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TITLE PAGE:

Analysis of multiple histamine H_4 receptor compound classes uncovers $G\alpha_i$ and β-arrestin2 biased ligands

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RUNNING TITLE PAGE:

Distinct pluridimensional efficacies of hH_4R ligands

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Abbreviations: GPCR – G protein-coupled receptor; hH_4R – human histamine H_4 receptor; CRE – cyclic AMP response element; PTx – pertussis toxin; ERK – extracellular signal-regulated kinase

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ABSTRACT:

Following the recent description of β-arrestin2 recruitment to the human histamine H_4 receptor (hH_4R) in response to the well-known H_4R antagonist JNJ 7777120 we evaluated in this study the efficacy of 31 known hH_4R ligands to induce $G\alpha_i$ protein signalling and β-arrestin2 recruitment by the hH_4R. The selected hH_4R ligands belong to 9 different structural classes that partly cover (pre-)clinical trial candidates. We have identified hH_4R ligands with a significant bias for the $G\alpha_i$ protein or β-arrestin2 pathway based on efficacy differences. In addition, hH_4R antagonists were found that did not show positive efficacy in either functional readouts. A common trend in pathway preference for the nine different ligand classes could not be observed. In particular, the isothiourea class shows very diverse results, varying from $G\alpha_i$ protein biased or β-arrestin2 biased to non-biased antagonists upon minor structural changes. The identified biased hH_4R ligands are important pharmacological tools to unravel the significance of biased hH_4R signalling in H_4R pharmacology.

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INTRODUCTION:

Since its discovery in 2000, the human histamine H_4 receptor (hH_4R) is the most recent addition to the histamine G protein-coupled receptor (GPCR) subfamily (Liu et al., 2001; Morse et al., 2001; Nakamura et al., 2000; Nguyen et al., 2001; Oda et al., 2000; Zhu et al., 2001). The exact function of hH_4R is still to be elucidated, but its predominant expression in cells of the hematopoietic lineage links this receptor to allergic and inflammatory responses (Leurs et al., 2011; Thurmond et al., 2008). During the last decade, hH_4R signalling has been the topic of many studies, in which $G\alpha_i$ proteins appear to be the key signal transducer in a variety of cell types (Liu et al., 2001; Morse et al., 2001; Oda et al., 2000; Zhu et al., 2001). Activation of the hH_4R leads to inhibition of adenylyl cyclase and a subsequent reduction in cAMP production (Lim et al., 2005; Liu et al., 2001), as well as increases in intracellular calcium levels, actin polymerization and chemotaxis (Baumer et al., 2008; Hofstra et al., 2003; Ling et al., 2004; O'Reilly, 2002). Moreover, in mouse mast cells the H_4R has been shown to signal to kinases such as ERK1/2 and AKT as well as PI3Kγ, leading to the production of IL6 (Desai and Thurmond, 2011). Besides ligand-induced signalling, hH_4R exhibits $G\alpha_i$-mediated signalling in the absence of histamine, i.e. the hH_4R shows constitutive activity (Lim et al., 2005; Schneider et al., 2009). These reported hH_4R activities are all pertussis toxin (PTx) sensitive, indicating $G\alpha_i$-mediated signalling routes. In addition, like many GPCR agonists, histamine stimulation of hH_4R leads to recruitment of β-arrestin2 and downstream ERK phosphorylation (Rosethorne and Charlton, 2011).

Recently, the well-explored reference H_4R antagonist JNJ 7777120 (Thurmond et al., 2004) was described to be the first biased H_4R ligand that selectively recruits β-arrestin2 in a $G\alpha_i$ protein-independent manner, as revealed by an enzyme fragment complementation (EFC) assay (Rosethorne and Charlton, 2011). Moreover, JNJ 7777120 induces ERK phosphorylation in a time–dependent manner typical for β-arrestin2-mediated signalling. In contrast, the inverse agonist thioperamide is not able to recruit β-arrestin2, but antagonizes the β-arrestin2 recruitment by both histamine and JNJ 7777120 (Rosethorne and Charlton, 2011). The ability of GPCR ligands to exhibit biased signalling is an emerging concept that has the potential to improve the efficacy, specificity and reduce the side effects of newly developed drugs (Galandrin et al., 2007; Kenakin, 2007; Rajagopal et al., 2010). Biased ligands that target the angiotensin II type 1 receptor have been shown to antagonize G protein signalling, but to induce β-arrestin2 recruitment as well as downstream phosphorylation events (Violin et al., 2010). *In vivo* this resulted in reduced blood pressure and favourable cardiac functioning, in

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contrast to non-biased ligands that decreased cardiac performance (DeWire and Violin, 2011; Violin et al., 2010).

The biased agonism of JNJ 7777120, together with the incomplete characterization of previous H_4R lead compounds, prompted us to fully explore the extent of ligand-biased signalling at the H_4R. Moreover, the identification of biased tool compounds will help to evaluate the impact of individual signalling pathways in H_4R-mediated (patho)-physiological responses. In recent years, both industry and academia have discovered and characterized many new hH_4R ligands, mainly on the basis of hH_4R binding affinity and specificity (Igel et al., 2009; Jablonowski et al., 2003; Lim et al., 2006; Lim et al., 2005; Liu et al., 2008; Schneider et al., 2010; Smits et al., 2010; Smits et al., 2008; Strakhova et al., 2009; Terzioglu et al., 2004; Thurmond et al., 2004). The therapeutic value of such hH_4R ligands is potentially very interesting for pathologies such as airway inflammation, allergic rhinitis, but also for other inflammatory indications (IBD, atopic dermatitis) (Leurs et al., 2011). Therefore, H_4R antagonists and inverse agonists are rapidly proceeding in preclinical tests and Palau Pharma has tested a first H_4R antagonist (UR-63325) already in clinical Phase II trials (NCT01260753).

In the present study we determined the efficacy of 31 known hH_4R ligands to induce $G\alpha_i$ protein-dependent signalling and β-arrestin2 recruitment. This broad selection of ligands partly covers the lead compounds that are currently tested in pre-clinical studies.

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MATERIALS AND METHODS:

Materials:

U2OS cell culture media were obtained from Gibco, Invitrogen. HEK293T cell culture media were purchased from PAA (Pasching, Austria). Forskolin was purchased from Sigma Aldrich. [^{3}H]-Histamine (10.6-13.4 Ci/mmol) was obtained from Perkin Elmer. Tested H_4R compounds were synthesized at the Medicinal Chemistry department of the VU University Amsterdam, except for UR-PI376 that was a kind gift of prof. dr. A. Buschauer, and histamine and clozapine that were obtained from Sigma-Aldrich.

Methods:

Cell Culture and transfection - PathHunter™ U2OS β-arrestin2:EA cells stably expressing the human histamine H_4 receptor (U2OS-H_4R) (Rosethorne and Charlton, 2011) were cultured in minimum essential media (MEM) containing L-glutamine supplemented with fetal bovine serum (10% v/v), penicillin (100 iu/mL), streptomycin (100 µg/mL), G418 Geneticin (500 µg/mL) and hygromycin (250 µg/mL) at 37□C, 5% CO_2. One day prior to the β-arrestin2 recruitment assay, 10,000 cells/well were seeded in white, clear bottomed 384-well ViewPlates (PerkinElmer, UK) in 20 µl MEM supplemented as described above and incubated at 37°C, 5% CO_2.

HEK293T (human embryonic kidney cells expressing the large T-antigen of simian virus 40) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and sodium pyruvate supplemented with foetal bovine serum (10% v/v), 50 iu/ml penicillin and 50 µg/ml streptomycin.

HEK293T cells were transiently co-transfected with 500ng hH4R/pcDEF3 and 2.5 µg pTNLC1-21 CRE luciferase (containing 21 cAMP-responsive elements upstream of the luciferase gene) by means of the linear polyethyleneimine (PEI, 25 kDa) method. The next day, 50,000 cells/well were transferred to white bottom 96-well plates (Greiner).

