Letter

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Fluorescent H_2 Receptor Squaramide-Type Antagonists: Synthesis, Characterization and Applications

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ABSTRACT: Fluorescence labeled ligands have been gaining importance as molecular tools, enabling receptor-ligand-binding studies by various fluorescence-based techniques. Aiming at red-emitting fluorescent ligands for the hH_2R, a series of squaramides labeled with pyridinium or cyanine fluorophores (**19**-**27**), was synthesized and characterized. The highest hH_2R affinities in radioligand competition binding assays were obtained in the case of pyridinium labeled antagonists **19**-**21** (pK_i: 7.71-7.76) and cyanine labeled antagonists **23** and **25** (pK_i: 7.67, 7.11). These fluorescent ligands proved to be useful tools for binding studies (saturation and competition binding as well as kinetic experiments), using confocal microscopy, flow cytometry and high content imaging. Saturation binding experiments revealed pK_d values comparable to the pK_i values. The fluorescent probes **21**, **23** and **25** could be used to localize H_2 receptors in HEK cells and to determine the binding affinities of unlabeled compounds.

KEYWORDS: histamine H_2 receptor, squaramides, fluorescence labeling, flow cytometry, high content imaging, confocal microscopy

The histamine H_2 receptor (H_2R), an aminergic GPCR, is one of the histamine receptor subtypes ($H_{1\text{-}4}R$) which mediate the action of the biogenic amine histamine (**1**). Activation of H_2R results e.g. in gastric acid secretion,[1,2] and positive inotropic and chronotropic effects[3]. In humans, the H_2R is located on parietal cells in the stomach,[2] in the brain,[4,5] on neutrophils and eosinophils[6] as well as on smooth muscle cells[7]. However, the (patho)-physiological role of the H_2R, especially in the brain, is still far from being understood.

During the past few decades, fluorescence labeled GPCR ligands have gained increasing importance as molecular tools for the investigation of ligand-receptor-interactions as they represent a complement or even an attractive alternative to radioligands with respect to waste disposal, safety protocols and costs.[8-11] Various fluorescent ligands for aminergic GPCRs have been reported, for example for muscarinic[12-14], adrenergic[15,16], histamine H_1[17,18] and H_3[19-21] receptors. There are different fluorophore core structures available for labeling[22], e.g. BODIPY, rhodamine, dansyl/NBD or cyanine.[8] Most of the fluorescent ligands reported for the H_2R are emitting at wavelengths below 550 nm.[23,24] The majority of reported fluorescent H_2R ligands consist of a piperidinomethylphenoxypropylamino pharmacophore, derived either from roxatidine (**2**), iodoaminopotentidine (**3**) or BMY 2536 (**4**), which were linked to the fluorophore by an alkyl chain (Figure 1). Labeling with relatively small chromophores such as the *N*-methylanthranilic acid amide or NBD-analogs resulted in fluorescent ligands with high affinity for the H_2R (**5**, **6**: pA_2 or pK_i > 7.0) (Figure 1).[23,24] However, **5** and **6** were inapplicable for cell-based methods e.g. flow cytometry due to the high cellular autofluorescence at the emission wavelength, giving low signal-to-noise ratios.[23,24] In order to expand the range of applications, well characterized fluorescent H_2R ligands labeled with red-emitting fluorophores (emission wavelength >600 nm) are required. First attempts to the development of such ligands were already made by labeling piperidinomethylphenoxypropylamino pharmacophores with the cyanine dye S0436 (**7**[25] and **8**[26] (Note to the paper authored by Petrache *et al.*[26], the ligands in that paper were prepared and pharmacologically characterized in our laboratories. The authors sketchily (compounds were "chemical synthesized"[26]) published very preliminary data without our knowledge and consent in a case of severe scientific misconduct)), BODIPY 650/665 (**9**[25]) or BODIPY 630/650 (**10**[27]) (Figure 1). AB118175, an aminopotentidine derivative labeled with BODIPY 630/650 (chemical structure not disclosed, alleged structure of **10** was deducted by the provided molecular formula ($C_{48}H_{56}BF_2N_9O_4S$)[27]), is the only commercially available fluorescent ligand for H_2R, but the exact chemical structure is elusive and the high costs compromise its appropriateness as a H_2R fluorescent ligand. Additionally, AB118175 and the other red-emitting H_2R fluorescent ligands lack a comprehensive pharmacological characterization (only functional data available, no subtype selectivity)

and the suitability for binding studies by fluorescence-based techniques (e.g. confocal microscopy, flow cytometry) was only partially reported.[25-27] Furthermore, ligands **8** and **9** were reported to have only moderate H_2R activities (pK_b: 6.89[26] and 6.59[25]).

