** were examined next. The N-alkylated analogs **6**-**11** were screened in a primary binding assay and none displayed >26% inhibition at a concentration of 10,000 nM (Table 1). As shown in Table 1, amine substitution was not tolerated. Even the simplest N-alkyl analog, **6**, lacked affinity (i.e., K_i > 10,000 nM).

The *des*-Cl analog of **1** (K_i = 209 nM), dihydroquinazoline **13**, resulted in a substantial decrease in affinity (K_i > 10,000 nM) indicating that the presence of a 6-Cl group is essential for **1** to bind at h5-HT_3A receptors. Changing the position of the 6-Cl group of **1** to a 5-Cl group (**16**, K_i = 718 nM; pK_i = 6.14 ± 0.07) or 7-Cl group (**15**, K_i = 1,975 nM)[6] resulted in diminished affinity compared to the parent **1**. A hybrid molecule of **15** and **16**, 5,7-diCl dihydroquinazoline **18** also binds with >2-fold lower affinity (K_i = 589 nM; pK_i = 6.2 ± 0.10) than **1**. It is apparent that both, the position and, even more so, the presence of the chloro group are essential for optimal affinity of **1** at h-5HT_3A receptors.

Functional Activity. Recently, we reported that 5-HT_3A receptor functional activity in the arylguanidinium series ligands depends on its substitution pattern.[14] The guanidinium side chain in the arylguanidinium series can exist in a number of conformations (i.e., as rotamers) in which the guanidinium group is approximately perpendicular to the plane of the aryl group. We tested one conformational extreme where the guanidinium side chain is constrained via a methylene bridge to the phenyl ring to form dihydroquinazolines, resulting in near co-planarity of the aryl and guanidinium moieties, a conformation not energetically feasible for arylguanidines. Introduction of the methylene bridge to **12**[14] resulted in a change of function from a partial agonist to an antagonist as seen with A6CDQ (**1**)[6] (Figure 2). The observed change in function could be due to the introduction of the methylene bridge, the position of the chloro substituent (i.e., **1** and **12** might be binding somewhat differently), or a combination of these. To examine the effect of the position of the chloro group we prepared a positional isomer of A6CDQ (**1**), A7CDQ (**15**). Dihydroquinazoline **15** can be also visualized as a conformationally constrained analog of the agonist 3-chlorophenylguanidine[14] (*m*CPG; **14**). There are two possible ways in which **14** (Figure 2) can be restricted to form a dihydroquinazoline ring. Thus, A5CDQ (**16**) with a 5-chloro substituent was synthesized as well. Although diminished, both A7CDQ (**15**) and A5CDQ (**16**) retained affinity at 5-HT_3 receptors and both behaved as antagonists (Figures 2 and 3). Thus, constraint of arylguanidine **14**[14] to dihydroquinazolines **15** and **16** also resulted in a change of function from an agonist to an antagonist (Figure 2).

Up to this point we were able to show that both a partial agonist and an agonist arylguanidine, when constrained via a methylene bridge to a quinazoline ring system, yielded functionally different 5-HT_3 ligands, antagonists. Next, we examined the effect of constraint on the superagonist arylguanidine **17**[14] and found that introduction of the methylene bridge also

converts superagonist **17** to antagonist **18** (Figures 2 and 3). Finally, to test the functional effect of the presence of the chloro group, we prepared *des*-chloro dihydroquinazoline **13** and found that, although binding with very low affinity (K_i > 10, 000 nM), it retained 5-HT_3 receptor antagonist action (Figures 2 and 3).

3-D Molecular Modeling. Prediction of favorable binding modes for the constrained dihydroquinazoline compounds was achieved using the same modeling protocol that was used for the unconstrained arylguanidines (see Methods section). The m5-HT_3A crystal structure[15] was also used for docking studies involving the dihydroquinazoline antagonists, as functional analysis showed that for this structure, "the channel conformation likely corresponds to that of an inhibited, non-conducting channel" compared to the recently-solved cryo-EM m5-HT_3A structure, in which "the ligand binding domain adopts a conformation reminiscent of the unliganded state".[16] A consistent binding mode was identified for the dihydroquinazolines, which bears both similarities and significant differences compared to the consistent binding mode previously identified[14] for the arylguanidine agonists (Figure 4).

In both the arylguanidine and dihydroquinazoline series, the guanidinium group is predicted to engage the 5-HT_3A receptor in a similar fashion: The N1 and N2 nitrogen atoms form hydrogen bonds with the side chain carboxylate group of E236, the N2 nitrogen forms a second hydrogen bond with the T181 side chain hydroxyl group, and the N3 nitrogen atom forms a hydrogen bond with the backbone carbonyl oxygen atom of S182. Thus, each N–H moiety in the guanidinium group is engaged in a hydrogen bond. This binding mode is therefore consistent with the current finding that dihydroquinazoline analogs produced by either 1) removal or replacement of any of

the three nitrogen atoms or 2) N-alkylation, exhibit substantial or complete loss of affinity and very weak activity.

The way in which the aryl portion of the ligands is predicted to interact with the receptor, however, differs significantly between the arylguanidine and dihydroquinazoline series. By bridging the guanidinium group back to the aryl ring, the guanidinium and aryl groups in the dihydroquinazoline compounds are locked in a near co-planar arrangement. This prevents them from simultaneously forming the aforementioned hydrogen bond interactions with E236, T181 and S182, and forming a thermodynamically favorable aromatic "sandwich" with residues W183 and W90 in the "aromatic box" region of the 5-HT_3A orthosteric binding site. We previously proposed, based on combined experimental and computational results for arylguanidines, that such simultaneous interactions involving both parts of the ligand are important for the agonist activity of the arylguanidines. The dihydroquinazoline compounds are not able to bury their aromatic moiety in the aromatic box (specifically, between W183 and W90), but instead the aromatic portion forms edge-to-face aromatic interactions only with W90 and is partially exposed to the solvent.

A potentially important factor that may influence the compounds' ability to interact with E236, and thus their measured binding affinity, is the basicity of the nitrogen-containing functional group, as measured by the pK_a value of the conjugate protonated acid. Of primary importance is the major microspecies that exist in the environment of the receptor binding site. The cationic conjugate acid form, with an additional proton available for H-bonding, is able to form a much more thermodynamically stable (i.e. favorable) interaction with the E236 carboxylate than the neutral free base. Although the basic functional groups of the reported compounds, particularly guanidines and amidines, tend to be inherently very strongly basic (p$K_a \geq 12$), their inclusion in

certain environments, such as conjugation with an aromatic ring, can dramatically lower their pK_a. For example, 3,4-dihydroquinazoline (pK_a = 9.19)[17] is nearly four log units lower than that of 1,4,5,6-tetrahydropyrimidine (pK_a = 13.0).[18] Thus, although it is possible that a substantial fraction of the low-affinity neutral form may exist for the compounds reported here, we expect that the protonated, cationic form will usually predominate at physiological pH = 7.4.

The modeling results are also generally consistent with our mutagenesis results (Tables 2 and 3).

Site-directed Mutagenesis Studies. Dihydroquinazoline antagonists **1** and **16** were examined, and the results were compared to their arylguanidine counterparts **12** (a partial agonist)[14] and **14** (a full agonist),[14] respectively. Two mutations in loop D were tested, W90F and R92A. The W90F mutation showed reduced affinity for both arylguanidines by about 10-fold, likely due to non-optimal interaction with the ligand via formation of pi-interactions with F compared to W in the putative aromatic "sandwich" (Table 2). The same mutation showed either no difference or a modest increase in affinity (~7-fold) for dihydroquinazolines **1** and **16**, respectively, perhaps due to a reduction of unfavorable steric interactions in the wild-type receptor, where the aryl portion of the molecule is located in a crowded location enclosed not only by W90, but also by R92, Y153, and F226 in a small area near the opening of the "aromatic box" (Figures 4 and S2). The R92A mutant showed only a small reduction in affinity (Table 2) for all four ligands, which is consistent with the prediction that, individually, R92 is not expected to have a large influence on the binding of the ligands due to its location at the edge of the orthosteric binding site. Similarly, the N128A loop A mutant showed negligible changes in binding affinity for the four ligands compared to the wild-type receptor (Table 2). Although N128 forms a small part of the

orthosteric binding site, its contribution to the binding of arylguanidines and dihydroquinazolines is not expected to be great.

