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Article

Design, Synthesis, and Pharmacological Characterization of Heterobivalent Ligands for the Putative 5-HT_{2A}/$mGlu_2$ Receptor Complex

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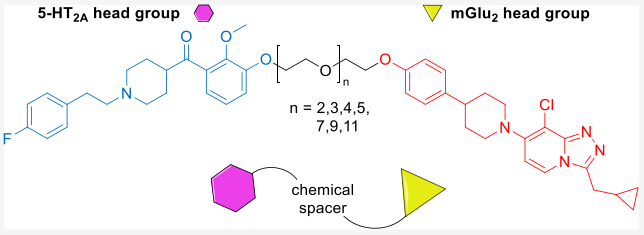
ABSTRACT: We report the synthesis of the first series of heterobivalent ligands targeting the putative heteromeric 5-HT_{2A}/$mGlu_2$ receptor complex, based on the 5-HT_{2A} antagonist **MDL-100,907** and the $mGlu_2$ ago-PAM **JNJ-42491293**. The functional properties of monovalent and heterobivalent ligands were characterized in 5-HT_{2A}-, $mGlu_2$/Gqo5-, 5-HT_{2A}/$mGlu_2$-, and 5-HT_{2A}/$mGlu_2$/Gqo5-expressing HEK293 cells using a Ca^{2+} imaging assay and a [^{3}H]ketanserin binding assay. Pronounced functional crosstalk was observed between the two receptors in 5-HT_{2A}/$mGlu_2$ and 5-HT_{2A}/$mGlu_2$/Gqo5 cells. While the synthesized monovalent ligands retained the 5-HT_{2A} antagonist and $mGlu_2$ ago-PAM functionalities, the seven bivalent ligands inhibited 5-HT-induced responses in 5-HT_{2A}/$mGlu_2$ cells and both 5-HT- and Glu-induced responses in 5-HT_{2A}/$mGlu_2$/Gqo5 cells. However, no definitive correlation between the functional potency and spacer length of the ligands was observed, an observation substantiated by the binding affinities exhibited by the compounds in 5-HT_{2A}, 5-HT_{2A}/$mGlu_2$, and 5-HT_{2A}/$mGlu_2$/Gqo5 cells. In conclusion, while functional crosstalk between 5-HT2A and mGlu2 was demonstrated, it remains unclear how these heterobivalent ligands interact with the putative receptor complex.



INTRODUCTION

G-protein-coupled receptors (GPCRs) mediate signaling of a diverse range of neurotransmitters and hormones and are divided into five major classes (class A, class B, class C, adhesion, and frizzled/taste receptors).[1] GPCRs exert their functions as monomers but may also assemble into functional dimeric or oligomeric complexes of homomeric or heteromeric compositions.[2] While the signaling properties of monomeric and homodimeric GPCRs are often difficult to dissect from one another, GPCR heterodimers have, in several cases, been reported to exhibit distinct functional properties compared to those of their two parent monomeric receptors.[2−7] Expression and unequivocal demonstration of the existence of GPCR dimer/oligomers *in vitro* are challenging because of the cell line-dependent receptor expression levels and other experimental factors.[8] Moreover, studies of GPCR dimerization/oligomerization *in vivo* are associated with additional challenges,[9] and with the exception of a few examples of truly well-established GPCR heterodimerization *in vivo*,[10,11] the actual extent and physiological importance of GPCR dimerization/oligomerization in native tissues are thus still largely unknown.

The present study focuses on the putative heteromeric complex of the serotonin 2A (5-HT_{2A}) receptor and the metabotropic glutamate 2 ($mGlu_2$) receptor.[12] As key mediators of neurotransmission mediated by serotonin (5-hydroxytryptamine, 5-HT) and glutamate (Glu) in the central nervous system, 5-HT_{2A} and $mGlu_2$ have both been pursued intensively as drug targets in several cognitive and psychiatric disorders over the years. Atypical antipsychotics act in part as 5-HT_{2A} antagonists, and augmentation of 5-HT_{2A} signaling has in recent years gained renewed therapeutic interest in light of remarkable effects exhibited by classical hallucinogens in clinical trials for depression and other psychiatric disorders.[13,14] The therapeutic potential in the augmentation of signaling through the $mGlu_2$ receptors within indications such as schizophrenia and depression has been investigated for $mGlu_{2/3}$ agonists in clinical trials.[15] In addition to the respective physiological functions and therapeutic potential of the two receptors, several lines of evidence from electrophysiological, biochemical, and behavioral studies have indicated substantial negative functional crosstalk between 5-HT_{2A} and $mGlu_2$ in the frontal cortex where the two receptors exhibit an overlapping distribution. While this crosstalk could arise from several different origins, including synaptic mechanisms and/or crosstalk between different stages of the signaling pathways activated through the two receptors,[16,17]

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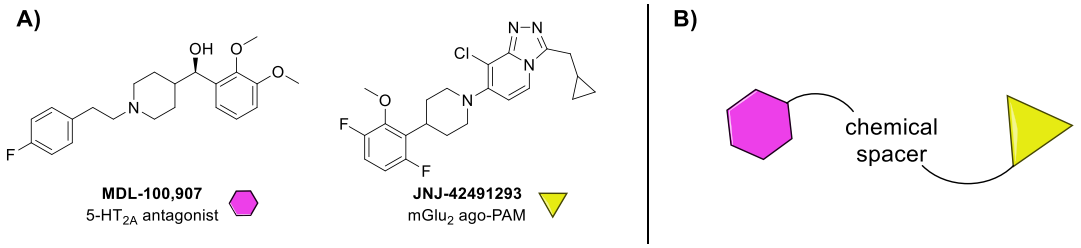
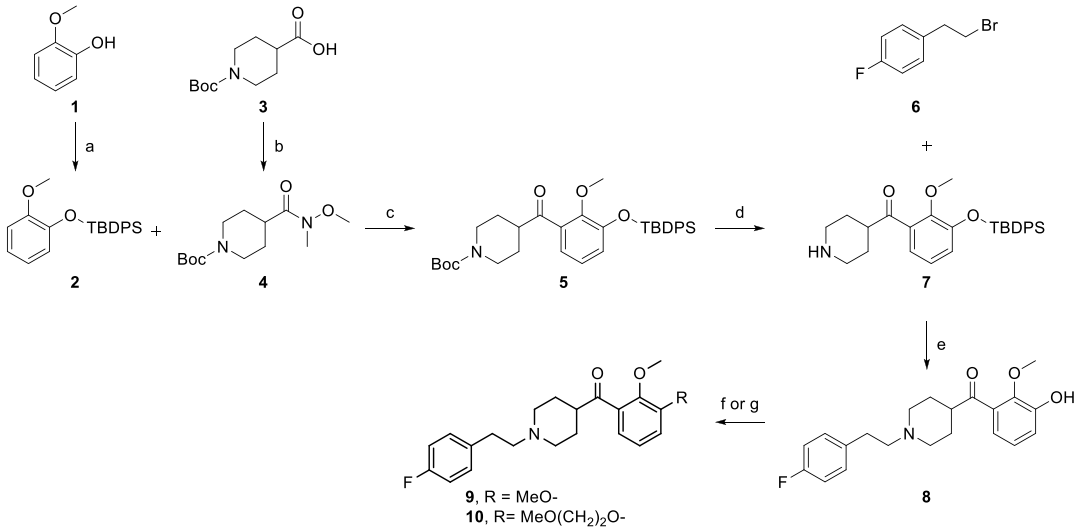


Figure 1. (A) Chemical structures of 5-HT_{2A} head group **MDL-100,907** and $mGlu_2$ head group **JNJ-42491293**. (B) Cartoon drawing of a heterobivalent ligand.

Scheme 1. Synthesis of 5-HT_{2A} Head Group[a]



[a]Reagents and conditions: (a) TBDPS-Cl, imidazole, DCM, rt, 20 h (96%); (b) *O*-methylhydroxylamine, EDCI, HOBt, Et_3N, DMF, rt, 18 h (94%); (c) (i) *n*BuLi, THF 0 °C → rt, 2 h and (ii) **4**, −42 °C → rt, 18 h (59%); (d) (i) TFA, DCM, 0 °C → rt, 2 h and (ii) NH_4OH (96%); (e) NaI, $NaHCO_3$, DMF, 65 °C, 16 h (48%); (f) MeOTs, K_2CO_3, DMF, 60 °C, 17 h (47%); and (g) KI, K_2CO_3, bromo-2-methoxyethane, DMF, 65 °C, 17 h (56%).

the existence of a heteromeric 5-HT_{2A}/$mGlu_2$ complex proposed by González-Maeso and colleagues in 2008 offered an attractive molecular mechanism by which this crosstalk could be explained.[12,18,19] Furthermore, both of these receptors are known to form homodimers[20] as well as heteromeric complexes with other receptors. For example, 5-HT_{2A} has been reported to form functional heterodimers with other class A GPCRs, specifically, the dopamine D_2 receptor[21,22] and the cannabinoid CB_1 receptor.[23] Analogous to other class C GPCRs, $mGlu_2$ is expressed as a constitutive dimer[24] and, in addition to its homodimeric assembly, it has also been shown to form heteromeric complexes with other mGlu receptors.[25−27] Although the reported efficient complex formation *in vitro* between two structurally different and distantly related GPCRs is quite remarkable, other family A and C GPCRs have also been proposed to assemble into heteromeric complexes.[28−30] Interestingly, the reported extent and characteristics of the functional crosstalk between the two receptors in this putative 5-HT_{2A}/$mGlu_2$ heteromer in heterologous expression systems have varied considerably in previous studies (this will be outlined in detail in Results and Discussion).[18,19,27,31,32] As a possible explanation for this variation, the relative expression ratio of the two receptors in the cells has been proposed to be important for the exact functional properties exhibited by the putative heteromer assembled from them.[16,18,19]

An attractive strategy to enable selective targeting of a heteromeric receptor complex over its two parent receptors is the design of a heterobivalent ligand, which comprises two receptor-selective head groups separated by a chemical spacer of suitable length and flexibility.[33−35] The bivalent strategy was pioneered by Portoghese and colleagues in their development of selective ligands for studies of opioid receptor dimerization[28,36,37] and has subsequently been pursued in the design of ligands for other GPCR dimers.[3] In the present work, we report the design, synthesis, and pharmacological characterization of a series of heterobivalent ligands aimed at the putative 5-HT_{2A}/$mGlu_2$ heteromer.

RESULTS AND DISCUSSION

Design. The heterobivalent ligands were designed to comprise the potent 5-HT_{2A}-selective antagonist **MDL-100,907** (volinanserin) as the 5-HT_{2A} head group (Figure 1A). This compound is attractive for this purpose, as it has previously been incorporated successfully into homobivalent 5-HT_{2A} ligands, using the *meta*-methoxy position as the anchor point for the spacer.[38−40] Furthermore, it has been reported that the secondary chiral alcohol in **MDL-100,907** can successfully be substituted for a carbonyl group.[40]

For the $mGlu_2$ head group, the low-nanomolar potent $mGlu_2$-selective allosteric agonist and positive allosteric modulator (ago-PAM) **JNJ-42491293** (Figure 1A) was

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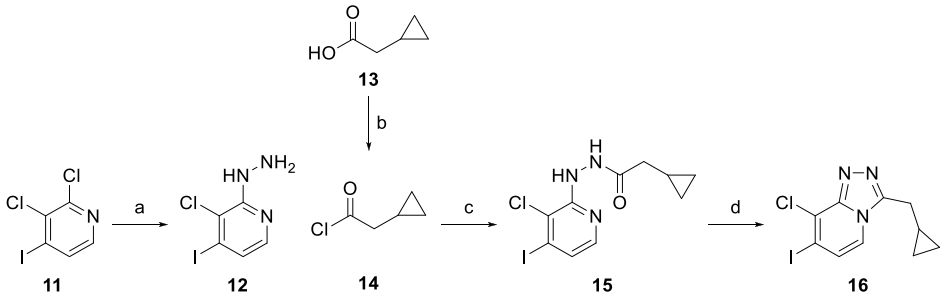
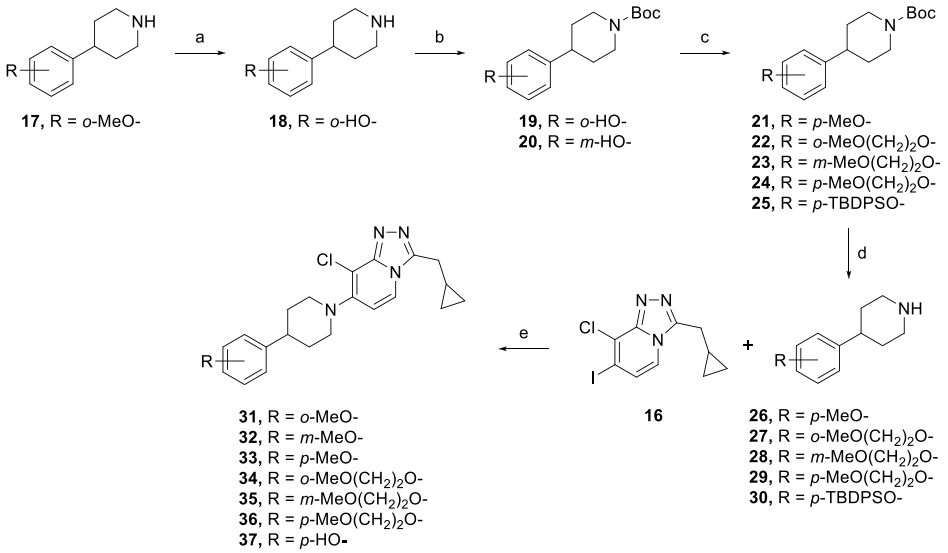
Scheme 2. Synthesis of Heterocycle 16[a]



[a]Reagents and conditions: (a) hydrazine·hydrate, dioxane, 70 °C, 16 h (64%); (b) oxalyl chloride, DCM, rt, 4 h (98%); (c) Et_3N, DCM, rt, 2 h (63%); and (d) dimethylsulfone, sulfolane, 160 °C, 3 h (68%).

Scheme 3. Synthesis of Methoxy and 2-Methoxyethoxy Derivatives of the $mGlu_2$ Head Group[a]



[a]Reagents and conditions: (a) BBr_3 (33 wt% in AcOH), reflux, 2 h (91%); (b) $(Boc)_2O$, Et_3N, DCM, rt, 18 h (94−96%); (c) KI, K_2CO_3, bromo-2-methoxyethane or MeI, DMF, 60 °C, 17 h (68−88%); (d) (i) TFA, DCM, rt, 2 h and (ii) NH_4OH (97−99%); and (e) 0.05 equiv $Pd(OAc)_2$, 0.1 equiv XantPhos, 2.4 equiv Cs_2CO_3, dioxane, reflux, 36 h (22−78%).

selected.[41] Extensive structure−activity relationship (SAR) information is available for this compound class.[41−50] Derived from all these data, it is apparent that the heterocycle of this compound class interacts deeply within the allosteric binding pocket in the 7-transmembrane domain of $mGlu_2$, and that the anisole moiety points toward the solvent. Nevertheless, we decided to extend the SAR by synthesizing analogues **31**−**36**, in order to determine the optimal orientation of the anisole moiety (described in detail below).

For the chemical spacer, a polyethylene glycol (PEG) chain was selected as it offers a balanced lipophilicity profile because of its alternating ethers and hydrocarbons and allows for significant flexibility between the two head groups.[33,51] Largely, because of the unknown structure of this putative heteromeric complex, the main focus of the present study was to address the optimal spacer length and, as such, we have selected seven spacer lengths ranging from 3 to 12 PEG units (9−36 atoms).

Chemistry. The synthesis of 5-HT_{2A} head group **8** followed the previously reported method,[52,53] and it commenced with the protection of commercially available guaiacol (**1**) as its *tert*-butyldiphenylsilyl (TBDPS) ether **2** (Scheme 1). Directed ortho metalation of **2** followed by quenching with the Weinreb amide **4**[54] (obtained from **3**) gave rise to **5** in 59% yield.[52,53] The product was obtained as a single regioisomer, which is in accordance with literature reports.[55] Subsequently, **5** was deprotected with trifluoroacetic acid (TFA) in dichloromethane (DCM) and converted to its corresponding free base **7**. Alkylation of **7** with bromide **6** was performed under previously reported conditions.[53] However, in our hands, the TBDPS ether was also cleaved, and head group **8** could be isolated directly without the need for an additional deprotection step.[52,53]

The methyl ether analogue **9** was now prepared by methylation of **8** with MeOTs, and 2-methoxyethoxy ether **10** was obtained using bromo-2-methoxyethane as the alkylating agent. Interestingly, no formation of the quaternary ammonium salt was observed, despite the fact that 2 equiv of the bromide alkylating agent was used.

The synthesis of $mGlu_2$ head group **37** was based on a previously reported work by Cid and colleagues,[50] and it commenced with the reaction of 2,3-dichloro-4-iodopyridine (**11**) with hydrazine·hydrate to give **12** (Scheme 2). Acid chloride **14** was prepared from 2-cyclopropylacetic acid (**13**) and oxalyl chloride and isolated by direct distillation of the reaction mixture. Acylation of **12** with **14** gave intermediate **15**, which was then set up for a cyclocondensation reaction.

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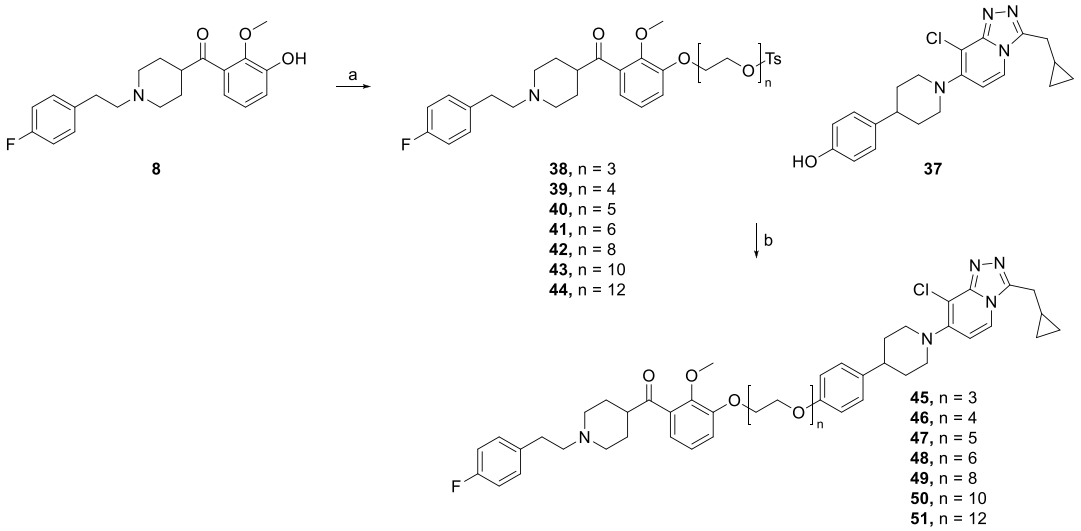
Scheme 4. Synthesis of Bivalent Ligands[a]



[a]Reagents and conditions: (a) NaH, ditosyl-(PEG)$_n$, THF, rt, 17 h (42−51%) and (b) NaH, DMF, rt, 17 h (10−23%).

Following the reported conditions, this step proceeded with low isolated yields (21%) of **16**. However, addition of dimethylsulfone and sulfolane greatly improved the isolated yield (68%).

The series of methoxy and 2-methoxyethoxy derivatives (**31**−**37**) (Scheme 3) were prepared directly by a Buchwald−Hartwig coupling of **16** with the corresponding aniline. The reported yield for this reaction is in the range of 20−50%,[50] for which reason, we sought to first optimize the reaction conditions using commercially available 4-(3-methoxyphenyl)-piperidine (**17**). From this study, we concluded that the best conditions were 0.05 equiv of $Pd(OAc)_2$, 0.1 equiv of XantPhos, 2.4 equiv of Cs_2CO_3, and dry dioxane stirred under reflux for 36 h under inert conditions, which gave an isolated yield of 78% (Scheme 3).

Free *ortho*-phenol **18** was obtained by deprotection of commercially available **17** by refluxing in 33 wt % hydrogen bromide/acetic acid (HBr/AcOH). The *ortho*- and (commercially available) *meta*-hydroxy analogues were both Boc-protected under standard conditions to give **19** and **20**, respectively. *tert*-Butyl-4-(4-hydroxyphenyl)piperidine-1-carboxylate was commercially available. With all the three Boc-protected phenol piperidines in hand, the 2-methoxyethoxy moieties could be installed, as well as the remaining *para*-methoxy analogue (**21**−**24**) (Scheme 3). The deprotection of the Boc groups was performed with TFA in DCM; the reaction mixtures were quenched with NH_4OH (25% in H_2O) and yielded the free amines in near quantitative yields (**26**−**29**). These secondary amines were cross-coupled with **16**, under the optimized reaction conditions established beforehand. Regrettably, the yield for these reactions varied significantly, with the yields of the 2-methoxyethoxy analogues being somewhat lower compared to their corresponding methoxy analogues (Scheme 3).