β-*arrestin2 recruitment assay* - U2OS-H_4R cells were stimulated with hH_4R ligands or DMSO (1%) for 2 h at 37°C, 5% CO_2 diluted in Hanks balanced salt solution (HBSS) supplemented with 20mM HEPES and 0.1% bovine serum albumin. 25µL Flash detection reagent (DiscoverR_X) was added and cells were further incubated for 15 minutes at room temperature on a table shaker. Luminescence was subsequently measured on the LeadSeeker™ imaging system (GE Healthcare, UK).

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CRE (cAMP-response element) luciferase reporter gene assay - Transiently transfected HEK293T cells were stimulated for 6 h with H_4R ligands or DMSO (1%) in serum-free DMEM supplemented with 1 µM forskolin at 37°C, 5% CO_2. After 6 hours, the stimulation medium was aspirated and 25 µl of luciferase assay reagent (0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM $MgCl_2$, 0.78 µM Na_2HPO_4, 38.9 mM Tris/HCl (pH 7.8), 0.39% glycerol, 0.03% Triton X-100 and 2.6 µM DTT (dithiothreitol)) was added to each well. Luminescence (1s/well) was measured after 30 minutes incubation at 37°C, 5% CO_2 in a Victor^3 1420 multilabel reader (PerkinElmer).

[^{3}H]-Histamine saturation and displacement binding experiments - Two days post transfection, cells were washed once with phosphate-buffered saline (PBS) and subsequently scraped from their culture dish in 1 ml of PBS. Cell pellets were collected by centrifugation at ~2000 g for 10 min at 4°C and dissolved in 50 mM Tris-HCl binding buffer (pH 7.4 at room temperature). The cell suspension was subsequently sonicated for 10 s to obtain a homogenous solution of crude membrane extracts. Both the displacement and saturation binding assays were performed on crude membrane extracts from H_4R transfected cells. Displacement binding was done on crude membrane extracts co-incubated with increasing amounts (10 pM – 100 µM) of H_4R ligand and ~10 nM ^{3}H-histamine in a total volume of 100µl/well. Saturation binding was performed on crude membrane extracts with increasing amounts of ^{3}H-histamine (0-40 nM) in the presence and absence of thioperamide. The reactions were incubated for 1.5hrs at room temperature on a shaking table (750rpm). Bound radioligand was separated from free radioligand via rapid filtration over a 0.5% PEI-pre-soaked glass fiber C plate (GF/C, Perkin Elmer). GF/C plates were subsequently washed three times with ice-cold 50 mM Tris-HCl wash buffer (pH 7.4 at 4°C). The retained radioactivity on the GF/C plates was counted by liquid scintillation counting in a Wallac Microbeta (Perkin Elmer).

Ligand selection - To evaluate the efficacy of structurally diverse hH_4R ligands we selected 31 ligands (K_i < 3 µM) from 9 scaffold classes: (I) histamine analogues (e.g. 4-methylhistamine), (II) triazoles, (III) guanidines (e.g. VUF8430), (IV) isothioureas (e.g. clobenpropit), (V) dibenzodiazepines (e.g. clozapine), (VI) aminopyrimidines, (VII) indolcarboxamides (e.g. JNJ 7777120), (VIII) quinoxalines and (IX) quinazoline sulfonamides (Figure 1; Supplementary Table I & II).

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Operational model calculations - Concentration response curves of both functional assays were fitted to the operational model (Black and Leff, 1983) (variable slope) in GraphPad Prism5. To analyse the data using global fitting, all concentration response curves for each assay were plotted in one graph. Coupling efficiency (τ) was calculated via the formula:

$$E = \frac{E_{max}\ \tau^n\ [A]^n}{([A] + K_i)^n + \tau^n\ [A]^n}$$

The pK_i values that were used to calculate the K_i input values can be found in supplementary table I & II. Subsequently, effective signalling (σ) was calculated with the formula as previously described (Rajagopal et al., 2011):

$$\sigma = log\left(\frac{\tau_{ligand}}{\tau_{histamine}}\right)$$

The σ values from both assays are graphically plotted against each other to visualize the ligand bias (Figure 7a). The ligand bias factor was calculated via the formula:

$$Bias\ factor = \frac{\sigma_{ligand}^{G\alpha i} - \sigma_{ligand}^{\beta-arrestin2}}{\sqrt{2}}$$

Data analysis – Curve fitting and statistical analysis were performed using GraphPad Prism (Version 5.0). Results shown are from pooled data (mean ± SEM) from at least three independent experiments performed in duplicate. Radioligand binding data were fitted to a one-site binding model (competition and saturation binding).

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RESULTS:

$G\alpha_i$ signalling and β-arrestin2 recruitment by the hH_4R – $G\alpha_i$ protein signalling by hH_4R in response to ligand stimulation (1 nM – 100 µM) was measured as CRE reporter gene activity, whereas β-arrestin2 recruitment was detected by β-galactosidase enzyme fragment complementation (DiscoveR$_X$ PathHunter assay). To confirm that observed responses were mediated through hH_4R, the CRE assay was first performed with histamine in the absence or presence of 10 µM selective hH_4R antagonist JNJ 7777120. Histamine inhibited 1 µM forskolin-stimulated CRE activity with a pEC_{50} value of 8.2±0.03 (n=3), which was slightly left shifted compared to its pK_i value of 7.8 (supplementary table II). JNJ 7777120 antagonized this histamine-induced hH_4R-modulation of CRE activity (Figure 2a). Moreover, pre-treatment with the $G\alpha_i$ inhibitor PTx also completely abolished the histamine responsiveness (Figure 2b). Using the PathHunter assay we could confirm that histamine was also able to recruit β-arrestin2 with a pEC_{50} value of 7.3±0.08 (n=3). The signal specificity of the hH_4R in recruiting β-arrestin2 as well as the ineffectiveness of PTx in this assay were previously published (Rosethorne and Charlton, 2011).

Ligand-induced responses were normalized to the observed activity of histamine (agonist in both assays, efficacy 100%) or thioperamide (inverse agonist in CRE assay, efficacy -100%). Consequently, histamine is defined as a non-biased, full agonist in both $G\alpha_i$ protein-dependent signalling and β-arrestin2 recruitment (Figure 3a). Concentration-response curves for the well-characterized hH_4R ligands, histamine, 4-methylhistamine (4-MeHA), VUF8430 and JNJ 7777120, were assessed to compare their potency and efficacy in both assays. Similar to histamine, 4-MeHA was a full agonist in both assays with a comparable 10-fold potency difference between the $G\alpha_i$ protein signalling and β-arrestin2 recruitment (Figure 3b). The potencies of histamine (pEC_{50}=7.3±0.08) and 4-methylhistamine (I) (pEC_{50}=6.9±0.06) for β-arrestin2 recruitment were comparable to the potencies (pEC_{50}=6.9 and 7.0, respectively) described for the Tango assay from Invitrogen (Invitrogen, 2008). In contrast, VUF8430 (III) tended to show a slightly higher efficacy (117%±3.8) toward β-arrestin2 recruitment compared to the $G\alpha_i$-mediated pathways (98%±5.5). Moreover, the potency difference between the two functional responses was larger (~30-fold) as compared to histamine or 4-MeHA (Figure 3c). The indolcarboxamide JNJ 7777120 (VII) was a weak inverse agonist in the employed CRE assay, but acted as a partial agonist (pEC_{50}=7.8±0.04) in the β-arrestin2 recruitment assay as reported previously (Rosethorne and Charlton, 2011) (Figure 3d).