In loop C, the conservative F226Y mutation resulted in modestly reduced affinity (5- to 10-fold) for the arylguanidines and essentially no change in affinity for the dihydroquinazolines (Table 3). The Y226 side chain hydroxyl group is predicted to be partially solvent exposed (see Figure S2 for the putative location of F/Y226), and because the residue at position 226 on loop C may also serve as a "lid" controlling access to the orthosteric binding site aromatic box, the presence of the water-soluble side chain hydroxyl group may cause the lid to exist more often in an open, solvated conformation. This effect would presumably be less deleterious to the binding affinity of the dihydroquinazolines than to the arylguanidines because the aryl portion of the dihydroquinazolines is predicted to be near the opening of the aromatic box, where it may occupy the same space as the lid (including F226) in its closed conformation (see Figure S1). Preventing complete and effective closure of loop C also provides a plausible mechanism by which the dihydroquinazolines exert their antagonistic effect. Loop C closure has been predicted to be related to the degree of agonist activity at the 5-HT_3 and other pentameric Cys-loop ligand-gated ion channels,[19] and also appears to be correlated with the ability of agonists to form an energetically favorable inter-subunit bridge between regions of the primary (including loop C) and complementary subunits comprising the orthosteric binding site.[20] Finally, two mutations at loop C position 236 (E236A and E236N) were evaluated. All ligands, with the exception of the weak partial agonist **12**, showed substantial reductions in binding affinity to these mutants (10- to 40-fold), highlighting the importance of this residue in hydrogen bond/salt bridge formation with the ligands. Compound **12** was not substantially affected by the mutations, which may be

related to the fact that **12** did not bind with high affinity even at the wild-type receptor (pK_i = 5.86; K_i = 1,400 nM), suggesting a degree of non-specific binding (Table 3).

Conclusions

We have shown that A6CDQ (**1**) binds with higher affinity than its deconstructed analogs **2**-**5**. It would appear that all nitrogen atoms of A6CDQ (**1**) contribute to an optimal receptor interaction indicating that an intact guanidine moiety is important for optimal binding affinity at h5-HT_3A receptors. Substituents at the terminal N2 nitrogen atom (even a single methyl group) are not tolerated by h5-HT_3A receptors; a primary amine seems optimal for binding at h5-HT_3A receptors. Ring closure ("methylene bridge" formation) of the guanidinium moiety is a dominating factor in the function of dihydroquinazolines. Furthermore, the antagonist action of dihydroquinazolines at 5-HT_3A receptors is substitution-pattern and substituent-presence independent.

Our previously-proposed hypothesis that explained the wide range of functional activity seen in the arylguanidine series (i.e. antagonist, partial agonist, full agonist, and superagonist) posited that "the arylguanidines that are able to simultaneously engage the primary and complementary subunits, thus keeping them in close proximity, have greater agonist character whereas those that are deficient in this ability are antagonists".[14] The inability of the dihydroquinazolines to completely occupy the orthosteric binding pocket likely hinders their ability to keep the C loop of the principal subunit in close proximity to the D, E, and F loops of the complementary subunit, so that the ring-constrained dihydroquinazoline analogs become antagonists, regardless of the functional activity of their unconstrained arylguanidine counterparts (see Figure 4 and compare panel A to panel B, C to D, and E to F).

Experimental Section

Synthesis. Melting points (°C) were taken in glass capillary tubes on a Mel-Temp or Thomas Hoover apparatus and are uncorrected. ^{1}H NMR were recorded with a Bruker ARX 400 MHz or Bruker AVANCE III 400 MHz spectrometer, and peak positions are given in parts per million (ppm) downfield from tetramethylsilane as internal standard. IR spectra were determined using a Thermo Nicolet iS10 FT-IR. MS were obtained using a Waters Acquity TQD (tandem quadrupole) spectrometer utilizing electrospray ionization in positive ion mode. Microanalyses were performed by Atlantic Microlab Inc. (Norcross, GA) for the indicated elements, and results are within 0.4% of calculated values. Chromatographic separations were performed on silica gel columns (silica gel 62, 60−200 mesh, Sigma-Aldrich). Flash chromatography was performed on a CombiFlash Companion/TS (Teledyne Isco Inc., Lincoln, NE) apparatus.

Reactions were monitored by thin-layer chromatography (TLC) on silica gel GHLF plates (250 μm, 2.5 × 10 cm^2; Analtech Inc., Newark, DE). Compound **1** HCl was previously synthesized by our laboratory.[6] Quinazoline analogs **2-8** HCl and **9-11** HI were prepared adapting literature procedures for preparation of similar compounds.

6-Chloro-3,4-dihydroquinazoline Hydrochloride (**2**)

A catalytic amount of conc H_2SO_4 (10 drops) was added to a solution of **20** (0.25 g, 1.59 mmol) in formic acid (5 mL), and the reaction mixture was heated at reflux overnight. The reaction mixture was cooled to room temperature, quenched by careful addition of 6N NaOH (to pH 11),

and extracted with $CHCl_3$ (3 x 10 mL). The combined organic portion was dried ($MgSO_4$) and evaporated to dryness under reduced pressure to yield 0.10 g of the free base of **2** as a white solid: mp 152-156 °C; ^{1}H NMR (DMSO-d_6) δ 4.51 (s, 1H, CH_2), 6.78 (d, J = 8.40 Hz, 1H, ArH), 7.01 (d, J = 2.00 Hz, 1H, ArH), 7.13 (m, 2H, ArH, NH=CH-NH). The hydrochloride salt was prepared by addition of a saturated solution of gaseous HCl in EtOAc to yield a white solid that was recrystallized from EtOH to give 0.10 g (83%) of **2** as a white solid: mp > 300 °C; ^{1}H NMR (DMSO-d_6) δ 4.75 (s, 2H, CH_2), 7.17 (d, J = 8.80 Hz, 1H, ArH), 7.32 (d, J = 2.00 Hz, 1H, ArH), 7.39 (dd, J = 2.40, 8.40 Hz,1H, ArH), 8.38 (s, 1H, NH=**CH**-NH), 10.75 (s, 1H, NH, D_2O ex), 12.35 (s, 1H, NH^+, D_2O ex); Anal. Calcd for ($C_8H_7ClN_2$·HCl) C, H, N.

2-Amino-6-chloro-3,4-dihydroquinoline Hydrochloride (3)

A solution of 6-chloro-3,4-dihydroquinoline-2(1H)-thione[8] (0.13 g, 0.66 mmol) in anhydrous THF (5 mL) was added to a solution of NH_4OH (5 mL) and $HgCl_2$ (0.18 g, 0.66 mmol) in anhydrous DMF (1 mL) and the reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with CH_2Cl_2 (15 mL), filtered, and acidified with 6N HCl (to pH 1). The aqueous portion was basified with 1N NaOH (to pH 9) and extracted with EtOAc (3 x 10 mL). The combined organic portion was evaporated under reduced pressure to yield 0.08 g of the free base of **3** as a pale yellow solid: mp 115-118 °C; ^{1}H NMR ($CDCl_3$) δ 2.39- 2.43 (m, 2H, CH_2), 2.73-2.76 (m, 2H,CH_2), 4.22 (br s, 1H, NH), 6.86 (d, J = 8.36 Hz, 1H, ArH), 6.99 (d, J = 2.20 Hz, 1H, ArH), 7.06- 7.08 (dd, J = 2.32, 8.32 Hz, 1H, ArH). The addition of a saturated solution of gaseous HCl in EtOAc (10 mL) yielded a white solid which upon recrystallization from EtOH/Et_2O gave 0.05 g (55%) of **3** as a white solid: mp 222-225 °C; ^{1}H NMR (DMSO-d_6) δ 2.86-2.94 (m, 4H, 2-CH_2), 7.15 (d, J = 8.48 Hz, 1H, ArH), 7.32 (dd, J = 2.40, 8.48 Hz, 1H,

ArH), 7.38 (d, J = 2.24 Hz, 1H, ArH), 9.07 (s, 1H, NH, D_2O ex), 9.92 (s, 1H, NH, D_2O ex), 12.36 (s, 1H, NH^+, D_2O ex). Anal. Calcd for ($C_9H_9ClN_2 \cdot HCl$) C, H, N.

