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# Model-based Discovery of Synthetic Agonists for the $Zn^{2+}$-sensing G Protein Coupled Receptor 39 (GPR39) Reveals Novel Biological Functions

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Keywords: GPR39 agonists, positive allosteric modulator, zinc-sensing receptor, antidiabetic treatment, virtual ligand screening, academic drug discovery

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## Abstract

The G-protein coupled receptor 39 (GPR39) is a G protein-coupled receptor activated by $Zn^{2+}$. We used a homology model-based approach to identify small-molecule pharmacological tool compounds for the receptor. The method focused on a putative binding site in GPR39 for synthetic ligands and knowledge of ligand binding to other receptors with similar binding pockets to select iterative series of mini-libraries. These libraries were cherry-picked from all commercially available synthetic compounds. A total of only 520 compounds were tested *in vitro,* making this method broadly applicable for tool compound development. The compounds of the initial library were inactive when tested alone, but lead compounds were identified using $Zn^{2+}$ as an allosteric enhancer. Highly selective, highly potent $Zn^{2+}$-independent GPR39 agonists were found in subsequent mini-libraries. These agonists identified GPR39 as a novel regulator of gastric somatostatin secretion.

## Introduction

7TM G protein-coupled receptors (GPCRs) collectively constitute the largest family of mammalian membrane proteins and are targets of a major fraction of both approved drugs and drugs under development[1]. However, the pharmaceutical companies follow rather common paths and focus their discovery and development processes on a relatively small number of the many hundred types of available receptor targets, which means that pharmacological ligands and tool compounds are only available for relatively few GPCRs. In fact, a surprisingly large fraction of GPCRs remain orphans, i.e., receptors without a known endogenous ligand and consequently without useful pharmacological tools[2]. In many cases, animal models in which the receptor has been genetically deleted or over-expressed may indicate certain potential physiological roles. However, these approaches are far from ideal and can be misleading due to the development of various types of genetic, cell biological and physiological compensatory mechanisms. Thus, there is a great need for efficient approaches to identify small-molecule synthetic ligands for GPCRs as useful tools to help determine the physiological importance of these receptors and to probe for their pharmacological potential as future drug targets[3], without requirement to screen several thousand compounds.

GPR39 is an example of a receptor that, due to a limited availability of suitable pharmacological tool compounds, has not yet been assigned a clear physiological role. Consequently, it has not been validated as a drug target, despite evidence indicating that it plays a major role in the control of many important endocrine and metabolic functions. GPR39 is a member of the ghrelin family of receptors. All other members of this receptor family bind ligands that are well-established peptide hormone and neuropeptide

regulators of metabolism: neurotensin, neuromedin U and motilin, in addition to ghrelin itself[4,5]. However, no peptide ligand has yet been identified for GPR39. In contrast, GPR39, which couples to Gαq, Gαs and β-arrestin, was found to act as a $Zn^{2+}$ sensor that is activated by physiological concentrations of $Zn^{2+}$ [6,7]. Similarly to several other members of this receptor family, GPR39 displays some degree of constitutive activity[7]. GPR39 is mainly expressed in endocrine and metabolic tissues[8], and based on studies in GPR39-deficient and GPR39-overexpressing mice, this receptor has been implicated in the control of glucose-induced insulin secretion[9], pancreatic β-cell differentiation[10] and control of lipolysis in white adipose tissue[11]. Based on similar studies, GPR39 has also been implicated in gastric fluid secretion, gastric and GI-tract motility[12], endothelial function and cell survival[13] and was recently found to have various functions in the central nervous system and depression[14–17], hemostatic regulation[18] and function as an extracellular pH regulator[19]. A general problem associated with GPR39 - and similar real orphan receptors - is a limited availability of pharmacological tool compounds. In the case of GPR39, apart from $Zn^{2+}$ itself, only a small number of ligands are currently known[20–22].