With both the 5-HT_{2A} and $mGlu_2$ head groups in hand, the construction of the heterobivalent ligands (**45**−**51**) was commenced (Scheme 4). Initial experimentation showed that the reaction between ditosyl-$(PEG)_4$ and $mGlu_2$ head group **37** proceeded with high conversion in dimethylformamide (DMF), but the product isolation proved tedious. On the other hand, the product of the reaction between ditosyl-$(PEG)_4$ and 5-HT_{2A} head group **8** could be readily isolated using preparative thin-layer chromatography (TLC). Thus, intermediates **38**−**44** were first prepared in 42−49% yield. Subsequently, phenolic $mGlu_2$ head group **37** was deprotonated with 2.0 equiv of NaH in DMF, followed by addition of appropriate intermediates **38**−**44**. The target bivalent ligands (**45**−**51**) were isolated by preparative high-performance liquid chromatography (HPLC) in 10−23% isolated yield as the corresponding TFA salts (Scheme 4).

Pharmacological Characterization. *Construction of Stable HEK293 Cell Lines Expressing 5-HT_{2A}, $mGlu_2$, and 5-HT_{2A}/$mGlu_2$.* In this study, we wanted to characterize the functional properties of the synthesized monovalent and bivalent ligands at cell lines expressing the putative 5-HT_{2A}/$mGlu_2$ heteromer and its two parent receptors using the same functional assay. For this purpose, four stable HEK293 cell lines expressing 5-HT_{2A} and $mGlu_2$ on their own or in combination were established, and a Ca^{2+} imaging assay using the fluorescent calcium dye Fluo-4 was applied. A previously constructed stable 5-HT_{2A}-HEK293 cell line was used to assay this receptor,[56] and the other parent receptor was assayed using a HEK293 cell line coexpressing $mGlu_2$ and the chimeric Gα-protein Gqo5 that directs the G$\alpha_{i/o}$-signaling of $mGlu_2$ into the G$\alpha_{q/11}$-pathway and intracellular Ca^{2+} mobilization.[57] To facilitate testing of the ligands at the putative 5-HT_{2A}/$mGlu_2$ complex, the following two cell lines coexpressing the two receptors were established: 5-HT_{2A}/$mGlu_2$- and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cells. The expressed receptors and the origin of intracellular Ca^{2+} mobilization measured in the four cell lines are illustrated in Figure 2A.

Basic Functional Characterization of 5-HT2A, mGlu2, and the Putative 5-HT2A/mGlu2 Complex. Prior to the testing of monovalent and bivalent compounds at 5-HT_{2A}, $mGlu_2$, and the putative 5-HT_{2A}/$mGlu_2$ heteromer in the four cell lines, we delineated the basic pharmacology of the receptors in the Ca^{2+}/Fluo-4 assay using reference ligands. 5-HT evoked robust responses in 5-HT_{2A}-HEK293 cells in a concentration-dependent manner (Figure 2B), and in a previous study, the functional properties exhibited by numerous reference 5-HT_{2A} agonists and antagonists on this cell line in the Ca^{2+}/Fluo-4 assay have been found to be in good agreement with literature

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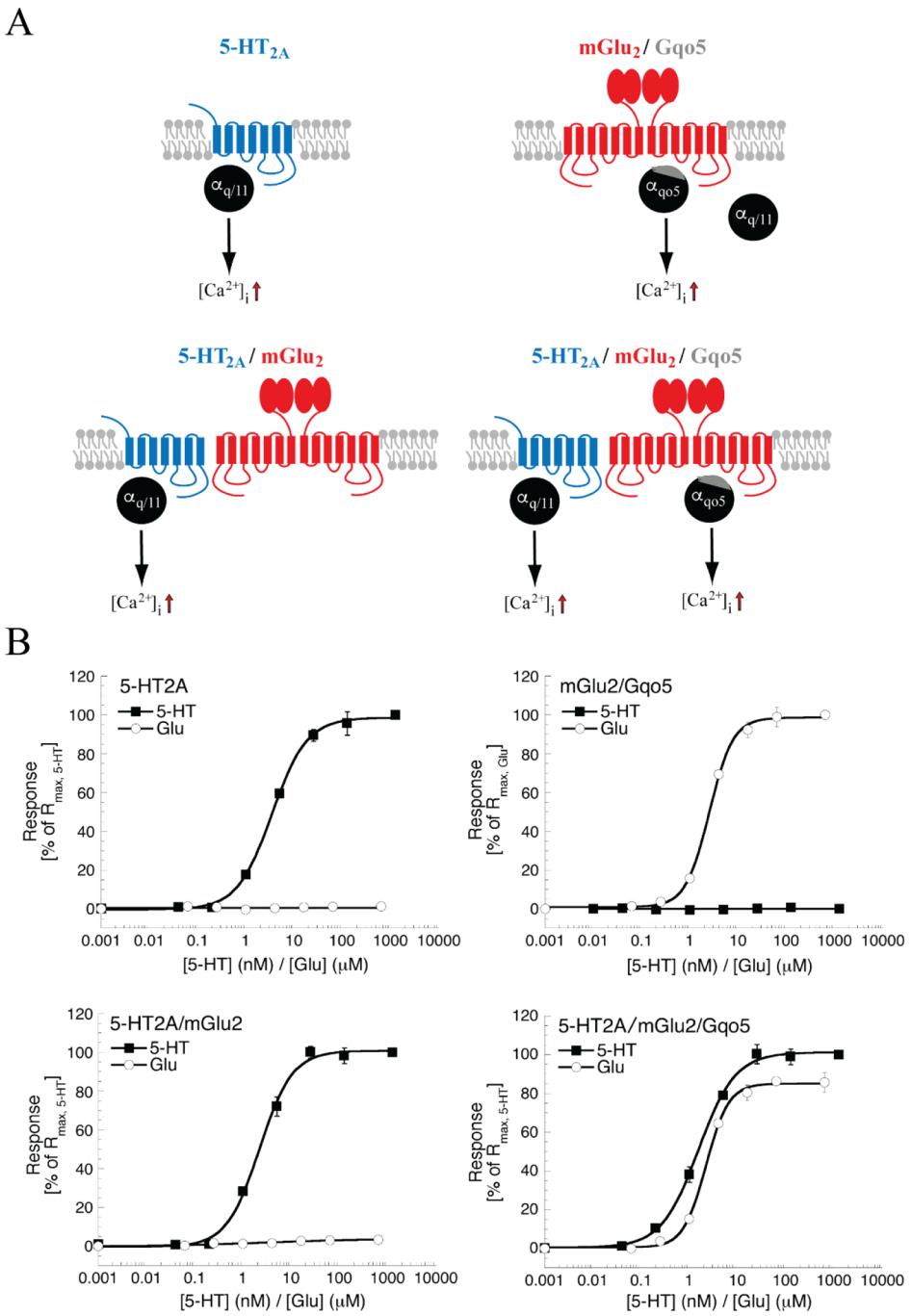

Figure 2. 5-HT_{2A}-, $mGlu_2$/Gqo5-, 5-HT_{2A}/$mGlu_2$-, and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cell lines. (A) Receptors and the origin of intracellular Ca^{2+} mobilization in the four cell lines. (B) Concentration−response relationships for 5-HT and Glu in the four stable HEK293 cell lines. 5-HT and Glu concentrations on the *X*-axis are given in nM and μM, respectively. Data are given in % of the R_{max} of 5-HT (5-HT_{2A}-, 5-HT_{2A}/$mGlu_2$-, and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293) or the R_{max} of Glu ($mGlu_2$/Gqo5-HEK293) in the same plate. Data are given as mean ± SD values from representative experiments out of a total of at least four independent experiments.

data.[56] Analogously, Glu mediated robust increases in intracellular Ca^{2+} levels *via* the chimeric Gα-protein Gqo5 in $mGlu_2$/Gqo5-HEK293 cells (Figure 2B), and a selection of reference $mGlu_2$ ligands [Glu, the $mGlu_{2/3}$-selective agonist (1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (**LY 354740**/eglumegad), the $mGlu_{2/3}$-selective competitive antagonist (1*S*,2*S*)-2-((*S*)-1-amino-1-carboxy-2-(9*H*-xanthen-9-yl)ethyl)cyclopropane-1-carboxylic acid (**LY 341495**), the $mGlu_2$-selective negative allosteric modulator (NAM) (*Z*)-1-(2-(cycloheptyloxy)-2-(2,6-dichlorophenyl)-vinyl)-1*H*-1,2,4-triazole (**Ro 64-5229**), and the ago-PAM *N*-(4′-cyano-[1,1′-biphenyl]-3-yl)-*N*-(pyridin-3-ylmethyl)-ethanesulfonamide (**CBiPES**)] also exhibited functional properties at the cell line in concordance with previously reported values (Figure S16 and Table S1).[58−62] Importantly, the 5-HT_{2A} agonists 5-HT and 1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine (DOI) (Figure S16) did not evoke significant responses in $mGlu_2$/Gqo5-HEK293 cells (shown for 5-HT in Figure 2B), and the 5-HT_{2A} antagonist 2-methyl-1,2,3,4,10,14b-hexahydrodibenzo[*c*,*f*]pyrazino[1,2-*a*]-

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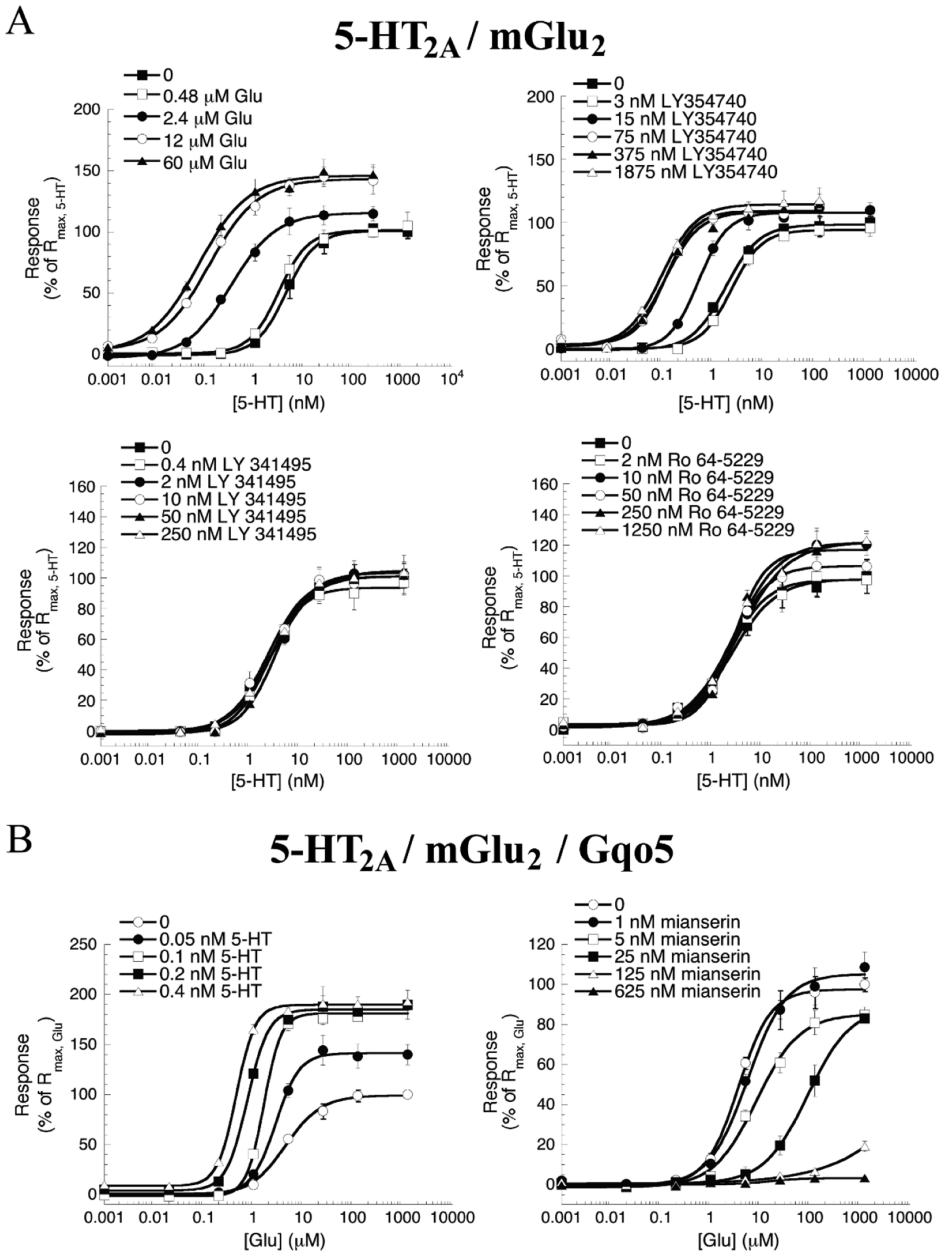

Figure 3. Functional crosstalk between 5-HT_{2A} and $mGlu_2$ in the 5-HT_{2A}/$mGlu_2$- and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cell lines. (A) Concentration−response relationships for 5-HT on the 5-HT_{2A}/$mGlu_2$-HEK293 cells in the absence and presence of various concentrations of the $mGlu_2$ ligands Glu, **LY 354740**, **LY 341495**, and **Ro 64-229**. The agonists Glu and **LY 354740** were coapplied together with 5-HT on the cells, whereas the antagonists **LY 341495** and **Ro 64-229** were applied on the cells prior to the application of 5-HT. (B) Concentration−response relationships for Glu on the 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cells in the absence and presence of various concentrations of the 5-HT_{2A} ligands 5-HT and mianserin. 5-HT was coapplied with Glu on the cells, whereas the antagonist mianserin was applied on the cells prior to the application of Glu. (A,B) Data in the figure are given as mean ± SD values from representative experiments for each cell line. Responses are given normalized to the R_{max} value of 5-HT (A) or Glu (B) in the absence of another ligand on the same plate, and data are given as mean ± SD values from representative experiments out of a total of 3−4 independent experiments.

azepine (mianserin) did not inhibit Glu-induced responses in the cells (data not shown). Conversely, neither Glu, **LY 354740** nor **CBiPES** mediated significant responses in 5-HT_{2A}-HEK293 cells (shown for Glu in Figure 2B), and **LY 341495** and **Ro 64-5229** did not inhibit 5-HT-induced responses in these cells (data not shown).

The concentration−response relationships exhibited by 5-HT as an agonist on 5-HT_{2A}/$mGlu_2$- and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cells in the Ca^{2+}/Fluo-4 assay did not differ substantially from those displayed by it on 5-HT_{2A}-HEK293 cells (Figure 2B). In contrast to its complete inactivity in 5-HT_{2A} cells, Glu induced small but significant responses in 5-HT_{2A}/$mGlu_2$ cells, although the response was very minute compared to that evoked by 5-HT (Figure 2B). As expected, Glu mediated robust responses in 5-HT_{2A}/$mGlu_2$/Gqo5 cells because of the Gqo5 expression in these cells, exhibiting an half maximal effective concentration (EC_{50}) value for these cells comparable to that at the $mGlu_2$/Gqo5 cell line (Figure 2B). Next, the crosstalk between 5-HT_{2A} and $mGlu_2$ in the two cell lines in the assay was investigated by determining the

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agonist properties of 5-HT and Glu in the presence of various ligands targeting $mGlu_2$ and 5-HT_{2A}, respectively. Co-application of 5-HT with Glu or **LY 354740** resulted in a substantial left shift in the 5-HT concentration−response relationship at the 5-HT_{2A}/$mGlu_2$-HEK293 cells (Figure 3A). In contrast, the 5-HT-evoked signaling in these cells was not affected significantly by the presence of the competitive antagonist **LY 341495** or the NAM **Ro 64-5229** (Figure 3A). In analogous experiments on the 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cell line, the putative effects of 5-HT and the 5-HT_{2A} antagonist mianserin on Glu-mediated signaling were investigated. The concentration−response relationship exhibited by Glu on the cells was substantially potentiated in a concentration-dependent manner by co-application of 5-HT, and Glu-induced signaling in the cells was inhibited in a concentration-dependent manner by mianserin (Figure 3B). While the substantial effects mediated by Glu and **LY 354740** on 5-HT-signaling in 5-HT_{2A}/$mGlu_2$ cells and by mianserin on Glu-signaling in 5-HT_{2A}/$mGlu_2$/Gqo5 cells were observed in the effective concentration ranges of the ligands at their respective target receptors (Figure 3A,B), 5-HT robustly enhanced both the Glu potency and efficacy on 5-HT_{2A}/$mGlu_2$/Gqo5 cells at concentrations below those displaying intrinsic activity on 5-HT_{2A}-HEK293 cells (Figure 2B).

Comparison of Basic Functional Properties of the Four Cell Lines with Findings from Previous Studies. As mentioned in the Introduction, the functional crosstalk observed between 5-HT_{2A} and $mGlu_2$ when coexpressed in heterologous expression systems, including HEK293 cells, have varied considerably in previous studies. González-Maeso and collaborators have reported inverse cross-signaling between 5-HT_{2A} and $mGlu_2$ in *Xenopus* oocytes with agonists for suppressing one receptor and antagonists for potentiating the signaling of the other receptor[31] and that $mGlu_{2/3}$ antagonists potentiate 5-HT_{2A} signaling in HEK293 cells,[18] although the latter observation was found to be highly dependent on receptor expression levels and cellular colocalization of the receptors. Moreno *et al.*[19] have found that both 5-HT- and $mGlu_{2/3}$-selective agonists induced robust $G\alpha_{q/11}$-mediated responses in 5-HT_{2A}/$mGlu_2$ cells, and in contrast to the lack of effect of the $mGlu_{2/3}$-selective antagonist **LY 341495** on the 5-HT-evoked response, the 5-HT_{2A} antagonist **MDL-100,907** inhibited $mGlu_{2/3}$ agonist-induced signaling in the cells. Delille *et al.*[27] observed no significant effects of coexpression of the two receptors in HEK293 cells on the pharmacological properties of $mGlu_2$ ligands in a cyclic adenosine monophosphate (cAMP) accumulation assay and of 5-HT_{2A} ligands in the Ca^{2+} imaging assay, but they did not investigate the effects of ligands of one receptor on the response evoked through the other. Finally, Murat *et al.* has recently reported that selective $mGlu_{2/3}$ agonist-stimulated phosphorylation of a specific serine residue in $mGlu_2$ requires coexpression of 5-HT_{2A} in HEK293 cells.[32] With this diversity of functional manifestations of the crosstalk between the two receptors in previous studies, the data presented in Figure 3 are bound to fit with some observations and not with others. We clearly do not see the inverse cross-signaling reported by some studies,[18,31] and although we do see a minute Glu-induced response in 5-HT_{2A}/$mGlu_2$ cells (Figure 2B), it is much less pronounced than that reported by Moreno *et al.*[19] Instead, we see a robust potentiation of signaling through each of the receptors being mediated by agonists of the other receptor (Figure 3). On the other hand, the inhibition of $mGlu_2$ signaling mediated by the 5-HT_{2A} antagonist and the ineffectiveness of $mGlu_2$ antagonists to inhibit 5-HT_{2A} signaling observed here are in excellent agreement with previous findings.[19]

The diverse types of functional cross-signaling reported for coexpressed 5-HT_{2A} and $mGlu_2$ in HEK293 cells in these studies are likely to arise from a complex background of differences in relative receptor expression levels, in the specific HEK293 strains used, and/or in the functional read-outs and specific characteristics of the assays used. For the purpose of this study, however, we argue that the robust functional crosstalk between the two receptors in the Ca^{2+}/Fluo-4 assay provides a solid foundation to characterize the functional properties of the bivalent 5-HT_{2A}/$mGlu_2$ ligands at the putative heteromeric complex.

Functional Characterization of Monovalent and Bivalent Ligands at 5-HT_{2A}, $mGlu_2$, and 5-HT_{2A}/$mGlu_2$. Monovalent Ligand SARs. First, we studied the SAR of the two head groups, $mGlu_2$ ago-PAM **31** and competitive 5-HT_{2A} antagonist **9**, on 5-HT_{2A}- and $mGlu_2$/Gqo5-HEK293 cell lines. This served the following two purposes: to identify the most optimal positions in the two head groups to introduce a linker without severely affecting their pharmacological activity and to be able to compare the functional properties of monomeric ligands at the respective receptors with those exhibited by the bivalent ligands.