2-Amino-7-chloro-1,4-dihydroisoquinoline Hydroiodide (4)

A solution of **25** (0.10 g, 0.29 mmol) in n-butanol (5 mL) at 0 °C (ice-bath) was charged with gaseous NH_3. The ice bath was removed and the reaction mixture was stirred at 100 °C for 2 h. The solution was allowed to cool, concentrated under reduced pressure, the resultant yellow oil was digested with petroleum ether, and the residue was collected by filtration and dried ($MgSO_4$) to yield 0.05 g (55%) of **4** as a yellow solid: mp 246-250 °C; ^{1}H NMR (DMSO-d_6) δ 3.87 (s, 2H, CH_2), 4.49 (s, 2H, CH_2), 7.36-7.42 (m, 2H, ArH), 7.50 (s, 1H, ArH), 8.63 (s, 1H, NH, D_2O ex), 9.05 (s, 1H, NH, D_2O ex), 9.72 (s, 1H, NH^+, D_2O ex); HRMS m/z (ESI^+) found $(M)^+$ 181.0525, $C_9H_{10}ClN_2^+$ (requires $(M)^+$ 181.0527); Anal. Calcd for ($C_9H_9ClN_2 \cdot HI \cdot 0.1C_4H_9OH$) C, H, N.

2-Amino-6-chlorotetralin Hydrochloride (5)

Sodium cyanoborohydride (0.08 g, 1.33 mmol) was added to a solution of 6-chloro-β-tetralone (0.20 g, 1.11 mmol) and NH_4OAc (0.85 g, 11.07 mmol) in MeOH (15 mL) at room temperature. The resulting solution was stirred for 37 h at room temperature. The reaction mixture was acidified with 10% HCl (to pH 2), concentrated under reduced pressure, and then extracted with CH_2Cl_2 (2 x 75 mL). The aqueous portion was basified with 6 N NaOH (to pH 10) and extracted with CH_2Cl_2 (3 x 75 mL). The combined organic portion was dried (Na_2SO_4) and evaporated under reduced pressure to yield 0.11 g of the free base of **5** as a greenish-grey oil. The hydrochloride salt was prepared by addition of saturated solution of gaseous HCl in absolute EtOH (10 mL). The resulting solid was collected by filtration and recrystallized from EtOH/Et_2O

to yield 0.05 g (38%) of **5** as a pink solid: mp >250 °C; ^{1}H NMR (DMSO-d_6) δ 1.69-1.79 (m, 1H, CH), 2.09-2.13 (m, 1H, CH), 2.74-2.91 (m, 3H, CH), 3.04-3.10 (m, 1H, CH), 3.41-3.45 (m, 1H, CH), 7.15-7.20 (m, 3H, ArH), 8.29 (br s, 3H, NH_3+, D_2O ex); Anal. Calcd for ($C_{10}H_{12}ClN{\cdot}HCl{\cdot}0.1H_2O$) C, H, N.

2-(*N*-Methylamino)-6-chloro-3,4-dihydroquinazoline Hydrochloride (6)

A complex of $BH_3{\cdot}THF$ (1M, 7 mL) was added in a dropwise manner to a stirred solution of **32** (0.35 g, 1.66 mmol) in anhydrous THF (6 mL) under an N_2 atmosphere at 0 °C (ice-bath). The stirred reaction mixture was heated at reflux overnight, cooled to room temperature, and quenched by addition of 6N HCl (to pH ~1). The mixture was basified with 6N NaOH (to pH ~13) and extracted with hot $CHCl_3$ (3 x 25 mL). The combined organic portion was dried ($MgSO_4$) and evaporated under reduced pressure to afford 0.09 g, of the free base of **6** as a white solid: mp 152-155 °C; ^{1}H NMR (DMSO-d_6) δ 2.88 (s, 3H, CH_3), 4.45 (s, 2H, CH_2), 6.81 (d, J = 8.4 Hz, 1H, ArH), 7.09 (d, J = 2.44 Hz, 1H, ArH), 7.16 (dd, J = 2.52, 8.40 Hz, 1H, ArH). A solution of the crude product (0.08 g, 0.41 mmol) in EtOAc (10 mL) was allowed to stir at 0 °C (ice-bath). A saturated solution of HCl gas in absolute EtOAc (10 mL) was added, and the reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated to yield a white solid which upon recrystallization from EtOH/Et_2O gave 0.06 g (38%) of **6** as a white solid: mp 230-233 °C; ^{1}H NMR (DMSO-d_6) δ 2.90 (d, J = 4.8 Hz, 3H, CH_3), 4.49 (s, 2H, CH_2), 7.12 (s, 1H,ArH), 7.31(d, J = 2.3 Hz,1H, ArH), 7.34 (s, 1H, ArH), 8.11 (br s, 1H, NH, D_2O ex), 8.70 (br s, 1H, NH, D_2O ex), 10.87 (br s, 1H, NH^+, D_2O ex). Anal. Calcd for ($C_9H_{10}ClN_3{\cdot}HCl{\cdot}0.35\ H_2O$) C, H, N.

2-(*N,N*-Dimethylamino)-6-chloro-3,4-dihydroquinazoline Hydrochloride (**7**)

An aqueous solution of dimethylamine (40% w/w; 60 mL) was added to a solution of **21** (1.00 g, 4.70 mmol) in absolute EtOH (10 mL) and the stirred reaction mixture was heated at reflux overnight. The dimethylamine hydrochloride was collected by filtration and the filtrate was allowed to cool to room temperature for 1 h. The precipitate was collected by filtration and dried to yield 0.18 g of a yellow solid: mp 168-170 ^{o}C; ^{1}H NMR (DMSO-d_6) δ 2.91(s, 6H, 2 x CH_3), 4.27 (s, 2H, CH_2), 6.64 (d, J = 8.32, 1H, ArH), 6.96 (m, 2H, ArH). A saturated solution of gaseous HCl in EtOH (5 mL) was added to form a hydrochloride salt as a white solid that was collected by filtration and recrystalized from EtOH/Et_2O to yield 0.13 g (69%) of **7** as a white solid: mp 280-282 ^{o}C; ^{1}H NMR (DMSO-d_6) δ 3.14 (s, 6H, 2-N(CH_3)$_2$), 4.46 (s, 2H, CH_2), 7.36 (m, 3H, ArH), 8.67 (s, 1H, NH), 10.61 (s, 1H, NH^+); Anal. Calcd for ($C_{10}H_{12}ClN_3$·HCl) C, H, N.

6-Chloro-2-(*N*-ethylamino)-3,4-dihydroquinazoline Hydroiodide (8)

An aqueous solution of ethylamine (70% w/w; 0.50 mL) was added to a solution of **21** (1.00 g, 4.70 mmol) in absolute EtOH (10 mL) and the stirred reaction mixture was heated at reflux overnight. The solvent was evaporated under reduced pressure to yield a residue that was recrystallized from EtOAc to afford 0.32 g (79%) of **8** as a white solid: mp 189-192 ^{o}C; ^{1}H NMR (DMSO-d_6) δ 1.16 (t, J = 7.16 Hz, 3H, CH_3), 3.26-3.31 (m, 2H, CH_2), 4.49 (s, 2H, CH_2), 7.08 (d, J = 8.36 Hz, 1H, ArH), 7.33-7.36 (m, 2H, ArH), 7.94 (s, 1H, NH), 8.40 (s, 1H, NH), 10.28 (s, 1H, NH^+); Anal. Calcd for ($C_{11}H_{12}ClN_3$·HI) C, H, N.