Here, we describe the synthesis and characterization of high affinity fluorescent H_2R antagonists with improved optical and physicochemical properties to gain access to a wide range of potential applications, in particular to confocal microscopy and to high throughput/content imaging.

Replacing the propionic acid amide of the high affinity radioligand [^{3}H]UR-DE257 (**11**) (K_d value: 31 nM)[28], a squaramide derived from BMY 2536 (**4**)[29], by red-emitting fluorophores was the starting point for the development of high affinity fluorescent hH_2R ligands. As the physicochemical properties of the fluorescent labels can considerably effect e.g. non-specific binding or internalization,[30] we chose two different types of red-emitting fluorophores with various electrical charge: the positively charged pyrilium dye (Py-5, **12**) and differently charged cyanine dyes (positive: S0223 (**13a**), neutral: S0436 (**14a**) or negative: S0386 (**15a**), free acids) (Scheme 1).

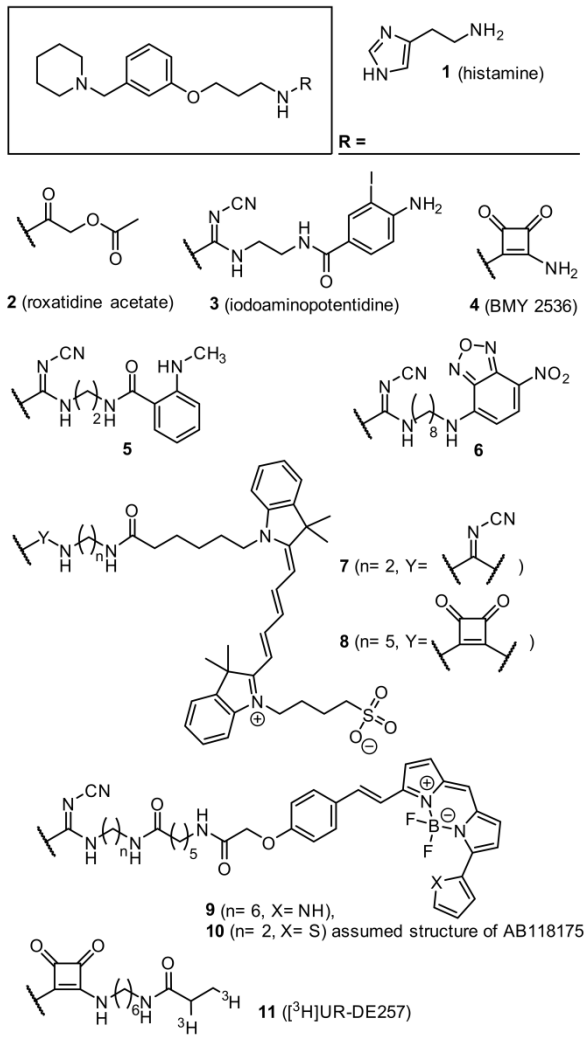


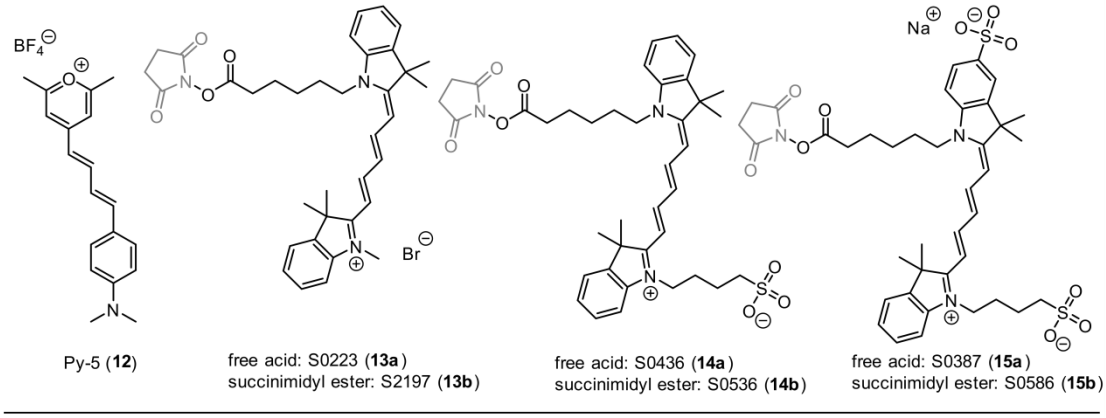
Figure 1. Chemical structures of histamine (**1**), reported H_2R standard antagonists (**2**-**4**), fluorescent H_2R ligands (**5**-**10**) and the radioligand [^{3}H]UR-DE257 (**11**).[5,23-29]