$mGlu_2$ Head Group. With its EC_{50} values of 37 nM (when tested as an agonist) and 5.2 nM (when tested as PAM) on the $mGlu_2$/Gqo5-cell line, the functional properties of **31** were in agreement with a previous report.[41] Analogous to what has been reported for its close structural analogue **JNJ-40411813**,[63] **31** displayed antagonist activity at 5-HT_{2A}, albeit its inhibitory potency at this receptor was 35- and 250-fold lower than its agonist and PAM potencies at $mGlu_2$, respectively (Table 1). The optimal orientation of the anisole moiety in the $mGlu_2$ head group was investigated using *meta*- and *para*-position analogues (**32**, **33**); both the analogues exhibited slightly reduced agonist potencies but similar PAM potencies compared to **31** at $mGlu_2$. The *meta*-substituted analogue **32** displayed a slightly reduced antagonist potency at 5-HT_{2A} compared to **31**, whereas **33** was completely devoid of activity at 5-HT_{2A} (Table 1). All three anisole analogues displayed comparable agonist potencies and slightly (3−5 fold) weaker PAM potencies on $mGlu_2$/5-HT_{2A}/Gqo5 cells compared to those on $mGlu_2$/Gqo5 cells. The corresponding 2-methoxyethoxy analogues (**34**−**36**) did not display significant decrease in the $mGlu_2$ ago-PAM activity compared to their parent methoxy analogues. **34** and **35** displayed slightly reduced antagonist potencies at 5-HT_{2A}, whereas **36**, analogous to **33**, was completely inactive at 5-HT_{2A} (Table 1). The introduction of a hydroxy substituent in the para position (**37**) resulted in a 16-fold decreased PAM potency at $mGlu_2$ compared to **31**. On $mGlu_2$/5-HT_{2A}/Gqo5 cells, this compound did not display significant agonism, but exhibited a slightly increased PAM potency compared to its PAM potency on $mGlu_2$/Gqo5 cells. None of the seven $mGlu_2$ head group analogues displayed significant agonist or PAM activity on $mGlu_2$/5-HT_{2A} cells but were weak antagonists of 5-HT-induced signaling in the cells (at mid-micromolar concentrations).

5-HT_{2A} Head Group. In concordance with the literature,[64] the carbonyl analogue of the 5-HT_{2A} antagonist **MDL-100,907** (**9**) displayed an half maximal inhibitory concentration (IC_{50}) value of 0.81 nM on 5-HT_{2A} cells in the Ca^{2+}/Fluo-4 assay

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Table 1. Functional Properties of Monovalent and Bivalent Ligands on the $mGlu_2$/Gqo5-, 5-HT_{2A}-, 5-HT_{2A}/$mGlu_2$-, and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 Cells in the Ca^{2+}/Fluo-4 Assay[a]

	$mGlu_2$/Gqo5				5-HT_{2A}	5-HT_{2A}/$mGlu_2$/Gqo5						5-HT_{2A}/$mGlu_2$
	agonist		PAM (Glu EC_{20})		antagonist (5-HT EC_{80})	agonist		PAM (Glu EC_{20})		antagonist (Glu EC_{80})	antagonist (5-HT EC_{80})	antagonist (5-HT EC_{80})
compound	EC_{50} (nM) [pEC_{50} ± SEM]	R_{max} ± SEM	EC_{50} (nM) [pEC_{50} ± SEM]	R_{max} ± SEM	IC_{50} (nM) [pIC_{50} ± SEM]	EC_{50} (nM) [pEC_{50} ± SEM]	R_{max} ± SEM	EC_{50} (nM) [pEC_{50} ± SEM]	R_{max} ± SEM	IC_{50} (nM) [pIC_{50} ± SEM]	IC_{50} (nM) [pIC_{50} ± SEM]	IC_{50} (nM) [pIC_{50} ± SEM]
Monovalent Ligands—5-HT_{2A}												
8	>50,000[b] [<4.3]		>50,000[b] [<4.3]		11 [7.96 ± 0.09]	>50,000[b] [<4.3]		n.d.		23 [7.64 ± 0.06]	22 [7.66 ± 0.06]	9.2 [8.04 ± 0.01]
9	>50,000[b] [<4.3]		>50,000[b] [<4.3]		0.81 [9.09 ± 0.09]	>50,000[b] [<4.3]		n.d.		24 [7.62 ± 0.06]	5.4 [8.26 ± 0.04]	6.2 [8.21 ± 0.07]
10	>50,000[b] [<4.3]		>50,000[b] [<4.3]		9.3 [8.03 ± 0.10]	>50,000[b] [<4.3]		n.d.		29 [7.54 ± 0.06]	23 [7.64 ± 0.08]	16 [7.79 ± 0.09]
Monovalent Ligands—$mGlu_2$												
31	37 [7.45 ± 0.11]	117 ± 8	5.2 [8.28 ± 0.08]	113 ± 8	1300 [5.89 ± 0.10]	210 [6.68 ± 0.09]	120 ± 11	19 [7.72 ± 0.06]	72 ± 9	n.d.	n.d.	~10,000[c] [~5.0]
32	160 [6.80 ± 0.07]	106 ± 6	9.9 [8.00 ± 0.09]	102 ± 10	4100 [5.38 ± 0.11]	170 [6.76 ± 0.08]	81 ± 4	25 [7.60 ± 0.07]	78 ± 7	n.d.	n.d.	~10,000[c] [~5.0]
33	300 [6.52 ± 0.10]	118 ± 8	7.9 [8.10 ± 0.08]	109 ± 8	>50,000[b] [<4.3]	210 [6.68 ± 0.11]	109 ± 10	24 [7.62 ± 0.10]	94 ± 11	n.d.	n.d.	~10,000[c] [~5.0]
34	210 [6.67 ± 0.07]	107 ± 11	9.0 [8.04 ± 0.14]	107 ± 6	3900 [5.40 ± 0.12]	120 [6.92 ± 0.10]	104 ± 9	31 [7.51 ± 0.11]	72 ± 4	n.d.	n.d.	~10,000[c] [~5.0]
35	230 [6.63 ± 0.11]	116 ± 6	12 [7.94 ± 0.11]	104 ± 6	6000 [5.22 ± 0.07]	280 [6.55 ± 0.07]	107 ± 7	32 [7.49 ± 0.06]	87 ± 5	n.d.	n.d.	~10,000[c] [~5.0]
36	290 [6.53 ± 0.09]	100 ± 5	8.7 [8.06 ± 0.12]	97 ± 4	>50,000[b] [<4.3]	260 [6.58 ± 0.06]	112 ± 9	43 [7.37 ± 0.09]	95 ± 5	n.d.	n.d.	~10,000[c] [~5.0]
37	440 [6.35 ± 0.03]	108 ± 9	79 [7.10 ± 0.09]	97 ± 8	720 [6.14 ± 0.12]	~10,000–50,000[c]		19 [7.72 ± 0.04]	85 ± 7	n.d.	n.d.	>50,000[b] [<4.3]
Heterobivalent Ligands												
45	900 [6.04 ± 0.02]	108 ± 10	320 [6.50 ± 0.05]	105 ± 3	1200 [5.92 ± 0.08]	~10,000–50,000[c]		n.d.		5300 [5.28 ± 0.11]	9900 [5.01 ± 0.08]	7900 [5.10 ± 0.11]
46	550 [6.26 ± 0.11]	107 ± 6	330 [6.49 ± 0.09]	114 ± 4	280 [6.54 ± 0.11]	~10,000–50,000[c]		n.d.		990 [6.00 ± 0.06]	800 [6.09 ± 0.08]	1700 [5.81 ± 0.06]
47	1600 [5.81 ± 0.04]	114 ± 6	510 [6.29 ± 0.08]	129 ± 2	450 [6.35 ± 0.07]	~10,000–50,000[c]		n.d.		1900 [5.72 ± 0.07]	2400 [5.62 ± 0.05]	1000 [5.98 ± 0.09]
48	1200 [5.91 ± 0.07]	121 ± 11	530 [6.27 ± 0.11]	131 ± 2	270 [6.56 ± 0.10]	~10,000–50,000[c]		n.d.		890 [6.05 ± 0.09]	420 [6.37 ± 0.07]	510 [6.29 ± 0.11]
49	1600 [5.81 ± 0.10]	119 ± 7	510 [6.29 ± 0.05]	123 ± 5	410 [6.39 ± 0.02]	~10,000–50,000[c]		n.d.		660 [6.18 ± 0.04]	470 [6.32 ± 0.11]	270 [6.57 ± 0.10]
50	1200 [5.92 ± 0.09]	112 ± 6	320 [6.49 ± 0.03]	121 ± 11	300 [6.52 ± 0.06]	~10,000–50,000[c]		n.d.		1200 [5.93 ± 0.10]	140 [6.86 ± 0.09]	300 [6.51 ± 0.08]
51	2500 [5.61 ± 0.09]	127 ± 7	480 [6.32 ± 0.11]	134 ± 7	520 [6.29 ± 0.04]	~10,000–50,000[c]		n.d.		700 [6.16 ± 0.03]	380 [6.42 ± 0.08]	1600 [5.80 ± 0.06]

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Table 1. continued

[a]Functional properties of **8**−**10**, **31**−**37**, and **45**−**51** at $mGlu_2$/Gqo5-HEK293, 5-HT_{2A}-HEK293, 5-HT_{2A}/mGlu2-Gqo5-HEK293, and 5-HT_{2A}/$mGlu_2$-HEK293 cell lines in the Ca^{2+}/Fluo-4 assay. EC_{50} values for agonists and PAMs and IC_{50} values for antagonists are given in nM with pEC_{50} ± SEM and pIC_{50} ± SEM values in brackets, respectively. R_{max} values for agonists and PAMs are given in % of R_{max} of Glu on the same plate. In the antagonist experiments, Glu EC_{80} or 5-HT EC_{80} was used as the agonist concentration, as indicated. In the PAM experiments, the compounds were coapplied with Glu EC_{20}. [b]Displayed no significant activity at concentrations up to 50 μM. [c]Displayed significant activity at micromolar concentrations, but the concentration−response curve was not completed at 50 μM; n.d.: not determined (the compound displayed activity at the receptors in another testing). All data are based on 3−5 independent experiments performed in duplicate.

(Table 1). Replacing the methoxy moiety in the 3-position with a 2-methoxyethoxy functionality (**10**) or a phenol (**8**) group resulted in ∼10-fold reduction in the inhibitory potency at 5-HT_{2A} in both cases (Table 1), which is also in agreement with previously reported values.[40] In concordance with these properties, all three analogues were antagonists of 5-HT EC_{80}-induced signaling in both the 5-HT_{2A}/$mGlu_2$ and 5-HT_{2A}/$mGlu_2$/Gqo5 cells, exhibiting comparable or slightly higher IC_{50} values for these cell lines compared to the 5-HT_{2A} cells. All three analogues were completely inactive on the $mGlu_2$/Gqo5 cell line and, in agreement with this, they did not exhibit activity as agonists or PAMs (co-applied with Glu EC_{20}) on the 5-HT_{2A}/$mGlu_2$/Gqo5 cell line. Interestingly, however, they exhibited potent antagonism (IC_{50} values of 23−29 nM) for Glu EC_{80}-induced signaling in 5-HT_{2A}/$mGlu_2$/Gqo5 cells (Table 1). Thus, analogous to the observed inhibition of Glu-induced signaling in the 5-HT_{2A}/$mGlu_2$/Gqo5 cells mediated by the reference 5-HT_{2A} antagonist mianserin (Figure 3B), binding of **8**−**10** to the orthosteric site in 5-HT_{2A} inhibited the signaling through $mGlu_2$ in these cells.

Based on these SAR data for the $mGlu_2$ head group, we have determined that the orientation of the anisole moiety only marginally reduced their potency as ago-PAMs for $mGlu_2$. Furthermore, the introduction of the 2-methoxyethoxy moiety, in any position, did not alter their potency. However, because of the weak 5-HT_{2A} antagonism of both *ortho*- and *meta*-anisole and the lack of any activity of *para*-anisole at the 5-HT_{2A} receptor, this orientation was selected as the appropriate anchor point for the spacer.

Heterobivalent Ligands. In the outline below, the functional properties exhibited by the bivalent 5-HT_{2A}-$mGlu_2$ ligands on the four cell lines will be compared with those of the two monovalent ligands that best represent the head groups in the bivalent ligands, **36** ($mGlu_2$) and **10** (5-HT_{2A}). All seven bivalent ligands (**45**−**51**) retained both the 5-HT_{2A} antagonist activity and the $mGlu_2$ ago-PAM activity of their two head groups on the 5-HT_{2A}- and $mGlu_2$/Gqo5-cell lines (Figure 4A,B, Table 1). However, they were considerably less potent as such, with the seven ligands exhibiting 30−120-fold higher IC_{50} values as antagonists than **10** at 5-HT_{2A} and 2−8- and 40−60-fold higher EC_{50} values as agonists and PAMs than **36** at $mGlu_2$ (Table 1). This is probably best explained by the entropic penalty caused by the large unbound section of the opposing head group in these ligands. Nevertheless, the qualitative functionalities of the two head groups at both 5-HT_{2A} and $mGlu_2$ were clearly retained in the bivalent ligands.

The functional properties exhibited by the seven bivalent ligands on the 5-HT_{2A}/$mGlu_2$ and 5-HT_{2A}/$mGlu_2$/Gqo5 cell lines differed substantially from their properties on the $mGlu_2$/Gqo5-cell line. None of the ligands displayed significant ago-PAM activities on 5-HT_{2A}/$mGlu_2$ or 5-HT_{2A}/$mGlu_2$/Gqo5 cells. Instead, they antagonized 5-HT-induced signaling in 5-HT_{2A}/$mGlu_2$ cells and both 5-HT- and Glu-induced signaling in 5-HT_{2A}/$mGlu_2$/Gqo5 cells (Figure 4C,D, Table 1). While **45** was the least potent antagonist, displaying IC_{50} values of 5−10 μM in the three antagonist assays (5-HT_{2A}/$mGlu_2$ with 5-HT as the agonist, 5-HT_{2A}/$mGlu_2$/Gqo5 with 5-HT or Glu as agonists), the other six bivalent ligands (**46**−**51**) displayed IC_{50} values in the high-nanomolar to low-micromolar range (140−2400 nM). The rank orders of antagonist potencies exhibited by these six bivalent ligands in the three antagonist assays were fairly similar and essentially comparable to their IC_{50} values at 5-HT_{2A} (Table 1).

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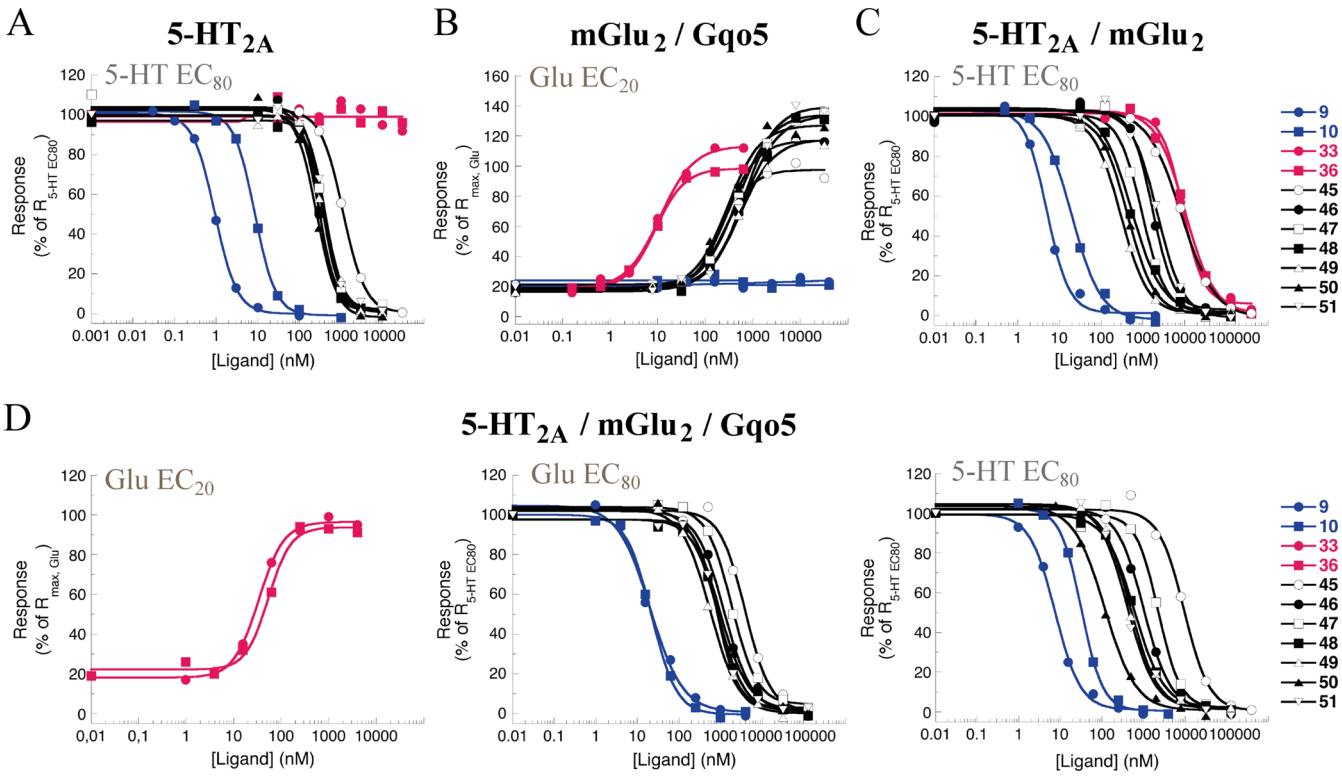

Figure 4. Functional properties displayed by monovalent and bivalent ligands in the 5-HT_{2A}-, $mGlu_2$/Gqo5-, 5-HT_{2A}/$mGlu_2$-, and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cell lines in the Ca^{2+}/Fluo-4 assay. Data for the monovalent 5-HT_{2A} (**9**, **10**) and $mGlu_2$ (**33**, **36**) head groups are given in blue and red, respectively, and data for the seven bivalent ligands (**45**−**51**) are in black. Data in the figure are from representative experiments performed in duplicate (error bars are omitted for reasons of clarity) out of a total of 3−5 independent experiments. Averaged data are given in Table 1. (A) Concentration−inhibition relationships for the ligands tested as antagonists at 5-HT_{2A}-HEK293 cells. The compounds were applied at the cells prior to the application of 5-HT EC_{80}. (B) Concentration−response relationships for the ligands tested as PAMs on $mGlu_2$/Gqo5-HEK293 cells. The compounds were coapplied with Glu EC_{20} on the cells, and responses are given normalized to the R_{max} value of Glu in the same plate. (C) Concentration−inhibition relationships for the ligands tested as antagonists on 5-HT_{2A}/$mGlu_2$-HEK293 cells. The compounds antagonists were applied to the cells prior to the application of 5-HT EC_{80}. (D) Concentration−inhibition relationships for the ligands on 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cells. Left: Concentration−response relationships for the ligands tested as PAMs (coapplied with Glu EC_{20} on the cells), and responses are given normalized to the R_{max} value of Glu on the same plate. Middle-Right: Concentration−inhibition relationships for the ligands tested as antagonists using Glu EC_{80} (middle) or 5-HT EC_{80} (right) as the agonist. The compounds were applied to the cells prior to the application of the agonist.

Binding Properties of Selected Monovalent and Bivalent Ligands at 5-HT_{2A} and 5-HT_{2A}/$mGlu_2$. To estimate the overall 5-HT_{2A} receptor expression levels in the 5-HT_{2A}-, 5-HT_{2A}/$mGlu_2$ and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cells and to determine the binding affinities of selected monovalent and bivalent ligands, we next performed saturation and competition binding experiments on membranes from the three cell lines using the 5-HT_{2A} antagonist radioligand [^{3}H]ketanserin. Because of the lack of the commercially available $mGlu_2$ radioligand, we were unable to perform analogous binding experiments focused on this receptor.

In the saturation binding experiments, [^{3}H]ketanserin displayed comparable K_D values (1.2−1.4 nM) for the 5-HT_{2A}, 5-HT_{2A}/$mGlu_2$, and 5-HT_{2A}/$mGlu_2$/Gqo5 cell membranes, and these values are in good agreement with previously reported K_D values for [^{3}H]ketanserin at 5-HT_{2A} (Table 2 and Figure 5A).[65,66] The B_{max} values exhibited by [^{3}H]ketanserin on the three cell lines were within a 2.5-fold range (1.37, 3.25, and 2.52 pmol/mg protein for 5-HT_{2A}, 5-HT_{2A}/$mGlu_2$, and 5-HT_{2A}/$mGlu_2$/Gqo5, respectively). Thus, while it is important to stress that the determined 5-HT_{2A} receptor levels represent both cell surface-expressed and intracellular receptor pools, it is reasonable to assume that 5-HT_{2A} receptor levels on the cell surface in the three cell lines are largely comparable.

The rank order of the binding affinities displayed by the monovalent 5-HT_{2A} head groups **9** and **10** and the seven bivalent ligands **45**−**51** at 5-HT_{2A}-, 5-HT_{2A}/$mGlu_2$-, and 5-HT_{2A}/$mGlu_2$/Gqo5-cell membranes in the [^{3}H]ketanserin competition binding assay was very much comparable, and the absolute K_i values displayed that each of the ligands across the three cell lines were also very similar (Table 2 and Figure 5B). Moreover, the binding properties of both monovalent and bivalent ligands aligned very well with their antagonist properties on the 5-HT_{2A}-containing cell lines. **9** and **10** exhibited substantially higher affinities (10−400-fold) for all three cell lines than **45**−**51**, and while **45** was a substantially weaker binder than the six other bivalent ligands, **46**−**51** exhibited essentially similar K_i values on the 5-HT_{2A}, 5-HT_{2A}/$mGlu_2$, and 5-HT_{2A}/$mGlu_2$/Gqo5 cells. Notably, none of the seven bivalent ligands exhibited significantly different K_i values on either of the two 5-HT_{2A}/$mGlu_2$-expressing cell lines compared to those on 5-HT_{2A} cells (Table 2 and Figure 5B). Thus, all in all, the binding affinities exhibited by the analogues corroborated their functional properties as antagonists on the three cell lines (Table 1 and Figure 4).