Most current drug discovery approaches depend on access to facilities capable of testing large libraries of chemical compounds, i.e., various types of high-throughput screening approaches[3]. In this study, we used a model based 'site-directed drug discovery' approach, which was originally introduced in the biotech industrial context[23], to identify small synthetic ligands for GPCRs. Through this approach, iterative series of mini-libraries of compounds are selected from the collective pool of commercially available screening compounds (Table S1), which are then probed in functional assays, which allow the use of both positive and negative information for the selection of compounds for subsequent mini-libraries. Based on the notion that many small agonists in GPCRs can act as both agonists and allosteric modulators[24–26], we tested the compounds both alone and in the presence of a submaximal dose of $Zn^{2+}$. Through this allosteric enhancer-assisted approach, we were able to identify sub-micromolar GPR39-agonist hit and lead compounds, which would not have been otherwise detected by functional assays. A complementary approach for discovering

allosteric modulators for GPCRs has been described by Huang and co-workers[27]. Importantly, selective, highly potent $Zn^{2+}$-independent GPR39 agonists were identified in subsequent series of ligand based optimized mini-libraries. Although expression analysis at an early stage demonstrated that GPR39 is strongly expressed in the stomach, its physiological role in the GI tract remained unclear. The novel GPR39 agonists now identify GPR39 as an important regulator of gastric somatostatin secretion.

## Results

### Structure-based discovery of agonists for the human GPR39 receptor

A large number of the currently available X-ray structures of 7TM GPCR-ligand complexes demonstrate that although the ligands can bind in many different ways to the GPCRs, the vast majority of ligands bind to what originally was proposed as the 'main ligand binding site' of GPCRs[28], i.e., to the cavity between the outer segments of TM-II, -III, -IV, -V and -VI[29]. To obtain agonist hit and lead compounds for GPR39 we used the so-called 'Site-Directed Drug Discovery' approach[23]. In brief, the process involves the analysis of the physicochemical properties of the binding pockets of GPCRs and the design of 3D pharmacophore search queries that exploit i) information from the structure of the binding pocket of GPR39 derived from comparative homology modeling based on the X-ray structures of structurally related GPCRs combined with ii) 3D structural information from small-molecule compounds, drugs and pharmacological tool compounds that are known to bind to 'binding pocket-related' receptors, i.e., receptors with binding pockets with similar physicochemical properties (Figs. 1a and 1b).

The analysis of amino acids located at 22 selected positions in the main ligand-binding pocket of GPR39 (Fig. 1a) identified other members of the ghrelin receptor family, i.e., the neurotensin receptors 1 and 2 ($NTS_1$ and $NTS_2$), the motilin receptor (MtlR) and the ghrelin receptor (GhrR), as well as phylogenetically non-related receptors, i.e., the chemokine receptors CCR1, CCR3 and CCR8, as being the closest 'binding pocket-related' receptors (Fig. 1a**)**. The availability of structural information of small-molecule ligands for GhrR, CCR3 and CCR8, combined with structural information on how these ligands bind in molecular models of

these receptors and how these binding sites correspond to interaction sites in GPR39 itself, were used as the basis for a 3D pharmacophore search query. The pharmacophore model was initially applied for the virtual screening of a GPCR-enriched chemical library of approx. 40 thousand compounds (Fig. 1b). Subsequent diversity selection (Suppl. methods) resulted in a focused library containing 197 compounds (Library 1), which was tested for agonist activity at concentrations of 1 and 10 µM in HEK293 cells transfected with the human GPR39 receptor to probe for $G\alpha_q$ coupling, as measured by an inositol phosphate accumulation assay. When tested alone, however, none of the compounds from Library 1 were able to increase GPR39 signaling (data not shown).