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Upon assessment of the other selected ligands we were able to identify hH_4R ligands that showed a bias toward one of the tested pathways. In particular clobenpropit (Figure 4a), VUF5222 (Figure 4b), VUF10778 (Figure 4c) and VUF10185 (Figure 4d) showed a bias for $G\alpha_i$ protein activation. VUF10185 showed only minor recruitment of β-arrestin2 (27%±12.5), therefore increasing concentrations of VUF10185 were added to antagonize the stimulatory effects of histamine (Figure 4e). Indeed, VUF10185 concentration-dependently right shifted the histamine concentration-response curves. The Schild plot that was constructed from the concentration ratios showed that VUF10185 is a competitive antagonist (pA_2 = 6.8) in the β-arrestin2 recruitment assay (Figure 4f).

Furthermore, in addition to JNJ 7777120, six of the 31 tested ligands (VUF10214, VUF10056, VUF6002, VUF11273, VUF11012 and VUF5223) showed efficacy toward β-arrestin2 recruitment, but possessed neutral or negative efficacy in $G\alpha_i$-dependent signalling (Figure 5, supplementary table I). Inverse agonism was percentualized to the hH_4R-mediated signalling in response to the inverse agonist thioperamide, hence compounds that were able to better inhibit the hH_4R constitutive activity showed efficacy >-100%. Interestingly, VUF10214 displayed a higher efficacy (86%±4.2) toward the β-arrestin2 pathway than the previously identified partial agonist JNJ 7777120 (60%±5.4).

A graphical overview of the relationship between affinity, potency and efficacy values in both functional screens of all tested hH_4R ligands is plotted in Figure 6. All ligands that have positive efficacy in both pathways were more potent (pEC_{50}) to activate $G\alpha_i$ protein-mediated signalling than to recruit β-arrestin2. The correlation between the agonist potencies for both assays was however linear (slope 1.2±0.1) (Figure 6a). The slope for the correlation between affinity versus potency in the β-arrestin2 recruitment or the CRE assay was respectively 1.0±0.1 and 1.2±0.1 (Figure 6b,c). However, no correlation was observed between the efficacies in $G\alpha_i$ protein-dependent signalling and β-arrestin2 recruitment for most ligands (Figure 6d). Interestingly, the majority of compounds had a higher efficacy toward the $G\alpha_i$ signalling pathway than toward the β-arrestin2 recruitment, and are situated in the upper-left corner of the β-arrestin2 versus $G\alpha_i$ protein efficacy plot (Figure 6d). These observed efficacies were not caused by differences in hH_4R expression levels, which were found to be comparable in HEK293T cells (1-3 pmol/mg protein) and U2OS-H4R cells (2.2±0.2 pmol/mg protein) (Rosethorne and Charlton, 2011). Compounds located near the x-axis of the graph show efficacy toward the β-arrestin2 recruitment assay, but do not or negatively affect $G\alpha_i$ protein activation. Hence these compounds are classified as β-arrestin2 biased (Figure 6d).

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Seven compounds induced neither $G\alpha_i$ protein activation nor β-arrestin2 recruitment (thioperamide, VUF9107, VUF11422, VUF10460, VUF10777, VUF10558 and VUF10519), although they have been shown to bind to hH_4R (supplementary table II). Consequently, these ligands are located around the origin of the efficacy plot Figure 6d.

Determination of ligand bias using the operational model for pharmacological agonism - The hH_4R ligands that show efficacy in both assays were further examined for biased signalling using the operational model for pharmacological agonism (Black and Leff, 1983). The coupling efficiency (τ) between each ligand/hH_4R complex and its downstream signalling components as well as the effective signalling (σ) was calculated (formulas are described in materials and methods section). The s values are graphically plotted against each other to visualize the ligand bias (Figure 7a). Ligands that are located at the right side of the unity line show a possible β-arrestin bias, whereas ligands located at the left side of this line show a possible $G\alpha_i$ bias. Finally, the ligand bias factor was calculated (Figure 7b). A positive bias factor indicated that a ligand is $G\alpha_i$ biased, whereas a negative value suggested β-arrestin2-bias (Figure 7b). Using this method we could identify additional $G\alpha_i$-biased ligands (i.e. VUF10192, UR-PI376, VUF4656 and clozapine) as well as a β-arrestin2-biased ligand (i.e. VUF5228), which were not readily apparent from their concentration-response curves in both assays (Figure 7c-k). Surprisingly, earlier identified $G\alpha_i$-biased ligands clobenpropit and VUF10185 did not show up as biased ligands based on these calculations.

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DISCUSSION:

Biased GPCR signalling is an emerging concept that has the potential to improve efficacy, specificity and reduce side effects of newly developed GPCR targeting drugs (Violin et al., 2010). Many hH_4R ligands have been developed in recent years, with the first promising candidates now progressing in preclinical and clinical studies (Leurs et al., 2011). Classically, hH_4R ligands were assessed for their ability to modulate $G\alpha_i$ protein-dependent signalling pathways. However, recently the widely used hH_4R neutral antagonist/inverse agonist JNJ 7777120 was identified as a partial agonist in an EFC-based β-arrestin2 recruitment assay. Moreover, JNJ 7777120 recruited β-arrestin2 in a $G\alpha_i$ protein-independent manner, which subsequently led to the phosphorylation of ERK1/2 (Rosethorne and Charlton, 2011).

In this study we investigated whether JNJ 7777120 is the only ligand displaying this biased agonism or that other hH_4R ligands exhibit this thus far hidden efficacy as well. Therefore, 31 hH_4R ligands of 9 different structural classes were examined for their ability to modulate $G\alpha_i$ protein-independent β-arrestin2 recruitment and $G\alpha_i$ protein-dependent CRE-luciferase activity. We compared ligand potency and efficacy in both pathways and determined if hH_4R ligands were biased toward one of the tested signalling pathways. Based on distinct efficacy values between the two assays, we identified hH_4R ligands that are biased toward the $G\alpha_i$-dependent pathway: two isothioreas (IV) clobenpropit and its analogue (VUF5222) (Istyastono et al., 2011), one aminopyrimidine (VI, VUF10778) and one triazole (II, VUF10185) (Wijtmans et al., 2011). Since the β-arrestin2 recruitment assay is novel for these hH_4R ligands, no comparison can be made with literature data. The potency of clobenpropit (pEC_{50} = 8.3; Efficacy = 132%) in the CRE assay was higher than previously reported by us in a SK-N-MC cell line that stably expressed hH_4R and CRE-β-galactosidase (pEC_{50} = 7.7; Efficacy = 80%) (Lim et al., 2005). On the other hand, the clobenpropit data does fit to literature in which histamine and clobenpropit have comparable efficacies (Buckland et al., 2003; Ling et al., 2004) or where clobenpropit has a higher efficacy than histamine (Gutzmer et al., 2009). The potency and efficacy of VUF10185 were comparable to previous results in HEK293T cells (Wijtmans et al., 2011). Interestingly, VUF10185 was identified as competitive antagonist for the histamine-induced β-arrestin2 recruitment, with a pA_2 value of 6.8 that almost equals its affinity (pK_i = 6.4). Some hH_4R ligands are known to interact with the other histamine receptor subtypes, especially with the $G\alpha_i$-coupled hH_3R (Lim et al., 2005). We therefore performed our CRE assay in the presence of the competitive H_4R antagonist JNJ 7777120 (Lim et al.,

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2005; Lim et al., 2006; Rosethorne and Charlton, 2011). Saturating concentrations (10 μM) of JNJ 777120 inhibited agonist-induced $G\alpha_i$-mediated signalling, confirming that signalling was mediated by hH_4R. Moreover, hH_4R-modulation of CRE activity can be fully inhibited by pre-incubation with PTx, a known inhibitor of $G\alpha_i$ proteins.