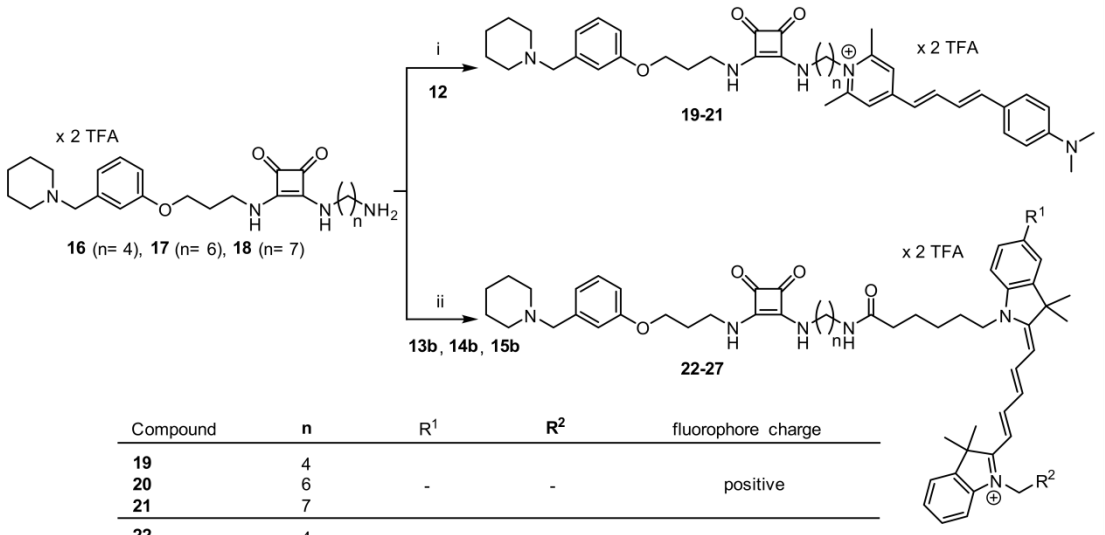
The synthesis of the amine precursors **16**-**18** and BMY 2536 (**4**) according to previously published procedures,[29,31] is described in the supporting information. The Py-5 labeled fluorescent ligands **19**-**21** were synthesized by direct coupling of Py-5 (**12**, chameleon label[32]) with the respective amine precursor **16**-**18** under basic conditions (Scheme 1). The reaction progressed rapidly accompanied by an immediate change in color from blue to red. The cyanine labeled fluorescent ligands **22**-**27** were derived from the amine precursors **16**-**17** by amide coupling using succinimidyl esters of the respective fluorescent dyes (**13b**-**15b**).

The fluorescence quantum yields of representative compounds (**20**, **21**, **23**, **25**, **27**) were determined in PBS at pH 7.4 and PBS containing 1% (w/v) of BSA (Table S1, Figure S61, Supporting Information). For all investigated compounds, fluorescence quantum yields, determined in PBS containing 1% BSA, were higher compared to the quantum yields determined in PBS devoid of protein. Fluorescence is strongly dependent on the environment of the fluorophore; this phenomenon can be explained by intermolecular hydrophobic and electrostatic interactions of the fluorescent ligand with proteins.[33] Additionally, ligands are flexible in solution and become more rigid upon binding, which generally leads to an increase in quantum yield.[33] Therefore, fluorescent ligands are not suitable for the determination of absolute values (e.g. number of specific binding sites B_{max}), but are valuable tools for the determination of dissociation constants (pK_d, and pK_i), which are accessible by measuring relative fluorescence intensities, by analogy with the determination of count rates (cpm/cps) in competition radioligand binding. The Py-5 labeled compounds **20** and **21** showed an excitation maximum at 481 nm and an emission maximum at 646 nm in the presence of BSA. The cyanine labeled compounds **23** and **25** showed an excitation maximum at 663-667 nm and an emission maximum at 672-676 nm in the presence of BSA. Therefore, the Py-5 labeled compounds **19**-**21** can be excited with an argon laser at 488 nm and the cyanine labeled compounds **22**-**27** by a red laser at 635 nm.

