Abstract

Photoswitchable ligands are promising pharmacological tools to optically modulate receptor signaling with high spatiotemporal precision. Recently, we developed photoswitchable antagonists VUF14738 and VUF14862 that upon photoisomerization from trans to cis increased or decreased their binding affinity for the histamine H3 receptor (H3R), respectively. In the current study, both photoswitchable antagonists were pharmacologically characterized and evaluated for future research applications. Schild analysis revealed that both trans and cis isomers of VUF14738 and VUF14862 act as competitive H3R antagonists. Site-directed mutagenesis combined with molecular modeling provided novel insights in the binding modes of VUF14738 and VUF14862 to the H3R. Based on these experimental data, we suggest that trans-VUF14738 binds in the vicinity of transmembrane (TM) helices 2, TM3, and TM7 using D114 as ionic anchor, while photoisomerization into the high affinity cis photoisomer changes its orientation towards TM5, TM6 and TM7, using E206 as ionic anchor instead of D114. Interestingly, alanine-substitution of Y91 in TM2 inverted the photo-induced affinity shift (PAS), by increasing the binding affinity of trans-VUF14738 but not cis. In contrast, both VUF14862 isomers bind in a similar orientation as VUF14738 by using E206 as ionic anchor. The aromatic interaction of trans-VUF14862 with Y394 is lost upon photoisomerization into cis, resulting in decreased binding affinity of the latter. In addition, PAS was determined for both VUF14738 and VUF14862 on the H3R of commonly used laboratory animals, and successfully validated for VUF14862 in dynamic inhibition of agonist-induced H3R activity in guinea pig ileum contraction. Hence, VUF14738 and VUF14862 are promising compounds to investigate H3R signaling with high spatiotemporal control.
Introduction

Photopharmacology is currently gaining attention with synthetic photoswitchable ligands as attractive tools to modulate cell function by using light of specific wavelengths to dynamically control their pharmacological properties such as binding affinity and/or efficacy, for their specific target [1,2]. Photoswitchable ligands might display less on-target side effects upon systemic administration in comparison to normal (non-photoswitchable) ligands, as localized illumination prevents binding and/or efficacy on their specific target elsewhere in the body [3].

The G protein-coupled receptor (GPCR) family comprises the largest class of drug targets and represent over 30% of prescribed drugs to date [4–6]. Recently, photoswitchable GPCR ligands have been developed by incorporation or extension of an photoactive moiety, predominantly an azobenzene, into known ligand pharmacophores [1,7]. Hitherto, this has resulted in photoswitchable ligands that can shift their affinity and/or efficacy upon photoisomerization between trans- and cis isomer [1,8–11]. Recently, we have reported the photoswitchable human histamine H₃ receptor (hH₃R) antagonists VUF14738 and VUF14862 that upon photoisomerization from trans to cis increased or decreased their binding affinity, respectively (Table 1). Moreover, based on their photo-induced affinity shift (PAS) these photoswitchable ligands dynamically antagonized histamine-induced G protein-coupled inwardly rectifying potassium (GIRK)-channel activation in *Xenopus leavis* oocytes that heterologously co-expressed the hH₃R [12]. The H₃R is predominantly expressed in various brain regions such as the cerebral cortex, striatum, hippocampus and amygdala, and regulates the release of histamine and several other neurotransmitters [13–16]. Consequently, the H₃R has been associated with various central nervous system (CNS) disorders such as, Alzheimer’s and Parkinson’s disease, memory and learning impairments, and sleeping disorders. Recently, the first H₃R drug pitolisant (Wakix®) has obtained clinical approval for the treatment of narcolepsy by the European Medicine Agency and Food and Drug Administration[17,18]. The complex physiological function of the H₃R in various brain regions is still not fully understood and light-mediated spatiotemporal control offered by these photoswitchable ligands might bring opportunities to locally modulate H₃R activity. Indeed, recent studies reported that local illumination of the amygdala rapidly modulated pain-behavior in mice by targeting photoswitchable ligands to metabotropic glutamate receptor 4 and 5 in this specific brain area [19,20].

In this study we further evaluate dynamic modulation of H₃R signaling by photoswitchable H₃R antagonists VUF14738 and VUF14862. Our previously reported hH₃R homology model identified two plausible binding modes for these photoswitchable antagonists in the vicinity of three tyrosine in which the piperidine-moiety anchored via ionic interactions via either D114 in TM3 or E206 in TM5 [12]. In the current study, we further detail the binding mode of these photoswitchable antagonists by utilizing a site-directed mutagenesis approach combined with molecular docking studies and, propose differential binding
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modes for VUF14738 and VUF14862 isomers. In addition, dynamic optical control of the antagonistic behavior of VUF14862 to modulate H₃R signaling is shown in organ tissue (guinea pig ileum) that endogenously expresses the H₃R.

Materials and Methods:

Reagents

N(alpha)-[methyl-³H]histamine ([³H]-NAMH; specific activity: 77.2-80.2 Ci/mmol) was purchased from PerkinElmer (Boston, MA, USA). The cDNA encoding for hH₃R (GenBank accession number AF140538) mutants were synthesized at Biomatik (Wilmington, DE, USA) and subcloned into the mammalian expression vector pcDE3. (R)-alpha-methylhistamine hydrochloride (RAMH) was purchased from Toronto research chemicals (North York, Canada). Immepip, VUF14738 and VUF14862 were synthesized in house as described previously [12,21].

Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells we cultured in Dulbecco’s modified Eagles medium (DMEM) supplement with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. 24 hours prior to transfection 2 million cells per 10 cm² dish were seeded. Cells were transfected with 5 µg cDNA and 20 µg linear 25 kDa polyethyleneimine as described previously [22].