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Table 2. Binding Properties of [^{3}H]Ketanserin and Monovalent and Bivalent Ligands on 5-HT$_{2A}$-, 5-HT$_{2A}$/mGlu$_2$-, and 5-HT$_{2A}$/mGlu$_2$/Gqo5-HEK293 Cell Membranes in the [^{3}H]Ketanserin Binding Assay[a]

	5-HT$_{2A}$	5-HT$_{2A}$/mGlu$_2$	5-HT$_{2A}$/mGlu$_2$/Gqo5
	[^{3}H]Ketanserin		
K_D (nM)	1.39 ± 0.09	1.36 ± 0.06	1.20 ± 0.09
B_{max} (pmol/mg protein)	1.37 ± 0.08	3.25 ± 0.12	2.52 ± 0.14
	K_i (nM) [pK_i ± SEM]		
	5-HT$_{2A}$	5-HT$_{2A}$/mGlu$_2$	5-HT$_{2A}$/mGlu$_2$/Gqo5
9	0.52 [9.28 ± 0.05]	3.0 [8.52 ± 0.08]	8.7 [8.06 ± 0.09]
10	3.0 [8.53 ± 0.07]	6.5 [8.19 ± 0.07]	13 [7.88 ± 0.05]
45 (PEG$_n$ = 3)	360 [6.44 ± 0.08]	840 [6.08 ± 0.05]	1040 [5.98 ± 0.04]
46 (PEG$_n$ = 4)	33 [7.48 ± 0.06]	100 [6.99 ± 0.08]	110 [6.94 ± 0.07]
47 (PEG$_n$ = 5)	130 [6.89 ± 0.05]	180 [6.75 ± 0.07]	320 [6.50 ± 0.03]
48 (PEG$_n$ = 6)	100 [7.00 ± 0.06]	120 [6.93 ± 0.08]	190 [6.71 ± 0.03]
49 (PEG$_n$ = 8)	110 [6.92 ± 0.08]	120 [6.94 ± 0.10]	160 [6.88 ± 0.08]
50 (PEG$_n$ = 10)	79 [7.10 ± 0.04]	70 [7.14 ± 0.08]	120 [6.93 ± 0.04]
51 (PEG$_n$ = 12)	200 [6.70 ± 0.04]	170 [6.78 ± 0.04]	170 [6.76 ± 0.01]

[a]Binding properties of [^{3}H]ketanserin, **9**–**10**, and **45**–**51** at 5-HT$_{2A}$-HEK293, 5-HT$_{2A}$/mGlu$_2$-HEK293, and 5-HT$_{2A}$/mGlu$_2$/Gqo5-HEK293 cell membranes in the [^{3}H]ketanserin binding assay. K_D ± SEM and B_{max} ± SEM values for [^{3}H]ketanserin are given in nM and pmol/mg protein, respectively. K_i values for the monovalent and bivalent ligands are given in nM (with pK_i ± SEM values in brackets). The saturation binding data are based on four independent experiments performed in duplicate, and the competition binding data are based on three independent experiments performed in duplicate.

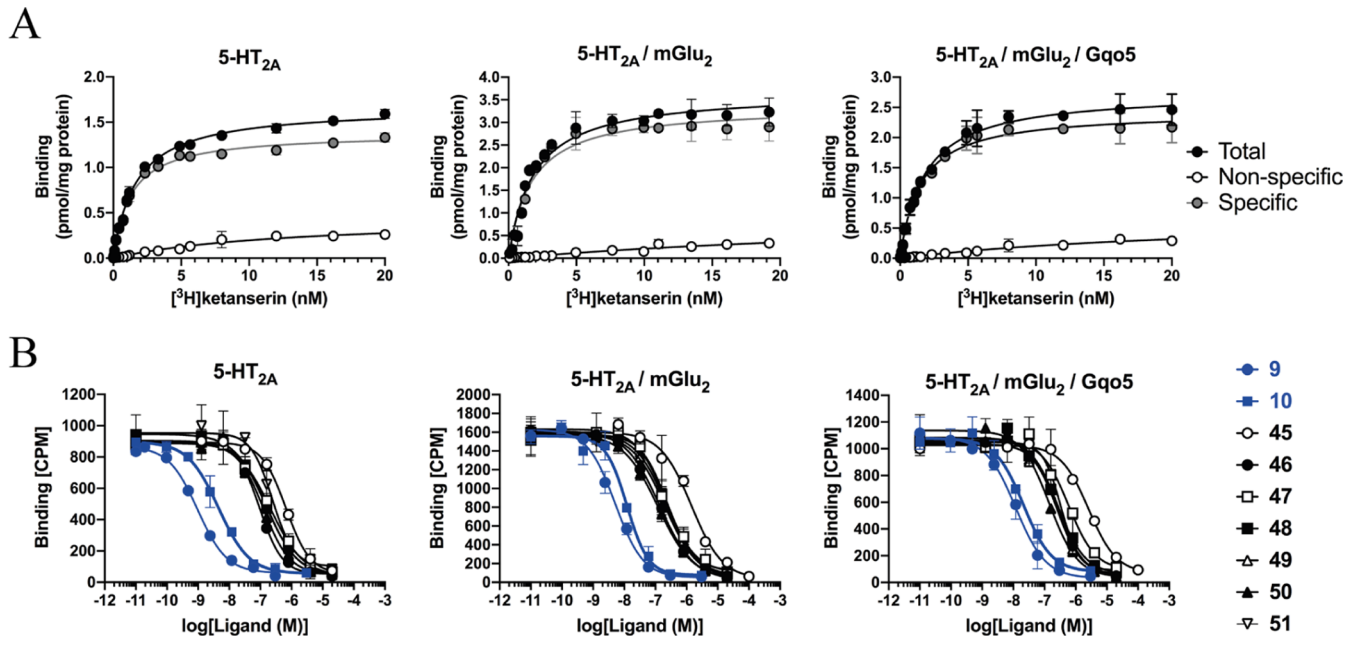

Figure 5. Binding properties displayed by [^{3}H]ketanserin and by monovalent and bivalent ligands on 5-HT$_{2A}$-, 5-HT$_{2A}$/mGlu$_2$- and 5-HT$_{2A}$/mGlu$_2$/Gqo5-HEK293 cell membranes in the [^{3}H]ketanserin binding assay. Data in the figure are from representative experiments performed in duplicate out of a total of 3–4 independent experiments. Averaged data are given in Table 2. (A) Saturation binding of [^{3}H]ketanserin at 5-HT$_{2A}$, 5-HT$_{2A}$/mGlu$_2$, and 5-HT$_{2A}$/mGlu$_2$/Gqo5. Data are from an individual experiment and given as mean ± SD values based on duplicate determinations. (B) Concentration–inhibition relationships exhibited by the monovalent 5-HT$_{2A}$ head groups (**9**, **10**, in blue) and the seven bivalent ligands (**45**–**51**, in black) on 5-HT$_{2A}$-, 5-HT$_{2A}$/mGlu$_2$-, and 5-HT$_{2A}$/mGlu$_2$/Gqo5-HEK293 cell membranes in the [^{3}H]ketanserin competition binding assay using a radioligand concentration of 1.52 nM on the day. Data are from an individual experiment and given as mean ± SD values based on duplicate determinations.

Overall Discussion of the SAR. The loss of activity displayed by the monovalent analogues **8**, **10**, **32**–**37** and the bivalent ligands (**45**–**51**) at the parent 5-HT$_{2A}$ and mGlu$_2$ receptors compared to the respective head group leads (**9** and **31**) was not surprising (Tables 1 and 2). The addition of the other head group in the bivalent ligands further reduced the 5-HT$_{2A}$ antagonist activity and the mGlu$_2$ ago-PAM activity at the two parent receptors. These observations are in line with previous works in which bivalent ligands have been developed from head groups already optimized for activity at their respective receptors, where introduction of a linker in the head group molecule or the fusion of two head groups *via* a linker most often have resulted in reduced receptor activity.[67,68]

The functional properties displayed by the monovalent analogues at the two cell lines expressing the putative 5-HT$_{2A}$/mGlu$_2$ heteromer were also very much in line with those at

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their respective parent receptors, with **8**−**10** being slightly less potent as antagonists of the 5-HT-induced responses in 5-HT_{2A}/$mGlu_2$ and 5-HT_{2A}/$mGlu_2$/Gqo5 cells and with **31**−**37** being slightly less potent as direct agonists and PAMs of the Glu-induced responses in the 5-HT_{2A}/$mGlu_2$/Gqo5 cells. As for the inhibition of the Glu-induced responses in 5-HT_{2A}/$mGlu_2$/Gqo5 cells mediated by the monovalent 5-HT_{2A} analogues **8**−**10**, on one hand, it is a strong reflection of the functional crosstalk between the two receptors in these cells, regardless of the basis, but on the other, it also presents a challenge for interpretation of the functional properties exhibited by the bivalent ligands.

The $mGlu_2$ PAM activity observed for the monovalent analogues **31**−**37** on 5-HT_{2A}/$mGlu_2$/Gqo5 cells is clearly eliminated in the bivalent ligands which all displayed antagonism for both 5-HT- and Glu-induced responses in these cells. However, in light of the antagonism of the Glu-induced response in 5-HT_{2A}/$mGlu_2$/Gqo5 cells mediated by monovalent 5-HT_{2A} analogues **8**−**10**, this cannot be taken as an unequivocal proof of the bivalent ligands acting through both the orthosteric site in 5-HT_{2A} and the allosteric site in $mGlu_2$. Considering the potencies displayed by the bivalent ligands compared to **8**−**10** as antagonists at 5-HT_{2A}, it is not necessarily a surprise that a similar pattern is observed in the 5-HT_{2A}/$mGlu_2$ and 5-HT_{2A}/$mGlu_2$/Gqo5 cells. On the other hand, fusion of the $mGlu_2$ head group to **8** in the bivalent ligands clearly does not increase the antagonist potencies at the putative 5-HT_{2A}/$mGlu_2$ complex nor does it increase the binding affinity to the 5-HT_{2A} receptor in the cells (Tables 1 and 2). It is clear from the functional properties of the bivalent ligand in the $mGlu_2$/Gqo5 cells that the $mGlu_2$ head group in this ligand is able to bind the allosteric binding site in $mGlu_2$ and that it actually does this in an ago-PAM binding mode. Regardless of whether the functional crosstalk observed between 5-HT_{2A} and $mGlu_2$ (Figure 3) arises from the formation of this putative heteromer or from another mechanism, concomitant binding of the bivalent ligand to the two binding sites in such as heteromer would be expected to result in substantially higher antagonist potency of it compared to the monovalent 5-HT_{2A} head group. The most obvious explanation for the lack of synergistic effect of the bivalent ligand compared to the monovalent ligand is that the two head groups in the ligand are unable to target the 5-HT_{2A} and $mGlu_2$ binding sites in the heteromer simultaneously. There could be several molecular explanations for this, including steric clashes between $mGlu_2$ and the $mGlu_2$ head group or the linker when the bivalent ligand is anchored through binding to the 5-HT_{2A} binding site. Alternatively, concomitant binding of the 5-HT_{2A} head group in the bivalent ligand to 5-HT_{2A} in the putative 5-HT_{2A}/$mGlu_2$ heteromer could be envisioned to limit the conformational flexibility of the $mGlu_2$ head group, thus disabling it from adopting the optimal binding orientation. Another possible explanation could be that binding of the bivalent ligands disrupts the heterodimer complex formation by shifting the equilibrium to monomeric complexes.

CONCLUSIONS

In summary, in the present work, we successfully synthesized both the 5-HT_{2A} and $mGlu_2$ head groups for bivalent ligands and confirmed the most suitable attachment points in them for the spacer. Moreover, four HEK293 cell lines expressing 5-HT_{2A}, $mGlu_2$, and the two receptors together were developed, thus, enabling functional profiling of ligands at the two parent receptors and the putative 5-HT_{2A}/$mGlu_2$ heteromer in assays measuring $G\alpha_{q/11}$-mediated signaling, and basic functional characterization of these cell lines revealed pronounced functional crosstalk between 5-HT_{2A} and $mGlu_2$ in 5-HT_{2A}/$mGlu_2$- and 5-HT_{2A}/$mGlu_2$/Gqo5-cell lines in a Ca^{2+}/Fluo-4 assay. Analogous to previous studies developing bivalent ligands for other GPCRs, the introduction of a spacer in the head groups as well as the fusion of the two head groups in bivalent ligands resulted in substantially reduced potencies compared to the head groups in the parent $mGlu_2$ and 5-HT_{2A} receptors. Importantly, however, the bivalent ligands exhibited the same 5-HT_{2A} antagonist and $mGlu_2$ ago-PAM functionalities as the respective head groups. In contrast, the bivalent ligands inhibited both the 5-HT- and the Glu-induced responses through the putative 5-HT_{2A}/$mGlu_2$ heteromer, suggesting a predominant role of the 5-HT_{2A} head group in the ligands. None of the bivalent ligands exhibited increased potencies as antagonists compared to the 5-HT_{2A} head group, and no definitive correlation between the functional potency and spacer length of the ligands could be observed. Thus, all in all, the observed functional crosstalk between 5-HT_{2A} and $mGlu_2$ in the 5-HT_{2A}/$mGlu_2$-cell lines in this study supports previous reports, even though the actual characteristics of it represents yet another phenotype of this crosstalk. As for the seven bivalent ligands designed for the putative 5-HT_{2A}/$mGlu_2$ complex, it remains a question whether they truly act by targeting binding sites in both receptors in this heteromer, or whether they have preference for the orthosteric 5-HT_{2A} site and thus mediates their inhibition of the 5-HT- and Glu-evoked responses in the 5-HT_{2A}/$mGlu_2$-expressing cells exclusively through binding to this receptor.

EXPERIMENTAL SECTION

Chemistry. All reactions involving dry solvents or sensitive agents were performed under a nitrogen atmosphere, and glassware was dried prior to use. Commercially available chemicals were used without further purification. Solvents were dried prior to use with an SG water solvent purification system or by standard procedures, and reactions were monitored by analytical TLC (Merck silica gel 60 F_{254} aluminum sheets). Dry-column vacuum chromatography (DCVC) was carried out using Merck silica gel 60A (15−40 μm),[69] and flash chromatography was carried out using Merck silica gel 60A (35−70 μm). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Bruker Avance III or 600 MHz Bruker Avance III HD and ^{13}C NMR spectra on a 101 MHz Bruker Avance III or 151 MHz Bruker Avance III HD. Analytical HPLC was performed using an UltiMate HPLC system consisting of an LPG-3400A pump (1 mL/min), a WPS-3000SL autosampler, and a 3000 diode array detector installed with a Gemini-NX C18 (250 × 4.60 mm, 3 μm) column. Solvent A: H_2O + 0.1% TFA; solvent B: MeCN−H_2O 9:1 + 0.1% TFA. For HPLC control, data collection, and data handling, Chromeleon software v. 6.80 was used. Preparative HPLC was carried out on an UltiMate Thermo Scientific HPLC system with an LPG-3200BX pump, a Rheodyne 9721i injector, a 10 mL loop, an MWD-3000SD detector (200, 210, 225, and 254 nm), and a Gemini-NX C18 (250 × 21.2 mm, 5 μm) column for preparative purifications or a Gemini-NX C18 (250 × 10.00 mm, 5 μm) column for semipreparative purifications. Solvent A: H_2O + 0.1% TFA; solvent B: MeCN−H_2O 9:1 + 0.1% TFA. For HPLC control, data collection, and data handling, Chromeleon software v. 6.80 was used. Chiral preparative HPLC was performed using the same instrumentation mentioned above. Ultraperformance liquid chromatography−tandem mass spectrometry (UPLC−MS) was performed using an Acquity UPLC H-Class Waters series solvent delivery system equipped with an autoinjector coupled to Acquity QDa and TUV detectors and

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installed with an Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm) column. Solvent A: 5% aq. MeCN + 0.1% HCO_2H; solvent B: MeCN + 0.1% HCO_2H. Usually, gradients of A/B from 1:0 to 1:1 (5 min) or A/B from 1:0 to 0:50 (5 min) were performed depending on the polarity of the compounds. For data collection and data handling, MassLynx software was used. Optical rotations were determined in a thermostated cuvette on an Anton Paar MCP 300 modular circular polarimeter. Compounds were dried under high vacuum or freeze-dried using a ScanVac CoolSafe freeze drier. The purity of compounds submitted for pharmacological characterization was determined by ^{1}H NMR and HPLC to be >95%, unless otherwise specified.

General Experimental Procedures. *General Procedure I.* To a solution of **37** (1.0 equiv) in DMF is added NaH (60%) (2.0 equiv) portion wise. The reaction is cooled to 0 °C and stirred for 10 min, followed by the addition of tosylate **38**–**44** (1.0 equiv). The reaction is allowed to warm to ambient temperature and stirred for an additional 15 h. The reaction is quenched with MeOH, diluted with TFA (0.1% v/v in H_2O), filtered, and subjected to preparative HPLC purification. The fractions containing the desired product were lyophilized.

General Procedure II. Phenol (1.0 equiv) was dissolved in dry DMF to which were added K_2CO_3 (2.0 equiv) and KI (2.0 equiv), followed by 1-bromo-2-methoxyethane (1.2 equiv). The reaction was stirred for 15 h at 60 °C. The crude was extracted with EtOAc (3×). The combined organic fractions were washed with H_2O (2×) and brine and dried over $MgSO_4$. The crude was purified by DCVC (5 → 25% EtOAc in heptane).

General Procedure III. *N*-Boc-arylpiperidine (1.0 equiv) was dissolved in dry DCM. An equal volume of TFA was added dropwise and the reaction was stirred for 2 h at room temperature (rt). The reaction was cooled to 0 °C and quenched with dropwise addition of NH_4OH (25% in H_2O) until pH 8–9. The crude was extracted with DCM (3×). The combined organic fractions were washed with brine and dried over $MgSO_4$. No additional purification was needed.

General Procedure IV. $Pd(OAc)_2$ (0.05 equiv), XantPhos (0.1 equiv), **16** (1.0 equiv), arylpiperidine (1.0 equiv), and Cs_2CO_3 (2.4 equiv) were placed in a closed vial and dry and degassed dioxane was added. The reaction was heated to reflux and stirred for 72 h, under an argon atmosphere. After cooling to rt, the reaction mixture was diluted with EtOAc and filtered over Celite. The Celite was washed with EtOAc (3×). The filtrate was concentrated, and the crude was purified by preparative HPLC. The isolated TFA salt was dissolved in HCl (1 M in H_2O, 3×) and concentrated. The concentrate was lyophilized.

General Procedure V. A solution of **8** (1.0 equiv) in dry tetrahydrofuran (THF) was cooled to 0 °C to which is added NaH (2.0 equiv). The reaction mixture was stirred for 10 min. Ditosyl-$(PEG)_n$ (5.0 equiv) was added to dry THF. The reaction mixture was allowed to warm to rt and stirred for 15 h. The reaction mixture was directly applied to a preparative TLC plate (100% EtOAc with 0.1% Et_3N).

Experimental Procedures and Characterization. *tert-Butyl(2-methoxyphenoxy)diphenylsilane (**2**).* Guaiacol (10.0 g, 80.55 mmol, 1.0 equiv) was dissolved in 250 mL of DCM and imidazole (13.7 g, 201.39 mmol, 2.5 equiv) was added, followed by *tert*-butyldiphenylchlorosilane (22.7 mL, 88.61 mmol, 1.1 equiv). The reaction mixture was stirred for 20 h at rt. H_2O (200 mL) was added and extracted with DCM (3 × 100 mL). The combined organic fractions were washed with H_2O (2 × 100 mL) and brine (250 mL) and dried over $MgSO_4$. The crude was purified by DCVC (heptane/EtOAc 9:1), which afforded the title compound (28.0 g, 77.23 mmol, 96%). MS (ESI) *m*/*z*: 363.6 $[M + H]^+$; R_f = 0.72 (heptane/EtOAc 4:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75–7.70 (m, 4H), 7.43–7.32 (m, 6H), 6.83 (ddd, *J* = 1.7, 7.2, 8.0 Hz, 1H), 6.78–6.71 (m, 2H), 6.65 (ddd, *J* = 1.7, 7.2, 7.9 Hz, 1H), 3.56 (s, 3H), 1.12 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 150.7, 145.3, 135.5, 133.8, 129.7, 127.6, 121.7, 120.8, 120.5, 112.6, 55.5, 26.8, 19.9.