The hH_4R ligands that display clear bias towards β-arrestin2 recruitment can be divided in three structural groups: one quinoxaline (VIII, VUF10214) (Smits et al., 2008), one isothiorea (IV, VUF5223) (Istyastono et al., 2011) and four indolcarboxamides (VII, VUF10056, VUF6002, VUF11273, VUF11012). These compounds are neutral antagonists or inverse agonists for $G\alpha_i$ protein-dependent signalling, but act as agonists in the β-arrestin2 recruitment assay. The 26% higher efficacy of VUF10214 compared to partial agonist JNJ 7777120 in this assay makes VUF10214 the most effective hH_4R biased agonist. hH_4R signal specificity in β-arrestin2 recruitment was previously shown via competitive antagonism experiments using the hH_3R/hH_4R antagonist thioperamide (Rosethorne and Charlton, 2011). Furthermore, this EFC-based assay relies on the specific interaction of two enzyme parts and thereby assures signal specificity through the tagged proteins.

The majority of hH_4R ligands were more potent in the CRE assay than in the β-arrestin2 recruitment assay. This discrepancy is most likely the consequence of a stoichiometry difference between the two assays. Where the β-arrestin2 recruitment assay depends on enzyme fragment complementation, CRE-luciferase is a reporter gene downstream of the second messenger cAMP. Consequently, the EFC assay has a 1:1 interaction stoichiometry between hH_4R and the β-arrestin2 protein, and is characterized by a lack of stimulus amplification. Consequently, in this study we found a linear correlation between hH_4R affinities and potencies. In contrast, the CRE assay is subjected to downstream signal amplification and can therefore yield higher potencies compared to the affinity values. Nonetheless, potency values of the tested ligands in both assays correlate in a linear way, indicating that the magnitude of signal amplification in the CRE assay at least is comparable for each ligand. The potential amplification in the CRE assay can cause an increase in efficacy compared to the β-arrestin2 recruitment, however, the lack of correlation between ligand efficacies in β-arrestin2 recruitment and CRE activity suggests that signal amplification in the latter is not causing the discrepancy in efficacies between the two functional readouts.

The hH_4R ligands that did not exhibit a positive efficacy in either the $G\alpha_i$ protein-dependent or -independent assay were the isothioreas (IV) VUF9107 and thioperamide; the aminopyrimidines (VI)

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VUF11422, VUF10460 and VUF10777 as well as the quinazoline sulphonamides (IX) VUF10558 and VUF10519. These ligands are currently considered to be hH_4R antagonists/inverse agonists. However, this is obviously only true for all pathways that have been investigated so far.

The hH_4R compounds that were effective in both assays, but did not show distinct efficacy differences, were further analysed using the 'operational model of pharmacological agonism' (Black and Leff, 1983) and calculation of sigma (Rajagopal et al., 2011; Rivero et al., 2012). Based on our definition that histamine is a full agonist in both assays, histamine and 4-MeHA are non-biased ligands (i.e. situated on unity line). All other tested compounds tend to show a preference for one of the two pathways. Subsequent calculation and analysis of the bias factor (Rajagopal et al., 2011; Rivero et al., 2012) revealed that VUF10192 (II), UR-PI376 (III), VUF5222 (IV), VUF10306 (IV), VUF4656 (IV), clozapine (V) and VUF10778 (VI) are $G\alpha_i$ biased ligands whereas VUF5228 (IV) is a β-arrestin2 biased ligand. Intriguingly, this ligand classification based on the operational model is impossible to identify from the individual concentration-response curves. Yet, clobenpropit and VUF10185, which were first classified as $G\alpha_i$ biased ligands based on the large efficacy difference between both pathways, show up as unbiased ligands based on the bias factor calculation using the operational model. This discrepancy for VUF10185 is probably caused by the relatively large error of one of its τ values, which is the consequence of the marginal efficacy in the β-arrestin2 recruitment assay. The reason for the different outcome that is observed for clobenpropit is not very clear and again emphasizes the caution that should be taken when interpreting ligand bias. A similar observation was reported for the beta 2 adrenergic receptor (β_2AR) and the angiotensin II type 1A. Three different methods for the determination of biased agonism resulted in three different outcomes (Rajagopal et al., 2011). This demonstrates the limitation of the current analysis methods and shows that the ultimate method to plot and calculate biased agonism still has to be identified.

In conclusion, we have tested a series of structurally diverse hH_4R ligands in a $G\alpha_i$ protein-dependent CRE assay and in a $G\alpha_i$ protein-independent β-arrestin2 recruitment assay. The previously reported β-arrestin2 biased agonism of JNJ 7777120 is clearly not limited to one hH_4R ligand, or even one chemical class. We have been able to identify several other β-arrestin2 biased H_4R ligands that cover diverse chemical structures. Additionally, we have demonstrated for the first time that $G\alpha_i$ protein-bias also exists for the hH_4R, with several ligands preferentially activating this pathway over β-arrestin2. This observed bias was not caused by assay differences (e.g. signal amplification, hH_4R expression

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levels) since potency values between both assays correlated linearly and receptor levels were comparable. Moreover, after fitting our data to the operational model of agonism we found additional biased hH_4R ligands. A general tendency in pathway preference could not be observed within the nine different ligand classes, except for the indolcarboxamides (VII i.e. JNJ 7777120 analogues) that all induced β-arrestin2 recruitment, but did not stimulate $G\alpha_i$ signalling, and the quinazoline sulphonamides (IX) that were antagonists in both assays. Particular the isothioureas (IV) were able to give a broad spectrum of efficacies upon minor structural changes, from $G\alpha_i$ protein biased (VUF5222), β-arrestin2 biased (VUF5223) to non-biased antagonist (VUF9107). The consequences of biased GPCR signalling are extensively studied, but not for all receptors completely understood. In the case of the hH_4R pre-clinical data should be analysed with care since biased hH_4R signalling is still an unexplored area that urgently needs clarification. The identified compounds may prove to be valuable tools for further investigations to unravel the contributions of these distinct pathways in H_4R (patho) physiology. Moreover, in light of the proceeding clinical trials and the high therapeutic interest of hH_4R ligands, the discovery of ligand-specific signalling bias might have important therapeutic implications. β-arrestin-biased ligands could be beneficial over a neutral antagonist in case of long-term treatments. Such a β-arrestin-biased ligand would potentially cause H_4R internalization and long-term desensitization without initiating traditional G protein-dependent signaling events.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Nijmeijer, Rosethorne, Vischer, Charlton and Leurs

Conducted experiments: Nijmeijer

Performed data analysis: Nijmeijer and Rosethorne

Wrote or contributed to the writing of the manuscript: Nijmeijer, Rosethorne, Vischer, Charlton and Leurs

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FOOTNOTES

SN, HFV and RL participate in the European COST BM0806

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LEGENDS FOR FIGURES

Figure 1. Selection of hH_4R ligands that belong to nine different chemical classes. I - Histamine analogues, II - Triazoles, III - Guanidines, IV - Isothioureas, V - Dibenzodiazepines, VI - Aminopyrimidines, VII - Indolcarboxamides, VIII - Quinoxalines and IX - Quinazoline sulphonamides.

Figure 2. **Inhibition of ligand-induced hH_4R signalling with JNJ 7777120 and PTx.** hH_4R and CRE-luciferase transfected cells were stimulated with histamine and A) co-incubated with buffer (○) or 10 μM hH_4R antagonist JNJ 7777120 (●). B) co-incubated with buffer (○) or 100 ng/ml PTx (●). Graphs shown are pooled data from of at least two experiments performed in duplicate. Data is normalized to histamine (100% efficacy). Error bars indicate SD values.

Figure 3. Functional responses of selected well-known hH_4R ligands in both CRE-luciferase (●) and β-arrestin2 recruitment (○) assays. Graphs shown are pooled data from at least three experiments performed in duplicate. Data is normalized to histamine (100% efficacy) or thioperamide (-100% efficacy) responses and error bars indicate SEM values. A) histamine B) 4-methylhistamine C) VUF8430 D) JNJ 7777120.