Membrane preparation

For radioligand binding assays, cell homogenates were prepared 48 hours after transfection by collecting the cells in PBS followed by centrifugation at 1900 g for 10 minutes at 4°C. Supernatant was removed and cell pellets were stored at -20°C until the day of experiment. For [³⁵S]-GTPyS assays, cells were collected 48 hours after transfection in PBS and centrifuged at 1300 g at 4°C. Subsequently, supernatant was removed and cells were resuspended in GTPyS membrane buffer (15 mM Tris-HCl, 1 mM EGTA, 0.3 mM EDTA, 2 mM MgCl₂, pH 7.4 at 4°C) and disrupted using a Branson sonifier 250 (Boom bv., Meppel, the Netherlands) for 10 seconds before centrifugation at 1900 g for 30 minutes at 4°C. Cell pellets were resuspended in 20 mM Tris-HCl with 250 mM sucrose (pH 7.4 at 4°C) and aliquots were stored at -80°C until day of experiment. Membrane protein concentrations were measured using the BCA (bicinchoninic acid) method (Pierce, Rockford, USA) [23].

Radioligand binding assays

[³H]-NAMH saturation and competition binding experiments on HEK293T cell homogenates transiently expressing wildtype (WT), mutant hH₃R, mouse (mH₃R, Kᵰ = 3.5
nM, GenBank accession number NM_133849.3), rat (rH3R, K<sub>D</sub> = 6.6 nM, GenBank accession number NM_053506.2), or guinea pig H3R (gpH3R, K<sub>D</sub> = 3.0 nM, GenBank accession number NM_001172737.1) were performed as previously described [11,12]. In brief, for saturation binding experiments H3R expressing cell homogenates (10-50 μg/well) were incubated with increasing concentrations of [3H]-NAMH in absence or presence of 10 μM thioperamide for 2 hours at 25°C. For competition binding assays H3R expressing cell homogenates (10-50 μg/well) were incubated with ~2 nM [3H]-NAMH and increasing concentrations of photoswitchable ligand for 2 hours at 25°C. Incubations were terminated by rapid filtration through 0.5% 750 kDa branched polyethyleneimine-coated GF/C filter with a PerkinElmer 96-well Filtermate-harvester (Perkin Elmer, Groningen, the Netherlands), followed by five washes with ice-cold 50 mM Tris-HCl (pH 7.4 at 4°C). Next, 25 μl Microscint-O scintillation liquid was added to dried filterplates to measure radioactivity with a Microbeta scintillation counter (Perkin Elmer, Groningen, the Netherlands) after a delay of 300 minutes.

[35S]-GTPγS accumulation assay

Cell homogenates (20 μg/well) expressing the hH3R were stimulated with increasing concentrations immepip in the absence or presence of increasing concentration of photoswitchable ligand in assay buffer (50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 4 μM GDP, 0.2 μg saponin, pH 7.4, 25°C) supplemented with ~0.5 nM [35S]-GTPγS for 1 hour at 25°C. Incubation was terminated by rapid filtration over a GF/B filter with a Perkin Elmer Filtermate-harvester, followed by five washes with ice-cold wash buffer (50 mM Tris-HCl, pH 7.4, 4°C). Next, 25 μl Microscint-O scintillation liquid was added to dried filter plates to measure radioactivity with a Microbeta scintillation counter (Perkin Elmer, Groningen, the Netherlands) after a delay of 500 minutes.

Guinea pig ileum contraction assay

All experiments with guinea pigs were performed in compliance with the Polish legislation concerning the protection and welfare of animals used for scientific purposes and followed the university guidelines. All animal procedures have been approved by the Local Ethical Committee for Animal Experiments in Lodz. Male guinea pigs (weighing 250-400 g) were sacrificed by a blow of the neck. The ileum (20-30 cm) was removed and placed in PBS [24], rinsed and cut into 1.5-2.0 cm segments (the terminal 5 cm segment was removed). Ileum segments were mounted isotonically (1 g tension) using a Hugo Sachs Hebel-Messvorsatz (TL-2)/HF-Modem (Hugo Sachs Electronik, Hugstetten, Germany) between two platinum electrodes in an organ bath with 20 ml Krebs buffer (118 mM NaCl, 5.6 mM KCl, 1.18 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1.28 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 5.5 mM glucose, and 10 μM indomethacin), continuously aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, at 36-37°C. The ileum segments were first equilibrated for 60 minutes by refreshing the Krebs-
buffer every 10 minutes, followed by stimulation at 15-20 Volts, continuously at a frequency of 0.1 Hz for 0.5 ms, with rectangular-wave electrical pulses using a Grass stimulator S88 (Grass instruments Co., Quincy, USA). After 10 minutes, 10 μM pyrilamine was added to block the H3R and after another 5 minutes ileum was ready for stimulation with H3R ligands. To study dynamic gpH3R modulation upon photoswitching of VUF14862, the ileum was pre-incubated with 10 nM trans-VUF14862 prior to addition of 100 nM RAMH, followed by alternating illumination of the ileum at 360 ± 20 nm or 434 ± 9 nm using the lamp described in the photochemistry section (see below).

Electrophysiology experiments

Two-electrode voltage clamp (TEVC) experiments to measure hH3R signaling to G protein-coupled inwardly rectifying potassium (GIRK) channels in *Xenopus laevis* oocytes were performed as previously reported [11,12]. Oocytes expressing hH3R WT or hH3R-Y91A were continuously perfused with 1 μM histamine in combination with 1 μM trans-VUF14738, or 10 μM histamine in combination with 0.1 μM trans-VUF14738, respectively, with alternating illumination at 360 ± 20 nm and 434 ± 9 nm.