**Use of $Zn^{2+}$ as an allosteric modulator to enhance hit and lead identification**

We previously have identified $Zn^{2+}$ as an agonist for GPR39. Two Histidine residues, namely $His^{17}$ and $His^{19}$, located in the N-terminal segment of the receptor are essential for the effect of the metal ion (Fig. 2)[30]. Based on the assumption that the zinc ion can act as an allosteric enhancer by stabilizing GPR39 in an active conformation and can thereby potentially increase the binding affinity and potency of other agonists[26], we re-tested Library 1 in the presence of submaximal doses of $Zn^{2+}$. As shown in Fig. 1c, in the presence of 10 µM $Zn^{2+}$, compounds **1** to **5** were found to activate GPR39**.** $Zn^{2+}$ increased not only the potency but also the efficacy of the compounds. In the presence of $Zn^{2+}$, the synthetic compounds generally reached a maximum efficacy approx. two-fold higher than that obtained with $Zn^{2+}$ alone (Fig. 1d). At a $Zn^{2+}$ concentration of 100 nM, no allosteric enhancement was observed for any of the compounds. However, by increasing the $Zn^{2+}$ concentration to 1 and 10 µM, both the potency and efficacy were dose-dependently improved, as is shown for **5** (TM-N973), which reached a maximal potency of 200 nM in the presence of $Zn^{2+}$ (Fig. 1d). Thus, using $Zn^{2+}$ as a positive allosteric modulator, it was possible to identify agonist hits for GPR39, which would not have been detected on their own.

**The synthetic small-molecule agonists bind to the main ligand-binding pocket of GPR39**

To test whether the identified compounds actually did bind to the presumed general ligand-binding pocket of GPR39, to which they were selected to bind, and to compare their binding mode with that of $Zn^{2+}$, we probed compound **5** in a library of GPR39 mutants (Fig. 2). Ala substitutions of $Glu^{90}$(II:20, 2.60), $Phe^{115}$(III:08, 3.32), $Glu^{116}$(III:09, 3.33) and $Glu^{330}$(VII:06, 7.39) (The residue numbering refers to the generic GPCR numbering system described by Schwartz[31] and Ballesteros[32], which is based on the actual location of the residues in each trans-membrane helix) revealed that these key residues located in the presumed binding pocket are all essential for the ability of **5** to stimulate GPR39 signaling (tested in the presence of 10 μM $Zn^{2+}$) (Fig. 2). These results are in agreement with the receptor-based pharmacophore model used for the initial virtual screening, where $Glu^{90}$(II:20, 2.60) and $Glu^{116}$(III:09, 3.33) were proposed to act as acceptor sites for hydrogen bond donor atoms. The central $Glu^{330}$(VII:09, 7.39) was modelled to associate with a hydrogen bond donor atom or a positively charged basic site on the ligand (Fig. 1b). Ala substitution of $Arg^{303}$(VI:20, 6.55) increased the potency of **5** approximately fivefold (Suppl. data 1). This could not be directly predicted from the pharmacophore model; however, one explanation could be that removal of the large side chain located in the middle of the binding pocket allowed this particular type of ligand to interact better with other residues in the binding pocket.

Although the effect of **5** was highly dependent on the presence of $Zn^{2+}$ (Fig. 1d), Ala substitutions of $His^{17}$ and $His^{19}$, which eliminated the effect of $Zn^{2+}$ when administered alone, surprisingly had a very limited effect on **5** ability to activate the GPR39 receptor in the presence of $Zn^{2+}$ (Fig. 2, upper panels). Thus, the mutational mapping results are in agreement with the notion that **5** binds to the main ligand-binding pocket of GPR39 but indicate that $Zn^{2+}$ binds differently when acting as an agonist alone than when acting as a positive allosteric modulator for novel synthetic agonists.