*tert-Butyl 4-(Methoxy(methyl)carbamoyl)piperidine-1-carboxylate (**4**).* 1-(*tert*-Butoxycarbonyl)piperidine-4-carboxylic acid (5.0 g, 21.81 mmol, 1.0 equiv) was dissolved in DMF. *N*,*O*-Dimethylhydroxylamine hydrochloride (3.2 g, 32.71 mmol, 1.5 equiv) was added, followed by Et_3N (6.1 mL, 43.62 mmol, 2.0 equiv). The reaction was stirred for 10 min, and subsequently hydroxybenzotriazole (HOBt; 3.5 g, 26.17 mmol, 1.2 equiv) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI; 4.63 mL, 26.17 mmol, 1.2 equiv) were added. The reaction mixture was stirred for an additional 18 h at rt. HCl (1 M in H_2O, 200 mL) was added and the crude was extracted with EtOAc (3 × 200 mL). The combined organic fractions were washed with sat. $NaHCO_3$ (100 mL) and brine (150 mL) and dried over $MgSO_4$. The crude was purified by DCVC (heptane/EtOAc 1:1), which afforded the title compound (5.6 g, 20.50 mmol, 94%). MS (ESI) *m*/*z*: 273.1 $[M + H]^+$; R_f = 0.21 (heptane/EtOAc 1:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.27–4.01 (m, 2H), 3.71 (s, 3H), 3.18 (s, 3H), 2.86–2.66 (m, 3H), 1.76–1.62 (m, 4H), 1.46 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 175.9, 154.8, 79.6, 61.7, 43.2, 38.3, 32.4, 28.6, 28.2.

*tert-Butyl 4-(3-((tert-Butyldiphenylsilyl)oxy)-2-methoxybenzoyl)-piperidine-1-carboxylate (**5**).* *n*-BuLi (1.71 M in hexanes) (15.78 mL, 26.98 mmol, 1.05 equiv) was added to a stirred solution of **2** (9.8 g, 26.98 mmol, 1.05 equiv) in THF (75 mL) at 0 °C. The reaction mixture was allowed to warm to rt and stirred for 2 h. After cooling to −42 °C, **4** (7.0 g, 25.70 mmol, 1.00 equiv) in THF (5 mL) was added dropwise to the stirred reaction mixture. The reaction mixture was allowed to warm slowly to rt and stirred for 2 h. The reaction was quenched with sat. NH_4Cl (5 mL). The crude was extracted with EtOAc (3 × 20 mL). The combined organic fractions were washed with brine (250 mL) and dried over $MgSO_4$. The crude was purified by DCVC (0 → 4% EtOAc in heptane), which afforded the title compound (7.7 g, 13.42 mmol, 59%). MS (ESI) *m*/*z*: 574.3 $[M + H]^+$; R_f = 0.40 (heptane/EtOAc 3:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.74–7.71 (m, 4H), 7.46–7.42 (m, 2H), 7.39–7.35 (m, 4H), 6.86 (dd, *J* = 1.6, 7.6 Hz, 1H), 6.87 (t, *J* = 7.9 Hz, 1H), 6.65 (dd, *J* = 1.6, 8.1 Hz, 1H), 4.16–3.98 (m, 2H), 3.92 (s, 3H), 3.19 (tt, *J* = 3.7, 11.0 Hz, 1H), 2.91–2.79 (m, 2H), 1.80 (dd, *J* = 2.4, 12.9 Hz, 2H), 1.57 (dd, *J* = 4.9, 8.8 Hz, 2H), 1.46 (s, 9H), 1.14 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 206.2, 154.9, 149.1, 148.7, 135.6, 134.8, 132.4, 130.2, 128.0, 124.1, 123.7, 121.1, 79.6, 62.2, 48.0, 28.6, 28.0, 26.7, 19.7.

*(3-((tert-Butyldiphenylsilyl)oxy)-2-methoxyphenyl)(piperidin-4-yl)methanone (**7**).* **5** (7.8 g, 13.58 mmol, 1.0 equiv) was dissolved in DCM (100 mL) and cooled to 0 °C. TFA (50 mL) was added slowly. After the addition, the reaction mixture was allowed to warm to rt and stirred for 1.5 h. The reaction mixture was cooled to 0 °C and quenched with NH_4OH (25% in H_2O) until pH 8–9. The crude was extracted with DCM (3 × 100 mL). The combined organic fractions were washed with brine (200 mL) and dried over $MgSO_4$, which afforded the title compound (6.18 g, 13.05 mmol, 96%). MS (ESI) *m*/*z*: 474.5 $[M + H]^+$; R_f = 0.29 (DCM/MeOH 5:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.74–7.69 (m, 4H), 7.47–7.41 (m, 2H), 7.37 (t, *J* = 7.4 Hz, 4H), 6.87 (dd, *J* = 1.6, 7.7 Hz, 1H), 6.70 (t, *J* = 7.9 Hz, 1H), 6.64 (dd, *J* = 1.6, 8.1 Hz, 1H), 3.92 (s, 3H), 3.72 (br s, 1H), 3.26–3.21 (m, 1H), 3.19 (dt, *J* = 3.9, 12.7 Hz, 2H), 2.78 (ddd, *J* = 3.0, 11.0, 12.4 Hz, 2H), 1.94–1.88 (m, 2H), 1.67 (dt, *J* = 3.7, 10.7 Hz, 2H), 1.13 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 205.9, 148.9, 148.6, 135.5, 134.5, 132.3, 130.1, 127.8, 124.0, 123.6, 121.0, 62.0, 47.2, 45.3, 27.9, 26.6, 19.5.

*(1-(4-Fluorophenethyl)piperidin-4-yl)(3-hydroxy-2-methoxyphenyl)methanone Hydrochloride (**8**).* 7 (3.80 g, 8.02 mmol, 1.0 equiv) was dissolved in DMF (50 mL) to which was added 1-(2-bromoethyl)-4-fluorobenzene (1.24 mL, 8.82 mmol, 1.1 equiv), followed by KI (2.66 g, 16.04 mmol, 2.0 equiv) and K_2CO_3 (2.22 g, 16.04 mmol, 2.0 equiv). The reaction mixture was heated to 85 °C and stirred for 17 h. The reaction was quenched with sat. $NaHCO_3$ (50 mL) and the crude was extracted with EtOAc (3 × 100 mL). The combined organic fractions were washed with H_2O (2 × 250 mL) and brine (300 mL) and dried over $MgSO_4$. The crude was purified by DCVC (50 → 100% EtOAc in heptane). The isolated free base was dissolved in HCl (2 M in dioxane, 40 mL) and concentrated, which afforded the title compound (1.52 g, 3.86 mmol, 48%). MS (ESI) *m*/

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z: 358.0 $[M + H]^+$; ^1H NMR (600 MHz, D_2O): δ 7.32 (dd, J = 5.5, 8.5 Hz, 2H), 7.19−7.07 (m, 5H), 3.81 (s, 3H), 3.72 (d, J = 12.7 Hz, 2H), 3.55 (tt, J = 3.7, 12.2 Hz, 1H), 3.42−3.34 (m, 2H), 3.17−3.10 (m, 2H), 3.09−3.02 (m, 2H), 2.21 (d, J = 14.6 Hz, 2H), 1.92−1.81 (m, 2H); ^{13}C NMR (151 MHz, MeOD): δ 207.4, 163.7, 162.0, 150.5, 146.7, 133.1, 133.0, 131.4, 131.3, 126.3, 122.0, 121.3, 116.7, 116.5, 62.9, 58.8, 53.1, 45.4, 30.0, 26.5.

*(2,3-Dimethoxyphenyl)(1-(4-fluorophenethyl)piperidin-4-yl)-methanone Hydrochloride (**9**).* **8** (30 mg, 0.08 mmol, 1.0 equiv) was dissolved in DMF (2 mL). K_2CO_3 (26 mg, 0.19 mmol, 2.5 equiv) was added, followed by methyl 4-methylbenzenesulfonate (16 mg, 0.08 mmol, 1.1 equiv). The reaction was heated to 65 °C and stirred for 15 h. The reaction was quenched with sat. $NaHCO_3$ (2 mL) and the crude was extracted with EtOAc (3 × 5 mL). The combined organic fractions were washed with H_2O (2 × 5 mL) and brine (10 mL) and dried over $MgSO_4$. The crude was purified by preparative HPLC. The isolated TFA salt was dissolved in HCl (1 M in H_2O, 3 × 15 mL) and concentrated. The concentrate was lyophilized, which afforded the title compound (15 mg, 0.04 mmol, 47%). MS (ESI) m/z: 372.1 $[M + H]^+$; ^1H NMR (600 MHz, MeOD): δ 7.35−7.29 (m, 2H), 7.23 (dd, J = 1.6, 8.2 Hz, 1H), 7.15 (t, J = 8.0 Hz, 1H), 7.08 (dd, J = 7.4, 10.0 Hz, 2H), 7.05 (dd, J = 1.6, 7.8 Hz, 1H), 3.90 (s, 6H), 3.77−3.70 (m, 2H), 3.54−3.45 (m, 1H), 3.37−3.32 (m, 2H), 3.19−3.09 (m, 2H), 3.09−3.01 (m, 2H), 2.24−2.17 (m, 2H), 1.93−1.81 (m, 2H); ^{13}C NMR (151 MHz, MeOD): δ 204.9, 164.3, 162.7, 154.4, 148.8, 133.9, 133.4, 131.7, 131.6, 125.6, 121.3, 117.4, 116.7, 116.6, 62.1, 59.1, 56.5, 53.5, 46.4, 30.5, 27.0.

*(1-(4-Fluorophenethyl)piperidin-4-yl)(2-methoxy-3-(2-methoxyethoxy)phenyl)methanone Hydrochloride (**10**).* **8** (50 mg, 0.13 mmol, 1.0 equiv) was dissolved in DMF (2 mL), NaH (60% in mineral oil) (5 mg, 0.13 mmol, 1.0 equiv) was added, and the reaction mixture was stirred for 10 min. 1-Bromo-2-methoxyethane (24 μL, 0.25 mmol, 2.0 equiv) was added and the reaction mixture was stirred for 6 h at rt. The reaction mixture was quenched with sat. $NaHCO_3$ (2 mL) and the crude was extracted with EtOAc (3 × 5 mL). The combined organic fractions were concentrated and the crude was purified by preparative HPLC. The isolated TFA salt was dissolved in HCl (1 M in H_2O, 3 × 15 mL) and concentrated. The concentrate was lyophilized, which afforded the title compound (32 mg, 0.07 mmol, 56%). MS (ESI) m/z: 416.1 $[M + H]^+$; ^1H NMR (600 MHz, MeOD): δ 7.33 (dd, J = 5.2, 8.4 Hz, 2H), 7.23 (dd, J = 1.6, 8.2 Hz, 1H), 7.13 (t, J = 7.9 Hz, 1H), 7.07 (q, J = 8.6 Hz, 3H), 4.24−4.17 (m, 2H), 3.95 (s, 3H), 3.83−3.78 (m, 2H), 3.74 (d, J = 12.4 Hz, 2H), 3.49 (td, J = 6.0, 11.8 Hz, 1H), 3.44 (s, 3H), 3.38−3.32 (m, 2H), 3.20−3.12 (m, 2H), 3.12−3.02 (m, 2H), 2.21 (d, J = 14.7 Hz, 2H), 1.97−1.83 (m, 2H); ^{13}C NMR (151 MHz, MeOD): δ 204.9, 164.3, 162.7, 153.5, 149.1, 134.0, 133.5, 133.4, 131.7, 131.6, 125.4, 121.7, 118.8, 116.7, 116.6, 72.2, 69.6, 62.2, 59.2, 59.1, 53.5, 46.5, 30.5, 27.0.

*3-Chloro-2-hydrazinyl-4-iodopyridine (**12**).* 2,3-Dichloro-4-iodopyridine (20.0 g, 73.02 mmol, 1.0 equiv) was dissolved in dioxane (500 mL) to which was added hydrazine·hydrate (21.25 mL, 438.13 mmol, 5.0 equiv). The reaction was heated to 70 °C and stirred for 16 h. After cooling to rt, NH_4OH (25% in H_2O, 250 mL) was added and the mixture was concentrated *in vacuo*. The crude was dissolved in warm EtOH and the residue was collected by filtration, which afforded the title compound (12.6 g, 46.74 mmol, 64%). MS (ESI) m/z: 269.8 $[M + H]^+$; R_f = 0.46 (heptane/EtOAc 1:3); ^1H NMR (600 MHz, $CDCl_3$): δ 7.72 (d, J = 5.2 Hz, 1H), 7.14 (d, J = 5.2 Hz, 1H), 6.34 (br s, 1H), 3.98 (br s, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 155.8, 145.6, 124.7, 119.4, 109.4, 67.2.

*2-Cyclopropylacetyl Chloride (**14**).* 2-Cyclopropylacetic acid (6.51 mL, 69.92 mmol, 1.0 equiv) was dissolved in DCM (30 mL). Oxalyl chloride (11.83 mL, 139.83 mmol, 2.0 equiv) was added dropwise to the solution and the reaction was stirred at rt for 4 h. The solvent and reagent were removed by Kugelrohr distillation (20 mbar, 25 °C), and the title compound was isolated by Kugelrohr distillation (20 mbar, 65 °C) (8.12 g, 68.49 mmol, 98%). ^1H NMR (600 MHz, $CDCl_3$): δ 2.78 (d, J = 7.0 Hz, 2H), 1.16−1.09 (m, 1H), 0.67−0.63 (m, 2H), 0.25 (dt, J = 4.9, 6.1 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 173.5, 52.0, 7.2, 4.7.

*N′-(3-Chloro-4-iodopyridin-2-yl)-2-cyclopropylacetohydrazide (**15**).* **12** (5.34 g, 19.82 mmol, 1.0 equiv) was dissolved in DMF (60 mL) and cooled to 0 °C. Et_3N (6.19 mL, 49.54 mmol, 2.5 equiv) was added, followed by **14** (2.58 g, 21.80 mmol, 1.1 equiv). The reaction was allowed to warm to rt and was stirred for 16 h. Sat. $NaHCO_3$ (25 mL) was added to quench the reaction and the crude was extracted with DCM (3 × 50 mL). The combined organic fractions were washed with H_2O (2 × 75 mL), followed by brine (100 mL), and dried over $MgSO_4$. No additional purification was needed and the title compound was obtained (4.39 g, 12.48 mmol, 63%). MS (ESI) m/z: 351.7 $[M + H]^+$; R_f = 0.26 (heptane/EtOAc 1:1); ^1H NMR (600 MHz, $CDCl_3$): δ 8.36 (d, J = 5.4 Hz, 1H), 7.68 (d, J = 5.2 Hz, 1H), 7.48 (d, J = 5.2 Hz, 1H), 7.25 (d, J = 5.2 Hz, 1H), 2.29 (d, J = 7.1 Hz, 2H), 1.14−1.04 (m, 1H), 0.69−0.62 (m, 2H), 0.28 (dt, J = 4.6, 6.03 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 170.7, 152.9, 145.5, 126.8, 120.4, 110.2, 39.6, 7.0, 4.9.

*8-Chloro-3-(cyclopropylmethyl)-7-iodo-[1,2,4]triazolo[4,3-a]-pyridine (**16**).* Dimethylsulfone (5.5 g) and sulfolane (0.7 g) were combined with **15** (4.1 g, 11.66 mmol, 1.0 equiv) and stirred for 3 h in a preheated flask at 160 °C. After cooling to rt, H_2O (100 mL) was added and the crude was extracted with DCM (3 × 150 mL). The combined organic fractions were washed with H_2O (2 × 100 mL) and brine (150 mL) and dried over $MgSO_4$. The crude was purified by DCVC (0 → 100% EtOAc in heptane), which afforded the title compound (2.7 g, 8.09 mmol, 68%). MS (ESI) m/z: 333.7 $[M + H]^+$; R_f = 0.45 (EtOAc); ^1H NMR (600 MHz, $CDCl_3$): δ 7.67 (d, J = 7.1 Hz, 1H), 7.16 (d, J = 7.1 Hz, 1H), 3.07 (d, J = 6.7 Hz, 2H), 1.23−1.12 (m, 1H), 0.68−0.59 (m, 2H), 0.33 (q, J = 5.3 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 148.9, 148.4, 128.2, 123.3, 120.5, 95.8, 29.6, 8.6, 5.3.

*2-(Piperidin-4-yl)phenol (**18**).* 4-(2-Methoxyphenyl)piperidine (400 mg, 2.09 mmol, 1.0 equiv) was dissolved in HBr (33 wt % in AcOH) (1.3 mL, 7.32 mmol, 3.5 equiv) and stirred under reflux for 2 h. After cooling to rt, the reaction mixture was neutralized to pH 8 with sat. Na_2CO_3. The crude was extracted with DCM (3 × 50 mL). The combined organic fractions were washed with brine (75 mL) and dried over $MgSO_4$. No additional purification was needed and the title compound was obtained (337 mg, 1.90 mmol, 91%). MS (ESI) m/z: 178.1 $[M + H]^+$; ^1H NMR (600 MHz, $CDCl_3$): δ 7.14 (dd, J = 1.6, 7.7 Hz, 1H), 7.04 (td, J = 1.5, 7.7 Hz, 1H), 6.84 (td, J = 1.4, 7.6 Hz, 1H), 6.70 (dd, J = 1.3, 7.8 Hz, 1H), 5.12−4.09 (br s, 2H), 3.26−3.06 (m, 2H), 3.13−2.99 (m, 1H), 2.89−2.71 (m, 2H), 1.84−1.72 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 155.2, 153.2, 132.0, 127.2, 127.1, 121.0, 115.5, 79.7, 31.9, 28.7.

*tert-Butyl 4-(2-Hydroxyphenyl)piperidine-1-carboxylate (**19**).* **18** (330 mg, 1.86 mmol, 1.0 equiv) was dissolved in DCM (15 mL). Et_3N (0.55 mL, 3.91 mmol, 2.1 equiv) was added, followed by di-*tert*-butyl dicarbonate (447 mg, 2.05 mol, 1.1 equiv). The reaction mixture was stirred for 8 h at rt. H_2O (20 mL) was added and the crude was extracted with DCM (3 × 25 mL). The combined organic fractions were washed with HCl (1 M in H_2O, 50 mL) and brine (75 mL) and dried over $MgSO_4$. No additional purification was needed and the title compound was obtained (495 mg, 1.78 mmol, 94%). MS (ESI) m/z: 178.2 $[M - Boc + H]^+$; R_f = 0.38 (heptane/EtOAc 3:1); ^1H NMR (600 MHz, $CDCl_3$): δ 7.13 (dd, J = 1.6, 7.7 Hz, 1H), 7.07 (td, J = 1.7, 7.7 Hz, 1H), 6.90 (td, J = 1.2, 7.5 Hz, 1H), 6.76 (dd, J = 1.1, 8.0 Hz, 1H), 4.24 (s, 2H), 3.04 (tt, J = 3.5, 12.2 Hz, 1H), 2.91−2.73 (m, 2H), 1.83 (d, J = 13.1 Hz, 2H), 1.67−1.58 (m, 2H), 1.49 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 155.2, 153.2, 132.0, 127.2, 127.1, 121.0, 115.4, 79.7, 35.8, 31.8, 28.7, 27.6.

*tert-Butyl 4-(3-Hydroxyphenyl)piperidine-1-carboxylate (**20**).* 3-(Piperidin-4-yl)phenol (4.05 g, 22.87 mmol, 1.0 equiv) was dissolved in DCM (100 mL) and Et_3N (3.51 mL, 25.16 mmol, 1.1 equiv) was added, followed by di-*tert*-butyl dicarbonate (5.49 g, 25.16 mol, 1.1 equiv). The reaction mixture was stirred for 8 h at rt. H_2O (100 mL) was added and the crude was extracted with DCM (3 × 100 mL). The combined organic fractions were washed with HCl (1 M in H_2O, 150 mL) and brine (200 mL) and dried over $MgSO_4$. No additional purification was needed and the title compound was obtained (6.09 g, 96%). 178.2 $[M - Boc + H]^+$; ^1H NMR (600 MHz, $CDCl_3$): δ 7.16

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(td, J = 1.4, 7.5 Hz, 1H), 6.76 (dt, J = 1.2, 7.5 Hz, 1H), 6.71−6.67 (m, 2H), 4.23 (br s, 2H), 2.79 (br s, 2H), 2.60 (tt, J = 3.6, 12.2 Hz, 1H), 1.81 (ddd, J = 2.6, 4.8, 12.8 Hz, 2H), 1.65−1.53 (m, 2H), 1.49 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 156.3, 155.1, 147.7, 129.8, 119.2, 113.7, 113.5, 79.9, 44.4, 42.7, 31.4, 28.6.