The fluorescent ligands **19**-**27**, BMY2536 (**4**) and the amine precursors **16**-**18** were investigated in equilibrium competition binding experiments on membrane preparations from *Sf*9 insect cells expressing the hH_2R-$G_{s\alpha S}$ fusion protein, co-expressing hH_1R and RGS4, or co-expressing either the hH_3R or the hH_4R and $G_{\alpha i2}$ and $G_{\beta 1\gamma 2}$ proteins (Table 1; Table S2, Figures S62 and S63, Supporting Information).[34] Radioligand competition binding experiments revealed that most of the fluorescent labels were tolerated with no or only a slight decrease in affinity. Exceptions were the cyanine labeled ligands **26** and **27,** in which the introduction of the S0387 fluorophore with a negative charge resulted in a decrease in hH_2R affinity (pK_i: **26**, 5.69; **27**, 5.88) compared to the corresponding amine-precursors (pK_i: **16**, 6.52; **17**, 7.87). The Py-5 labeled ligands **19**-**21** showed, irrespective of linker length, high hH_2R affinities (pK_i: 7.71-7.76) in the same range as the parent compound BMY 25368 (**4**) (pK_i: 7.80). In case of **19**, the hH_2R affinity even increased with labeling (pK_i of amine precursor **16**: 6.52, pK_i of **19**: 7.75). In the cyanine dye series, ligand **23**, precursor **17** (n=6, Table 1) labeled with fluorophore S0223 (positive charge), and ligand **25**, precursor **17** (n=6, Table 1) labeled with fluorophore S0436 (no charge), showed the highest hH_2R affinity (pK_i: **23**, 7.67; **25**, 7.11). Interestingly, labeling with Py-5, S0223 and S0436 led to an

Scheme 1. Chemical structures of Py-5 (**12**), the free acids S0223 (**13a**), S0436 (**14a**), S0387 (**15a**) and the succinimidyl esters S2197 (**13b**), S0S0536 (**14b**), S0586 (**15b**), as well as the synthesis of the fluorescent ligands **19**-**27**.





Compound	n	R^1	R^2	fluorophore charge
19	4			
20	6	-	-	positive
21	7			
22	4	H	H	positive
23	6			
24	4	H	$-(CH_2)_3-SO_3^-$	neutral
25	6			
26	4	$-SO_3^-$	$-(CH_2)_3-SO_3^-$	negative
27	6			

Reagents and conditions: i) DMF, TEA or DIPEA, rt, 90-120 min, 24-32%; ii) DMF, DIPEA, rt, 45-90 min, 18-44%.

increase in hH_1R and hH_3R receptor affinity up to two orders of magnitude compared to the corresponding amine precursor (Table S2, Figure S62, Supporting Information). The pyridinium labeled ligands **19**-**21** and the cyanine labeled ligands **23** and **25** showed a slight preference for the hH_2R over the hH_1R and hH_3R. In case of the cyanine labeled ligand **24**, precursor **16** (n=4) labeled with fluorophore S0436 (no charge), the preference changed in favor of the hH_3R. Interestingly, cyanine ligand **22**, precursor **16** (n=4) labeled with fluorophore S0223 (positive charge), showed a high hH_1R affinity (pK_i of 7.88) with a 20-fold selectivity for the hH_1R over the hH_2R. The compounds **19**-**21**, **23** and **25**, which were investigated for hH_4R affinity, showed a 70 to 150-fold selectivity for the hH_2R over hH_4R (Table S2, Figure S63, Supporting Information).

The amine precursors **16**-**18**, BMY 25368 (**4**) and representative fluorescent ligands (**20**, **21**, **23** and **25**) were functionally characterized in a GTPγS binding assay on membrane preparations of Sf9 insect cells expressing the hH_2R-$G_{s\alpha S}$ fusion protein (Table 1; Figure S64, Supporting Information).[33] The investigated compounds proved antagonists with the calculated pK_b values being in a good agreement with the pK_i values from radioligand competition binding.

Furthermore, representative fluorescent ligands (**20**, **21**, **23** and **26**) were investigated for hH_2R agonism in a β-arrestin2 recruitment assay on HEK293T-β-Arr2-hH_2R cells (Figure S65, Supporting Information). None of the investigated ligands showed any β-arrestin2 recruitment, indicating that no β-arrestin2 mediated internalization of the receptor-ligand-complex took place.

The hH_2R binding of the fluorescent ligands **21**, **23** and **25** was also investigated by confocal microscopy (Figure 2). After 20 min of incubation, all investigated ligands were still localized at the cell membrane of HEK293T-hH_2R-qs5 cells.

Table 1. H_2R affinities and antagonistic activities as well as HR selectivity profiles.

