RESEARCH ARTICLE

View Article Online
View Journal | View Issue Cite this: *Med. Chem. Commun.*,
2017, 8, 647Multivalent ligands for the serotonin 5-HT₄ receptor†‡Federica Castriconi,^a Marco Paolino,^a Alessandro Donati,^a Germano Giuliani,^a Maurizio Anzini,^a Laura Mennuni,^b Chiara Sabatini,^b Marco Lanza,^b Gianfranco Caselli,^b Francesco Makovec,^b Maria Sbraccia,^c Paola Molinari,^c Tommaso Costa^c and Andrea Cappelli^{*a}

5-HT₄ receptors are known to form constitutive dimers in membranes. To explore whether multivalency can enhance ligand interactions and/or efficacy in 5-HT₄ receptors, the structure of the partial agonist ML10302 was modified with oligo(ethylene glycol) chains, thus generating, by a gradual approach, short and long tethered bivalent or tetravalent ligands and the corresponding spanner-linked monovalent controls. Both bivalent and tetravalent ligands displayed a 10–20-fold increase in binding affinity compared to appropriate controls, but no multivalent ligand showed greater binding energy than ML10302 itself. Furthermore, the direct assessment of receptor–Gs interaction and studies of cAMP signalling indicated that multivalency does not enhance the efficacy of ML10302.

Received 8th August 2016,
Accepted 7th February 2017

DOI: 10.1039/c6md00458j

rsc.li/medchemcomm

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter involved in many physiological, behavioural, and cognitive functions.¹ Its physiological effects are mediated by seven receptor subtypes belonging to the G-protein coupled receptor (GPCR) super family, with the exception of the 5-HT₃ receptor, which is a ligand-gated ion channel.² Since its discovery, the 5-HT₄ receptor (5-HT₄R) has catalysed great interest,^{3,4} and has been considered as an interesting target for drug discovery and development. Medicinal chemists from both pharmaceutical companies and academia have produced a large number of 5-HT₄R ligands (representative compounds 1–6 are reported in Fig. 1).⁵ Some 5-HT₄R agonists have entered the market as gastrointestinal drugs used in the therapy of functional bowel illnesses such as constipation, irritable bowel syndrome (IBS), gastroparesis, and gastroesophageal reflux disease (GERD).⁶

Data suggesting that the 5-HT₄R may form constitutive homodimers in plasma membranes⁷ have encouraged the design of new agents that may exploit the close distance between binding sites in a dimeric receptor assembly. For instance, Berque-Bestel and co-workers developed a large series of bivalent 5-HT₄R ligands consisting of two ML10302 (2) pharmacophores linked by spacers of different lengths and attached at the 4-position of the piperidine moiety.^{8,9}

Fig. 1 Structures of some significant serotonin 5-HT₄R ligands.

^a Dipartimento di Biotecnologie, Chimica e Farmacia and European Research Centre for Drug Discovery and Development, Università degli Studi di Siena, Via A. Moro 2, 53100 Siena, Italy. E-mail: andrea.cappelli@unisi.it;

Fax: +39 0577 234333; Tel: +39 0577 234320

^b Rottapharm Biotech S.r.l., Via Valosa di Sopra 3, 20900 Monza, Italy

^c Dipartimento di Farmacologia, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy

† Electronic supplementary information (ESI) available: Experimental details for the synthesis and characterization of target compounds and their intermediates. The experimental procedures used in the biological studies. See DOI: 10.1039/c6md00458j

‡ The authors declare no competing interests.

Consistent with our research on development of 5-HT receptor ligands,^{11–17} we evaluated a series of new multivalent 5-HT_{2R} ligands. Following the guidance of Berque-Bestel *et al.*,^{8,9} we used the same protomeric unit (*i.e.*, 2, tethered at the 4-position of the piperidine moiety) as an active entity, because a partial agonist would facilitate detection of a potential increase of efficacy. However, we adopted a different strategy for spanner design by using long flexible chains of oligo(ethylene glycol) (OEG), as flexibility may be useful under conditions in which there is weak dependence of the free energy of multivalent binding on spacer length.¹¹ Moreover, favourable physico-chemical properties, such as hydrophilicity and flexibility, may help minimize undesirable interactions of the spacer with membrane lipids or hydrophobic pockets of the receptor. Assuming that dimeric receptor forms might be closely packed in the membrane, we also examined both bivalent and tetravalent versions of the ligands. A total of eight ligands (Fig. 2) were designed, including 4 monomeric derivatives (7a (ref. 19)–d) carrying spacers of dif-