Photochemistry

The Lambda LS with a 300 W full-spectrum lamp connected to a Lambda 10-3 optical filter changer (Sutter instruments) was used for illumination in all experiments. The light intensity was 0.77 mW/mm² for the 360 ± 20 nm filter and 0.57 mW/mm² for the 434 ± 9 nm filter, as measured using a Thorlabs PM16–401 power meter. Illuminations for all pharmacological experiments were performed in cylindrical clear glass vials with a volume of 4.5 ml. The typical distance between light source and vial was 2 cm. Photoisomerization from trans to cis with 360 ± 20 nm resulted in photostationary state area percentage (PSS) for VUF14738 of >90% cis and VUF14862 reached >96% cis at PSS as determined by LCMS analysis. Hence, when in this paper we refer to the cis isomer of our photoswitchable ligands, we in fact refer to the condition in which the cis isomer has reached its PSS. The light source was positioned at 5 cm from the chamber and organ bath containing the oocyte and guinea pig ileum segment, respectively. In the TEVC experiments, the focused beam of the light source had a diameter of 1.8 cm and was aimed to illuminate the entire oocyte. In the guinea pig ileum contraction assays, the focused beam was aimed to maximally illuminate the organ bath.

Molecular dynamic simulations

The homology hH3R models and initial binding modes were created as previously described [12]. Based on these models the cis- and trans-isomers of VUF14738 and VUF14862 were docked into the homology models but this time the sidechains of Y91, Y94 and Y394 were treated as flexible. The hH3R model of the Y91A mutant was created using
MOE (v2016.0802; Chemical Computing Group, Inc., Montreal, Quebec, Canada) and VUF14738 isomers were subsequently docked into this model using again flexible sidechains for the three aforementioned tyrosines. PLANTS version 1.2 was used for docking (settings: speed 1, scoring function ChemPLP, 5-fold docking, 25 proposed poses per fold) into each of the models [26]. The resulting docking poses were filtered using interaction fingerprint (IFP) on either and ionic and H-bond interaction with D114 or E206 from which the top 5 ChemPLP-scored poses were kept and visually inspected [27].

Data analysis

All data were analyzed using GraphPad Prism 7.03 (GraphPad software inc, San Diego, USA). Data are presented as mean ± SD of at least three independent experiments. The number of independent experimental replicates (n) is indicated in the table and figure legends. Competition binding experiments were fitted using non-linear regression to a one-site binding model and obtained IC$_{50}$ values were converted into pKi values using the Cheng-Prusoff equation [28]. Dose-response curves were fitted using non-linear regression and antagonist affinity values (pA$_{2}$) were determined by Schild-plot analysis using equation 1.

$$\log DR - 1 = \log[B] + pA_{2} \quad (1)$$

DR is the equiactive dose ratio: $[A']/[A]$ were A is de EC$_{50}$ of agonist and $A'$ is de EC$_{50}$ of agonist in the presence of antagonist and [B] is de concentration of antagonist.

Photo-induced affinity shift (PAS) was calculated as the ratio between the lowest and highest affinity between trans- and cis isomers.

Statistical analysis was performed using GraphPad Prism 7.03. A Student’s t test was used to compare differences between the means of two groups and, for multiple comparisons One-Way ANOVA followed by the Holm-Sidak method was used. P<0.05 was considered to be statistically significant.

Results

Competitive antagonism of hH$_{3}$R signaling by VUF14738 and VUF14862

As previously reported, photoswitchable antagonists VUF14738 and VUF14862 display a 12.0-fold increase and an 11.2-fold decrease in binding affinity for the hH$_{3}$R, respectively, upon photoisomerization in competition binding assay (Table 1). In addition, we have previously shown that photoswitchable antagonists VUF14738 and VUF14862 antagonize histamine-induced hH$_{3}$R signaling to GIRK channels in Xenopus laevis oocytes in dynamic manner due to their bidirectional PAS [12]. To confirm that photoswitchable ligands VUF14738 and VUF14862 are competitive hH$_{3}$R antagonists, we measured their effect on
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Table 1. Affinity of cis and trans isomers of photoswitchable antagonists VUF14738 and VUF14862 for the hH3R. Affinities were determined by [3H]-NAMH competition binding assay (pKi) and Schild regression analysis (pA2) in a agonist-induced G protein activation assay as measured by [35S]-GTPγS binding. Affinity data and slopes of Schild regression are reported as mean ± SD of (n) experiments performed in duplicate ([35S]-GTPγS) or triplicate (radioligand competition binding assay).

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<th>Binding</th>
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<tr>
<td></td>
<td>pKi</td>
</tr>
<tr>
<td>trans</td>
<td>6.25 ± 0.32 (6)</td>
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<tr>
<td>PSS cis</td>
<td>7.33 ± 0.32 (6)</td>
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<tr>
<td>PASb</td>
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<td>trans</td>
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<tr>
<td>PSS cis</td>
<td>7.71 ± 0.18 (4)a</td>
</tr>
<tr>
<td>PASb</td>
<td>-11.2</td>
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a pKi determined in radioligand displacement assays as previously reported by Hauwert et al., (2018). b The photo-induced affinity shift (PAS) was calculated as fold difference between the affinity (Ki) values of the trans and cis isomers.

immepip-induced [35S]-GTPγS binding to activated Gαi proteins in hH3R-expressing cells homogenates (Bmax = 1425 ± 878 fmol / mg protein, as determined using [3H]-NAMH binding. The full agonist immepip was used instead of histamine as it displays a 10-fold higher potency in this assay (i.e. pEC50 = 7.80 ± 0.45, n=6, and pEC50 = 6.84 ± 0.22, n=3, respectively). Both trans and cis isomers of VUF14738 and VUF14862 produced concentration-dependent parallel rightward shifts of immepip concentration-response curves in [35S]-GTPγS accumulation to hH3R-expressing cell homogenates (Figure 1 A, B, D, E). Schild-regression analysis of these data yielded pA2 values for the cis and trans isomers of VUF14738 and VUF14862 that are comparable to their pKi values observed in radioligand competition binding assays (Table 1, Figure 1 C, F). Hence, the PAS observed in receptor binding is conserved for VUF14738 and 1.9-fold higher for VUF14862 antagonism of hH3R-induced [35S]-GTPγS accumulation in response to immepip (Table 1). Moreover,
the Schil regression slopes suggest that these ligands competitively antagonized immepip on the hH₃R (Table 1).