compd.	n[a]	Fluorophore	hH_2R					HR selectivity profile
			pK_i[b]	pK_d flow cytom.[c]	pK_d high content imaging[d]		pK_b (pEC_{50})[e]	ratios of K_i H_1R / H_2R / H_3R / H_4R[f]
					automated cell imaging	imaging flow cytometry		
His (**1**)	-	-	6.53 ± 0.04	n.a.	n.a.	n.a.	(5.80 ± 0.06)	- / 1 / 0.5 / 0.1
4	-	-	7.80 ± 0.01[g]	n.a.	n.a.	n.a.	7.03 ± 0.02	- / 1 / 1300 / -
16	4	-	6.52 ± 0.04	n.a.	n.a.	n.a.	5.76 ± 0.22	33 / 1 / 36 / 33
17	6	-	7.87 ± 0.02	n.a.	n.a.	n.a.	6.73 ± 0.08	740 / 1 / 620 / 740
18	7	-	7.86 ± 0.02	n.a.	n.a.	n.a.	7.06 ±0.13	380 / 1 / 550 / 720
19	4	Py-5	7.75 ± 0.02	7.55 ± 0.02	7.13 ± 0.01	7.06 ± 0.03	n.d.	6 / 1 / 20 / 70
20	6	Py-5	7.71 ± 0.04	7.84 ± 0.07	n.d.	n.d.	7.21 ± 0.04	5 / 1 / 4 / 120
21	7	Py-5	7.76 ± 0.01	7.73 ± 0.09	7.05 ± 0.04	7.19 ± 0.03	7.85 ± 0.10	4 / 1 / 6 / 160
22	4	S0223	6.57 ± 0.02	6.54 ± 0.05	6.92 ± 0.06	n.d.	n.d.	1 / 20 / 8 / 180
23	6	S0223	7.67 ± 0.07	7.89 ± 0.07	7.82 ± 0.10	n.d.	7.73 ± 0.04	2 / 1 / 3 / 50
24	4	S0436	6.49 ± 0.04	6.25 ± 0.21	6.77 ± 0.09	n.d.	n.d.	2 / 2 / 1 / -
25	6	S0436	7.11 ± 0.01	7.32 ± 0.02	7.79 ± 0.13	n.d.	6.49 ± 0.03	6 / 1 / 3 / 130
26	4	S0387	5.69 ± 0.08	n.d.	n.d.	n.d.	n.d.	- / 1 / 2 / -
27	6	S0387	5.88 ± 0.09	n.d.	6.35 ± 0.04	n.d.	n.d.	n.d.

[a]Length of the linker given as the number of carbon atoms. [b]Determined by radioligand competition binding with [^{3}H]UR-DE257 (K_d= 12.2 nM, c= 20 nM) at membrane preparations of Sf9 insect cells expressing the hH_2R-$G_{s\alpha S}$ fusion protein; mean values ± SEM from three independent experiments (each performed in triplicate). [c]Determined by flow cytometric saturation binding at HEK293T-hH_2R-qs5 cells; mean values ± SEM from three independent experiments (each performed in duplicate). [d]Determined by high-content imaging saturation binding at HEK293T-hH_2R-qs5 cells; mean values ± SEM from 2-3 experiments (each performed in duplicate). [e]Determined by GTPγS binding assay at membrane preparations of Sf9 insect cells expressing the hH_2R-$G_{s\alpha S}$ fusion protein; pK_b values of neutral antagonists were determined in the antagonist mode versus histamine (c= 1 µM); mean values ± SEM from 2-3 independent experiments (each performed in triplicate). [f] Calculated from the K_i values obtained by conversion of the respective pK_i value (Table S2, Supporting Information). [g]Data was previously reported as K_i value by Baumeister *et al.*[28] and the raw data was re-analyzed to give the pK_i values. [c,d]Non-specific binding was determined in the presence of famotidine (300-fold excess). The incubation period was 60 min (*b*, *d*, *e*, *f*) or 90 min (*c*). n.d.: not determined. n.a.: not applicable.

The ligands **21,** comprising a positively charged pyridinium moiety, and **25,** comprising an electroneutral cyanine moiety, showed low non-specific binding at a concentration of 100 nM in the presence of famotidine (300-fold excess), whereas **23,** comprising a positively charged cyanine moiety, showed higher non-specific binding (Figure 2).

Fluorescent ligands **19**-**25**, which showed moderate to high hH_2R affinity (pK_i >6.0) were used for flow cytometric binding studies at HEK293T-hH_2R-qs5 cells (Table 1; Figure S66-S68, Supporting Information). All investigated ligands afforded pK_d values which were in good agreement with the pK_i values. Within the pyridinium labeled ligands **19**-**21**, non-specific binding was low (<10% of total binding around the K_d). The cyanine labeled ligands **22** and **23** with a positive charge of the fluorophore showed slightly higher non-specific binding (~20%). The introduction of a sulfonic acid group into the cyanine moiety resulting in an uncharged fluorophore (**24**, **25**), had a positive effect regarding non-specific binding (~10%).