**Design of iterative GPR39-focused mini-libraries to improve agonist drug-like properties and zinc independency**

Having obtained appropriate agonist lead compounds through the initial library (Fig. 1c), we used a ligand-based virtual screening to generate the following iterative series of mini-libraries of compounds to attempt to broaden the number of active chemo-types, to optimize their potency and, in particular, to attempt to identify compounds that can activate GPR39 in the absence of $Zn^{2+}$ and present appropriate ADME properties (steps 1-4 in Fig. 3a). In this process, we used structure-activity relationships (SARs) of both active and inactive compounds to guide the design of the subsequent three focused mini-libraries containing 176, 96 and 58 compounds, respectively (Supplementary tables, Table S2, S3, and S4), which were obtained from the collective pool of commercially available synthetic compounds (Table S1). In the design of the individual libraries, compounds with physicochemical properties similar to those of orally active approved drugs, were prioritized over compounds containing undesired chemical functionalities, such as potential reactive groups[33], and novel chemotypes were prioritized by discarding compounds that resembled small-molecules ligands known to be active on other protein or receptor targets (Compared to more than 1.25 million chemicals associated with biological activities in the ChEMBL database). Notably, compounds **3** and **4** contain a hydroxyphenyl hydrazone moiety, which has been recognized to be a potential covalent modifier and a sequester of metal ions (when present in other chemotypes[3]) and reported to be able to inactivate proteins and yield false signals across a variety of assays. While such compounds may be valuable as 'hits' for the optimization and development of new ligands, the hydroxyphenyl hydrazone moiety is certainly not preferred in more advanced tool compounds. Hence, we deliberately rejected compounds containing this and other promiscuous moieties in the subsequent libraries (Tables S2 – S4). Thus, in the search for improved and chemically attractive chemotypes, the zinc-dependent agonists **4** and **5** from Library 1 (Fig. 1c) were used as seed structures in an initial "scaffold hopping" similarity approach based on pharmacophore multiplets (Suppl. methods) to yield a second-generation library collected from four chemical vendors (Library 2 - 176 compounds).

In Library 2, the first two zinc-independent agonists, which presented low potency and low efficacy when tested without $Zn^{2+}$, were identified, as exemplified by **6** (TM-N1034, library 2, Table S2). Compound **6** also presented acceptable drug-like physicochemical properties (Table S2; Fig. 3b, upper panel). In the presence of increasing $Zn^{2+}$ concentrations, the potency of **6** could be increased by almost three orders of magnitude and presented an $E_{max}$ that was approx. two-fold higher than that obtained with $Zn^{2+}$ alone (Fig. 3b, lower panel).

To specifically attempt to improve the zinc-independent potential, we used **6** in a query based on similarity and substructure searches (Suppl. methods) to acquire a third-generation library (Library 3 – 96 compounds, Table S3). Among a number of zinc-dependent potent agonists, we discovered six purine-substituted variants, exemplified by **7** (TM-N1230, library 3, Table S3), with improved zinc-independent activation of the human (potency of 2.7 μM) and mouse (0.38 μM) GPR39 receptor compared with the 2,4-depyrimidin-substituted lead compound, **6** (Fig. 3b). **7** was highly efficacious and had a potency of 18 nM in the presence of 10 μM $Zn^{2+}$ (Fig. 3b, middle panel, Suppl. data 8 and Table S3).

In the design of a fourth-generation library, we made use of the observation that substituents in the 2 and 4 positions of the right-side phenyl improved the potency compared with substituents in the 3 position, which often resulted in partial or significant loss of activity (Table S3 and S4). Among the 58 purine-substituted analogous constituting Library 4, we identified several highly efficacious GPR39 agonists displaying single-digit nanomolar potencies in the presence of $Zn^{2+}$ and, importantly, a number of agonists that exhibited high potency in the absence of $Zn^{2+}$ (Fig. 3b, Table S4). The most promising compound of this class, **8** (TM-N1324), had a rigid structure and a reasonably low molecular weight (MW=397.08) and presented generally improved drug-like physicochemical properties compared with the original zinc-independent agonist lead, **6**. Importantly, **8** activated human GPR39 with high efficacy and potencies of 280 nM and 9 nM in the absence and presence of $Zn^{2+}$, respectively (Fig. 3b). The compound had similar potencies on murine GPR39, 180 nM and 5 nM (Suppl. data 8). Thus, the combined ligand-based and

allostery-assisted approach has led to the discovery of several novel, potent and efficacious GPR39 agonist chemo-types that present double-digit nanomolar potencies in the absence of the allosteric enhancer $Zn^{2+}$.