General procedure for synthesis of compounds (9-11)

In a sealed tube, the appropriate amine (0.34 mmol) was added to a solution of **21** (0.31 mmol) in anhydrous MeCN, and the reaction mixture was heated at 80 °C overnight, and allowed to cool to room temperature. The solvent was evaporated to dryness under reduced pressure to yield a crude solid followed by recrystallization from EtOH/Et_2O.

2-(*N*-Propylamino)-6-chloro-3,4-dihydroquinazoline Hydroiodide (9)

Yield: 58%; white solid: mp 176-178 °C; ^{1}H NMR (DMSO-d_6) δ 0.91 (t, J = 7.2 Hz, CH_3), 1.51-1.60 (m, 2H, CH_2), 3.20 (t, J = 7.2 Hz, CH_2), 4.48 (s, 2H, CH_2), 7.08 (d, J = 8 Hz, 1H, ArH), 7.32-7.35 (m, 2H, ArH), 7.93 (s, 1H, NH), 8.40 (s, 1H, NH), 10.24 (s, 1H, NH^+); Anal. Calcd for ($C_{11}H_{14}ClN_3$·HI) C, H, N.

2-(*N-iso*Propylamino)-6-chloro-3,4-dihydroquinazoline Hydroiodide (10)

Yield: 48%; white solid: mp 140-142 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (t, J = 6.4 Hz, 2-CH_3), 3.82-3.87 (m, 1H, CH), 4.47 (s, 2H, CH_2), 7.09 (d, J = 8.4 Hz, 1H, ArH), 7.32-7.39 (m, 2H, ArH), 7.91 (s, 1H, NH), 8.30 (s, 1H, NH), 10.11 (s, 1H, NH^+); Anal. Calcd for ($C_{11}H_{14}ClN_3$·HI·0.1H_2O) C, H, N.

2-(N-Benzylamino)-6-chloro-3,4-dihydroquinazoline Hydroiodide (11)

Yield: 62%; white solid: mp 230-233 °C; ^{1}H NMR (DMSO-d_6) δ 4.50 (s, 2H, CH_2), 4.57 (s, 2H, CH_2), 7.09 (d, J = 8 Hz, 1H, ArH), 7.30-7.45 (m, 7H, ArH), 8.39 (s, 1H, NH), 8.55 (s, 1H, NH), 10.47 (s, 1H, NH^+); Anal. Calcd for ($C_{15}H_{14}ClN_3$ · HI) C, H, N.

2-Amino-3,4-dihydroquinazoline Hydrochloride (13)

The hydrochloride salt of **13**, was prepared by addition of a saturated solution of gaseous HCl in absolute EtOH to its free base[12] and isolated as a white solid that upon recrystallization from EtOH yielded 74% of **13** as an off-white solid: mp 158-160 °C; ^{1}H NMR (DMSO-d_6) δ 4.49 (s, 2H, CH_2), 6.96-6.98 (d, 1H, ArH), 7.07-7.10 (t, 1H, ArH), 7.17-7.19 (d, 1H, ArH), 7.24-7.27 (t, 1H, ArH), 7.63 (br s, 2H, NH_2, D_2O ex), 8.51 (br s, 1H, NH, D_2O ex), 10.83 (br s, 1H, NH^+, D_2O ex); IR (solid, cm^{-1}): 3261, 3088, 2963, 2870, 1671, 1626; Anal. Calcd for ($C_8H_9N_3$·HCl·0.25 EtOH·0.25H_2O) C, H, N.

2-Amino-5-chloro-3,4-dihydroquinazoline Hydrochloride (16)

The free base of **16**[13] was dissolved in absolute EtOH (2 mL) and converted to the hydrochloride salt by the addition of 1 N HCl/MeOH. Precipitation was forced by adding Et_2O; the precipitate was collected by filtration and recrystallized from EtOH to yield 45% of the desired target as white crystals: mp 238-239 °C; ^{1}H NMR (DMSO-d_6) δ 4.5 (s, 2H, CH_2), 6.93 (dd, J = 7.98 Hz, J = 0.74 Hz, 1H, ArH), 7.18 (dd, J = 8.12 Hz, J = 0.92 Hz, 1H, ArH), 7.28 (t, J = 8.06 Hz, 1H, ArH); IR (Diamond) cm^{-1}: 1618, 3192 (NH), 3285 (NH_2); Anal Calcd for ($C_8H_8ClN_3$·HCl) C, H, N.

2-Amino-5,7-dichloro-3,4-dihydroquinazoline Hydrochloride (**18**)

A solution of BH_3·THF complex (2.9 mL, 1.0 M) was added in a dropwise manner to 2-amino-5,7-dichloroquinazolin-4(3*H*)-one (**31**) under a N_2 atmosphere. The green reaction mixture was heated at reflux for 1 h. A solution of 6 N HCl (1 mL) was added in a dropwise manner at 0 °C (ice-bath) to hydrolyze the borate complex and excess reagent. The dark-blue suspension was basified with 6 N NaOH (1.5 mL). The reaction mixture was concentrated under reduced pressure and the resulting residue was extracted with hot $CHCl_3$ (2 x 15 mL). The crude product

precipitated when the combined organic extracts cooled to room temperature. The combined organic extracts were filtered. The filtrate was concentrated and the product was recrystallized from $CHCl_3$. The combined solid was dried in an Abderhalden over toluene heated at reflux for 8 h and yielded 0.09 g (59%) of the free base of **18** as an off-white solid: mp 207-208 ^oC; ^1H NMR (DMSO-d_6): δ 4.34 (s, 2H, CH_2), 5.87 (br s, 2H, NH_2, D_2O ex), 6.41 (br s, 1H, NH, D_2O ex), 6.48-6.49 (d, 1H, ArH), 6.78-6.79 (d, 1H, ArH); IR (solid, cm^{-1}): 3466, 3309, 3162, 1648, 1607.

The hydrochloride salt was formed by addition of a saturated solution of HCl in EtOH (5 mL). The resulting off-white solid was recrystallized from abs EtOH to yield 0.04 g (68%) of **18** as an off-white solid: mp 272-273 ^oC; ^1H NMR (DMSO-d_6) δ 4.48 (s, 2H, CH_2), 7.03-7.04 (d, 1H, ArH), 7.36-7.37 (d, 1H, ArH), 7.85 (br s, 2H, NH_2, D_2O ex), 8.69 (br s, 1H, NH, D_2O ex), 11.22 (br s, 1H, NH^+, D_2O ex); IR (solid, cm^{-1}): 3251, 3026, 2972, 2915, 2837, 1662, 1618; Anal Calcd for ($C_8H_7Cl_2N_3 \cdot HCl \cdot 0.25\ EtOH \cdot 0.25\ H_2O$) C, H, N.

2-Amino-5-chlorobenzylamine (20)

A solution of 2-amino-5-chlrobenzonitrile (**19**) (3.00 g, 19.76 mmol) in anhydrous THF (20 mL) was added in a dropwise manner to a suspension of $LiAlH_4$ (1.27 g, 33 mmol) in anhydrous THF (50 mL) under an N_2 atmosphere and cooled to 0 ^oC (ice-bath). The stirred reaction mixture was heated at reflux for 2 h, cooled to 0 ^oC (ice-bath), and quenched by the careful addition of H_2O (2 mL), 5% NaOH (2 mL), and H_2O (6 mL). The white precipitate was collected by filtration and the filtrate was extracted with EtOAc (3 x 25 mL). The combined organic portion was dried ($MgSO_4$) and evaporated under reduced pressure to give 2.90 g (94%) of a crude product as a yellow solid: mp 97-99 ^oC; ^1H NMR (DMSO-d_6) δ 1.70 (br s, 2H, NH_2), 3.57 (s, 2H, CH_2), 5.20

(br s, 2H, NH_2), 6.59 (d, J = 8.44 Hz, 1H, ArH), 6.94 (dd, J = 2.56, 8.40 Hz, 1H, ArH), 7.08 (d, J = 2.52 Hz, 1H, ArH).