Figure 4. Functional responses of $G\alpha_i$ protein-biased hH_4R ligands in both CRE-luciferase (●) **and β-arrestin2 recruitment** (○) **assays.** Pooled data are shown from at least three experiments performed in duplicate. Data is normalized to histamine responses (100% efficacy) and error bars indicate SEM values. A) clobenpropit B) VUF5222 C) VUF10778 D) VUF10185. Increasing amounts: 0 (○), 316 nM (◇), 1 μM (△), 3.16 μM (□) and 10 μM (▽) of VUF10185 were added to a concentration-response curve of histamine (E) in a β-arrestin2 recruitment assay to finally construct a Schild Plot from the calculated concentration ratios (F).

Figure 5. Functional responses of β-arrestin2-biased hH_4R ligands in both CRE-luciferase (●) **and β-arrestin2 recruitment** (○) **assays.**

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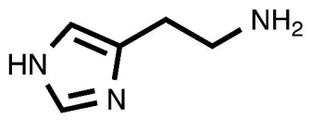
Graphs shown are pooled data from at least three experiments performed in duplicate. Data are normalized to histamine (100% efficacy) or thioperamide (-100% efficacy) responses and error bars indicate SEM values. A) VUF10214 B) VUF10056 C) VUF6002 D) VUF5223

Figure 6. Correlation plots between affinity, potency and efficacy values for all tested hH_4R compounds. Efficacies in each assay were normalized to histamine (efficacy 100%) or thioperamide (-100% in CRE assay) responses. A) Potency values between different assays show a linear relation. B) linear correlation between potency in β-arrestin2 recruitment assay and affinity values. C) linear correlation between potency in CRE reporter gene assay and affinity values. D) Comparison of efficacy values between different assays shows that few ligands display equal efficacy toward the two pathways. Dotted lines in panels A, B and C represent regression lines, the continuous line in panel D is the unity line. Data are mean +/- SEM values of at least three experiments performed in duplicate. pK_i values were previously published or determined via a heterologous competition binding experiment.

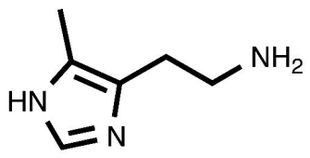
Figure 7. Evaluation of hH_4R ligand bias: operational model versus efficacy comparisons. A) Effective signalling (σ) was calculated using τ values obtained from an operational model fit of pooled data. Error bars represent SEM values. Ligands shown are: ✳ histamine; ⊗ VUF4656; O 4-MeHA; ☐ VUF8430; ◇ VUF10192; ⟐ VUF10306; △ VUF6884; ☒ Clozapine; ⊙ UR-PI376; ▽ VUF4704; ★ VUF8328 and **X** VUF5228; ■ Clobenpropit; ● VUF10185; ▲ VUF5222; ▼ VUF10778. B) Bar graph of Bias factor. Positive values indicate a possible $G\alpha_i$ protein bias, negative values indicate a possible β-arrestin2 bias. Statistical differences were determined using an unpaired two tailed t-test (* $p<0.05$; ** $p<0.01$). C-H) CRE-luciferase (●) and β-arrestin2 recruitment (○) assay data for possible $G\alpha_i$-biased compounds based on operational model fit. I-K) CRE-luciferase (●) and β-arrestin2 recruitment (○) assay data for possible β-arrestin2-biased compounds based on operational model fit.

FIGURE 1

I

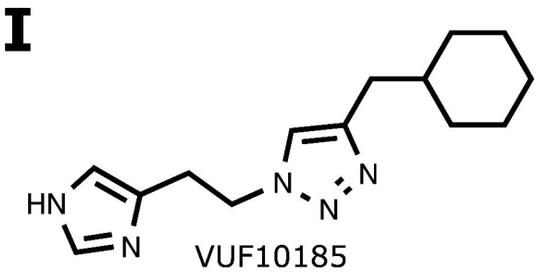


Histamine

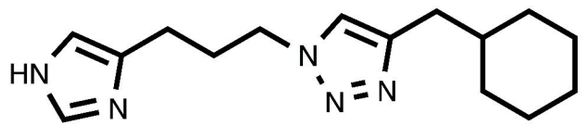


4-Methylhistamine

II



VUF10185



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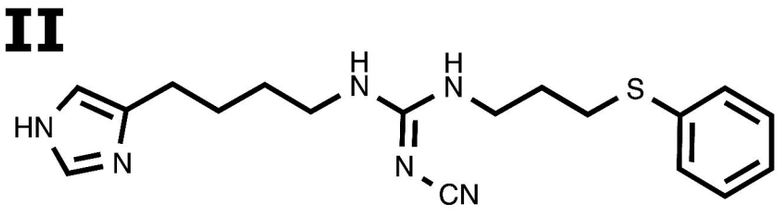
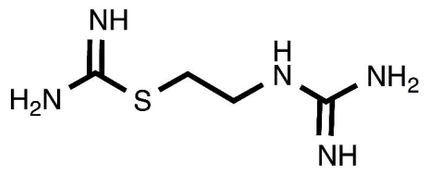
III



UR-PI376



VUF8430

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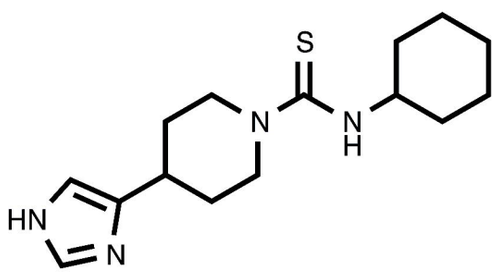
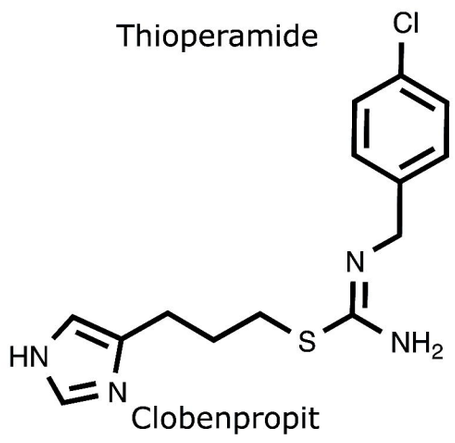
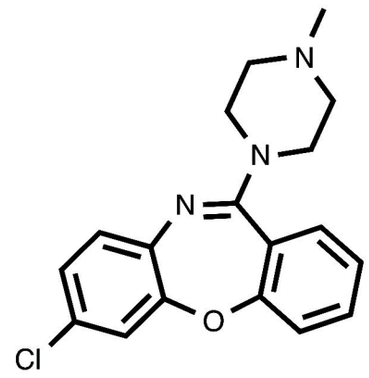


Thioperamide



Clobenpropit

V



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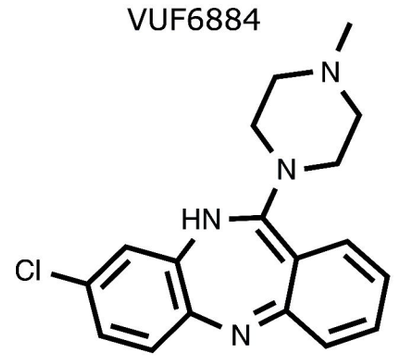
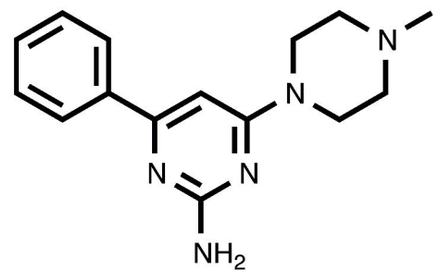
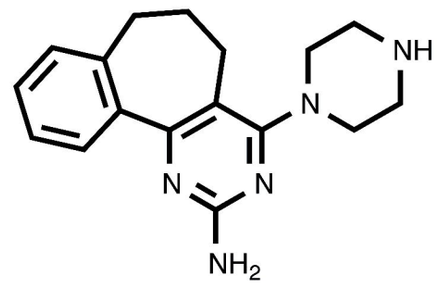