**Further improvements in properties through SAR-guided synthesis**

Having exhausted the existing commercially available chemical libraries, such as, for example, all of the available preferred right-hand-side substituents from Library 2 and the left-hand-side purine moiety from Library 3 and 4, we resorted to the synthesis of a final library of compounds in an attempt to further improve the properties of this chemical series and create truly novel, patentable compounds. Based on the collective knowledge of the SARs of the compounds in Libraries 2 to 4, we synthesized a fifth library (Library 5 – containing 30 compounds, Table 1) as described in the supplementary methods. In this library, we maintained the purine moiety at the left-hand side, which enabled good agonist activity in Library 3 to 4, exemplified by **10** (TM-N1231, Table S4) and combined it with a diverse set of chemical modifications in position 4 of the right-hand side phenyl. These modifications included distinct primarily two-to-three-atom linkers to aryl-substituted moieties, which had enabled the first observation of zinc-independent activity of **6** in Library 2 (Fig. 4a), which is key for activity when compared to **9.** As shown in Table 1 and Fig. 4b, approximately half of the compounds presented improved potency with respect to the zinc-independent activation of GPR39 compared to **8**, and eight compounds (**13**, **17**, **18**, **19**, **26**, **27**, **28** and **39**) had potencies below 100 nM. Notably, the most potent compound **26** (TM-N1562) had a potency of 30 nM to activate human GPR39 in the absence of $Zn^{2+}$, i.e., an improvement of approx. nine-fold compared to that of compound **8**. **26** had a potency of 1.5 nM in the presence of $Zn^{2+}$. It activated murine GPR39 with a similar potency of 2.6 nM in the presence of $Zn^{2+}$ (Suppl. data 8)

Both compounds **8** and **26** were further analyzed for their stimulation of cAMP signaling, as a measurement for G$\alpha$s coupling. Both compounds, in the presence of 3 µM $Zn^{2+}$, increased the intracellular cAMP level in a dose-dependent manner with potencies of 17 nM and 12 nM, respectively (Fig. 4d, lower panel). When

tested without $Zn^{2+}$ however, neither compound caused a marked increase in cAMP. Hence, in contrast to the Gαq coupling, none of the compounds induced efficient $Zn^{2+}$ independent Gαs coupling.

**Drug-like properties of the discovered GPR39 agonists**

Since chemical novelty was one of the selection criteria, both **8** and the in-house synthesized **26** were clearly chemically distinct from known pharmacological ligands, as determined through comparisons with more than 1.25 million ChEMBL chemicals associated with biological activities of all major target classes, i.e., they both had Tanimoto coefficients less than 0.7 (Fig. 4c).

Compound **8** and **26** were also probed for off-target activities using a panel of 165 GPCRs, i.e., the Millipore GPCR-profiler service (Suppl. data 2), and neither compound showed relevant agonist activities. Even when tested in-house using signal transduction assays, the compounds did not exhibit agonist activities neither on closely related receptors, such as the ghrelin, motilin and NMU2 receptors, nor on receptors with similarities in the binding pocket, such as the chemokine CCR1 and CCR5 receptors (Suppl. data 3).