*tert-Butyl 4-(4-Methoxyphenyl)piperidine-1-carboxylate (**21**).* *tert*-Butyl 4-(4-hydroxyphenyl)piperidine-1-carboxylate (500 mg, 1.80 mmol, 1.0 equiv) was dissolved in DMF (3 mL), to which were added K_2CO_3 (498 mg, 3.61 mmol, 2.0 equiv) and methyl iodide (0.12 mL, 1.98 mmol, 1.1 equiv). The reaction mixture was stirred for 15 h at 60 °C. The crude was extracted with EtOAc (3 × 25 mL). The combined organic fractions were washed with H_2O (2 × 50 mL) and brine (50 mL) and dried over $MgSO_4$. The crude was purified by DCVC (5 → 25% EtOAc in heptane), which afforded the title compound (414 mg, 79%). MS (ESI) m/z: 192.4 [M − Boc + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.12 (d, J = 8.59 Hz, 2H), 6.85 (d, J = 8.60 Hz, 2H), 4.23 (br s, 2H), 3.79 (s, 3H), 2.79 (br s, 2H), 2.59 (tt, J = 3.62, 12.17 Hz, 1H), 1.79 (d, J = 13.05 Hz, 2H), 1.63−1.55 (m, 2H), 1.48 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 158.2, 155.0, 138.2, 127.8, 114.0, 79.5, 55.4, 44.6, 42.0, 33.6, 28.6.

*tert-Butyl 4-(2-(2-Methoxyethoxy)phenyl)piperidine-1-carboxylate (**22**).* The title compound was prepared according to general procedure II (319 mg, 0.95 mmol, 88%). MS (ESI) m/z: 236.3 [M − Boc + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.19−7.12 (m, 2H), 6.93 (t, J = 7.5 Hz, 1H), 6.86 (d, J = 8.1 Hz, 1H), 4.23 (br s, 2H), 4.13 (t, J = 4.8 Hz, 2H), 3.76 (t, J = 4.8 Hz, 2H), 3.45 (s, 3H), 3.11 (tt, J = 3.5, 12.2 Hz, 1H), 2.82 (br t, J = 13.0 Hz, 2H), 1.81 (d, J = 12.8 Hz, 2H), 1.59 (qd, J = 4.2, 12.8 Hz, 2H), 1.48 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 156.2, 155.1, 134.4, 127.2, 126.7, 121.2, 112.0, 79.5, 71.4, 67.9, 59.4, 44.8, 35.7, 31.9, 28.6.

*tert-Butyl 4-(3-(2-Methoxyethoxy)phenyl)piperidine-1-carboxylate (**23**).* The title compound was prepared according to general procedure II (246 mg, 0.75 mmol, 68%). MS (ESI) m/z: 236.3 [M − Boc + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.21 (t, J = 8.1 Hz, 1H), 6.81−6.78 (m, 2H), 6.78−6.75 (m, 1H), 4.23 (br s, 2H), 4.13−4.09 (m, 2H), 3.78−3.71 (m, 2H), 3.45 (br s, 3H), 2.78 (s, 2H), 2.60 (tt, J = 3.6, 12.2 Hz, 1H), 1.81 (d, J = 13.1 Hz, 2H), 1.64−1.57 (m, 2H), 1.48 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 159.1, 155.0, 147.6, 129.5, 119.6, 113.8, 112.1, 79.6, 71.2, 67.3, 59.4, 42.9, 28.6.

*tert-Butyl 4-(4-(2-Methoxyethoxy)phenyl)piperidine-1-carboxylate (**24**).* The title compound was prepared according to general procedure II (507 mg, 1.51 mmol, 84%). MS (ESI) m/z: 236.3 [M − Boc + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.10 (d, J = 8.6 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 4.22 (br s, 2H), 4.12−4.07 (m, 2H), 3.76−3.71 (m, 2H), 3.44 (s, 3H), 2.78 (br t, J = 10.7, 21.3 Hz, 2H), 2.58 (tt, J = 3.6, 12.2 Hz, 1H), 1.82−1.75 (m, 2H), 1.57 (qd, J = 4.4, 12.8, 13.7 Hz, 2H), 1.48 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 157.4, 155.0, 138.4, 127.7, 114.7, 79.5, 71.2, 67.4, 59.3, 44.6, 42.0, 33.5, 28.6.

*tert-Butyl 4-(4-((tert-Butyldiphenylsilyl)oxy)phenyl)piperidine-1-carboxylate (**25**).* *tert*-Butyl 4-(4-hydroxyphenyl)piperidine-1-carboxylate (2.5 g, 9.01 mmol, 1.0 equiv) and imidazole (1.53 g, 22.53 mmol, 2.5 equiv) were dissolved in DCM (50 mL), followed by *tert*-butyldiphenylchlorosilane (2.54 mL, 9.91 mmol, 1.1 equiv). The reaction mixture was stirred for 16 h at rt. The crude was extracted with DCM (3 × 50 mL). The combined organic fractions were washed with brine (100 mL) and dried over $MgSO_4$. The crude was purified by DCVC (0 → 20% EtOAc in heptane), which afforded the title compound (4.64 g, 8.99 mmol, quantitative). MS (ESI) m/z: 416.1 [M − Boc + H]$^+$; R_f = 0.49 (heptane/EtOAc 3:1); ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.73−7.70 (m, 4H), 7.44−7.40 (m, 2H), 7.38−7.34 (m, 4H), 6.91 (d, J = 8.5 Hz, 2H), 6.69 (d, J = 8.5 Hz, 2H), 4.19 (br s, 2H), 2.74 (br s, 2H), 2.50 (tt, J = 3.6, 12.1 Hz, 1H), 1.74 (dd, J = 3.9, 13.4 Hz, 2H), 1.51 (td, J = 4.4, 12.7 Hz, 2H), 1.46 (s, 9H), 1.09 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 155.0, 154.1, 138.4, 135.7, 133.3, 123.0, 127.9, 127.5, 119.7, 79.5, 44.6, 42.0, 33.5, 28.7, 26.7, 26.7, 19.6.

*4-(4-Methoxyphenyl)piperidine (**26**).* The title compound was prepared according to general procedure III (255 mg, 1.33 mmol, 97%). MS (ESI) m/z: 192.3 [M + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.13 (d, J = 8.6 Hz, 2H), 6.84 (d, J = 8.5 Hz, 2H), 5.24 (br s, 1H), 3.77 (s, 3H), 3.30 (dt, J = 2.7, 12.5 Hz, 2H), 2.81 (td, J = 2.7, 12.5 Hz, 2H), 2.61 (tt, J = 3.7, 12.1 Hz, 1H), 1.87 (d, J = 12.7 Hz, 2H), 1.76 (qd, J = 4.0, 12.9 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 157.1, 136.9, 126.6, 112.9, 54.3, 45.2, 40.3, 32.2.

*4-(2-(2-Methoxyethoxy)phenyl)piperidine (**27**).* The title compound was prepared according to general procedure III (149 mg, 0.63 mmol, 99%). MS (ESI) m/z: 236.1 [M + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.18 (ddt, J = 2.0, 4.2, 6.0 Hz, 2H), 6.95 (td, J = 1.2, 7.5 Hz, 1H), 6.86 (dd, J = 1.2, 8.5 Hz, 1H), 6.37 (br s, 1H), 4.15−4.10 (m, 2H), 3.78−3.72 (m, 2H), 3.48−3.39 (m, 5H), 3.23−3.14 (m, 1H), 2.96 (td, J = 3.9, 12.3 Hz, 2H), 2.00−1.88 (m, 4H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 155.0, 132.1, 126.6, 125.7, 120.3, 111.0, 70.3, 66.8, 58.3, 44.6, 33.5, 29.0.

*4-(3-(2-Methoxyethoxy)phenyl)piperidine (**28**).* The title compound was prepared according to general procedure III (48 mg, 0.49 mmol, quantitative). MS (ESI) m/z: 236.1 [M + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.21 (t, J = 8.1 Hz, 1H), 6.84−6.80 (m, 2H), 6.78−6.74 (m, 1H), 4.14−4.09 (m, 2H), 3.77−3.72 (m, 2H), 3.45 (s, 3H), 3.21 (dt, J = 2.9, 12.2 Hz, 2H), 2.75 (td, J = 2.5, 12.3 Hz, 2H), 2.59 (tt, J = 3.7, 12.1 Hz, 1H), 2.43 (br s, 1H), 1.84 (d, J = 13.1 Hz, 2H), 1.66 (qd, J = 4.0, 12.5 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 159.0, 148.3, 129.5, 119.6, 113.7, 112.0, 71.2, 67.3, 59.4, 47.1, 43.1, 34.3.

*4-(4-(2-Methoxyethoxy)phenyl)piperidine (**29**).* The title compound was prepared according to general procedure III (313 mg, 1.33 mmol, 97%). MS (ESI) m/z: 236.1 [M + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.12 (d, J = 8.6 Hz, 2H), 6.86 (d, J = 8.6 Hz, 2H), 4.10−4.07 (m, 2H), 3.90 (br s, 1H), 3.74−3.71 (m, 2H), 3.43 (s, 3H), 3.24 (dt, J = 3.1, 12.3 Hz, 2H), 2.77 (td, J = 2.6, 12.4 Hz, 2H), 2.57 (tt, J = 3.7, 12.1 Hz, 1H), 1.83 (d, J = 13.8 Hz, 2H), 1.69 (dtd, J = 4.0, 12.1, 13.3 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 157.3, 138.7, 127.7, 114.7, 71.2, 67.4, 59.3, 46.8, 41.8, 34.0.

*4-(4-((tert-Butyldiphenylsilyl)oxy)phenyl)piperidine (**30**).* The title compound was prepared according to general procedure III (3.27 g, 7.87 mmol, 74%). MS (ESI) m/z: 416.2 [M + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.75−7.69 (m, 4H), 7.44−7.38 (m, 2H), 7.39−7.33 (m, 4H), 6.93 (d, J = 8.5 Hz, 2H), 6.69 (d, J = 8.5 Hz, 2H), 3.18 (dt, J = 3.0, 12.4 Hz, 2H), 2.71 (td, J = 2.5, 12.4 Hz, 2H), 2.49 (tt, J = 3.7, 12.1 Hz, 1H), 2.21 (br s, 1H), 1.77 (ddq, J = 2.3, 4.30, 12.8 Hz, 2H), 1.63−1.53 (m, 2H), 1.09 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 153.9, 139.0, 135.7, 135.0, 133.3, 129.9, 127.8, 119.6, 47.0, 42.0, 34.3, 26.7, 19.6.

*8-Chloro-3-(cyclopropylmethyl)-7-(4-(2-methoxyphenyl)-piperidin-1-yl)-[1,2,4]triazolo-[4,3-a]pyridine Hydrochloride (**31**).* The title compound was prepared according to general procedure IV (184 mg, 0.42 mmol, 47%). MS (ESI) m/z: 397.0 [M + H]$^+$; ^{1}H NMR (600 MHz, MeOD): δ 8.48 (d, J = 7.5 Hz, 1H), 7.35 (d, J = 7.5 Hz, 1H), 7.23−7.17 (m, 2H), 6.96 (d, J = 7.7 Hz, 1H), 6.92 (td, J = 1.1, 7.5 Hz, 1H), 4.22 (dt, J = 2.2, 13.1 Hz, 2H), 3.85 (s, 3H), 3.37 (td, J = 2.5, 12.6 Hz, 2H), 3.07 (d, J = 6.9 Hz, 2H), 2.05−1.96 (m, 2H), 1.92 (qd, J = 3.7, 12.3 Hz, 3H), 1.35−1.22 (m, 1H), 0.73−0.66 (m, 2H), 0.39 (dt, J = 4.6, 6.0 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 158.3, 154.2, 149.4, 147.1, 134.1, 128.5, 127.4, 125.1, 121.8, 113.8, 111.7, 98.3, 55.8, 52.3, 36.4, 33.2, 29.7, 8.4, 5.5.

*8-Chloro-3-(cyclopropylmethyl)-7-(4-(3-methoxyphenyl)-piperidin-1-yl)-[1,2,4]triazolo-[4,3-a]pyridine Hydrochloride (**32**).* The title compound was prepared according to general procedure IV (98 mg, 0.23 mmol, 78%). MS (ESI) m/z: 397.0 [M + H]$^+$; ^{1}H NMR (600 MHz, MeOD): δ 8.50 (d, J = 7.6 Hz, 1H), 7.37 (d, J = 7.6 Hz, 1H), 7.23 (t, J = 7.9 Hz, 1H), 6.87 (d, J = 7.7 Hz, 1H), 6.84 (t, J = 2.1 Hz, 1H), 6.8 (dd, J = 2.17, 8.22 Hz, 1H), 4.24 (dp, J = 1.9, 13.3 Hz, 2H), 3.79 (s, 3H), 3.37 (td, J = 2.4, 12.7 Hz, 2H), 3.07 (d, J = 6.9 Hz, 2H), 2.89 (tt, J = 3.8, 12.1 Hz, 1H), 2.04 (ddd, J = 1.9, 4.0, 12.6 Hz, 2H), 1.92 (qd, J = 3.8, 12.8 Hz, 2H), 1.35−1.23 (m, 1H), 0.74−0.65 (m, 2H), 0.40 (dt, J = 4.6, 6.1 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 161.4, 154.4, 149.4, 148.1, 146.9, 130.6, 125.2, 120.1, 113.9, 113.9, 112.6, 98.1, 55.6, 52.0, 43.3, 34.6, 29.7, 8.4, 5.5.

*8-Chloro-3-(cyclopropylmethyl)-7-(4-(4-methoxyphenyl)-piperidin-1-yl)-[1,2,4]triazolo-[4,3-a]pyridine Hydrochloride (**33**).* The title compound was prepared according to general procedure

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IV (35 mg, 0.08 mmol, 31%). MS (ESI) m/z: 397.0 $[M + H]^+$; ^1H NMR (600 MHz, MeOD): δ 8.48 (d, J = 7.6 Hz, 1H), 7.35 (d, J = 7.7 Hz, 1H), 7.22−7.16 (m, 2H), 6.89−6.85 (m, 2H), 4.23 (dp, J = 2.1, 13.2 Hz, 2H), 3.77 (s, 3H), 3.35 (td, J = 2.4, 12.7 Hz, 2H), 3.06 (d, J = 6.9 Hz, 2H), 2.85 (tt, J = 3.8, 12.1 Hz, 1H), 2.04−1.98 (m, 2H), 1.89 (qd, J = 3.8, 12.6 Hz, 2H), 1.33−1.23 (m, 1H), 0.72−0.66 (m, 2H), 0.39 (dt, J = 4.6, 6.1 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 159.8, 154.4, 149.4, 146.9, 138.6, 128.7, 125.3, 115.0, 114.0, 98.0, 55.7, 52.1, 42.4, 34.9, 29.7, 8.4, 5.5.

*8-Chloro-3-(cyclopropylmethyl)-7-(4-(2-(2-methoxyethoxy)-phenyl)piperidin-1-yl)-[1,2,4]triazolo[4,3-a]pyridine Hydrochloride (**34**).* The title compound was prepared according to general procedure IV (34 mg, 0.08 mmol, 26%). MS (ESI) m/z: 441.1 $[M + H]^+$; ^1H NMR (600 MHz, MeOD): δ 8.33 (d, J = 7.5 Hz, 1H), 7.24 (dd, J = 1.6, 7.6 Hz, 1H), 7.20−7.16 (m, 1H), 7.15 (d, J = 7.5 Hz, 1H), 6.98−6.91 (m, 2H), 4.18−4.13 (m, 2H), 3.94 (dq, J = 2.5, 12.2 Hz, 2H), 3.80−3.77 (m, 2H), 3.44 (s, 3H), 3.26 (ddd, J = 7.3, 9.6, 15.9 Hz, 1H), 3.23−3.17 (m, 2H), 3.06 (d, J = 6.9 Hz, 2H), 2.00−1.94 (m, 4H), 1.28−1.21 (m, 1H), 0.69−0.62 (m, 2H), 0.36 (dt, J = 4.6, 6.1 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 157.6, 154.4, 149.4, 146.9, 134.5, 128.5, 127.7, 125.2, 122.1, 113.9, 113.1, 97.8, 72.4, 68.8, 59.3, 52.3, 36.9, 33.1, 29.7, 8.4, 5.5.

*8-Chloro-3-(cyclopropylmethyl)-7-(4-(3-(2-methoxyethoxy)-phenyl)piperidin-1-yl)-[1,2,4]triazolo[4,3-a]pyridine Hydrochloride (**35**).* The title compound was prepared according to general procedure IV (108 mg, 0.22 mmol, 54%). MS (ESI) m/z: 441.1 $[M + H]^+$; ^1H NMR (600 MHz, MeOD): δ 8.47 (d, J = 7.4 Hz, 1H), 7.34 (d, J = 7.4 Hz, 1H), 7.23 (t, J = 7.8 Hz, 1H), 6.90−6.85 (m, 2H), 6.80 (dd, J = 2.1, 8.2 Hz, 1H), 4.19 (d, J = 14.1 Hz, 2H), 4.13−4.09 (m, 2H), 3.76−3.72 (m, 2H), 3.42 (s, 3H), 3.39−3.33 (m, 2H), 3.07 (d, J = 6.9 Hz, 2H), 2.88 (tt, J = 3.7, 12.2 Hz, 1H), 2.07−1.99 (m, 2H), 1.92 (qd, J = 3.7, 12.5 Hz, 2H), 1.36−1.22 (m, 1H), 0.72−0.65 (m, 2H), 0.39 (dt, J = 4.6, 5.9 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 160.5, 153.9, 149.4, 148.2, 147.3, 130.6, 125.0, 120.4, 114.6, 113.7, 113.3, 99.0, 72.3, 68.3, 59.2, 52.1, 43.3, 34.6, 29.7, 8.5, 5.5.

*8-Chloro-3-(cyclopropylmethyl)-7-(4-(4-(2-methoxyethoxy)-phenyl)piperidin-1-yl)-[1,2,4]triazolo[4,3-a]pyridine Hydrochloride (**36**).* The title compound was prepared according to general procedure IV (42 mg, 0.09 mmol, 22%). MS (ESI) m/z: 441.1 $[M + H]^+$; ^1H NMR (600 MHz, MeOD): δ 8.50 (d, J = 7.3 Hz, 1H), 7.37 (d, J = 7.3 Hz, 1H), 7.23−7.17 (m, 2H), 6.93−6.87 (m, 2H), 4.24 (d, J = 12.8 Hz, 2H), 4.12−4.07 (m, 2H), 3.76−3.71 (m, 2H), 3.42 (s, 3H), 3.41−3.33 (m, 2H), 3.07 (d, J = 6.8 Hz, 2H), 2.86 (tt, J = 3.7, 12.1 Hz, 1H), 2.07−1.95 (m, 2H), 1.97−1.84 (m, 2H), 1.38−1.22 (m, 1H), 0.75−0.63 (m, 2H), 0.45−0.35 (m, 2H); ^{13}C NMR (151 MHz, MeOD): δ 158.9, 154.4, 149.4, 146.9, 138.9, 128.7, 125.2, 115.7, 114.0, 97.9, 72.3, 68.4, 59.2, 52.1, 42.4, 34.9, 29.7, 8.4, 5.5.

*4-(1-(8-Chloro-3-(cyclopropylmethyl)-[1,2,4]triazolo[4,3-a]-pyridin-7-yl)piperidin-4-yl)phenol Hydrochloride (**37**).* The title compound was prepared according to general procedure IV (42 mg, 0.09 mmol, 27%). MS (ESI) m/z: 420.1 $[M + H]^+$; ^1H NMR (600 MHz, MeOD): δ 8.37 (d, J = 7.6 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.23−7.14 (m, 2H), 6.84 (dt, J = 2.8, 9.2 Hz, 2H), 4.21 (d, J = 12. Hz, 2H), 3.23−3.14 (m, 2H), 3.05 (d, J = 7.5 Hz, 2H), 2.89−2.80 (m, 1H), 1.99 (d, J = 13.2 Hz, 2H), 1.85 (dd, J = 11.0, 14.8 Hz, 2H), 1.26−1.18 (m, 1H), 0.71−0.61 (m, 2H), 0.36 (dt, J = 5.1, 7.6 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 155.1, 154.0, 149.4, 146.5, 138.5, 128.9, 124.5, 116.3, 113.9, 97.5, 51.7, 49.4, 41.6, 34.3, 29.3, 7.9, 5.3.

*3,6,9,12,15,18,21-Heptaoxatricosane-1,23-diyl Bis(4-methylbenzenesulfonate) (**52**).* 3,6,9,12,15,18,21-Heptaoxatricosane-1,23-diol (1000 mg, 2.70 mmol, 1.0 equiv) was dissolved in dry DCM (150 mL). Tosyl chloride (1081 mg, 5.67 mmol, 2.1 equiv) was added followed by 4-dimethylaminopyridine (DMAP) (693 mg, 5.67 mmol, 2.1 equiv) and the reaction was stirred for 16 h at rt. The reaction mixture was quenched with sat. NH_4Cl (100 mL), diluted with H_2O (100 mL) and was extracted with DCM (3 × 100 mL). The combined organic fractions were washed with brine (100 mL) and dried over $MgSO_4$. The crude was purified by DCVC, which afforded the title compound (1295 mg, 1.91 mmol, 71%). MS (ESI) m/z: 679.3 $[M + H]^+$; ^1H NMR (600 MHz, $CDCl_3$): δ 7.79 (d, J = 8.3 Hz, 4H), 7.33 (d, J = 8.2 Hz, 4H), 4.17−4.12 (m, 4H), 3.67−3.59 (m, 20H), 3.57 (s, 8H), 2.44 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 144.9, 133.2, 130.0, 128.1, 70.9, 70.74, 70.69, 70.6, 69.4, 68.8, 21.8.