Clozapine

VI



VUF10460



VUF11422

VII

JNJ 7777120

VUF6002

VIII

VUF10214

VUF10148

IX

VUF10558

VUF10159

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FIGURE 2

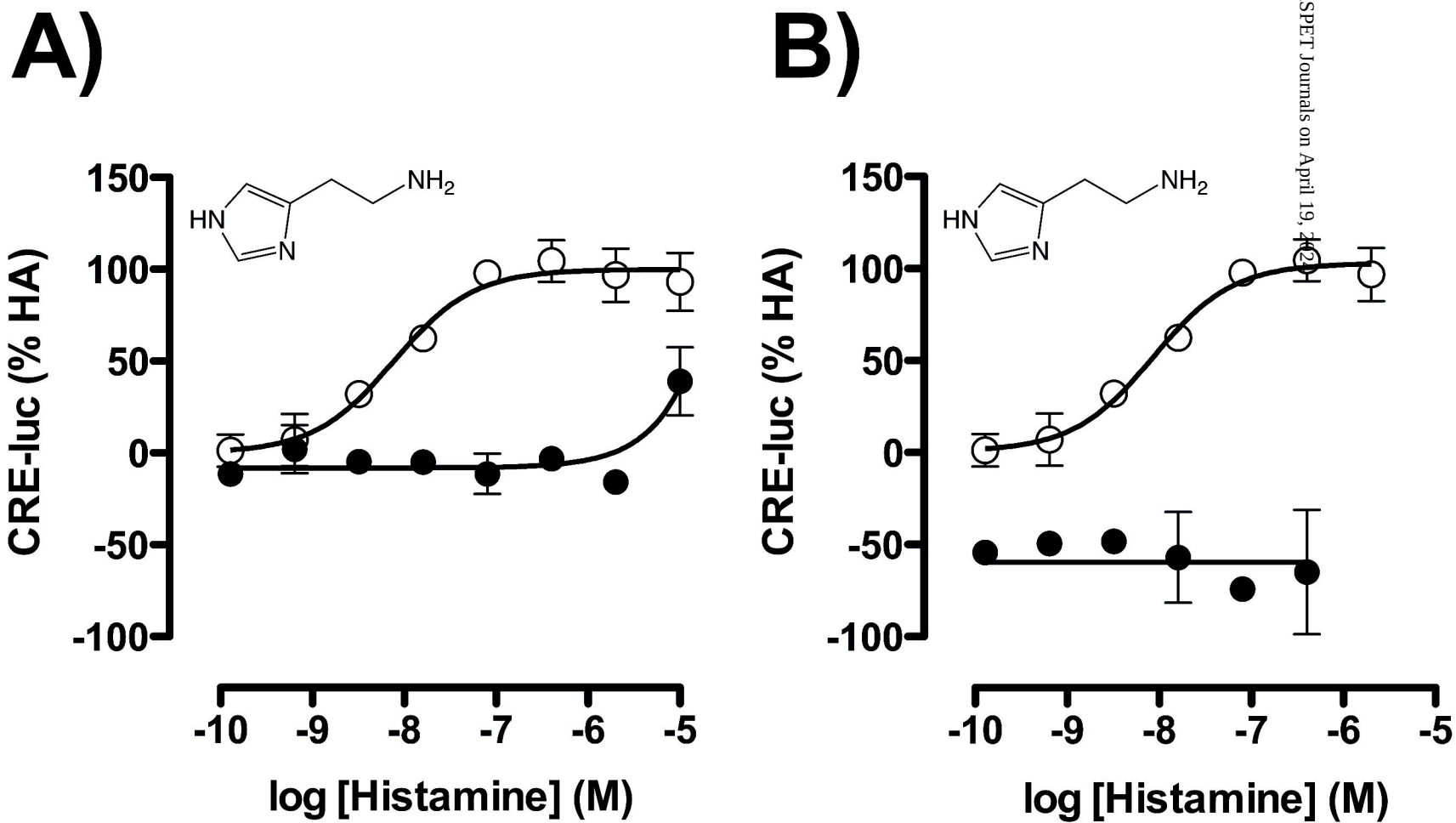

FIGURE 3

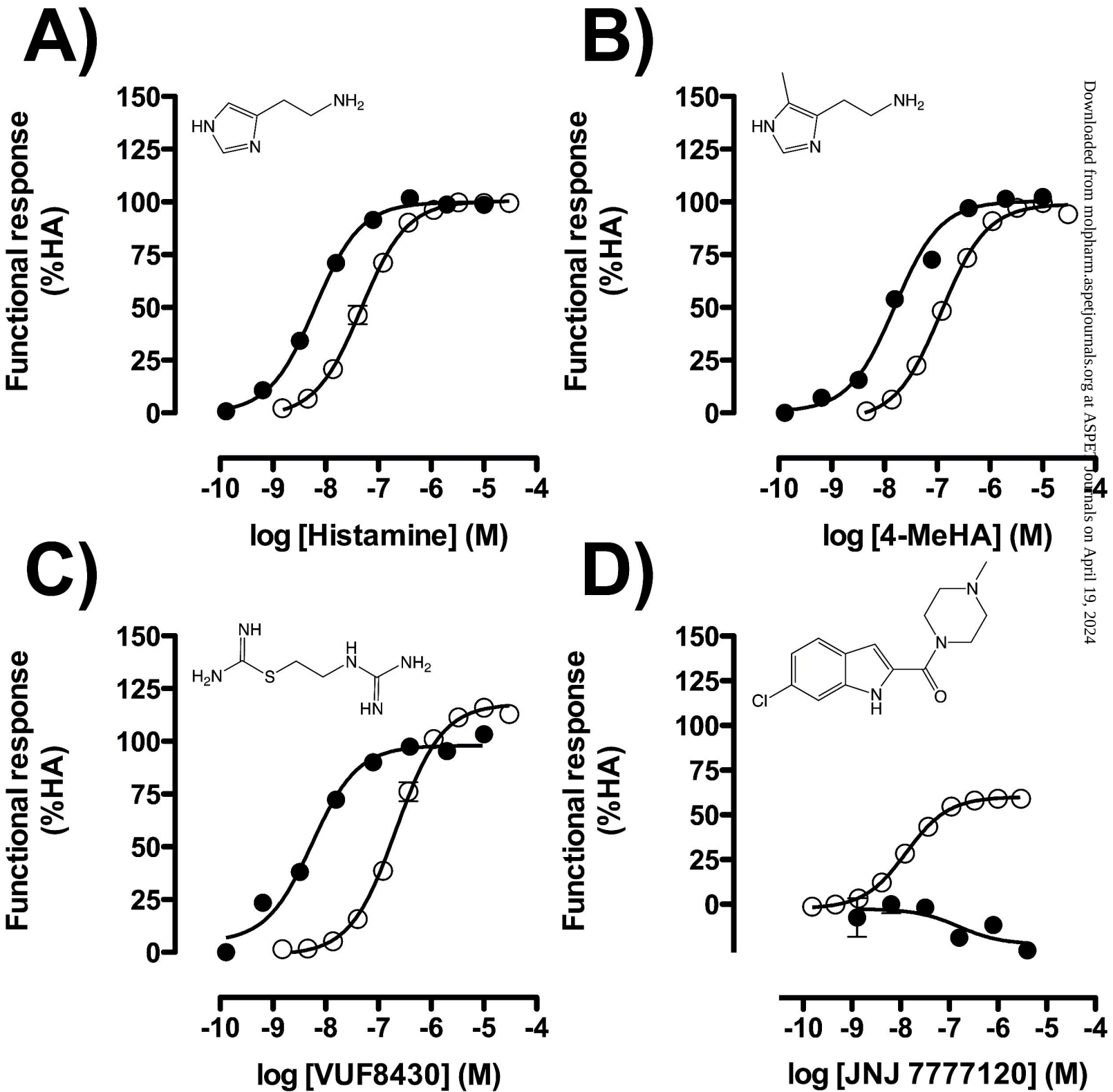

FIGURE 4

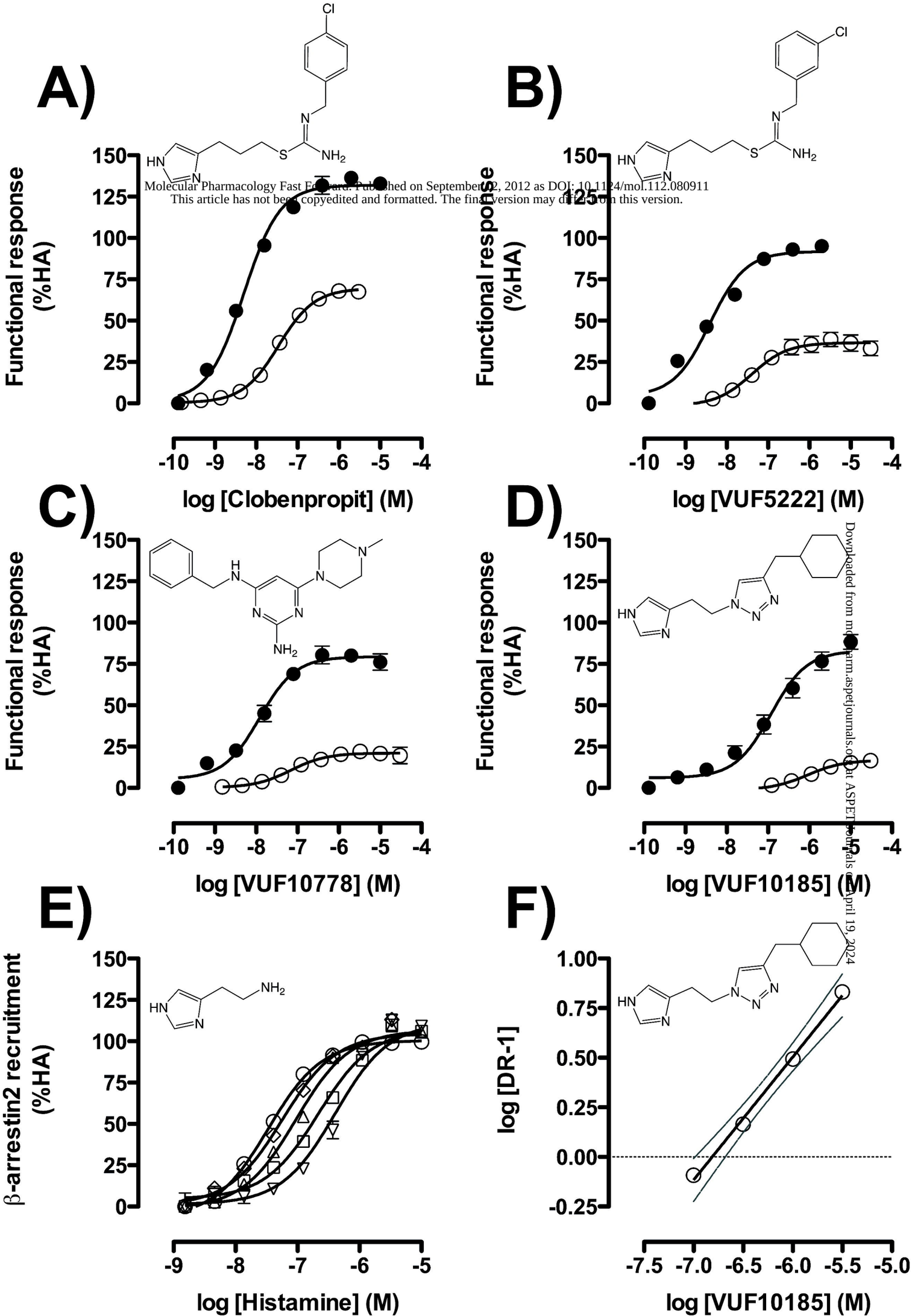

FIGURE 5

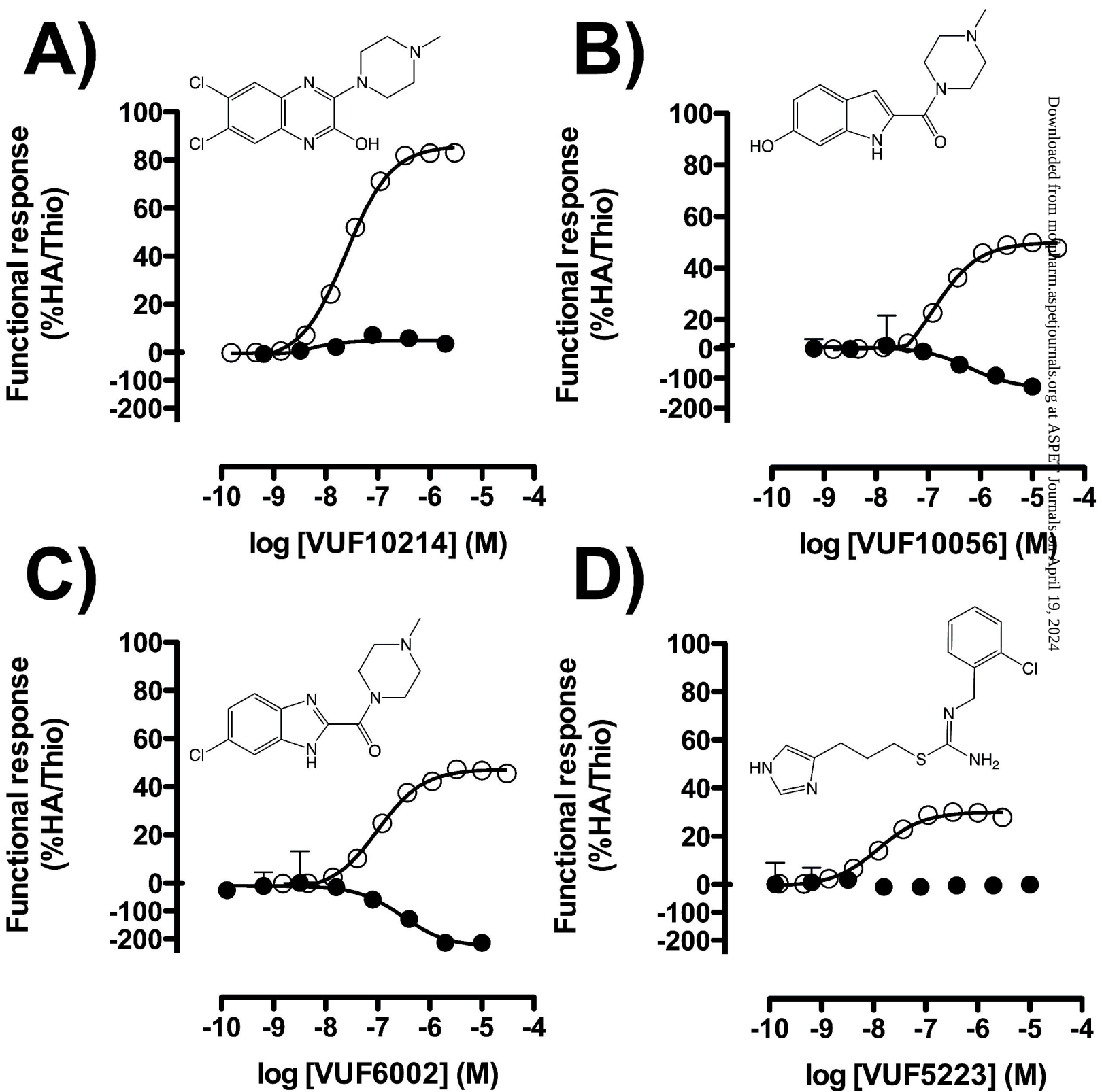

FIGURE 6