Furthermore, compound **8** was found to have promising *in vitro* ADME properties. Although an early compound **7**, exhibited low solubility, **8** proved to have reasonably good aqueous solubility (65 µM at pH 7.0) (Suppl. data 4). The compound also demonstrated high membrane permeability, as measured by the rate of transport across Caco-2 cells derived from a human colon carcinoma in the A-B direction ($P_{app}$ A-B ~ 25 x $10^{-6}$ cm/s), with good recoveries in both directions (Suppl. data 5). In addition, with respect to *in vitro* metabolic clearance in human liver microsomes, **8** was superior to **7** (CLint of 58.3 vs. 23.7 and $t_{1/2}$ of 58 min vs. 24 min, respectively) (Suppl. data 6), implying that the 2-chloro-4-fluoro-benzene substituents improve stability by blocking potential metabolically liable oxidation sites and/or affect the molecular conformation.

Importantly, the concentration of compound **8** in the plasma after oral administration of two different doses (30 mg/kg and 60 mg/kg) reached micro molar concentrations after 30–240 minutes, which is sufficient for maximal activation of the GPR39 receptor (Suppl. data 7).

**Probing the physiological functions of GPR39 using the novel synthetic agonist**

*Effect on insulin secretion* - GPR39 is highly expressed in insulin-producing β-cells of the endocrine pancreas and has been proposed to be involved in a $Zn^{2+}$-mediated autocrine feed-back control of insulin secretion based on the impaired glucose tolerance and insulin secretion observed in GPR39-deficient mice[9,34]. Furthermore, novel agonist ligands discovered by HTS efforts also suggest an improved insulin secretion from artificial β-cell and *in vivo* studies[21]. However, although **7** apparently weakly stimulates insulin secretion from murine islets isolated on five independent days each including of 2 to 3 mice, a similar response was observed in islets isolated from GPR39-deficient mice (Fig 5a,b). In contrast, no consistent increase in insulin secretion was observed for **8** (Fig 5c,d). Because these compounds are clearly efficacious with respect to stimulating GPR39 Gαq signaling *in vitro* it can be concluded that the role of GPR39 in the control of β-cell function remains unclear.

*GPR39 function in gastric endocrine cells* - GPR39 is known to be highly expressed in the stomach[8], but no function has yet been found for GPR39 in the ventricle. As part of the systematic characterization of receptors controlling the secretion of ghrelin[35], we tested the effect of **8** and **26** on the release of ghrelin from primary cultures of gastric mucosal cells. As shown in **Fig. 6a**, the GPR39 agonists at doses of 1 μM in the presence of 10 μM $Zn^{2+}$ inhibited basal ghrelin secretion to 79 and 61 % of the basal level, respectively, i.e., which is similar to the efficiency obtained with other inhibitors in this system[35]. This finding was surprising in view of the fact that GPR39 is a Gαq-coupled receptor and would thus be expected not to inhibit but rather stimulate hormone secretion. Because primary cultures of gastric mucosal cells also contain somatostatin cells, we tested the effect of broad-spectra somatostatin receptor antagonists, which completely eliminated the inhibitory effect of the two GPR39 agonists on the secretion of ghrelin and even showed a trend toward a higher release in the presence of the antagonist (Fig. 6a). Measurements of somatostatin confirmed that the GPR39 agonists **8** and **26** increased somatostatin release, i.e., by 53 and 82 %, respectively (Fig. 6d). The stimulatory effect of the two GPR39 agonists on somatostatin release was

eliminated in cells isolated from GPR39-deficient mice (Fig. 6d and e). In accordance with the observation that GPR39 activation increases somatostatin secretion and accordingly decreases the secretion of ghrelin, we found that the fasting plasma levels of ghrelin were increased in GPR39-deficient mice (Fig. 6c). Finally we tested that the GPR39 agonists acted through Gαq in a dose dependent manner also on the gastric cells and found similar potency on the gastric cells compared what we observed on the heterologous expression system (Fig. 6f). Thus, the novel GPR39 agonists enabled identification of novel important physiological functions of GPR39 as a regulator of gastric somatostatin secretion (Fig 6g).

## Discussion

In the study, we here used a structure-based approach to discover a number of highly potent and highly efficacious pharmacological GPR39 agonist tool compounds. In the process, which was greatly facilitated by the use of the low-potency endogenous agonist $Zn^{2+}$ as an allosteric enhancer to detect the early hit series, only 520 compounds were tested in *in vitro* signaling assays.