![Graphs](image)

Figure 1. VUF14738 and VUF14862 competitively antagonize hH₃R-induced G protein activation in response to immepip. Inhibition of immepip-induced hH₃R signaling by trans-VUF14738 (A), cis-VUF14738 (B), trans-VUF14862 (D), and cis-VUF14862 (E) as determined by [³²P]-GTPγS binding to H₂R-expressing HEK293T cell homogenates. Schil plots of photoswitchable VUF14738 (C) and VUF14862 (F) antagonists. The cis samples were illuminated to their respective photostationary states at 360 ± 20 nm, generally containing >90% cis. Representative concentration-response curves of n≥3 experiments performed in duplicate and pooled Schil plots are shown.

**Differential interaction of VUF14738 and VUF14862 isomers with three tyrosines in the extracellular vestibule**

VUF14738 and VUF14862 only differ in the rigidity and positioning of the linker on the azobenzene-moieity, but show an opposite PAS on the hH₃R upon isomerization from *trans* to *cis* [12]. Previous docking studies in an hH₃R homology model proposed both photoswitchable antagonists VUF14738 and VUF14862 to interact with a triad of tyrosine residues (*i.e.* Y91, Y94 and Y394) in the extracellular vestibule of the hH₃R [12]. To evaluate the role of these tyrosine residues in binding of the two photoswitchable antagonists we mutated these tyrosines individually or in combination to alanine. The Y94A, Y394A, and Y94A-Y394A mutants have comparable affinities as wild-type hH₃R for both the agonist radioligand [³H]NAMH and the natural agonist histamine, while reduced agonist affinities (6.5- to 10-fold) were observed for Y91A, Y91A-Y94A and Y91A-Y394A (Table S1). No specific [³H]-NAMH binding was detected to the triple mutant Y91A-Y94A-Y394A (Table S1).

The Y94A, Y394A, and Y94A-Y394A mutants have comparable affinity for *trans*-VUF14738 as the wild-type hH₃R, while their affinity for *cis*-VUF14738 are reduced (21-, 14-, and 27-fold, respectively) to values that are similar to those of the *trans*-isomer. Consequently, the PAS of the photoswitchable VUF14738 is abolished on these three hH₃R mutants.
Figure 2. Effect of site-directed mutagenesis of tyrosine residues on binding affinity and photo-induced affinity shift (PAS) of VUF14738 (A) and VUF14862 (B) isomers for wild-type (WT) and mutant hH3R. The cis and trans isomers are indicated in magenta and cyan, respectively. Data shown are mean ± SD of at least three experiments performed in triplicate. Significant difference compared to wild-type * P<0.05, *** and p<0.001 as determined by unpaired Students t-test with multiple comparison using the Holm-Sidak method. Full detail on affinity and n-values is reported in Supplemental table 2.

(Figure 2A and Table S2). Interestingly, trans-VUF14738 has a 107-fold higher affinity for the Y91A mutant as compared to wild-type hH3R, while the binding affinity of cis-VUF14738 isomer is not affected by this mutation. Consequently, the PAS of VUF14738 is reversed from a light-induced 12-fold increase in affinity for hH3R upon trans to cis photoisomerization, into a 9.3-fold decrease in affinity on the Y91A mutant (Figure 2A) upon trans to cis photoisomerization.

The double mutant Y91A-Y94A displays similar binding affinities for cis- and trans-VUF14738 as wild-type hH3R, while the double mutant Y91A-Y394A has a 12-fold increased affinity for trans-VUF14738 in combination with a 4-fold reduced affinity for the cis isomer, resulting in a reversed PAS (4.5-fold) as compared to wild-type hH3R (Figure 2A, Table S2). These double mutants suggest that both Y94 and Y394 contribute to the observed increase in affinity for trans-VUF14738 if Y91 is Ala-substituted.

Alanine-substitution of Y91, Y394, and Y91-Y394 had minor effects (<2.5-fold) on binding affinities of both cis- and trans-VUF14862, while Y94A and Y91A-Y94A mutations equally decreased binding affinities of both cis- and trans-VUF14862 (Figure 2B and Table S2).
Nonetheless, the PAS of VUF14862 upon trans to cis isomerization was reduced (<4-fold) for these mutants as compared to wild-type hH3R. Alanine-substitution of Y94 in combination with Y394 did not affect cis-VUF14862 binding but resulted in a 11.5-fold decreased affinity for trans-VUF14862 as compared to wild type hH3R (Figure 2B and Table S2). Consequently, no PAS was observed for trans- and cis-VUF14862 on this double mutant.