Fig. 2 Design of multivalent 5-HT₄R ligands **8a** and **b** and **9** from **2** through monovalent **7a–e**.

ferent sizes, 2 bivalent compounds linked by spacers of different lengths (**8a** with 14 atom and **8b** with 79 atom spacers), and a tetravalent ligand **9** (79 and 84 atom spacers).

2. Results and discussion

Compounds **7a** (ref. 19)–**d**, **8a** and **b**, and **9** were synthesized²⁰ as described in the ESI† and characterized by first evaluating their binding affinity for 5-HT₄R. Apparent binding constants were measured in radioreceptor binding assays using concentration–inhibition curves of the ligands in competition for the binding of radiolabeled [³H]GR113808 ([1-[2-(methylsulfonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole-3-carboxylate) to 5-HT₄R_s in guinea pig striatum membranes.²¹ The specific binding of the reference ligands **1**–**4** was also measured for comparison (Table 1, 1st column).

The urea moiety in position 4 of the piperidine function in **2**, as originally proposed by Berque-Bestel and co-workers,^{8,9} was introduced as the first building block for spanner extension, thus representing the starting point of subsequent development in engineering the multivalent molecules. The simple urea derivative **7b** retained a nanomolar receptor binding constant, indicating that this modification does not interfere with the interaction of ML10302 with the receptor's binding pocket. The binding affinities of **7d** and

Table 1 Interaction of monovalent (**7a–e**), bivalent (**8a** and **b**), and tetravalent (**9**) ligands with 5-HT₄R

Compd	$K_i \pm \text{SEM}^a$ (nM)	Effect on 5-HT ₄ R–G protein coupling	Effect on cAMP production relative to 5-HT $\pm$ SEM
7a	30 $\pm$ 4.0	Partial agonist	0.21 $\pm$ 0.030
7b	13 $\pm$ 2.0	Partial agonist	0.24 $\pm$ 0.029
7c	4.0 $\pm$ 0.4	Partial agonist	0.15 $\pm$ 0.032
7d	61 $\pm$ 7.0	Partial agonist	0.21 $\pm$ 0.032
7e	121 $\pm$ 3.0	Partial agonist	0.18 $\pm$ 0.033
8a	4.0 $\pm$ 1.5	Partial agonist	0.28 $\pm$ 0.033
8b	9.0 $\pm$ 1.0	Partial agonist	0.16 $\pm$ 0.033
9	4.5 $\pm$ 2.5	Partial agonist	0.14 $\pm$ 0.034
1	20 $\pm$ 5.5	Partial agonist	0.58 $\pm$ 0.033
2	6.0 $\pm$ 1.5	Partial agonist	0.20 $\pm$ 0.032
3	3.9 $\pm$ 0.55	Partial agonist	0.21 $\pm$ 0.030
4	19 $\pm$ 3.5	Partial agonist	0.09 $\pm$ 0.030

^a Values are means $\pm$ SEM of 3 independent determinations performed in duplicate and represent the apparent dissociation constant assessed with [³H]GR113808 (final concentration: 0.2 nM) specific binding assays in guinea pig striatum membranes.

7e, both representing monomeric units of ligand **2** tethered to spacers of different sizes, were 10–20-fold lower than those of **2** or **7b**. Thus, the addition of spacers (either 14 or 29 atoms long) can impose a moderate but significant penalty to the interaction of ML10302 with the 5-HT₄R binding site. However, phenyl ring addition at the shorter spacer end in **7c** fully restored the binding affinity to a level comparable to that of **2**. The dissociation constants of the two bivalent ligands with shorter (**8a**) or longer (**8b**) spacers and the tetravalent ligand **9** did not differ, and matched that of **2**.