The association and dissociation kinetics of **21**, **23** and **25** were determined on HEK293T-hH_2R-qs5 cells at 37 °C using flow cytometry (Figure S68, Table S3, Supporting Information). The fluorescent ligands showed a fast (k_{on} (**21**): 0.0043 min^{-1} nM^{-1}) or moderate (k_{on} (**23**, **25**): 0.0009-0.002 min^{-1} nM^{-1}) association rate and incomplete dissociation. A similar dissociation behavior was reported for the closely related radioligand [^{3}H]UR-DE257.[28] A possible explanation for the (pseudo-)irreversible binding of these ligands is a slow dissociation from the receptor or binding to two different receptor states: a fast reversible and a tight-binding state (for more detailed information, see Supporting Information).[28] The estimated $pK_{d(kin)}$ values of **21**, **23** and **25**, were consistent with the pK_d values determined in saturation binding experiments, suggesting that binding followed at least in part the law of.

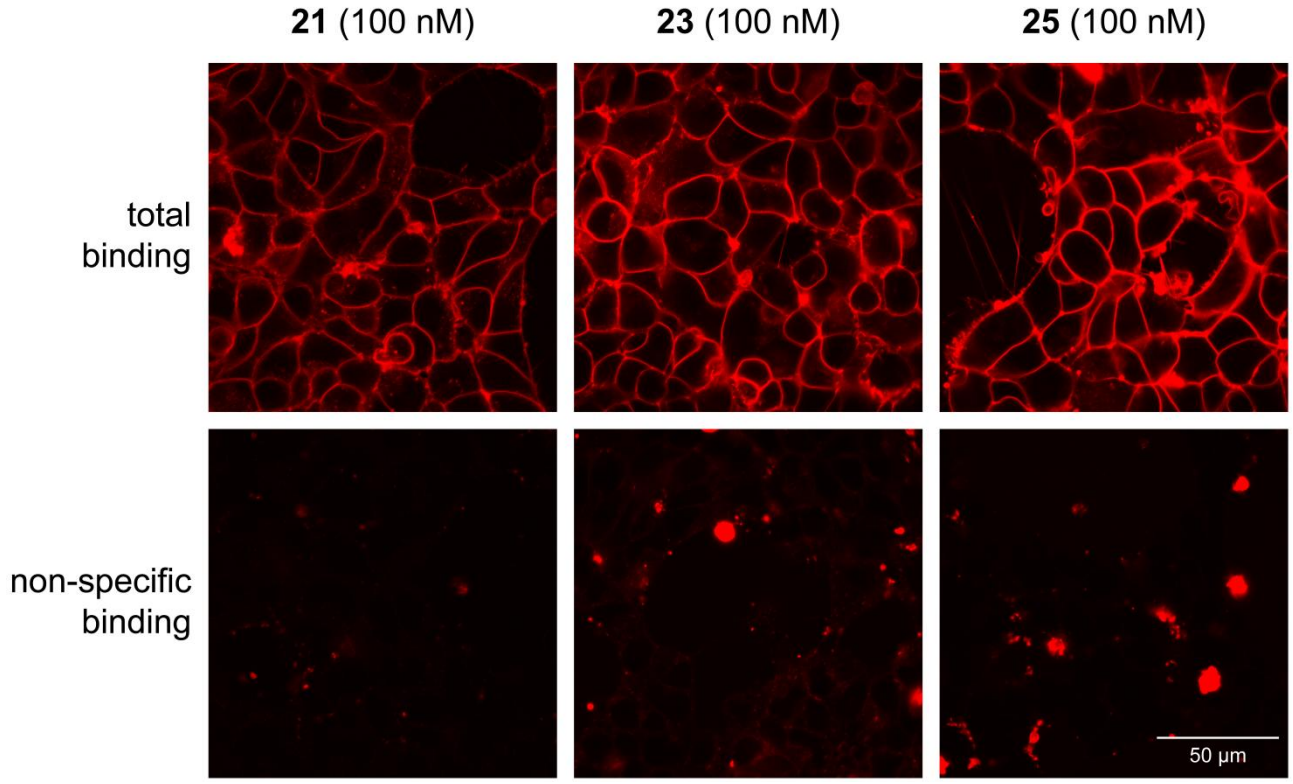


Figure 2. Visualization of binding of the fluorescent ligands **21**, **23** and **25** (all 100 nM) to the membrane of HEK293T-hH_2R-qs5 cells determined by confocal microscopy after 20 min of incubation at rt. Non-specific binding was determined in the presence of famotidine (300-fold excess). Images were acquired with a Zeiss Axiovert 200M microscope equipped with the LSM 510 Laser scanner. A 63x/1.40 oil immersion objective was used.

mass action. The (pseudo-)irreversibility of binding might be suboptimal for e.g. competition binding experiments, but might be advantageous in microscopy or even in in vivo (imaging) experiments The fluorescent ligands **19**, **21**-**25** and **27** were also analyzed by automated cell imaging, enabling measurement of hH_2R binding on live and adherent HEK293T-hH_2R-qs5 cells. Figure 3 shows representative images after incubation of the cells with the fluorescent ligands **21** (250 nM), **23** (75 nM), **25** (75 nM) and **27** (500 nM) at room temperature for 60 min, followed by a washing step. All fluorescent ligands were localized at the cell membrane. The pK_d values determined by saturation binding by automated cell imaging were generally in good agreement with those obtained by flow cytometry (Table 1; Figure S69-S70, Supporting Information).