*3,6,9,12,15,18,21,24,27-Nonaoxanonacosane-1,29-diyl Bis(4-methylbenzenesulfonate) (**53**).* To a solution of 3,6,9,12,15,18,21,24,27-nonaoxanonacosane-1,29-diol (250 mg, 0.55 mmol, 1.0 equiv) in THF, tosyl chloride (312 mg, 1.64 mmol, 3.0 equiv) was added at rt and then cooled to 0 °C. To this mixture, potassium hydroxide (6.6 equiv) in H_2O (0.9 g/mL) was added dropwise over 1 h. Then the reaction mixture was kept at 0 °C for 30 min and some white precipitation appeared. Afterward this mixture was further stirred at rt until TLC analysis indicated complete consumption of the diol. The reaction mixture was neutralized with a saturated ammonium chloride solution and concentrated *in vacuo*. The residue was absorbed on the silica gel and further purified by column chromatography to obtain the pure ditosylate (241 mg, 0.31 mmol, 58%). MS (ESI) m/z: 767.3 $[M + H]^+$; ^1H NMR (400 MHz, $CDCl_3$): δ 7.82−7.77 (m, 4H), 7.37−7.32 (m, 4H), 4.18−4.13 (m, 4H), 3.70−3.67 (m, 4H), 3.63 (d, J = 7.85 Hz, 24H), 3.58 (s, 8H), 2.45 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 144.8, 133.1, 129.8, 128.0, 70.8, 70.6, 70.6, 70.5, 69.2, 68.7, 21.6.

*3,6,9,12,15,18,21,24,27,30,33-Undecaoxapentatriacontane-1,35-diyl Bis(4-methylbenzenesulfonate) (**54**).* 3,6,9,12,15,18,21,24,27,30,33-Undecaoxapentatriacontane-1,35-diol (1000 mg, 1.83 mmol, 1.0 equiv) was dissolved in dry DCM (150 mL). Tosyl chloride (732 mg, 3.84 mmol, 2.1 equiv) was added, followed by DMAP (469 mg, 3.84 mmol, 2.1 equiv), and the reaction was stirred for 16 h at rt. The reaction mixture was quenched with sat. NH_4Cl (100 mL), diluted with H_2O (100 mL), and extracted with DCM (3 × 100 mL). The combined organic fractions were washed with brine (100 mL) and dried over $MgSO_4$. The crude was purified by DCVC, which afforded the title compound (1192 mg, 1.40 mmol, 76%). MS (ESI) m/z: 855.2 $[M + H]^+$; ^1H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, J = 8.3 Hz, 4H), 7.34 (d, J = 8.1 Hz, 4H), 4.19−4.11 (m, 4H), 3.69−3.60 (m, 36H), 3.58 (s, 8H), 2.44 (s, 6H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 144.9, 133.2, 130.0, 128.1, 70.9, 70.8, 70.73, 70.70, 70.66, 69.4, 68.8, 21.8.

*2-(2-(2-(3-(1-(4-Fluorophenethyl)piperidine-4-carbonyl)-2-methoxyphenoxy)ethoxy)ethoxy)ethyl 4-Methylbenzenesulfonate (**38**).* The title compound was prepared according to general procedure V (27 mg, 0.04 mmol, 42%); MS (ESI) m/z: 644.3 $[M + H]^+$, ^1H NMR (600 MHz, $CDCl_3$): δ 7.78 (d, J = 8.2 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.16−7.12 (m, 2H), 7.07−7.00 (m, 2H), 6.96 (dddd, J = 2.0, 6.7, 8.7, 15.10 Hz, 3H), 4.18−4.12 (m, 4H), 3.88 (s, 3H), 3.86 (d, J = 4.7 Hz, 2H), 3.71−3.64 (m, 4H), 3.61 (dd, J = 3.3, 5.8 Hz, 2H), 3.08 (tt, J = 3.8, 11.1 Hz, 1H), 2.97 (dt, J = 3.8, 11.8 Hz, 2H), 2.78−2.73 (m, 2H), 2.57−2.52 (m, 2H), 2.43 (s, 3H), 2.10 (td, J = 2.62, 11.8 Hz, 2H), 1.93−1.86 (m, 2H), 1.78−1.70 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 206.6, 162.3, 160.7, 152.0, 147.5, 145.0, 134.6, 133.1, 130.2, 130.0, 128.01 124.3, 120.9, 116.7, 115.3, 71.0, 70.9, 69.9, 69.3, 68.9, 68.6, 61.8, 60.8, 53.4, 48.3, 33.0, 28.2, 21.8.

*2-(2-(2-(2-(3-(1-(4-Fluorophenethyl)piperidine-4-carbonyl)-2-methoxyphenoxy)ethoxy)ethoxy)ethoxy)ethyl 4-Methylbenzenesulfonate (**39**).* The title compound was prepared according to general procedure V (32 mg, 0.05 mmol, 46%); MS (ESI) m/z: 688.3 $[M + H]^+$; ^1H NMR (600 MHz, $CDCl_3$): δ 7.79 (dd, J = 2.1, 8.5 Hz, 2H), 7.33 (dd, J = 3.6, 8.4 Hz, 2H), 7.17−7.12 (m, 2H), 7.07−7.00 (m, 2H), 7.00−6.91 (m, 3H), 4.16 (ddd, J = 4.0, 5.7, 18.1 Hz, 4H), 3.90−3.89 (m, 1H), 3.88 (s, 3H), 3.73−3.70 (m, 2H), 3.70−3.65 (m, 3H), 3.65−3.61 (m, 2H), 3.60−3.57 (m, 4H), 3.08 (tt, J = 3.9, 11.1 Hz, 1H), 2.97 (dt, J = 3.9, 11.3 Hz, 2H), 2.79−2.73 (m, 2H), 2.59−2.46 (m, 2H), 2.43 (s, 3H), 2.11 (t, J = 10.8 Hz, 2H), 1.90 (d, J = 13.9 Hz, 2H), 1.79−1.70 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 206.6, 162.3, 160.7, 152.0, 147.5, 144.9, 134.6, 133.2, 130.2, 129.9, 128.01, 124.3, 120.8, 116.6, 115.3, 70.9, 70.9, 70.8, 70.7, 69.8, 69.3, 68.8, 68.6, 61.8, 60.8, 53.4, 48.2, 33.0, 28.2, 21.8.

*14-(3-(1-(4-Fluorophenethyl)piperidine-4-carbonyl)-2-methoxyphenoxy)-3,6,9,12-tetra Oxatetradecyl 4-Methylbenzenesulfonate (**40**).* The title compound was prepared according to general procedure V (29 mg, 0.04 mmol, 40%); MS (ESI) m/z: 732.4 [M

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+ H]$^+$, ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.78 (dt, J = 1.5, 2.0, 8.6 Hz, 2H), 7.33 (dt, J = 1.4, 7.8 Hz, 2H), 7.17−7.11 (m, 2H), 7.06−6.99 (m, 2H), 6.99−6.91 (m, 3H), 4.20−4.14 (m, 4H), 3.88 (s, 3H), 3.74−3.69 (m, 2H), 3.69−3.65 (m, 4H), 3.64−3.60 (m, 5H), 3.57 (s, 4H), 3.07 (tt, J = 3.9, 11.1 Hz, 1H), 2.96 (dt, J = 3.7, 11.7 Hz, 2H), 2.76 (dd, J = 6.2, 10.0 Hz, 2H), 2.59−2.50 (m, 2H), 2.45−2.42 (m, 4H), 2.16−2.05 (m, 2H), 1.95−1.83 (m, 2H), 1.78−1.69 (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 206.6, 162.7, 160.3, 152.0, 147.45 144.9, 134.6, 133.2, 130.2, 129.9, 128.1, 124.3, 120.8, 116.6, 115.3, 70.9, 70.9, 70.8, 70.7, 70.7, 70.7, 69.8, 69.4, 68.8, 68.6, 61.8, 60.9, 53.5, 48.3, 33.0, 28.2, 21.8.

*17-(3-(1-(4-Fluorophenethyl)piperidine-4-carbonyl)-2-methoxyphenoxy)-3,6,9,12,15-pentaoxaheptadecyl 4-Methylbenzenesulfonate (**41**).* The title compound was prepared according to general procedure V (38 mg, 0.05 mmol, 49%); MS (ESI) m/z: 776.3; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.78 (d, J = 8.3 Hz, 2H), 7.33 (d, J = 7.9 Hz, 2H), 7.14 (dd, J = 5.6, 8.4 Hz, 2H), 7.07−7.00 (m, 2H), 6.99−6.92 (m, 3H), 4.18−4.14 (m, 4H), 3.88 (s, 3H), 3.73−3.70 (m, 2H), 3.69−3.65 (m, 5H), 3.64−3.62 (m, 5H), 3.62−3.59 (m, 4H), 3.58−3.56 (m, 4H), 3.07 (tt, J = 3.8, 11.1 Hz, 1H), 2.96 (dt, J = 3.5, 11.8 Hz, 2H), 2.78−2.72 (m, 2H), 2.56−2.51 (m, 2H), 2.43 (s, 3H), 2.10 (t, J = 10.8 Hz, 2H), 1.92−1.82 (m, 2H), 1.77−1.68 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 206.6, 162.3, 160.7, 152.0, 147.5, 144.9, 134.6, 133.2, 130.2, 129.9, 128.1, 124.3, 120.8, 116.6, 115.3, 70.9, 70.9, 70.8, 70.8, 70.7, 70.7, 70.7, 70.6, 69.8, 69.4, 68.8, 68.6, 61.8, 60.8, 53.4, 48.3, 33.0, 28.2, 21.8.

*23-(3-(1-(4-Fluorophenethyl)piperidine-4-carbonyl)-2-methoxyphenoxy)-3,6,9,12,15,18,21-heptaoxatricosyl 4-Methylbenzenesulfonate (**42**).* The title compound was prepared according to general procedure V and purified by flash chromatography (0 → 3% MeOH in DCM) (109 mg, 0.13 mmol, 45%). MS (ESI) m/z: 864.3 [M + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.78 (d, J = 8.3 Hz, 2H), 7.33 (d, J = 7.8 Hz, 2H), 7.17−7.11 (m, 2H), 7.07−6.99 (m, 2H), 6.98−6.90 (m, 3H), 4.17 (dd, J = 5.7, 4.1 Hz, 2H), 4.16−4.12 (m, 2H), 3.91−3.86 (m, 5H), 3.74−3.70 (m, 2H), 3.68−3.57 (m, 20H), 3.57 (s, 4H), 3.08 (t, J = 11.4 Hz, 1H), 2.97 (dt, J = 11.7, 3.9 Hz, 2H), 2.81−2.74 (m, 2H), 2.61−2.51 (m, 2H), 2.43 (s, 3H), 2.22−2.08 (m, 2H), 1.91 (d, J = 12.6 Hz, 2H), 1.81−1.71 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 206.5, 162.3, 160.7, 152.0, 147.5, 144.9, 136.1, 134.5, 133.2, 130.2, 130.1, 129.9, 128.1, 124.3, 120.8, 116.7, 115.3, 115.1, 72.7, 71.5, 70.92, 70.86, 70.8, 70.74, 70.73, 70.69, 70.6, 69.8, 69.4, 68.8, 68.6, 61.7, 60.7, 53.3, 48.1, 42.8, 32.9, 28.0, 21.7.

*29-(3-(1-(4-Fluorophenethyl)piperidine-4-carbonyl)-2-methoxyphenoxy)-3,6,9,12,15,18,21,24,27-nonaoxanonacosyl 4-Methylbenzenesulfonate (**43**).* The title compound was prepared according to general procedure V and purified by flash chromatography (0 → 3% MeOH in DCM) (33 mg, 0.03 mmol, 43%). MS (ESI) m/z: 952.1 [M + H]$^+$, 447.0 [M + 2H]$^{2+}$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.81−7.77 (d, J = 12.0 Hz 2H), 7.33 (d, J = 8.0 Hz, 2H), 7.16−7.12 (m, 2H), 7.06−7.01 (m, 2H), 6.99−6.92 (m, 3H), 4.18 (t, J = 4.9 Hz, 2H), 4.16 (t, J = 4.9 Hz, 2H), 3.90−3.88 (m, 5H), 3.72 (dd, J = 3.7, 5.9 Hz, 2H), 3.69−3.60 (m, 28H), 3.57 (s, 4H), 3.09 (ddt, J = 4.2, 7.7, 11.1 Hz, 1H), 2.98 (dt, J = 3.9, 11.7 Hz, 2H), 2.77 (dd, J = 6.3, 10.0 Hz, 2H), 2.57 (dd, J = 6.0, 10.0 Hz, 2H), 2.44 (s, 3H), 2.19−2.08 (m, 2H), 1.91 (d, J = 13.4 Hz, 2H), 1.82−1.70 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 206.4, 162.2, 160.6, 151.9, 147.3, 144.8, 136.0, 134.4, 133.0, 130.1, 130.0, 129.8, 128.0, 124.2, 120.7, 116.5, 115.2, 115.0, 70.8, 70.7, 70.7, 70.6, 70.6, 70.6, 70.6, 70.5, 70.5, 70.5, 69.7, 69.2, 68.7, 68.4, 61.6, 60.6, 53.2, 48.0, 32.8, 29.7, 28.0, 21.6.

*35-(3-(1-(4-Fluorophenethyl)piperidine-4-carbonyl)-2-methoxyphenoxy)-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl 4-Methylbenzenesulfonate (**44**).* The title compound was prepared according to general procedure V and purified by flash chromatography (0 → 3% MeOH in DCM) (148 mg, 0.14 mmol, 51%). MS (ESI) m/z: 1040.4 [M + H]$^+$, 520.7 [M + 2H]$^{2+}$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.76 (d, J = 8.4 Hz, 2H), 7.31 (d, J = 8.3 Hz, 2H), 7.14−7.09 (m, 2H), 7.04−6.98 (m, 2H), 6.96−6.89 (m, 3H), 4.15 (dd, J = 5.7, 4.1 Hz, 2H), 4.14−4.10 (m, 2H), 3.88−3.84 (m, 5H), 3.74−3.56 (m, 38H), 3.55 (s, 4H), 3.06 (tt, J = 10.9, 3.7 Hz, 1H), 2.94 (dt, J = 10.8, 3.8 Hz, 2H), 2.77−2.72 (m, 2H), 2.56−2.50 (m, 2H), 2.41 (s, 3H), 2.09 (t, J = 11.0 Hz, 2H), 1.91−1.84 (m, 2H), 1.77−1.67 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 206.4, 162.2, 160.6, 151.9, 147.4, 144.8, 136.1, 134.5, 133.1, 130.1, 130.0, 129.9, 128.0, 124.2, 120.7, 116.6, 115.2, 115.0, 72.6, 71.4, 70.83, 70.77, 70.68, 70.65, 70.64, 70.60, 70.5, 70.4, 69.7, 69.3, 68.7, 68.5, 61.73, 61.65, 61.6, 60.7, 53.3, 48.1, 42.8, 32.9, 28.0, 21.7.

*(3-(2-(2-(2-(4-(1-(8-Chloro-3-(cyclopropylmethyl)-[1,2,4]triazolo-[4,3-a]pyridin-7-yl)piperidin-4-yl)phenoxy)ethoxy)ethoxy)ethoxy)-2-methoxyphenyl)(1-(4-fluorophenethyl)piperidin-4-yl)methanone Bis(2,2,2-trifluoroacetate) (**45**).* The title compound was prepared according to general procedure I (4 mg, 0.003 mmol, 10%); MS (ESI) m/z: 584.6 [M + H]$^+$; ^{1}H NMR (600 MHz, MeOD): δ 8.46 (d, J = 7.6 Hz, 1H), 7.39 (d, J = 7.4 Hz, 1H), 7.35−7.30 (m, 3H), 7.26 (dd, J = 1.1, 7.9 Hz, 1H), 7.16−7.03 (m, 5H), 6.88−6.83 (m, 2H), 4.32 (t, J = 4.2 Hz, 2H), 4.28−4.11 (m, 2H), 4.10−4.06 (m, 2H), 3.91−3.88 (m, 3H), 3.85 (s, 1H), 3.83−3.81 (m, 2H), 3.75−3.57 (m, 10H), 3.56−3.38 (m, 4H), 3.07 (d, J = 6.9 Hz, 2H), 2.80 (tt, J = 3.9, 12.2 Hz, 1H), 2.27 (t, J = 12.2 Hz, 2H), 2.07−1.91 (m, 4H), 1.85 (qd, J = 3.4, 12.7, 13.3 Hz, 2H), 1.36−1.23 (m, 3H), 0.72−0.65 (m, 2H), 0.39 (dt, J = 4.6, 6.0 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 202.1, 164.3, 162.7, 162.5, 158.8, 153.9, 149.4, 147.6, 147.4, 139.1, 133.4, 131.70, 131.67, 128.8, 125.0, 124.7, 122.7, 122.0, 116.9, 116.8, 116.6, 115.9, 113.6, 99.2, 71.8, 71.8, 70.9, 70.9, 70.8, 70.7, 70.1, 69.6, 68.8, 68.7, 59.2, 52.1, 50.0, 42.4, 34.9, 30.5, 29.7, 8.5, 5.5.

*(3-(2-(2-(2-(2-(4-(1-(8-Chloro-3-(cyclopropylmethyl)-[1,2,4]-triazolo[4,3-a]pyridin-7-yl)piperidin-4-yl)phenoxy)ethoxy)ethoxy)-ethoxy)ethoxy)-2-methoxyphenyl)(1-(4-fluoro phenethyl)piperidin-4-yl)methanone Bis(2,2,2-trifluoroacetate) (**46**).* The title compound was prepared according to general procedure I (10 mg, 0.008 mmol, 20%) (purity 90%); MS (ESI) m/z: 898.3 [M + H]$^+$; ^{1}H NMR (600 MHz, MeOD): δ 8.45 (d, J = 7.6 Hz, 1H), 7.30 (dt, J = 3.0, 8.4 Hz, 3H), 7.22 (dd, J = 1.6, 8.1 Hz, 1H), 7.21−7.16 (m, 2H), 7.15−7.02 (m, 4H), 6.92−6.85 (m, 2H), 4.22−4.17 (m, 2H), 4.19−4.12 (m, 2H), 4.11−4.07 (m, 2H), 3.95 (s, 3H), 3.91−3.78 (m, 5H), 3.75−3.56 (m, 12H), 3.47 (dd, J = 10.5, 13.9 Hz, 1H), 3.36−3.31 (m, 1H), 3.16−3.00 (m, 6H), 2.82 (tt, J = 3.8, 12.0 Hz, 1H), 2.33−1.95 (m, 4H), 1.94−1.79 (m, 4H), 1.29 (tdd, J = 1.6, 3.2, 8.1 Hz, 1H), 0.68 (dt, J = 4.8, 6.1, 8.0 Hz, 2H), 0.39 (q, J = 4.7, 6.1 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 204.9, 164.3, 162.7, 158.9, 153.8, 153.5, 149.4, 147.5, 139.0, 133.9, 133.44, 133.41, 131.7, 131.6, 128.8, 125.4, 124.9, 121.7, 118.8, 116.8, 116.6, 115.8, 113.5, 99.4, 71.8, 71.7, 71.6, 70.9, 70.8, 69.8, 68.7, 62.3, 59.1, 53.5, 52.1, 46.8, 42.4, 34.9, 30.5, 29.7, 27.0, 8.5, 5.5.

*(3-((14-(4-(1-(8-Chloro-3-(cyclopropylmethyl)-[1,2,4]triazolo[4,3-a]pyridin-7-yl)piperidin-4-yl)phenoxy)-3,6,9,12-tetraoxatetradecyl)oxy)-2-methoxyphenyl)(1-(4-fluorophen-ethyl)-piperidin-4-yl)methanone Bis(2,2,2-trifluoroacetate) (**47**).* The title compound was prepared according to general procedure I (5 mg, 0.004 mmol, 12%); MS (ESI) m/z: 942.3 [M + H]$^+$; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.46 (d, J = 7.6 Hz, 1H), 7.36−7.27 (m, 3H), 7.24−7.15 (m, 3H), 7.11 (t, J = 8.3 Hz, 1H), 7.09−7.01 (m, 3H), 6.88 (d, J = 8.6 Hz, 2H), 4.23−4.14 (m, 4H), 4.12−4.04 (m, 2H), 3.97−3.92 (m, 3H), 3.92−3.75 (m, 6H), 3.75−3.57 (m, 16H), 3.57−3.41 (m, 2H), 3.14−3.09 (m, 1H), 3.09−3.00 (m, 4H), 2.82 (tt, J = 3.9, 12.3 Hz, 1H), 2.19 (d, J = 14.3 Hz, 2H), 1.99 (dd, J = 3.7, 13.8 Hz, 2H), 1.93−1.80 (m, 3H), 1.32−1.24 (m, 2H), 0.71−0.65 (m, 2H), 0.42−0.35 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): δ 204.9, 164.3, 162.7, 158.9, 154.0, 153.5, 149.4, 147.2, 138.9, 134.0, 133.5, 133.4, 131.7, 131.6, 128.8, 125.4, 125.0, 121.7, 118.8, 116.7, 116.6, 115.8, 113.7, 98.7, 71.74, 71.68, 71.66, 71.61, 71.60, 71.58, 71.56, 71.5, 70.9, 70.7, 69.8, 69.8, 68.7, 62.3, 62.1, 59.1, 53.5, 52.1, 46.5, 42.4, 34.9, 30.5, 29.7, 27.0, 8.4, 5.5.