*Trans-VUF14738 has a different hH3R binding pose in comparison to cis-VUF14738 and both VUF14862 isomers*

Two possible binding modes for both VUF14738 and VUF14862 were previously predicted in docking studies on an hH3R homology model [12]. In these docking studies the basic nitrogen of the piperidine moiety of both photoswitchable ligands interacted with either D114 or E206 as main ionic anchor point in transmembrane helix (TM) 3 and 5, respectively [12]. To include our site-directed mutagenesis results, we docked the trans and cis isomers of VUF14738 and VUF14862 again in hH3R homology models, but this time keeping Y91, Y94 and Y394 side-chains flexible. In line with our previous model, trans-VUF14738 was ionically anchored to D114 and positioned upwards via TM2 and TM3 along Y91 and Y94 into the extracellular vestibule so that the azobenzene moiety interacts hydrophobically with Y394 in TM7 (Figure 3A, B). In contrast to trans-VUF14738, the affinity of cis-VUF14738 is predominantly affected by Y94A and Y394A but not by the Y91A mutation. Moreover, alanine substitution of Y94 or Y394 reduced the binding affinity for cis similar to the affinity of trans-VUF14738, which suggests that cis-VUF14738 has a different binding pose in the hH3R binding pocket in comparison to the trans isomer.

Docking of cis-VUF14738 into our refined hH3R homology model suggests that the observed interactions with Y94 and Y394 were best explained when cis-VUF14738 is ionically anchored to E206 in TM5 instead of D114 in TM3. In this binding pose, cis-VUF14738 is positioned upwards via TM5, TM6, and TM7, so that one of the azobenzene rings shows aromatic stacking with Y394, while the 3-carboxamide engages into H-bonding with Y94, possibly explaining its higher hH3R affinity compared to its trans isomer. Following these studies we propose that photoisomerization results in reorientation of VUF14738 in the binding pocket from a position via TM2, TM3 and TM7 to a pose via TM5, TM6 and TM7. We hypothesize that the reduced binding affinity of cis-VUF14738 for the Y94A and Y394A mutants disfavors a binding mode via E206, suggesting that cis-VUF14738 interacts with D114 and consequently adopts a binding pose similar to its trans isomer. Indeed, this might explain the similar binding affinities of the isomers on these mutants.

To rationalize the 107-fold gain in affinity of trans-VUF14738 but not cis-VUF14738 for the Y91A mutant, we docked both VUF14738 isomers into a homology model of the hH3R that harbors this mutation. Alanine-substitution of Y91 opens up a sub-pocket underneath Y94, allowing trans-VUF14738 to shift into this region and to engage into π-π stacking with Y94.
Figure 3. Proposed binding modes of the *cis* (magenta sticks) and *trans* (cyan sticks) isomers of VUF14738 (A, B) and VUF14862 (E, F) in an hH3R homology model (yellow cartoon and sticks) based on mutation data and MD simulations. Proposed binding mode of VUF14738 docked in the Y91A (C, D) based on an hH3R homology model. Figures are shown as side (left panels) and top view (right panels). H-bond and π-π stacking interactions are shown as dashed lines, the magenta and cyan lines represent interactions with the *cis* and *trans* isomer, respectively.
and hydrogen bonding with Y394 (Figure 3C, D). These newly acquired interactions might explain the increase in affinity of trans-VUF14738 as alanine-substitution of either Y94 or Y394 in combination with Y91 completely or partially abolished the observed increase in trans-VUF14738 affinity, respectively (Figure 3A, Table S2). Binding of cis-VUF14738 was not affected by the Y91A mutation, which is in line with the proposed different binding mode as compared to trans-VUF14738.

Similar to cis-VUF14738, both VUF14862 isomers were unaffected by Y91A mutation, while affinity was over 10-fold reduced by Y94A, which suggests that VUF14862 adopts a binding pose similar to cis-VUF14738. Indeed, docking suggests that both VUF14862 isomers adopt a similar binding mode as cis-VUF14738 by interacting with E206 in TM5 and extending into the extracellular vestibule via TM 5, TM 6, and TM 7 (Figure 3C, F). For trans-VUF14862 aromatic stacking of the azobenzene moiety with Y394 is observed and this antagonists forms an additional hydrogen-bond via its 3-carboxamide with Y94. In contrast, the azobenzene moiety of cis-VUF14862 is not able to form aromatic interactions with Y394, which might explain its 10-fold lower hH₃R affinity.

**Optical modulation of hH₃R and Y91A by VUF14738 is reversed**

To confirm the observed PAS reversal of VUF14738 on the Y91A mutant upon photoisomerization, optical modulation of histamine-induced GIRK channel activity by this photoswitchable antagonist was measured in *Xenopus laevis* oocytes using Two Electrode Voltage Clamp (TEVC). Histamine has a 16-fold lower potency to induce GIRK channel activation via Y91A (pEC₅₀ = 7.0 ± 0.4) as compared to hH₃R (pEC₅₀ = 8.2 ± 0.2), which corroborates with their difference in affinity for histamine (Table S1 and Figure S1). GIRK channel activation in hH₃R-expressing oocytes that are perfused with 1 µM histamine and 1 µM trans-VUF14738 is more antagonized upon photoisomerization into the higher affinity cis isomer by illumination at 360 nm, which can be dynamically reversed by illumination at 434 nm, as previously described (Hauwert et al., 2018). Oppositely, histamine-induced GIRK channel activation in Y91A mutant-expressing oocytes was less antagonized upon photoisomerization of trans-VUF14738 into the lower affinity cis-VUF14738 by illumination at 360 nm, while illumination at 434 nm restored antagonism of histamine-mediated signaling by trans-VUF14738 (Figure 4).