The background fluorescence (binding to plastics) was low in case of fluorescent ligands with no or with negative charge of the fluorophore (**24**, **25, 27**: <15% of total binding around the K_d), but relatively high, if the ligand contained a fluorophore with positive charge (**21**: 25%; **22**, **23**: 30-40%). An exception was compound **19**, which was also labeled with a positively charge Py-5, but showed a lower background fluorescence than **21** (~12%).

Investigation of the association and dissociation kinetics of the cyanine labeled ligand **25** with automated cell imaging revealed a much faster association (k_{on}: 0.0098 min^{-1} nM^{-1}) compared to flow cytometry, but also an incomplete dissociation (Figure S71-S72, Table S3, Supporting Information). The residual fluorescent ligand was preferentially located at the cell membrane, and there was only very low fluorescence in the cytoplasm.

Results from saturation binding studies with **19** and **21** on suspended HEK293T-hH_2R-qs5 cells using an imaging flow cytometer were in good agreement with the results from automated cell imaging (Table 1; Figure S73-74, Supporting Information). The applicability of fluorescent ligands **21** and **25** for the determination of binding affinities of unlabeled ligands was demonstrated by flow cytometric and high content imaging competition binding assays (a more detailed description is provided in the Supporting information, Figure S75, Table S4).

For none of the investigated fluorescent ligands was a negative influence on cell viability during the incubation periods of the applied assays observed (flow cytometry and high content imaging (Figures S76-S78, Supporting Information).

Taken together, we showed that the presented fluorescent ligands are useful molecular tools for non-radioactive binding studies using different techniques such as confocal microscopy, (imaging) flow cytometry and automated cell imaging. The cyanine dye labeled ligands proved to be useful candidates for in vivo imaging as a high tissue permeability requires excitation and emission wavelengths in the far-red / near-infrared (>650 nm)[35] and the pyridinium labeled ligands **19**-**21** might be suitable molecular tools (BRET acceptors) for Nano-BRET assays[36].