6- Chloro-2-(methylthio)-3,4-dihydroquinazoline Hydroiodide (21)

A solution of CS_2 (2 mL, 31.61 mmol) and KOH (1.18 g, 21.03 mmol) was added to a solution of **20** (3.30 g, 21.07 mmol) in EtOH (25mL) and the reaction mixture was stirred at room temperature for 2 h, then heated at reflux for 16 h. The precipitate was collected by filtration and dried to yield 3.60 g of a yellow solid: mp > 250 ^oC; ^1H NMR (DMSO-d_6) δ 4.36 (s, 2H, CH_2), 6.93 (dd, J = 2.12, 7.20 Hz, 1H, ArH), 7.21 (s, 1H, ArH), 7.24 (d, J = 2.32 Hz, 1H, ArH), 8.67 (s, 1H, NH), 10.47 (s, 1H, NH). A solution of MeI (3.70 mL, 60.40 mmol) in absolute EtOH was added to a solution of the yellow solid (3.00 g, 15.10 mmol) in absolute EtOH (50 mL) and the reaction mixture was heated at reflux for 2 h. The solid was collected by filtration and recrystallized from EtOH to give 4.00 g (78%) of **21** as a pale-yellow solid: mp 236-240 ^oC; ^1H NMR (DMSO-d_6) δ 2.74 (s, 3H, CH_3), 4.72 (s, 2H, CH_2), 7.08 (d, J = 8.12 Hz, 1H, ArH), 7.39-7.42 (m, 2H, ArH); Anal. Calcd for ($C_9H_9ClN_2S{\cdot}HI$) C, H, N.

7-Chloro-1,2,3,4-tetrahydroisoquinolin-3-one (23)[10]

A mixture of 4-chlorophenylacetamide (1.5 g, 10 mmol), paraformaldehyde (0.33 g, 15 mmol) and pyrophosphoric acid (20 g, 112 mmol) was heated at 200 ^oC for 1 h, cooled to room temperature and quenched by the careful addition of saturated aqueous solution of $NaHCO_3$ and extracted with $CHCl_3$ (3 x 25 mL). The combined organic portion was washed with H_2O, dried ($MgSO_4$), and evaporated to dryness under reduced pressure to yield 1.3 g (72%) of **23** as a yellow-colored powder: mp 166-170 ^oC (lit.[10] mp 209-211 ^oC); ^1H NMR (DMSO-d_6) δ 3.42 (s,

2H, CH_2), 4.32 (s, 2H, CH_2), 7.22 (d, J = 8 Hz, 1H, ArH), 7.28 (dd, J = 2, 8 Hz, 1H, ArH), 7.39 (s, 1H, ArH), 8.06 (s, 1H, NH).

7-Chloro-1,2,3,4-tetrahydroisoquinolin-3-thione (24)

A solution of Lawesson's reagent (1.3 g, 3.3 mmol) in anhydrous THF (10 mL) was added in a dropwise manner to a stirred solution of **23**[10] (0.6 g, 3.3 mmol) in anhydrous THF (10 mL) at room temperature. The reaction mixture was then heated at reflux overnight, cooled to room temperature and the solvent was evaporated under reduced pressure to yield a crude product which was purified by column chromatography (silica gel; $CHCl_3$/MeOH; 100:0 to 80:20) to afford 0.3 g (47%) of **24** as a yellow solid: mp 256-260 ^oC; ^1H NMR (DMSO-d_6) δ 3.92 (s, 2H, CH_2), 4.41 (s, 2H, CH_2), 7.27 (d, J = 8 Hz, 1H, ArH), 7.34 (dd, J = 2, 8 Hz, 1H, ArH), 7.45 (s, 1H, ArH), 10.65 (br s, 1H, NH).

7-Chloro-3-(methylthio)-1,4-dihydroisoquinoline Hydroiodide (25)

Methyl iodide (0.15 mL, 3.00 mmol) was added to a solution of **24** (0.15 g, 0.76 mmol) in MeCN (20 mL) and the reaction mixture was heated at reflux for 2 h, cooled to room temperature and the residue was collected by filtration and dried to yield 0.17 g (66%) of **25** as a pale yellow solid: mp 216-220 ^oC; ^1H NMR (DMSO-d_6) δ 2.70 (s, 3H, CH_3), 4.16 (m, 2 H, CH_2), 4.81 (s, 2H, CH_2), 7.34 (d, J = 8.4 Hz, 1H, ArH), 7.43 (d, J = 7.6 Hz, 1H, ArH), 7.54 (s, 1H, ArH).

6-Chloro-2-(*N*-methylamino)-3,4-dihydroquinazolin-4-(3H)-one (28)

N,S-dimethylthioisourea hydroiodide (1.17 g, 5.06 mmol) and Na_2CO_3 (0.58 g, 5.51 mmol) were added to a stirred solution of isatoic anhydride (1.00 g, 5.06 mmol) in aq. MeCN (25 mL, 80%) and the resulting yellow solution was heated at reflux for 24 h. The reaction mixture was allowed to cool to room temperature for 1.5 h. The resulting precipitate was collected by filtration and washed successively with aq. MeCN (3 x 25 mL; 80%) and H_2O (75 mL). The solid was dried under reduced pressure and recrystallized from anhydrous THF to give 0.90 g (47%) of **28** as a white solid: mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 2.85 (d, J = 4.68 Hz, 3H, CH_3), 6.28 (br s, 1H, NH), 7.28 (d, J = 8.76 Hz, 1H, ArH), 7.57 (dd, J = 2.64, 6.12 Hz, 1H, ArH), 7.80 (d, J = 2.56 Hz, 1H, ArH), 11.20 (br s, 1H, NH).

2-Amino-5,7-dichloroquinazolin-4(3*H*)-one (31). *S*-Methylthioisourea sulfate (0.30 g, 1.07 mmol) and Na_2CO_3 (0.11 g, 1.07 mmol) were added to a solution of 4,6-dichloroisatoic anhydride (0.25 g, 1.07 mmol) (**29**)[21] dissolved in aq MeCN (11 mL, 80%) and heated at reflux for 23 h. The reaction mixture was allowed to cool to room temperature over a period of 30 min, H_2O (20 mL) was added, and the reaction mixture was allowed to stir for 2 h. The reaction mixture was filtered and the precipitate was washed with aq 80% MeCN (3 x 15 mL). The solid was dried in an Abderhalden over toluene heated at reflux for 24 h to yield 0.17 g (70%) of **31** as a beige solid: mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 6.64 (br s, 2H, NH_2, D_2O ex), 7.13-7.15 (m, 2H, ArH), 11.10 (br s, 1H, NH, D_2O ex); IR (solid, cm^{-1}): 3385, 2925, 2257, 2130, 1652.

Radioligand Binding Assay. Assays were carried out with h5-HT_3 receptors expressed in HEK293 cells using [^{3}H]GR65630 as radioligand and zacopride as reference compound.[22] In brief: target compounds were subjected to a primary binding assay in which compounds were

examined at a single concentration (10 μM) in quadruplicate in 96-well plates; compounds that showed a minimum of 50% inhibition at h5-HT_3A receptors were evaluated in secondary radioligand binding assays to determine equilibrium binding affinity at 5-HT_3 receptors. In the secondary binding assay, selected compounds were tested at 11 concentrations (0.1, 0.3, 1, 3, 10, 30, 100, 300 nM, 1, 3, 10 μM) in triplicate. K_i values were obtained from the following equation: $Ki = IC_{50}/(1+ [radioligand]/K_d)$.