Both the bivalent ligand **8b** (K_i = 9 nM) and the tetravalent ligand **9** (K_i = 4.5 nM) exhibit a significant enhancement (13–27-fold) of affinity when compared with the corresponding monovalent counterpart **7e** (K_i = 121 nM). This gain in binding energy might suggest that multivalent ligands engage in additional interactions with the proximal binding pockets of oligomeric receptors, thus off-setting the penalty imposed by the linker. However, a similar enhancement of affinity (15-fold) was observed for the bivalent ligand with a shorter linker chain (**8a** vs. **7d**), suggesting that this phenomenon is independent of the maximal distance between pharmacophore units. In addition, as observed for **7c**, a simple aromatic ring was as effective as a second pharmacophoric unit in generating that increase of affinity, implying that the additional interaction may not be site-specific or might involve an accessory site of the same receptor interacting with the aromatic moiety of **2**. Altogether, both observations cast a fair doubt on the idea that the affinity increase of multivalent ligands may be caused by multitopic binding to vicinal orthosteric sites of the receptor dimer.

Regardless of mechanistic considerations, the data show that none of the investigated multivalent compounds gained any significant improvement in binding avidity compared to ML10302. These results are in agreement with previous data based on a much larger series of divalent compounds,^{8,9} but also expand on those results by showing that even a tetravalent ligand does not achieve significant gains in binding energy.

We next examined whether bivalent (**8a** and **b**) and tetravalent (**9**) ligands, despite lacking differences in K_i values, may exhibit differences in receptor activation. We used two different assays to assess the functional properties of these molecules.

First, we measured the effects of the ligands on the direct interaction between 5-HT₄R and G protein in isolated plasma membranes using a cell-free BRET assay. In these studies, the binding of ligand-occupied receptors to G α s subunits produces a decrease in distance between the receptor C-terminal and the N-terminal region of the G $\beta\gamma$ subunit. This is measured as enhanced BRET between the bioluminescent Renilla luciferase (Rluc) donor fused to the C-terminus of human 5-HT₄R and the fluorescent Renilla GFP acceptor tethered at the N-terminus of the G β subunit.^{15,22} Membranes prepared from neuroblastoma cells SH-SY5Y virally co-transduced²² with RGFP-G β_1 and Rluc-5-HT₄R were used for these experiments (Fig. 3). When tested at receptor saturating concentrations, no ligand showed a level of intrinsic activity significantly greater than that of ligand **2**, suggesting no improvements in the efficiency of Gs interaction (Fig. 3a). The bivalent compound **8b** displayed a reduced activation ability, which was also observed, however, in the monovalent congeners **7c** and **7d**, implying that this effect may likely be related to the linked spacers. Since these ligands retained the weak partial agonism of ML10302, we also used this BRET assay to compare the relative ability of a monovalent and a bivalent ligand to inhibit the receptor–Gs interaction induced by the full agonist serotonin (Fig. 3b). Bivalent **8a** and the corresponding spanner-tethered control **7d** diminished the agonist-induced 5-HT₄–Gs association to a lower level in a concentration-dependent manner, reflecting their intrinsic coupling effect, as expected for partial agonism. The “functional” K_i s measured from the curves (Fig. 3b, *legend*), and the enhancement of potency (15-fold) of bivalent vs. monovalent molecules, are in good agreement with the binding data shown in Table 1, suggesting that the partial agonism of the ligand does not interfere with a correct measure of affinity in the radioreceptor assay. In conclusion, direct coupling

Fig. 3 Effect of ligands on 5-HT₄R coupling to Gs measured by BRET in SH-SY5Y cell membranes. **a**) All test ligands and reference compounds (1 cisapride and 2 ML10302) were tested at 10 μ M in comparison with 5-HT (100 μ M). The BRET signal, subtracted from the basal value, is shown as % of the 5-HT effect. Data are means with SEM bars from 6 determinations. * indicates $P < 0.05$ in paired t -tests evaluating the difference from 2. **b**) Concentration-inhibition curves of the receptor-Gs interaction induced by 100 nM 5-HT obtained with the indicated ligands. Data were fitted with a 4-parameter logistic model (solid lines). The IC₅₀ values were 34.3 nM for 7d and 2.1 nM for 8a. Data are means ($n = 3$) of an experiment repeated twice with similar results.

between the receptor and Gs indicates that neither bivalency nor tetravalency can provide the ligand an ability to activate 5-HT₄R to a greater extent than the pharmacophore 2. Thus, the level of agonism in these compounds remains similar to that of ML10302 and significantly lower than that of the stronger partial agonist cisapride (Fig. 3a).