**Affinity of VUF14738 and VUF14862 isomers for H₃R orthologs**

Next, we determined whether the binding affinities and PAS of the two H₃R photoswitchable antagonists are conserved for various H₃R orthologs. Trans-VUF14738 has a slightly lower (2- to 4-fold) affinity for mouse, rat and guinea pig H₃Rs as compared to hH₃R (Table 2). In contrast, VUF14738 cis has 8.5- to 30-fold lower affinity for these species orthologs. Consequently, ~3- to 4-fold smaller PAS values were observed for VUF14738 on these orthologs in comparison to hH₃R (Table 2). Binding affinities of trans-
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Figure 4. Representative tracing of light-induced modulation of histamine-mediated GIRK activation. A) Modulation of VUF14738 (1µM or 0.1µM) induced antagonism of histamine (1 µM or 10 µM) mediated GIRK activation on the hH₃R (n=3) and Y91A (n=3), respectively, as measured by TEVC using a continuous perfused system.

Table 2. Affinity of VUF14738 and VUF14862 for H₃R orthologs as determined by [³H]-NAMH competition binding assays. Data are mean ± SD of (n) experiments performed in triplicate.

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<tr>
<th>species</th>
<th>pKi&lt;sub&gt;trans&lt;/sub&gt;</th>
<th>pKi&lt;sub&gt;cis&lt;/sub&gt;</th>
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<td>hH₃R</td>
<td>6.25 ± 0.32 (6)</td>
<td>7.33 ± 0.32 (6)</td>
<td>12.0</td>
<td>8.76 ± 0.19 (4)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.71 ±0.18 (4)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-11.2</td>
</tr>
<tr>
<td>mH₃R</td>
<td>5.78 ± 0.15 (3)</td>
<td>6.42 ± 0.09 (3)</td>
<td>4.36</td>
<td>7.55 ± 0.23 (3)</td>
<td>6.76 ± 0.29 (3)</td>
<td>-6.17</td>
</tr>
<tr>
<td>rH₃R</td>
<td>5.64 ± 0.38 (3)</td>
<td>6.13 ± 0.30 (3)</td>
<td>2.82</td>
<td>7.39 ± 0.15 (3)</td>
<td>6.30 ± 0.17 (3)</td>
<td>-12.3</td>
</tr>
<tr>
<td>gpH₃R</td>
<td>5.77 ± 0.03 (3)</td>
<td>6.26 ± 0.04 (4)</td>
<td>3.02</td>
<td>8.24 ± 0.02 (3)</td>
<td>6.97 ±0.11 (3)</td>
<td>-18.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> pKi at photostationary state upon illumination with 360 ± 20 nm at 1mM in DMSO-d₆. <sup>b</sup> Photo-induced affinity shift (PAS) was calculated as fold difference between the affinity (K) values of the trans and cis isomers. <sup>c</sup> pKi values as reported by [12].

and cis-VUF14862 are 9- to 26-fold lower for mH₃R and rH₃R, resulting in a slightly reduced PAS on mH₃R (Table 2). Trans- and cis-VUF14862 have a 3.3- and 5.5-fold lower affinity for gpH₃R as compared to hH₃R, resulting in a 1.6-fold larger PAS on gpH₃R (Figure 5 and Table 2).
Figure 5: Binding of VUF14738 (A, C) and VUF14862 (B, D) isomers to hH3R (A, B) and gpH3R (C, D) transiently expressed in HEK293T cells. Pooled data are shown as mean ± SD of A) n=6, B) n=4, C) n=4 and D) n=3 experiments. [3H]NAMH binding in the absence of antagonist was set at 100% and in presence of 10µM histamine was defined as 0%.

Light-induced H3R modulation of electrically stimulated guinea pig ileum contraction

We next investigated whether our photoswitchable antagonist can optically control agonist-induced H3R-mediated response in an isolated organ in a dynamic manner. Since the affinity and PAS of VUF14862 isomers on the gpH3R best resembled the hH3R as compared to other orthologs (vide supra), the guinea pig ileum was selected to study optical modulation of endogenously expressed H3Rs in a functional assay (Figure 6A). Stimulation of endogenously expressed gpH3R inhibits the electrically-induced contraction of the guinea pig ileal smooth muscle by reducing the release of the neurotransmitter acetylcholine from the myenteric plexus [29]. Indeed, application of the specific H3R agonist RAMH (100 nM) to the organ bath attenuated electrically-induced contraction of ileum within 2 minutes (Figure S2A). This effect of the H3R agonist was fully antagonized within 4 minutes by the addition of 1µM trans-VUF14862 (Figure S2A). Pre-incubation of ileal segments with 10 nM trans-VUF14862 did not affect electrically-induced ileum contractions (Figure 6B), whereas subsequent addition of 100 nM RAMH rapidly reduced the ileum contraction to an intermediate steady-state level due to partial receptor blockade by VUF14862. Photoisomerization of VUF14862 into the lower affinity cis-isomer by in situ illumination at 360 nm increased the RAMH-mediated reduction of ileum...
contraction within 2 min. Subsequent illumination at 434 nm photoisomerizes cis-VUF14862 into the higher-affinity trans isomer returned the RAMH-induced attenuation of ileum contraction back to the intermediate steady-state level within 4.7 minutes. Alternating illumination at 360 and 434 nm to decrease (cis) and increase (trans) the affinity of photoswitchable antagonist VUF14862, respectively, allowed dynamic modulation of RAMH-induced attenuation of ileum contraction by antagonizing the ghH3R. Importantly, illumination at 360 nm or 434 nm did not affect ileum contraction in the absence or presence of RAMH (Figure S4B, C).

Discussion
We previously identified two photoswitchable antagonists, VUF14738 and VUF14862 that gain (12-fold) or loose (11-fold) affinity for the hH3R, respectively, upon illumination at 360 nm (Hauwert et al. 2018). For both photoswitchable ligands, similar PAS values were obtained in functional experiments looking at the competitive antagonism of agonist-induced [35S]-GTPγS accumulation to activated Gi proteins assay in hH3R-expressing HEK-293T cell homogenates.