Electrophysiology. Compounds **13**, **16** and **18** were evaluated functionally using two-electrode voltage clamp of *Xenopus* oocytes expressing murine 5-HT_3A receptors using previously published procedures.[6, 14]

Oocytes were surgically excised, then defolliculated by shaking for 90 min in 1.5% collagenase dissolved in Ca^{2+}-free OR-II medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl2 and 5 mM HEPES, pH 7.4). cRNA was synthesized in vitro using an appropriate cDNA and the mMESSAGE MACHINE mRNA synthesis kit (Ambion). Each oocyte was injected with 50 nL of mRNA (0.3 μg/μL concentration) and incubated at 19 °C for at least 24 h prior to electrophysiological recording. An oocyte was placed in the oocyte recording chamber and perfused with ND-96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1mM MgCl2, 2 mM phosphate, pH 7.4) at a flow rate of 20 mL/min. Two electrodes (1-2 MΩ filled with 3 M KCl) corresponding to the current and voltage electrodes were inserted and the holding potential was clamped at −60 mV using a Warner instruments amplifier. Ligands were prepared in ND-96 buffer then injected into the chamber. Current responses were recorded using a Axon Instruments A/D board and PClamp software. A dose response curve was determined using pooled peak amplitudes of individual responses and analyzed using nonlinear curve fitting and GraphPad

Prism software. All experiments were repeated a minimum of 4 times using oocytes from at least 2 different frogs. Compounds were initially applied alone, in the absence of 5-HT to determine if they were capable of activating the receptors (agonist action). Compounds that do not activate the receptors were further evaluated for their ability to inhibit 5-HT induced responses (antagonist action). For determination of antagonist K_i values, the IC_{50} was first determined by inhibition of 5-HT induced responses by increasing concentrations of the antagonist (compound **13**, **16** and **18**). The K_i was calculated from the IC_{50} value and 5-HT concentration using the Cheng−Prusoff relationship. A concentration of 5-HT equal to the EC_{50} and the EC_{90} for 5-HT stimulation of the receptor was used for inhibition and agonist experiments, respectively.

Site-Directed Mutagenesis and Ligand Binding. The generation of mutants and analysis of ligand binding to the homomeric murine 5-HT_3A(b) receptor was carried as previously described.[14] Briefly, membranes from transfected cells were incubated for 2 h at 37 °C in a total volume of 0.5 mL of 154 mM NaCl, 20 mM Tris-HCl, pH 7.4 containing the appropriate concentrations of the competing unlabeled ligand (e.g., *m*CPG; **14**) and radioligand ([^{3}H] granisetron; PerkinElmer, 85 Ci/mmol). Binding was terminated by rapid vacuum filtration onto GF/B filters pretreated with 50 mM Tris-HCl, pH 7.4, 0.2% polyethyleneimine and the filters were washed with 10 mL of cold 50 mM Tris-HCl, pH 7.4 per sample. Nonspecific binding was defined as that binding not displaced by 10 μM *m*CPBG (*meta*-chlorophenylbiguanide). IC_{50} values for the various compounds were determined by fitting the data to Eq. 1 using a Levenberg–Marquardt algorithm in a commercially available software package (Igor Pro, WaveMetrics, Oswego, OR):

$$\theta = (1 + ([I]/IC_{50})^{n})^{-1} \qquad \text{(Eq. 1)}$$

where θ is the fractional amount of [^{3}H]granisetron bound in the presence of the antagonist at concentration [I] compared to that in the absence of antagonist, IC_{50} is the concentration of antagonist at which θ=0.5, and n is the apparent Hill coefficient. All experiments were carried out with a [^{3}H]granisetron concentration equal to its experimentally-determined dissociation constant for the particular receptor (WT: 2.2 nM; W90F: 11 nM; R92A: 7.5 nM; N128A:1.7 nM; F226Y: 1.6 nM).

Molecular Modeling. Modeling studies were performed as described previously.[14] and are briefly described here. The ligands were sketched using SYBYL-X 2.1.1 (Certara USA, Inc., Princeton, NJ) and energy-minimized using the Tripos Force Field (TFF) with Gasteiger–Hückel charges and a distance-dependent dielectric constant of 4.0 D/Å to an energy gradient cut-off of 0.05 kcal(mol×Å)$^{-1}$.

Models representing the extracellular ligand binding domains (LBDs) of two adjacent subunits of the homopentameric mouse 5-HT_3A receptor were generated using the A (primary) and E (complementary) subunits from the m5-HT_3A receptor crystal structure (PDB ID: 4PIR).[15] The orthosteric ligand binding site, consisting of loops A–G, was modeled at the interface of subunits A and E. Residues preceding T35 and following P247 were not included in the models. To model the flexibility of the C loop, a population of 17 models of the LBD were generated with varying degrees of C loop openness. Although the receptor modeling was performed using the mouse 5-

HT_3A LBD crystal structure, some of the experimental results reported here were determined using human 5-HT_3A. We have previously shown[14] that the primary amino acid sequences of mouse and human 5-HT_3A LBDs are very similar, and we would also expect their 3-D structures to show a corresponding high degree of similarity. Importantly, there are no significant differences in the orthosteric binding site, and no differences in amino acid composition of the "aromatic box", the putative binding site for ligands described here. We would thus not expect significant differences between human and mouse receptors, especially in the molecular recognition of ligands at the orthosteric binding site.

GOLD Suite v.5.5 (Cambridge Crystallographic Data Centre, Cambridge, UK)[23] was used to dock the ligands into the orthosteric binding site of each member of the population to generate candidate binding modes. The in-house clustering algorithm[14] developed to identify common and disparate binding modes among the ligands was used to post-process the GOLD-docked solutions. A cutoff RMSD value of 2.0 Å was used to define clusters of binding modes based on a common phenylguanidine fragment shared by all ligands. The common binding modes were then manually evaluated. A common binding mode for the dihydroquinazoline antagonists was identified with a loop opening of 15–16 Å that was different than the binding mode previously identified for the arylguanidine ligands.[14] This binding mode was selected for further study based on its consistency with experimentally-derived data, namely mutagenesis and ligand SARs. The resulting receptor–ligand complexes were then subjected to a short 500-iteration energy-minimization in SYBYL-X 2.1.1 under the same conditions as described above for the unbound ligands. SYBYL-X 2.1.1 was also used to prepare the images in Figure 4.

Supporting Information

C, H, N analysis for compounds **2**–**11**, **13**, **16**, and **18** (Table S1). Traces and dose response curves for antagonists **1**, **13**, **15**, **16** and **18** (Figure S1). Putative binding modes of 5-HT_3 ligands showing the disposition of F226 (Figure S2).

Supplementary data related to this article can be found at http://...

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Author Contributions

M. D. conceived the idea, supervised the work and prepared the first draft of the manuscript; A. S. K., O. I. A., and G. S. A. synthesized 2-aminodihydroquinazolines and analogs under the supervision of M. D.; S. K. performed electrophysiological studies at m5-HT_3A receptors under the supervision of M. K. S.; H. L. N. performed site-directed mutagenesis and radioligand binding studies at m5-HT_3A receptors under the supervision of M. M. W.; P. D. M. generated homology models and carried out docking studies.

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Notes

The authors declare no competing financial interest.

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5-HT_3 receptor K_i values for compounds **1-11** and **13, 15, 16** and **18** were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, # HHSN-271-2013-00017-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda MD, USA. We thank Dr. Dong Yan for his contribution in generating some site-directed mutagenesis and radioligand binding data at m5-HT_3A receptors under the supervision of Prof. Michael M. White. Ahmed S. Abdelkhalek was supported by a fellowship from the Egyptian Cultural and Educational Bureau, the Egyptian Channel Program coordinated by Prof. Mohamed Elhusseny Elsadek. The authors are grateful to Prof. Richard A. Glennon for discussions and proof reading of the manuscript.

ABBREVIATIONS

A5CDQ (2-amino-5-chlorodihydroqinazoline); A6CDQ (2-amino-6-chlorodihydroqinazoline); A7CDQ (2-amino-6-chlorodihydroqinazoline); *m*CPG (*N*-(3-chlorophenylguanidine)); *m*CPBG (*meta*-chlorophenylbiguanide); 5-HT (serotonin); SAR (structure-activity relationship).