Finally, the pharmacological profile of the newly synthesized ligands was also evaluated as enhancement of cAMP production in cells co-transfected with plasmids encoding human 5-HT₄R and a luminescent biosensor of intracellular cAMP concentration. As summarized in Table 1, these results agree with those of receptor-G protein interactions: all ligands had intrinsic activity values indistinguishable from that of 2. Although the short-spaced bivalent ligand 8a exhibited significantly greater activity (0.28) than the longer-spaced bivalent 8b (0.16) or the tetravalent ligand 9 (0.14) in this assay, such differences are of negligible relevance when compared with the gap in efficacy existing between ML10302 (2) and the higher efficacy agonist cisapride (1). Thus, cAMP assays confirm the notion that multivalency does not provide the ligands any improved ability to trigger Gs-mediated signalling in the receptor.

3. Conclusions

Here, we have re-examined the impact of multivalency on the interaction of ligands with 5-HT₄R. Following a strategy that proved useful in previous studies on 5-HT₃R,¹⁶ we designed a small series of bivalent and tetravalent ligands with properly matched monovalent controls, using a versatile conjugation approach that allows building of variably spaced bi- and multivalent molecules without changing the linker properties. Neither the strength of the ML10302-receptor interaction nor

the ability to activate receptors was significantly altered by assembling this ligand into bivalent or tetravalent architectures. This result agrees with previous work in which a larger series of bivalent ligands was investigated.^{8,9} Considering the existing evidence that 5-HT₄R form dimeric structures in membranes,^{7,10} it is surprising that no bivalent ligand shows the expected increase in binding energy.¹⁸ Based on BRET studies of inter-receptor proximity, some bivalent ligands were proposed to induce a conformational change requiring dual-site occupation of dimeric receptors, even if that double interaction did not result in enhanced affinity.⁹ Yet, this interpretation remains unproven, since no BRET analysis in a homologous dimer can tell if a conformational change is mediated by molecular contacts with a single subunit or both subunits. Whatever mechanism is involved, this and previous studies^{8,9} collectively show that multivalent analogs of 2 have negligible differences in effects on the functional chemistry and biological output of the receptor compared to unaltered 2. This result stands in contrast with our previous studies on 5-HT₃R, where the same synthetic strategy resulted in a tetravalent ligand that was orders of magnitude more potent than the corresponding monovalent ligand.¹⁶ However, 5-HT₃R is a member of the ligand-gated ion channel family in which the multimeric structure, unlike in class A GPCRs, plays a crucial role in function.

Author contributions

Synthesis and preliminary characterization: F. C., M. P., G. G., and M. A.; NMR studies: A. D.; binding assays: L. M., C. S., M. L., and G. C.; functional studies: M. S., P. M., and T. C.; design of experiments, analysis of results, supervision, and writing of the paper: A. C. and F. M.

Acknowledgements

We thank Dr. Laura Salvini (Toscana Life Sciences, Siena) for HRMS measurements, Prof. Maria Cristina Menziani and Prof. Francesca De Rienzo (Università di Modena e Reggio Emilia) for the preliminary molecular modelling experiments, and Prof. Gianluca Giorgi (Università di Siena) for useful suggestions.