Intrigued by the opposite PAS of VUF14738 and VUF14862 upon illumination, the previously proposed binding modes of both photoisomers were validated using site-directed mutagenesis [12]. The D114 residue in TM3 is generally seen as a key anchor point for binding of ligands to aminergic GPCRs by forming an ionic interaction with basic amine groups in aminergic ligands [30]. However, in contrast to most other aminergic GPCRs the ligand-binding pocket of H3R contains a second ionic anchor E206 in TM5. Indeed, many H3R ligands typically possess two basic moieties that are considered to interact with both D114 and E206 [30,31]. Molecular docking of VUF14738 and VUF14862 isomers into hH3R homology models suggested that these ligands could interact either with D114 or E206, but initial molecular dynamic (MD) simulations indicated that these ligands interact with D114 [12]. Our mutational data indeed supported this binding mode for VUF14738 trans via D114, but indicated that cis-VUF14738 and both VUF14862 isomers are more likely to form a salt bridge with their piperidine moiety to E206 in TM5. Similar to our photoswitchable antagonists, the piperidine moiety of pitolisant and derived analogues was suggested to interact with E206 rather than D114 [32,33]. Furthermore, E206 was found to best explain binding of dibasic H3R antagonists that lack an imidazole-moiety and, in agreement with our findings, antagonist positioned perpendicular to the membrane or in a L-shape form toward residues in extracellular vestibule [34]. A binding mode similar to cis-VUF14738 has also been suggested for H3R antagonist A349821, which aligned vertically via TM 5, and with aromatic interaction via Y394 towards Y94 [35]. A similar tyrosine cluster was also proposed for binding of photoswitchable muscarinic M1 receptor ligands and suggested to stabilize the ligand via both H-bond and aromatic interactions with the azobenzene [36]. It should be emphasized that elucidation of the binding mode of these photoswitchable ligands remains challenging, as their linear form
and flexibility allows them to possibly adopt a new binding-mode upon mutation of binding site residue(s).

Figure 6: Schematic representation of guinea pig ileum experimental setup with illumination (A). Representative tracings of electrically-induced ileal smooth muscle contraction. The first arrow indicates addition of 10 nM VUF14862 (B, n=3) and the second arrow indicates the addition of 100 nM of (R)-α-methylhistamine (RAMH). The colors behind the trace show applied illumination at 360 ± 20 nm (magenta) and 434 ± 9 nm (cyan).

VUF14738 and VUF14862 isomers show reduced binding affinities for mH3R, rH3R and gpH3R orthologs, despite the fact that several residues involved in the binding of these ligands to hH3R are conserved in the H3R orthologs. Moreover, decreased PAS values were observed for most H3R orthologs, with the exception of VUF14862 on rH3R and gpH3R. Variation in binding affinity has previously already been observed for other H3R ligands, and H3R pharmacology most similar on gpH3R as compared to the other rodent orthologues [37,38]. It is known that residues T119 (A in rat and mouse) and A122 (V in rat mouse and guinea pig) play an important role in H3R species differences [39–41]. The species differences observed with our photoswitchable antagonist illustrate that it is highly recommended to test the affinity of photo-isomers on relevant species orthologs before moving such a photoswitchable tool from a human protein to an ex or in vivo animal model.

Since VUF14862, but not VUF14738, retained its high affinity and photoswitching characteristics at the gpH3R, we evaluated the ability of this photoswitchable antagonist to dynamically inhibit RAMH-induced H3R activity in the electrically-stimulated guinea pig ileum contraction assay. Real-time detection of electrically-induced ileal smooth muscle contraction showed rapid modulation of RAMH-induced attenuation of muscle contraction by alternating illumination of VUF14862 with 360 nm and 430 nm light. This illustrates that photoswitchable H3R ligands facilitate optical control over endogenous receptor activity in guinea pig ileum tissue by modulating the release of neurotransmitter
acetylcholine from the myenteric plexus. Organ experiments were previously applied to illustrate potential of photoswitchable ligands to induce optically control over insulin secretion by beta cells in isolated human or mouse islet [42] and to provide optical control over endogenously expressed metabotropic glutamate receptor 5 activity in brain slices with temporal and spatial resolution [43]. Moreover, photomodulation of endogenous H3Rs by photoswitchable antagonists in isolated organs provides a first indication of their potential to modulate H3R signaling in vivo.

Dynamic optical modulation of VUF14862-mediated antagonism of RAMH-induced gpH3R response in ileal smooth muscle sections revealed slower responses for trans-VUF14862 as compared to its cis isomer despite similar isomerization rates in the organ bath to reach photostationary state (PSS) (Figure S3) and better tissue penetration of 434 nm light. Therefore, we speculate that diffusion of the better aqueous soluble cis isomer into and out of the guinea pig ileum tissue is faster as for the less polar trans isomer, resulting in a more rapid response upon illumination with 360 nm [44].