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FIGURE CAPTIONS

Figure 1. h5-HT_3A receptor affinity (pK_i ± SD) of A6CDQ (**1**) and the analogs where one (i.e., **2**-**4**) or two (i.e., **5**) of the N atoms have been removed or replaced.

Figure 2. Introduction of a "methylene bridge" to arylguanidines (the 5-HT_3 receptor partial agonist **12**, agonist **14**, and superagonist **17**) resulted in conformationally-constrained antagonists. Potency as agonists or antagonists (EC_{50} or IC_{50} ± SEM, respectively) are provided. Lack of SEM indicates data previously reported.[6, 14]

Figure 3. Dose response curves for **13, 16** (A5CDQ) and **18**. The fractional response (Y-axis) represents inhibition of 5-HT (2.5 μM) induced currents by increasing concentrations of each compound. IC_{50} values: **13**, 7.2 ± 0.90 μM; **16**, 2.6 ± 0.82 μM; **18**, 1.2 ± 0.87 μM.

Figure 4. Putative binding mode of phenylguanidine agonists and their bridged dihydroquinazoline counterparts (ball-and-stick rendering; agonists, white carbon atoms; antagonists, orange carbon atoms) in the orthosteric binding site (CPK rendering; primary subunit, tan carbon atoms; complementary subunit, yellow carbon atoms). (A) Partial agonist **12** and (B) antagonist **1** (A6CDQ). (C) Full agonist **14** (mCPG) and (D) antagonists **15** (A7CDQ) and **16** (A5CDQ). (E) Superagonist **17** and (F) antagonist **18**. To enhance clarity, loop C is labeled and residue F226 (loop C), which forms the "lid" of the aromatic box, is not shown (but see Figure S2).

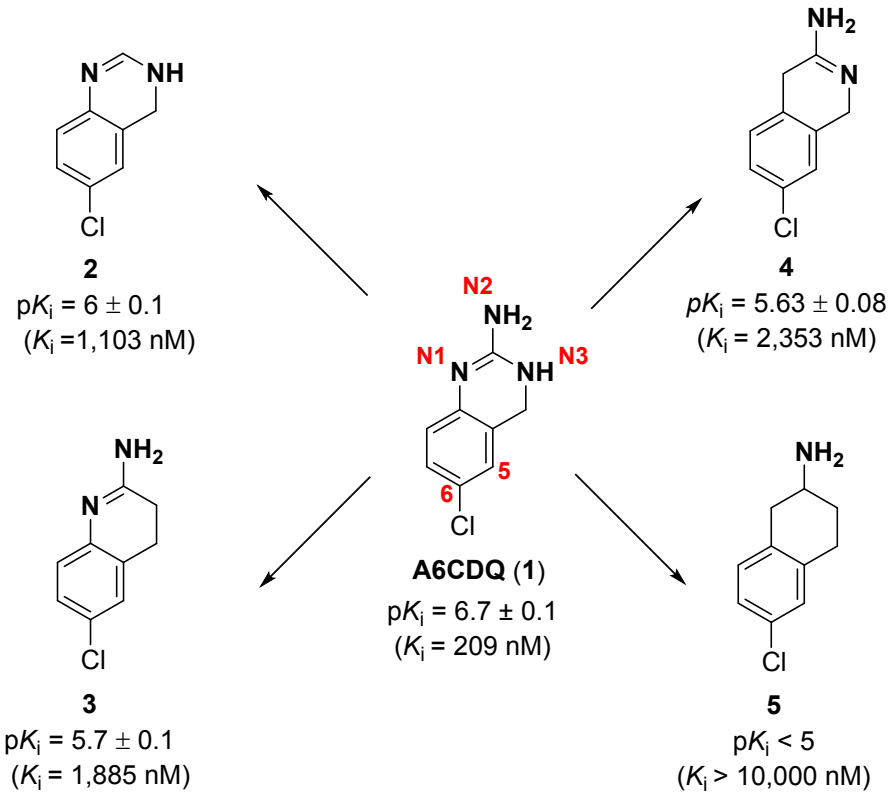
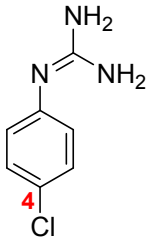

N
NH
Cl
2
pKi = 6 ± 0.1
(Ki =1,103 nM)
NH2
N
Cl
3
pKi = 5.7 ± 0.1
(Ki = 1,885 nM)
N2
NH2
N1
N
NH
N3
5
6
Cl
A6CDQ (1)
pKi = 6.7 ± 0.1
(Ki = 209 nM)
NH2
N
Cl
4
pKi = 5.63 ± 0.08
(Ki = 2,353 nM)
NH2
Cl
5
pKi < 5
(Ki > 10,000 nM)

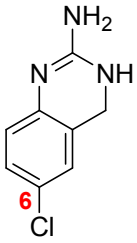
Figure 1.



12

EC_{50} = 53.89 μM

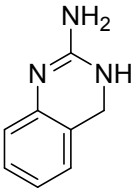
(partial agonist)



1

IC_{50} = 0.26 μM

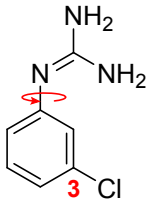
(antagonist)



13

IC_{50} = 7.2 ± 0.90 μM

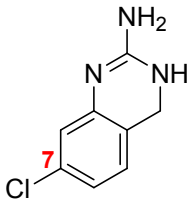
(antagonist)



14 (***m*CPG**)

EC_{50} = 3.1 μM

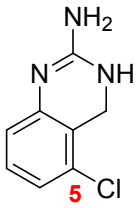
(agonist)



15

IC_{50} = 5.7 μM

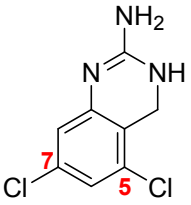
(antagonist)



16

IC_{50} = 2.6 ± 0.82 μM

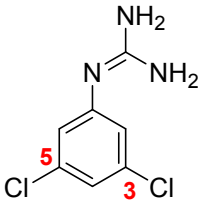
(antagonist)



18

IC_{50} = 1.2 ± 0.87 μM

(antagonist)



17

EC_{50} = 0.8 μM

(superagonist)

Figure 2.

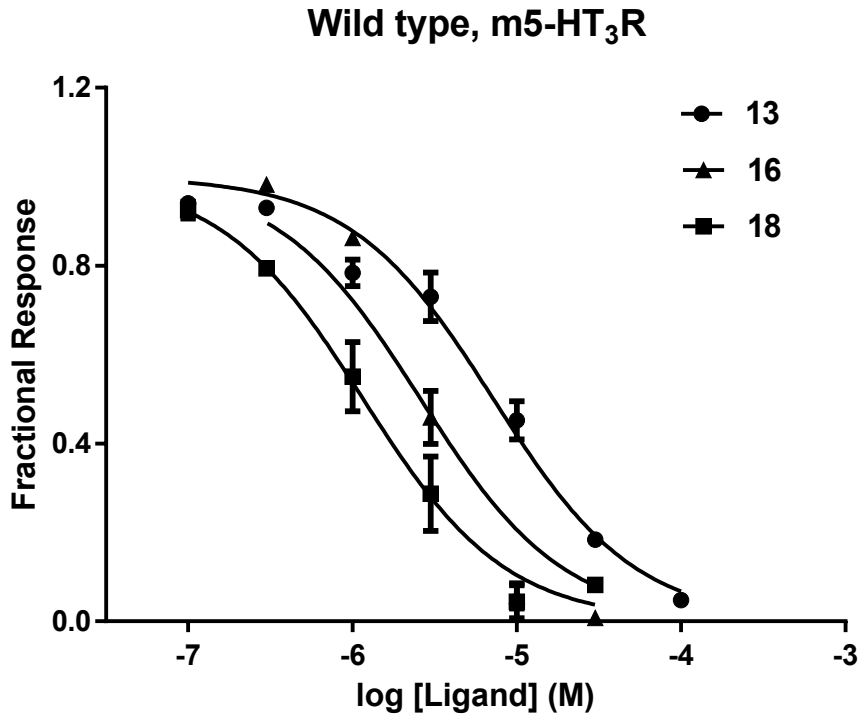


Figure 3.