Notes and references

- 1 J. Hannon and D. Hoyer, *Behav. Brain Res.*, 2008, **195**, 198.
- 2 D. E. Nichols and C. D. Nichols, *Chem. Rev.*, 2008, **108**, 1614.
- 3 A. Dumuis, M. Sebben and J. Bockaert, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 1989, **340**, 403.
- 4 J. Bockaert, S. Claeyen, V. Compan and A. Dumuis, *Neuropharmacology*, 2008, **55**, 922.
- 5 M. Langlois and R. Fischmeister, *J. Med. Chem.*, 2003, **46**, 319.
- 6 M. J. Lee, S. Choi and W. Im, *Int. J. Gastroenterol. Disord. Ther.*, 2014, **1**, 108.
- 7 M. Berthouze, M. Ayoub, O. Russo, L. Rivail, S. Sicsic, R. Fischmeister, I. Berque-Bestel, R. Jockers and F. Lezoualc'h, *FEBS Lett.*, 2005, **579**, 2973.
- 8 J. L. Soulier, O. Russo, M. Giner, L. Rivail, M. Berthouze, S. Onger, B. Maigret, R. Fischmeister, F. Lezoualc'h, S. Sicsic and I. Berque-Bestel, *J. Med. Chem.*, 2005, **48**, 6220.
- 9 O. Russo, M. Berthouze, M. Giner, J. L. Soulier, L. Rivail, S. Sicsic, F. Lezoualc'h, R. Jockers and I. Berque-Bestel, *J. Med. Chem.*, 2007, **50**, 4482.
- 10 L. P. Pellissier, G. Barthet, F. Gaven, E. Cassier, E. Trinquet, J.-P. Pin, P. Marin, A. Dumuis, J. Bockaert, J.-L. Baneres and S. Claeyen, *J. Biol. Chem.*, 2011, **296**, 9985.
- 11 A. Cappelli, M. Anzini, S. Vomero, L. Mennuni, F. Makovec, M. Hamon, P. G. De Benedetti and M. C. Menziani, *Curr. Top. Med. Chem.*, 2002, **2**, 599.
- 12 A. Cappelli, S. Butini, A. Brizzi, S. Gemma, S. Valenti, G. Giuliani, M. Anzini, L. Mennuni, G. Campiani, V. Brizzi and S. Vomero, *Curr. Top. Med. Chem.*, 2010, **10**, 504.
- 13 A. Cappelli, M. Manini, M. Paolino, A. Gallelli, M. Anzini, L. Mennuni, M. Del Cadia, F. De Rienzo, M. C. Menziani and S. Vomero, *ACS Med. Chem. Lett.*, 2011, **2**, 571.
- 14 A. Cappelli, M. Manini, S. Valenti, F. Castriconi, G. Giuliani, M. Anzini, S. Brogi, S. Butini, S. Gemma, G. Campiani, G. Giorgi, L. Mennuni, M. Lanza, A. Giordani, G. Caselli, O. Letari and F. Makovec, *Eur. J. Med. Chem.*, 2013, **63**, 85.
- 15 F. Castriconi, M. Paolino, G. Giuliani, M. Anzini, G. Campiani, L. Mennuni, C. Sabatini, M. Lanza, G. Caselli, F. De Rienzo, M. C. Menziani, M. Sbraccia, P. Molinari, T. Costa and A. Cappelli, *Eur. J. Med. Chem.*, 2014, **82**, 36.
- 16 M. Paolino, L. Mennuni, G. Giuliani, M. Anzini, M. Lanza, G. Caselli, C. Galimberti, M. C. Menziani, A. Donati and A. Cappelli, *Chem. Commun.*, 2014, **50**, 8582.
- 17 M. Paolino, H. Komber, L. Mennuni, G. Caselli, D. Appelhaus, B. Voit and A. Cappelli, *Biomacromolecules*, 2014, **15**, 3985.
- 18 R. S. Kane, *Langmuir*, 2010, **26**, 8636.
- 19 I. Berque-Bestel, J. L. Soulier, M. Giner, L. Rivail, M. Langlois and S. Sicsic, *J. Med. Chem.*, 2003, **46**, 2606.
- 20 S. Galeazzi, T. Hermans, M. Paolino, M. Anzini, L. Mennuni, A. Giordani, G. Caselli, F. Makovec, E. W. Meijer, S. Vomero and A. Cappelli, *Biomacromolecules*, 2010, **11**, 182.
- 21 C. J. Grossman, G. J. Kilpatrick and K. T. Bunce, *Br. J. Pharmacol.*, 1993, **109**, 618.
- 22 P. Molinari, I. Casella and T. Costa, *Biochem. J.*, 2008, **409**, 251.