Dynamic photo-modulation of ileum contraction by our photoswitchable H3R antagonist is promising for future applications, even though tissue penetration is low with the applied wavelengths. Recent advances in photopharmacology have shown the possibility to control cardiac signaling in vivo by targeting the M2 muscarinic acetylcholine receptor (M2R) with photoswitchable ligands using 365 nm and visible light [45]. Moreover, application of the 2-photon excitation of these ligands enabled dynamic control of M2R activity utilizing near infrared light, thereby improving tissue penetration [45]. Moreover, photoswitchable sphingosine 1 phosphate (S1P) receptor ligands, i.e. sphingosine-1-phosphate (photoS1P) and photo-sphingosine, could not only modulate S1P receptor activity but, could also be used to optically regulate sphingolipid metabolism in mice by application of 365 nm and 460 nm light [46]. Hence, photopharmacology might be an improved strategy to obtain dynamic control over neuronal activity via endogenous GPCRs than the commonly used optogenetics that utilizes genetically modified receptors [47]. The H3R functions as auto- and heteroreceptor in the brain [48,49], and spatiotemporal control provided by our photo-tools might bring opportunities to explore the complex consequences of local H3R signaling.

Taken together, photoswitchable antagonists VUF14738 and VUF14862 competitively antagonize the H3R in [35S]-GTPγS accumulation assay while maintaining the affinity shift. VUF14862 shows excellent photopharmacology properties for dynamic H3R modulation in tissue, endogenously expressing H3Rs. Moreover, these photoswitchable antagonists open novel avenue’s to investigate functional consequences of H3R signaling in selective brain regions which could be interesting for unraveling its involvement in H3R associated pathologies such as neuropathic pain and sleeping disorders [50].
Supplementary information

Table S1: Wildtype and mutant human H3R expression levels (B\text{max}) and affinities for [\textsuperscript{3}H]NAMH and histamine (K\text{D} and pK\text{i}). Data shown are mean ± SD (n).

<table>
<thead>
<tr>
<th>mutant</th>
<th>pK\text{D} (nM)</th>
<th>B\text{max} (fmol/mg)</th>
<th>pK\text{i} histamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>8.9 ± 0.1 (5)</td>
<td>1425 ± 878 (3)</td>
<td>7.9 ± 0.2 (7)</td>
</tr>
<tr>
<td>Y91A</td>
<td>7.8 ± 0.1 (3)</td>
<td>822 ± 237 (3)</td>
<td>7.0 ± 0.3 (3)</td>
</tr>
<tr>
<td>Y94A</td>
<td>8.4 ± 0.0 (3)</td>
<td>1352 ± 93 (3)</td>
<td>7.7 ± 0.2 (5)</td>
</tr>
<tr>
<td>Y394A</td>
<td>8.7 ± 0.1 (3)</td>
<td>1475 ± 296 (3)</td>
<td>7.8 ± 0.1 (5)</td>
</tr>
<tr>
<td>Y91A-Y94A</td>
<td>8.1 ± 0.1 (3)</td>
<td>227 ± 15 (3)</td>
<td>7.0 ± 0.1 (3)</td>
</tr>
<tr>
<td>Y91A-Y394A</td>
<td>8.0 ± 0.1 (3)</td>
<td>566 ± 432 (3)</td>
<td>7.1 ± 0.3 (3)</td>
</tr>
<tr>
<td>Y94A-Y394A</td>
<td>8.4 ± 0.1 (3)</td>
<td>544 ± 45 (3)</td>
<td>7.5 ± 0.1 (3)</td>
</tr>
<tr>
<td>Y91A-Y94A-Y394A</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: not applicable
Table S2: Affinity of VUF14738 and VUF14862 on wild-type and mutant hH3R. Data are mean ± SD of at least (n) experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>VUF14738</th>
<th>VUF14862</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pK_i trans</td>
<td>pK_i cis</td>
</tr>
<tr>
<td>Wild-type hH3R</td>
<td>6.25 ± 0.32 (6)</td>
<td>7.33 ± 0.32 (6)</td>
</tr>
<tr>
<td>Y91A</td>
<td>8.28 ± 0.18 (3)</td>
<td>7.31 ± 0.12 (3)</td>
</tr>
<tr>
<td>Y94A</td>
<td>5.89 ± 0.14 (4)</td>
<td>6.01 ± 0.26 (4)</td>
</tr>
<tr>
<td>Y394A</td>
<td>6.07 ± 0.15 (3)</td>
<td>6.18 ± 0.20 (3)</td>
</tr>
<tr>
<td>Y91A-Y94A</td>
<td>6.25 ± 0.26 (3)</td>
<td>7.28 ± 0.30 (3)</td>
</tr>
<tr>
<td>Y91A-Y394A</td>
<td>7.33 ± 0.25 (3)</td>
<td>6.75 ± 0.07 (3)</td>
</tr>
<tr>
<td>Y94A-Y394A</td>
<td>6.02 ± 0.16 (3)</td>
<td>5.90 ± 0.26 (3)</td>
</tr>
</tbody>
</table>

* pK_i at photostationary state, NA: not acquired. Photo-induced affinity shift (PAS) was calculated as fold difference between the affinity (K_i) values of the trans and cis isomers. **pK_i values as reported by [12].

Figure S1: Concentration-response curves of histamine as determine in TEVC on Xenopus laevis oocytes co-expressing GIRK in combination with wildtype-H3R (n=5) or hH3R-Y91264A (n=10). Pooled data are shown as mean ± SD for indicated number of experiments.
Figure S2: Inhibition of guinea pig ileum contraction by endogenous gpH3R signaling in response to 100 nM RAMH stimulation (arrow). A) Addition 1µM VUF14862 trans (second arrow) antagonized RAMH-induced (first arrow) inhibition of guinea pig ileum. Effect of irradiation with 360 (magenta) or 434 nm (cyan) on electrically-induced contraction of ileal smooth muscle B) before or C) after stimulation with 100nM RAMH. Data shown are representative tracings of 2 experiments.

Figure S3: Isomerization of VUF14862 360 ± 20 nm (A) and 434 ± 9 nm (B) in organ bath.
References


Molecular insight in dynamic modulation of H3R signaling by photoswitchable antagonists