*(3-((17-(4-(1-(8-Chloro-3-(cyclopropylmethyl)-[1,2,4]triazolo[4,3-a]pyridin-7-yl)piperidin-4-yl)phenoxy)-3,6,9,12,15-pentaoxaheptadecyl)oxy)-2-methoxyphenyl)(1-(4-fluorophenethyl)piperidin-4-yl)methanone Bis(2,2,2-trifluoroacetate) (**48**).* The title compound was prepared according to general procedure I (11 mg, 0.009 mmol, 20%); MS (ESI) m/z: 986.3 [M + H]$^+$; ^{1}H NMR (600 MHz, MeOD): δ 8.46 (d, J = 7.5 Hz, 1H), 7.34−7.27 (m, 3H), 7.22 (dd, J = 1.6, 8.2 Hz, 1H), 7.21−7.17 (m, 2H), 7.12 (t, J = 8.0 Hz, 1H), 7.10−7.02 (m, 3H), 6.92−6.87 (m, 2H), 4.21−4.15 (m, 4H), 4.09 (t, J = 4.9 Hz, 2H), 3.95 (s, 3H),

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3.90−3.84 (m, 3H), 3.82 (t, J = 4.7 Hz, 2H), 3.75−3.67 (m, 6H), 3.66−3.59 (m, 14H), 3.55−3.42 (m, 2H), 3.35−3.33 (m, 1H), 3.14−3.03 (m, 5H), 2.83 (tt, J = 3.7, 12.1 Hz, 1H), 2.31−2.13 (m, 2H), 2.02−1.95 (m, 2H), 1.90−1.81 (m, 3H), 1.37−1.25 (m, 2H), 0.71−0.66 (m, 2H), 0.39 (dt, J = 4.7, 6.2 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 204.9, 164.3, 162.7, 158.9, 154.0, 153.5, 149.4, 147.3, 138.9, 134.0, 133.4, 133.4, 131.6, 131.6, 128.8, 125.4, 125.0, 121.7, 118.8, 116.8, 116.6, 115.8, 113.6, 98.2, 71.75, 71.70, 71.62, 71.59, 71.58, 71.56, 71.5, 70.9, 70.8, 69.8, 68.7, 62.3, 59.1, 53.5, 52.1, 46.5, 42.4, 34.9, 30.5, 29.7, 27.0, 8.5, 5.5.

*(3-((23-(4-(1-(8-Chloro-3-(cyclopropylmethyl)-[1,2,4]triazolo[4,3-a]pyridin-7-yl)piperidin-4-yl)phenoxy)-3,6,9,12,15,18,21-heptaoxatricosyl)oxy)-2-methoxyphenyl)(1-(4-fluorophenethyl)-piperidin-4-yl)methanone Bis(2,2,2-trifluoroacetate) (**49**).* The title compound was prepared according to general procedure I (3 mg, 0.002 mmol, 9%). MS (ESI) m/z: 1074.7 $[M + H]^+$, 538.0 $[M + 2H]^{2+}$; ^{1}H NMR (600 MHz, MeOD): δ 8.40 (d, J = 7.6 Hz, 1H), 7.34−7.28 (m, 2H), 7.25−7.22 (m, 2H), 7.21−7.18 (m, 2H), 7.12 (t, J = 7.9 Hz, 1H), 7.10−7.02 (m, 3H), 6.91−6.87 (m, 2H), 4.25−4.17 (m, 4H), 4.13−4.09 (m, 2H), 3.95 (s, 3H), 3.91−3.85 (m, 2H), 3.84−3.79 (m, 2H), 3.77−3.67 (m, 6H), 3.67−3.57 (m, 20H), 3.52−3.44 (m, 2H), 3.38−3.32 (m, 3H), 3.18−3.10 (m, 2H), 3.08−3.01 (m, 4H), 2.80 (tt, J = 12.0, 3.9 Hz, 1H), 2.25−2.15 (m, 2H), 2.01−1.95 (m, 2H), 1.94−1.79 (m, 4H), 1.37−1.23 (m, 1H), 0.71−0.63 (m, 2H), 0.38 (dt, J = 6.0, 4.6 Hz, 2H). ^{13}C NMR (151 MHz, MeOD): δ 204.9, 164.3, 162.7, 158.9, 154.0, 153.5, 149.4, 147.4, 139.1, 134.0, 133.40, 133.38, 131.7, 131.6, 128.8, 125.5, 124.5, 121.7, 118.8, 116.8, 116.6, 115.8, 113.0, 98.4, 71.8, 71.73, 71.67, 71.60, 71.59, 71.57, 71.56, 70.9, 70.8, 69.8, 68.7, 62.3, 59.1, 53.5, 52.2, 46.5, 42.5, 35.0, 30.5, 29.7, 27.0, 8.7, 5.4.

*(3-((29-(4-(1-(8-Chloro-3-(cyclopropylmethyl)-[1,2,4]triazolo[4,3-a]pyridin-7-yl)piperidin-4-yl)phenoxy)-3,6,9,12,15,18,21,24,27-nonaoxanonacosyl)oxy)-2-methoxyphenyl)(1-(4-fluorophenethyl)-piperidin-4-yl)methanone Bis(2,2,2-trifluoroacetate) (**50**).* The title compound was prepared according to general procedure I (6 mg, 0.005 mmol, 12%). MS (ESI) m/z: 1162.9 $[M + H]^+$, 582.4 $[M + 2H]^{2+}$; ^{1}H NMR (600 MHz, MeOD): δ 8.47 (d, J = 7.6 Hz, 1H), 7.35−7.28 (m, 3H), 7.24 (dd, J = 8.2, 1.6 Hz, 1H), 7.23−7.17 (m, 2H), 7.13 (t, J = 7.9 Hz, 1H), 7.11−7.03 (m, 3H), 6.93−6.87 (m, 2H), 4.23−4.16 (m, 4H), 4.13−4.08 (m, 2H), 3.95−3.94 (m, 3H), 3.92−3.88 (m, 2H), 3.85−3.80 (m, 2H), 3.74 (d, J = 12.9 Hz, 2H), 3.72−3.68 (m, 4H), 3.67−3.60 (m, 28H), 3.47 (tt, J = 12.0, 3.40 Hz, 1H), 3.37−3.32 (m, 4H), 3.16−3.08 (m, 2H), 3.07−3.04 (m, 4H), 2.75 (tt, J = 12.1, 3.8 Hz, 1H), 2.21 (dd, J = 14.4, 3.6 Hz, 2H), 2.05−1.97 (m, 2H), 1.87 (pd, J = 3.8, 13.2, 14.2 Hz, 4H), 1.32−1.26 (m, 1H), 0.72−0.66 (m, 2H), 0.39 (dt, J = 6.1, 4.6 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 204.9, 164.3, 162.7, 158.9, 154.1, 153.5, 149.4, 149.2, 147.2, 138.9, 134.0, 133.45, 133.43, 131.7, 131.6, 128.8, 125.5, 125.0, 121.7, 118.8, 116.8, 116.6, 115.8, 113.7, 98.8, 71.8, 71.7, 71.65, 71.62, 71.59, 71.54, 71.53, 71.47, 70.9, 70.8, 69.8, 68.7, 62.3, 59.1, 53.5, 52.1, 46.4, 42.4, 34.9, 30.5, 29.7, 27.0, 24.6, 8.5, 5.5.

*(3-((35-(4-(1-(8-Chloro-3-(cyclopropylmethyl)-[1,2,4]triazolo[4,3-a]pyridin-7-yl)piperidin-4-yl)phenoxy)-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontyl)oxy)-2-methoxyphenyl)(1-(4-fluorophenethyl)piperidin-4-yl)methanone Bis(2,2,2-trifluoroacetate) (**51**).* The title compound was prepared according to general procedure I (18 mg, 0.012 mmol, 23%). MS (ESI) m/z: 625.9 $[M + 2H]^{2+}$, 418.0 $[M + 3H]^{3+}$; ^{1}H NMR (600 MHz, MeOD): δ 8.44 (d, J = 7.6 Hz, 1H), 7.33−7.26 (m, 3H), 7.23 (dd, J = 8.2, 1.6 Hz, 1H), 7.22−7.18 (m, 2H), 7.12 (t, J = 7.9 Hz, 1H), 7.10−7.02 (m, 3H), 6.92−6.87 (m, 2H), 4.24−4.18 (m, 2H), 4.17−4.07 (m, 4H), 3.96−3.93 (m, 3H), 3.91−3.87 (m, 2H), 3.85−3.81 (m, 2H), 3.78−3.68 (m, 6H), 3.68−3.55 (m, 34H), 3.56−3.43 (m, 2H), 3.37−3.32 (m, 2H), 3.30−3.27 (m, 2H), 3.20−3.09 (m, 2H), 3.09−3.02 (m, 4H), 2.82 (tt, J = 12.1, 3.8 Hz, 1H), 2.30−2.08 (m, 3H), 2.05−1.95 (m, 2H), 1.95−1.82 (m, 4H), 1.30−1.24 (m, 1H), 0.70−0.65 (m, 2H), 0.38 (dt, J = 6.2, 4.6 Hz, 2H); ^{13}C NMR (151 MHz, MeOD): δ 204.9, 164.3, 162.7, 158.9, 153.55, 153.49, 149.4, 149.1, 147.7, 139.0, 134.0, 133.47, 133.45, 131.7, 131.6, 128.8, 125.4, 124.8, 121.7, 118.8, 116.7, 116.6, 115.8, 113.4, 99.9, 71.8, 71.72, 7166, 71.59, 71.56, 71.55, 71.54, 70.9, 70.8, 69.8, 68.7, 62.3, 59.1, 53.5, 52.2, 46.5, 42.5, 34.9, 30.5, 29.7, 27.0, 8.6, 5.4.

Pharmacology. *Materials.* Culture media, serum, antibiotics, and buffers for cell culture were obtained from Invitrogen (Paisley, UK). The Fluo-4/AM dye and pluronic acid were obtained from Molecular Probes (Eugene, OR). Glu, 5-HT, and probenecid were purchased from Sigma-Aldrich (St. Louis, MO), whereas mianserin, clozapine, **LY 354740**, **LY 341495**, **CBiPES**, and **Ro 62-5229** were obtained from Tocris Cookson (Bristol, UK). [^{3}H]Ketanserin (specific activity: 41.9 Ci/mmol) was obtained from PerkinElmer (Waltham, MA). The h5-HT$_{2A}$-pCDNA3.1neo and Gqo5-pCDNA3neo plasmids were kind gifts from Drs. H. Bräuner-Osborne and B. R. Conklin, respectively. The construction of the stable 5-HT$_{2A}$-HEK293 cell line has been described previously.[56]

Molecular Biology. The complementary deoxyribonucleic acid (cDNA) encoding for the human mGlu$_2$ receptor was cloned from the IMAGE clone 8322671 (Source Bioscience, Nottingham, UK) and subcloned into the human cytomegalovirus (CMV) promoter-associated multicloning site in the bistronic mammalian expression vector pBudCE4.1 (Thermo Fisher Scientific, Waltham, MA) by use of the restriction enzymes *Hind*III and *Xba*I, yielding the mGlu$_2$-pBudCE4.1 construct. The cDNA for the human 5-HT$_{2A}$ receptor was subcloned from 5-HT$_{2A}$-pCDNA3.1neo into the EF-1α promoter-associated multicloning site in mGlu$_2$-pBudCE4.1 by use of the restriction enzymes *Not*I and *Xho*I, yielding the 5-HT$_{2A}$/mGlu$_2$-pBudCE4.1 construct. The integrity and the absence of unwanted mutations in all cDNAs created by the polymerase chain reaction were verified by DNA sequencing (Eurofins MWG Operon, Martinsried, Germany).

Construction and Culture of Stable Cell Lines. Stable HEK293 cell lines coexpressing mGlu$_2$ and Gqo5 (termed mGlu$_2$/Gqo5-HEK293); 5-HT$_{2A}$ and mGlu$_2$ (5-HT$_{2A}$/mGlu$_2$-HEK293); and 5-HT$_{2A}$, mGlu$_2$, and Gqo5 (5-HT$_{2A}$/mGlu$_2$/Gqo5-HEK293) were constructed using the mGlu$_2$-pBudCE4.1, 5-HT$_{2A}$/mGlu$_2$-pBudCE4.1, and Gqo5-pCDNA3neo constructs. HEK293 cells were transfected with various combinations of these plasmids using the PolyFect transfection reagent (Qiagen, Hilden, Germany) and maintained in a culture medium (Dulbecco's modified Eagle's medium-Glutamax-I with penicillin [100 U/mL], streptomycin [100 μg/mL], and 5% dialyzed fetal bovine serum) supplemented with 100 μg/mL zeocin (5-HT$_{2A}$/mGlu$_2$-HEK293), or with 100 μg/mL zeocin and 1 mg/mL G418 (mGlu$_2$/Gqo5-HEK293 and 5-HT$_{2A}$/mGlu$_2$/Gqo5-HEK293). After approximately 3 weeks of culture, antibiotic-resistant colonies were picked up and cultured further with selected antibiotics. Various clones were screened for activity by determination of concentration−response relationships for Glu (mGlu$_2$/Gqo5-HEK293), 5-HT (5-HT$_{2A}$/mGlu$_2$-HEK293), and both Glu and 5-HT (5-HT$_{2A}$/mGlu$_2$/Gqo5-HEK293). The three cell lines and the 5-HT$_{2A}$-HEK293 cell line[56] were cultured under a humidified atmosphere at 37 °C and 5% CO_2 in a culture medium supplemented with 1 mg/mL G-418 (5-HT$_{2A}$-HEK293), with 100 μg/mL zeocin (5-HT$_{2A}$/mGlu$_2$-HEK293) or with 100 μg/mL zeocin and 1 mg/mL G418 (mGlu$_2$/Gqo5-HEK293 and 5-HT$_{2A}$/mGlu$_2$/Gqo5-HEK293).

Ca^{2+}/Fluo-4 Assay. Reference ligands and test compounds were characterized functionally with various cell lines in the fluorescence-based Ca^{2+}/Fluo-4 assay essentially as previously described.[56] Briefly, the cells were split into poly-D-lysine-coated black 96-well plates with a clear bottom (6×10^4 cells/well). The following day, the culture medium was aspirated and the cells were incubated in 50 μL of assay buffer (Hank's Balanced salt solution containing 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2.5 mM probenecid; pH 7.4) supplemented with 6 mM Fluo-4/AM at 37 °C for 1 h. Then, the buffer was aspirated, the cells were washed once with 100 μL of assay buffer, and then 100 μL of assay buffer was added to the cells (in the antagonist experiments, the antagonist was added at this point). The 96-well plate was assayed in a FLEXStation^3 (Molecular Devices, Crawley, UK) measuring emission [in fluorescence units] at 525 nm caused by excitation at 485 nm before and up to 90 s after the addition of 33.3 μL of agonist solution in assay buffer. To characterize the PAM activity of ago-PAMs, the compounds were coapplied with

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the agonist EC_{10}, and the antagonist activity was tested by adding the compounds on the cells in the assay buffer prior to agonist application. The compounds were characterized for various forms of activities in duplicate at least 3 times at the receptors.

[^{3}H]Ketanserin Binding Assay. The 5-HT_{2A} receptor expression levels in 5-HT_{2A}-, 5-HT_{2A}/$mGlu_2$-, and 5-HT_{2A}/$mGlu_2$/Gqo5-HEK293 cells and the binding properties of selected monovalent analogues and all seven bivalent analogues at these cell lines were characterized by the [^{3}H]ketanserin binding assay essentially as previously described.[70] The three cell lines were cultured until ∼90% confluent, scraped into ice-cold assay buffer (50 mM Tris−HCl, 10 mM $MgCl_2$, and 0.1 mM ethylenediaminetetraacetic acid; pH 7.4), homogenized on ice using an IKA T18 Basic Ultra-Turrax for 10 s, and centrifuged for 20 min at 50.000*g* at 4 °C. Cell pellets were resuspended in a fresh assay buffer, homogenized on ice, and centrifuged at 50.000*g* at 4 °C for another 20 min, after which the membranes were stored at −80 °C until use.

On the day of the assay, cell membranes were resuspended in the assay buffer, and protein concentrations were determined using the Bradford protein assay. The membranes were incubated with various concentrations of [^{3}H]ketanserin (determined by scintillation counting) and test compounds in a total assay volume of 225 μL in the wells in the 96-well plate, and the amount of membrane protein added to wells was adjusted so that the bound/free ratio of [^{3}H]ketanserin was always <10%. In the saturation binding experiments, 0.03−20 nM [^{3}H]ketanserin were incubated with cell membranes in the absence (total binding) or presence of 20 μM clozapine (nonspecific binding). In the competition binding experiments, a fixed [^{3}H]ketanserin concentration ∼1.5 nM (1.3−1.8 nM) was incubated with the cell membranes in the presence of various concentrations of test compounds. The reactions were incubated for 2 h at rt while shaking, and binding reactions were terminated by rapid filtration through UniFilter 96-well GF/C plates (PerkinElmer, Waltham, MA) using a FilterMate Harvester (PerkinElmer), followed by four washes of each with ∼300 μL of ice-cold wash buffer (0.9% w/v NaCl and 50 mM Tris−HCl; pH 7.4). Then, the filters were dried at least for 1 h at 50 °C and 25 μL of MicroScint-O (PerkinElmer) was added to each well in the filters, which were then shaken gently overnight. The following day, the amount of bound radioactivity on the filters was determined using a TopCount NXT scintillation counter (PerkinElmer).

Data Analysis. The data from the Ca^{2+}/Fluo-4 assay was analyzed using KaleidaGraph 3.6 (Synergy Software). The concentration−response and concentration−inhibition relationships exhibited by various ligands in the Ca^{2+}/Fluo-4 assay were extracted as the difference in relative fluorescence units (ΔRFU) between the maximum fluorescence level measured after ligand application and the basal level measured before ligand application. Concentration−response curves for compounds tested as agonists and PAMs were fitted to a nonlinear regression curve fit with a variable slope [Response = Bottom + (Top − Bottom)/(1 + $10^{((\log EC_{50}-X)\times n_H)}$)], where the Top and Bottom values are plateaus in units of the response axis, X is the concentration of the ligand, EC_{50} is the concentration of the ligand that gives a response half way between Bottom and Top, and n_H is the Hill slope. Concentration−inhibition curves for compounds tested as antagonists were fitted to a nonlinear regression curve fit with variable slope Response = Bottom + (Top − Bottom)/(1 + $10^{((\log IC_{50}-X)\times n_H)}$), where Top and Bottom values are plateaus in units of the response axis, X is the concentration of the ligand, IC_{50} is the concentration of the ligand that gives a response half way between Bottom and Top, and n_H is the Hill slope.

The data from the [^{3}H]ketanserin binding assay were analyzed using GraphPad Prism 7.0c (GraphPad Software, Inc. La Jolla, CA). Specific binding from the saturation binding experiments was analyzed with a nonlinear regression one site-specific binding model given by the equation: $Y = B_{max} \times X/(K_D + X)$, where Y is the specific [^{3}H]ketanserin binding, B_{max} is the total amount of binding, X is the [^{3}H]ketanserin concentration, and K_D is the dissociation constant. The competition binding data were analyzed with a nonlinear regression one site-fit log IC_{50} model given by the following equation: Y = Bottom + (Top − Bottom)/(1 + $10^{((\log IC_{50}-X)\times n_H)}$), where Y is the specific radioligand binding, Top and Bottom are the plateau values of the curves, X is the test compound concentration, IC_{50} is the equilibrium affinity of the test compound, and n_H is the Hill slope. K_i values were calculated from the determined IC_{50} values using the Cheng−Prusoff equation:[71] $K_i = IC_{50}/(1 - ([RL]/K_D))$, where [RL] is the radioligand concentration used for the specific experiment and K_D is the dissociation constant determined in the saturation binding experiments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01058.

Analytical HPLC traces of all tested compounds; chemical structures of the reference ligands; basal characterization of the mGlu2/Gqo5-HEK293 cell line; and graphs showing the potency/affinity−spacer length correlations (PDF)

Molecular formula strings with pharmacological data (CSV)

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C.B.M.P., medicinal chemistry and manuscript preparation; N.L., medicinal chemistry; A.A.J., pharmacology and manuscript preparation; L.B., medicinal chemistry and manuscript preparation.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

5-HT_{2A}R, serotonin 2A receptor; CNS, central nervous system; DCVC, dry-column vacuum chromatography; HEK, human embryonic kidney; $mGlu_2$R, metabotropic glutamate 2

receptor; PEG, polyethylene glycol; TBDPS, *tert*-butyldiphenylsilyl

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