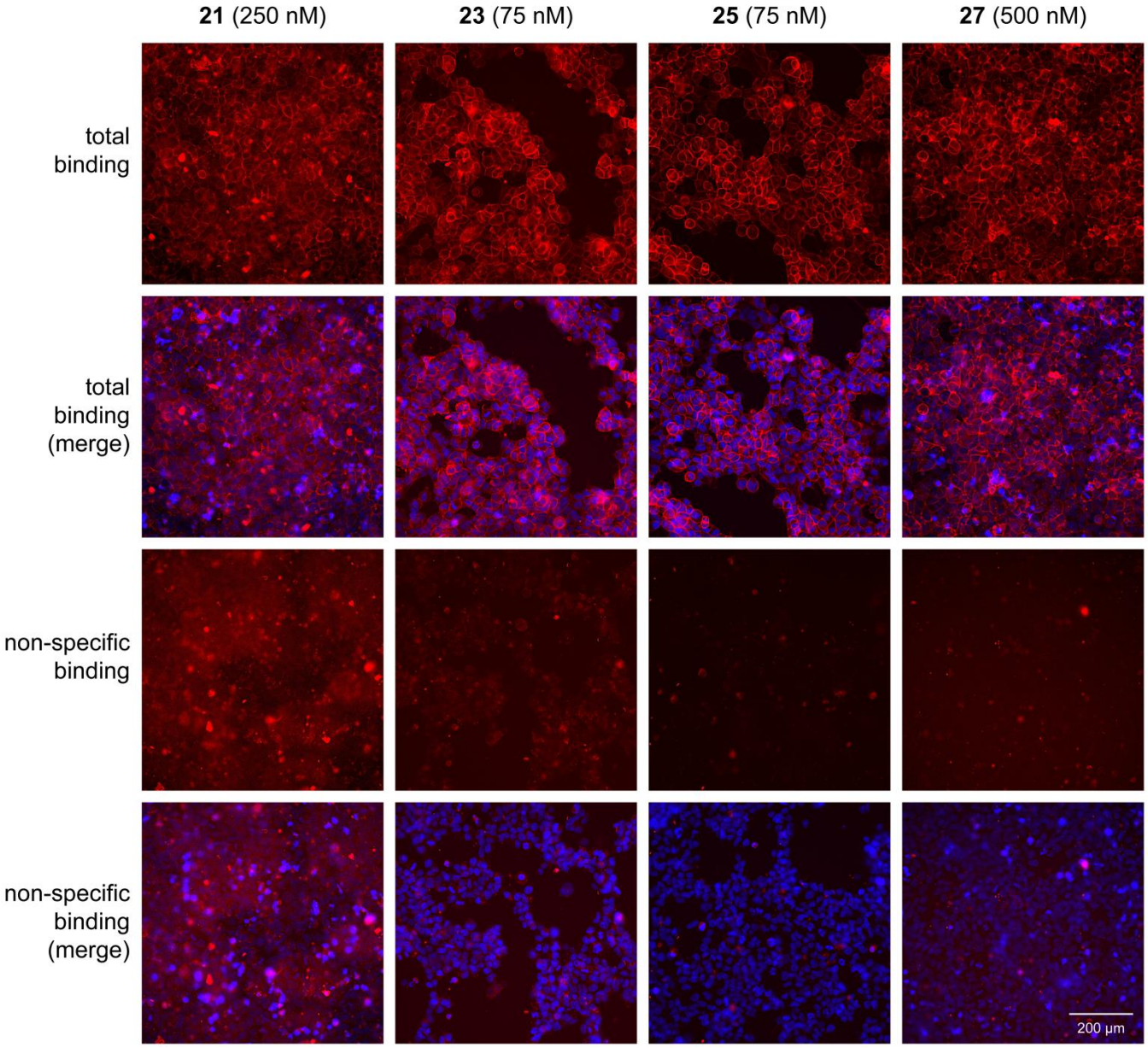


Figure 3. Visualization of binding of the fluorescent ligands **21** (250 nM), **23** (75 nM), **25** (75 nM) and **27** (500 nM) to the membrane of HEK293T-hH_2R-qs5 cells (red) determined by automated cell imaging after 60 min of incubation at rt. Hoechst 33342 was used as a nuclear stain (blue). Fluorescence was mainly associated with the plasma membrane. Non-specific binding was determined in the presence of famotidine (300-fold excess). Images were acquired with a IN Cell Analyzer 2000.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Synthesis of the intermediates **30**-**36**, BMY 25368 (**4**) and the amine precursors **16**-**18** (including Schemes S1 and S2); Experimental section (including Figures S1-S9); ^{1}H and ^{13}C-NMR spectra of **30**-**36**, **4**, **16**-**27** (including Figures S10-S37); HPLC chromatograms of **16**-**27** and BMY 2536 (**4**) (including Figures S38-S40); HRMS spectra of compounds **30**-**36**, **4**, **16**-**27** (including Figures S41-S60), Determination of quantum yields (including Figure S61 and Table S1); Radioligand competition binding studies and GTPγS binding assay (including Figures S62-S64 and Table S2); Investigation of β-arrestin2 recruitment by a split luciferase assay at HEK-hH_2R cells (including Figure S65); Binding studies of **19**-**25** at HEK293T-hH_2R-qs5 cells studied by flow cytometry (including Figures S66-S68 and Table S3); Binding of **19**, **21**-**25** and **27** at HEK293T-hH_2R-qs5 cells studied by automated cell imaging (including Figures S69-S72); Saturation binding of **19** and **21** at HEK293T-hH_2R-qs5 cells studied by imaging flow cytometry (including Figures S73 and S74); Competition binding studies with **21** and **25** (including Figure S75 and Table S4); Rough estimate of cell viability (including Figures S76-S78).

Molecular formula strings (XLSX)

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Author Contributions

S.B., D.E., K.T. and M.C. performed the synthesis. S.B., I.A., T.L., A.F.M. and M.G-L. performed functional and binding studies. M.T. cloned the vector hH_2R-ELucC/ELucN-β-arrestin2 under supervision of T.O.. G.B. and A.B. initiated and planned the project. G.B., M.K., M.L. and A.B. supervised the research. S.B. and G.B. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.
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ABBREVIATIONS

BSA, bovine serum albumin; BRET, bioluminescence energy transfer; cpm/cps, counts per minute/counts per second; DIPEA, diisopropylethylamine; GPCR, G-protein coupled receptor; GTPγS, guanosine 5´-thiotriphosphate; HEK293T, human embryonic kidney 293T; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; PBS, phosphate buffered saline; pK_b, negative logarithm of the dissociation constant obtained from functional assays; pK_d, negative logarithm of the dissociation constant obtained from saturation binding experiments; pK_i, negative logarithm of the dissociation constant obtained from competition binding experiments; Sf9, Spodoptera frugiperda cells, TEA, triethylamine; TFA, trifluoroacetic acid.

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