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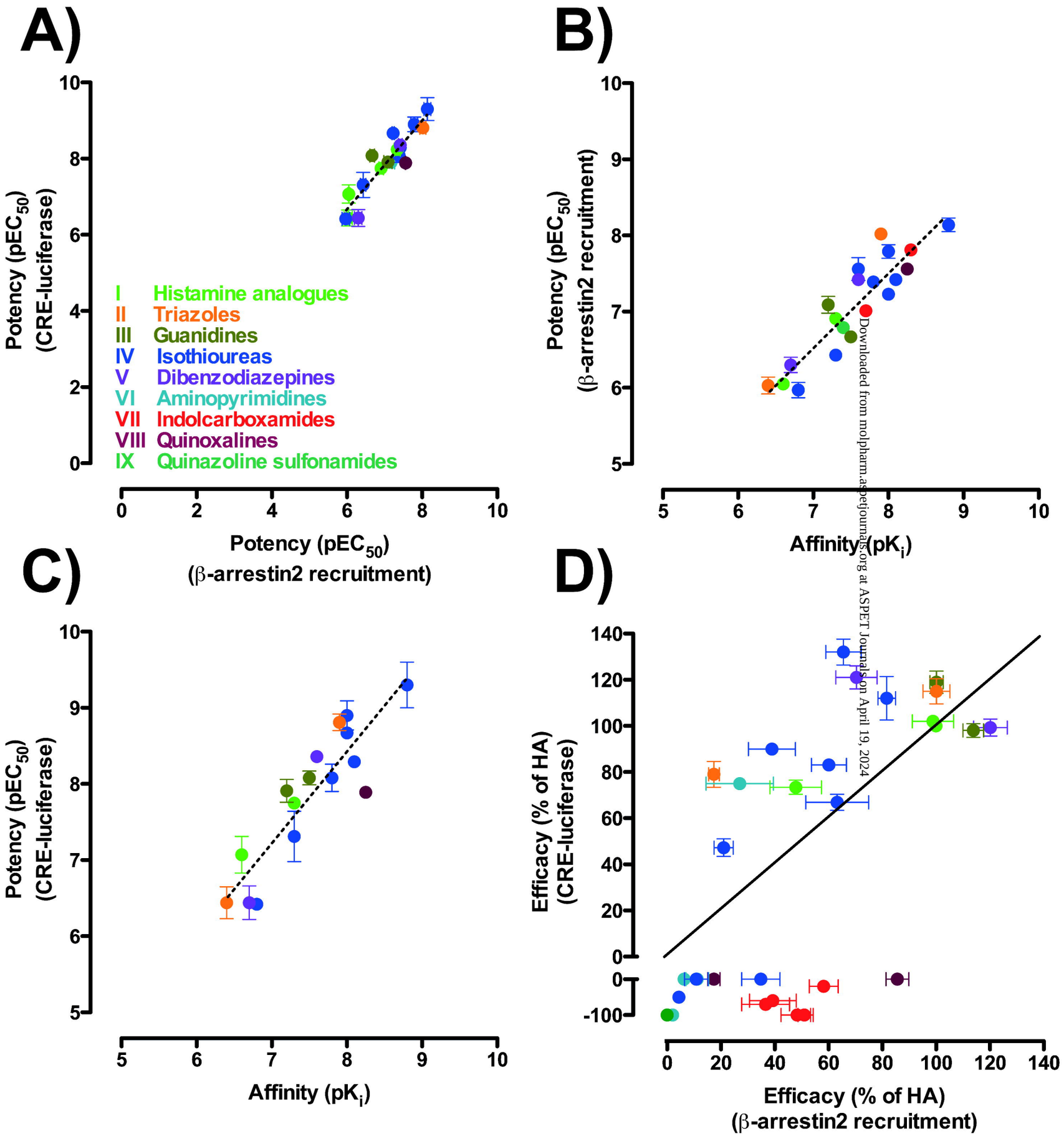
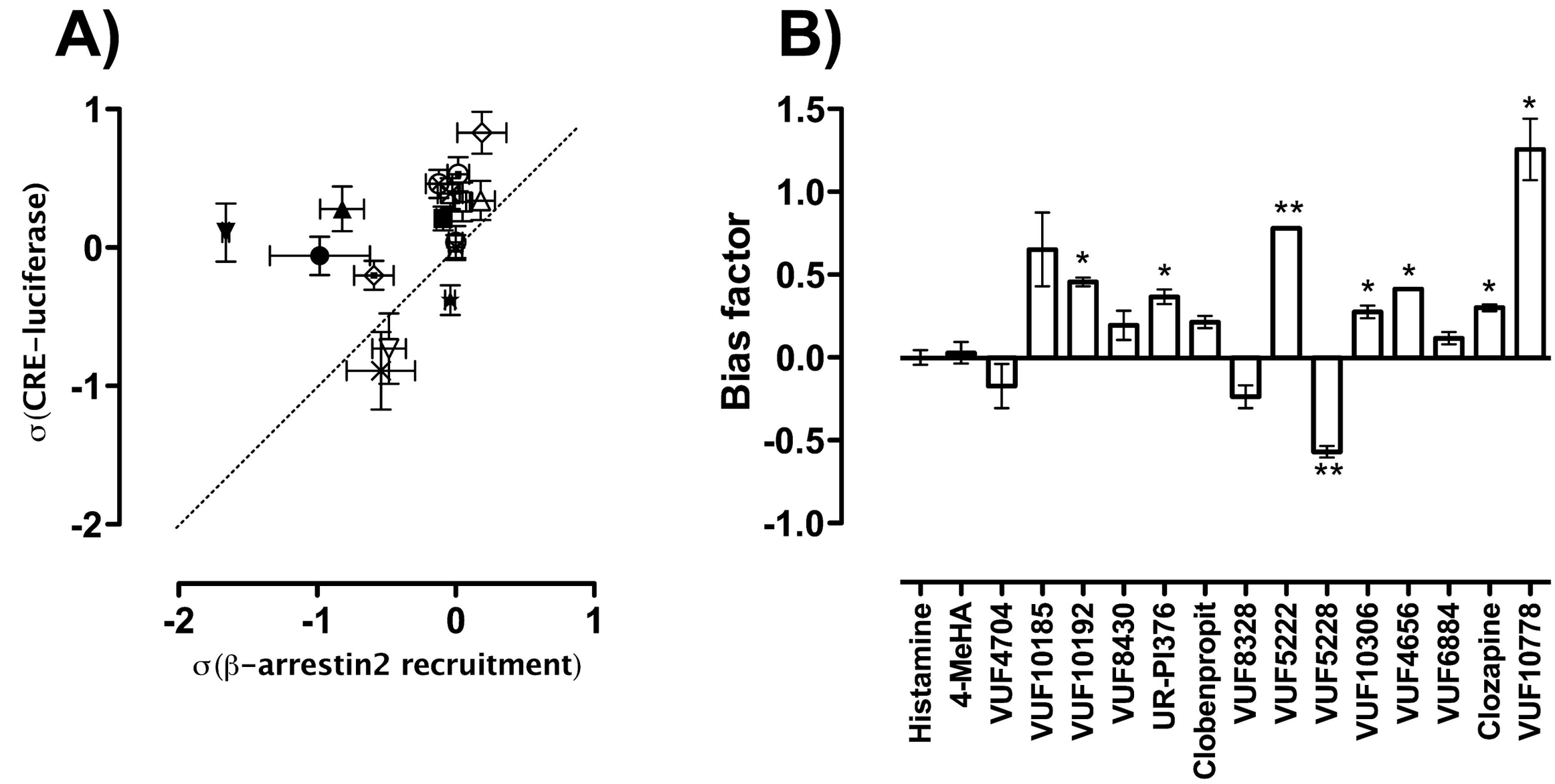


FIGURE 7



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