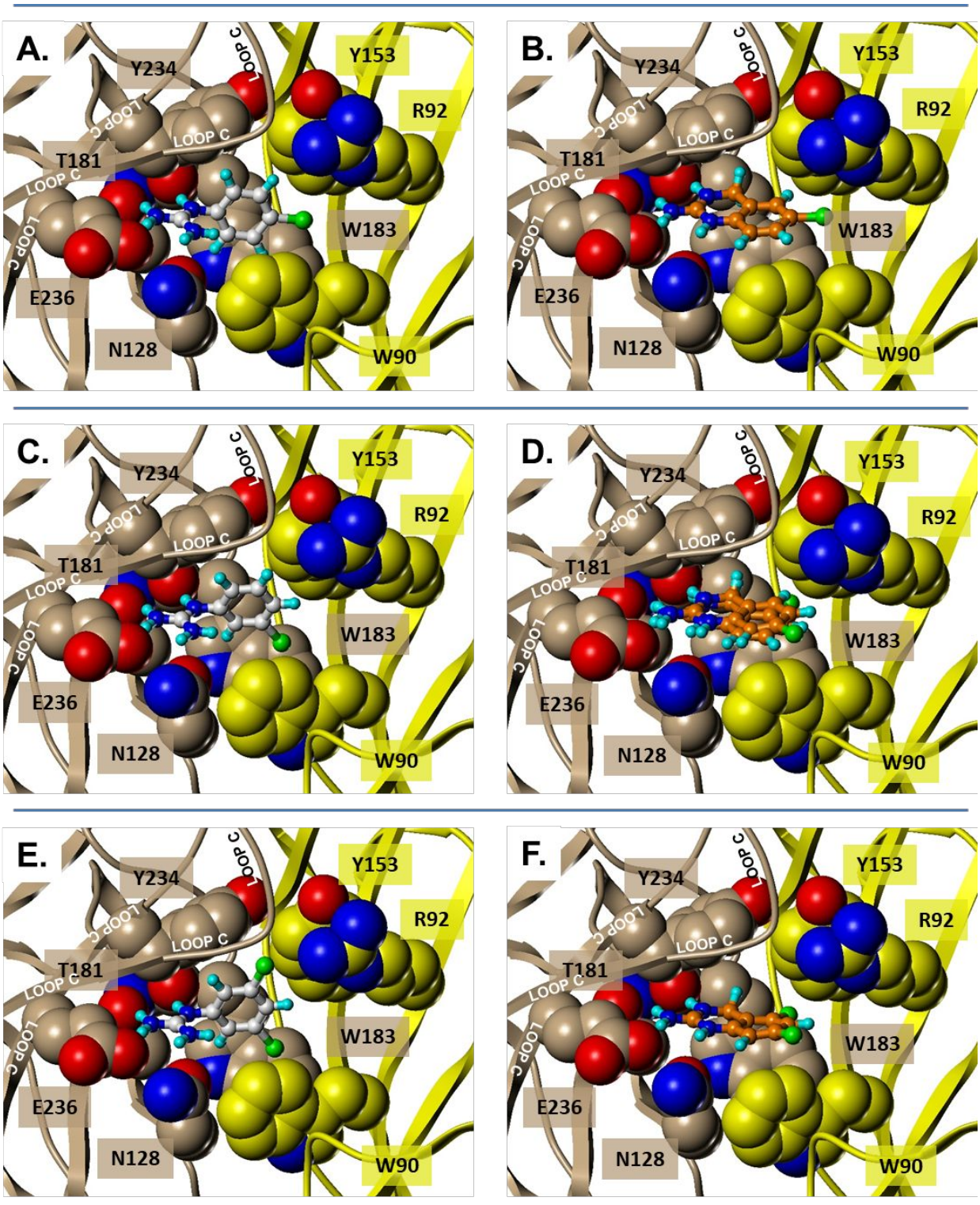
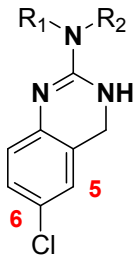

A.
Y234
LOOP C
Y153
R92
LOOP C
T181
LOOP C
W183
LOOP C
E236
N128
W90
B.
Y234
LOOP C
Y153
R92
LOOP C
T181
LOOP C
W183
LOOP C
E236
N128
W90
C.
Y234
LOOP C
Y153
R92
LOOP C
T181
LOOP C
W183
LOOP C
E236
N128
W90
D.
Y234
LOOP C
Y153
R92
LOOP C
T181
LOOP C
W183
LOOP C
E236
N128
W90
E.
Y234
LOOP C
Y153
R92
LOOP C
T181
LOOP C
W183
LOOP C
E236
N128
W90
F.
Y234
LOOP C
Y153
R92
LOOP C
T181
LOOP C
W183
LOOP C
E236
N128
W90

Figure 4.

Table 1. Primary radioligand binding (% inhibition) for primary amine **1**, secondary amine **6**, **8-11**, and tertiary amine **7** at h5-HT_3A receptors.



1, **6-11**

compound	R_1	R_2	primary binding % inhibition[a]
A6CDQ (1)	H	H	89.5[b]
6	H	CH_3	25.3
7	CH_3	CH_3	-5.6
8	H	CH_3CH_2	23.5
9	H	$CH_3CH_2CH_2$	13.3
10	H	$CH(CH_3)_2$	3.0
11	H	$CH_2(C_6H_5)$	16.9

[a]Examined at a concentration of 10,000 nM (n = 4); [b]K_i = 209 nM (Figure 1).

Table 2. Affinity (pK_i ± SD) values for representative arylguanidines (agonist 1**2**; partial agonist **14**) and corresponding antagonist quinazolines (**1** and **16**) for the wild-type and mutant m5-HT_3A receptors.

	pK_i ± SD				
compound	**wild-type[a]**	**W90F Loop D**	**R92A Loop D**	**N128A Loop A**	**F226Y Loop C**
1	6.91 ± 0.05	7.19 ± 0.07*	6.40 ± 0.05*	7.07 ± 0.05*	6.44 ± 0.07*
12[b]	6.52 ± 0.04	5.51 ± 0.14*	6.19 ± 0.07*	6.46 ± 0.03	5.77 ± 0.08*
14 (*m*CPG)[b]	7.29 ± 0.06	6.30 ± 0.07*	6.66 ± 0.07*	7.07 ± 0.06*	6.25 ± 0.04*
16	5.94 ± 0.05	6.79 ± 0.02*	5.63 ± 0.04*	5.91 ± 0.06	5.42 ± 0.04*

[a]Mutations of the m5-HT_3 receptor were constructed in two separate experiments, and binding affinity at the wild-type receptor was determined for each experiment as a control. [b]Data previously reported.[14] Asterisks represent a statistically-significant increase (red) and decrease (black) in affinity relative to wild-type.

Table 3. Affinity pK_i (± SD) values for representative arylguanidines (agonist 1**2**; partial agonist **14**) and corresponding antagonist quinazolines (**1** and **16**) for the wild-type and mutant m5-HT_{3A} receptors.

compound	pK_i ± SD		
	wild-type[a]	E236A Loop C	E236N Loop C
1	7.28 ± 0.03	5.85 ± 0.05*	5.65 ± 0.05*
12[b]	5.86 ± 0.03	5.77 ± 0.10	5.31 ± 0.10*
14 (*m*CPG)[b]	7.41 ± 0.06	6.48 ± 0.60*	5.84 ± 0.09*
16	6.15 ± 0.01	5.07 ± 0.07*	4.51 ± 0.04*

[a]Mutations of the m5-HT_3 receptor were constructed in two separate experiments, and binding affinity at the wild-type receptor was determined for each experiment as a control. As a consequence, the values might differ from those provided in Table 2. [b]Data previously reported.[14] Asterisks represent a statistically-significant decrease in affinity relative to wild-type.

Table of Contents graphics