A large number of the currently available high-resolution X-ray structures of GPCR-ligand complexes support the original hypothesis that although ligands bind in many different modes to their different target receptors, they nevertheless mostly interact with residues located in a common, main ligand-binding pocket between the extracellular segments of the transmembrane helices[36,37]. Structure-based docking screens using the actual ligand-binding sites of available high-resolution X-ray structures of GPCR-ligand complexes have been highly efficient for the identification of novel and potent ligands, with hit-rates as high as 20 to 70 %[38–41]. In this study we successfully used a structure-and-knowledge-based approach taking advantages of both molecular models of the presumed binding sites for small molecules in molecular models of the target receptor (GPR39) and structures of ligands binding to receptors with binding pockets of similar physicochemical properties[23,42]. Related approaches have been used in the pharmaceutical industry to search for hits and leads in their large in-house chemical libraries[43,44]. For example, Klarbunde and coworkers used a similar approach in a combined retrospective and prospective screen to identify

potent agonists for the complement component 3a receptor 1 (C3AR1)[44]. Importantly, we propose that the knowledge-based discovery process described in this manuscript is applicable in different settings, because we selected and experimentally tested an iterative series of focused mini-libraries of compounds, which were cherry-picked from the collective repertoire of chemicals available from commercial chemical vendors. The approach complements the High-Throughput Screening (HTS) or "random" experimental testing of large compound collections, which is currently becoming available in a number of academic institutions[3]. Recently, GPR39 agonists were identified by Boehm and coworkers in the industrial setting through the screening of 30,000 compounds selected from the Pfizer chemical collection[45]. The computational approach also allows for bias in the search strategy toward specific aims, such as finding novel chemotypes ('scaffold-jumping') or enhancing certain physiochemical and/or pharmacological properties of a particular chemotype – in this requiring only limited chemical synthesis.

The use of $Zn^{2+}$ as an allosteric enhancer in the search for agonists for GPR39 was based on the hypothesis that the zinc ion would stabilize the receptor in an agonist-prone conformation and thereby facilitate the discovery of small-molecule agonists. Despite its small size, $Zn^{2+}$ is a bona fide, albeit relatively low-potency agonist for GPR39, as measured in several different signal transduction pathways, including $G\alpha q$, $G\alpha s$ and arrestin mobilization[6,19]. Over the last decade, it has become clear that many agonists, despite the traditional pharmacological view[46], can act as allosteric enhancers for other agonists[26,47–50]. For GPR39, co-administration with $Zn^{2+}$ was found to be essential for the identification of the initial hits from the virtual screening because none of the compounds in Library 1 could activate the receptor alone. A complementary approach to identify allosteric ligands for GPR68 and GPR65 has been described recently[27]. Notably, Fjellström and co-workers at AstraZeneca recently discovered $Zn^{2+}$ modulated GPR39 agonists by a high throughput screen, followed by a medicinal chemistry program[22]. However, in the subsequent mini-libraries, we were gradually able to discover compounds that were both potent and efficacious in the absence of $Zn^{2+}$, and even more potent in the presence of $Zn^{2+}$. Zinc increased both the potency and the efficacy of the compounds in accordance with the classical properties of an ago-allosteric agonist[26].

Interestingly, $Zn^{2+}$ has been described to act as an allosteric modulator for a number of GPCRs[25,51–54] and can conceivably be used as an enhancer in the search for novel ligands for receptors other than GPR39. Importantly, we believe that the allostery-enhanced approach is much more generally applicable. Due to the chemical nature of the two types of ligands, we use the term 'allosteric enhancer' for $Zn^{2+}$ and 'agonists' for the novel synthetic compounds, although $Zn^{2+}$ may really be the natural, endogenous agonist for GPR39[6,7]. We propose that the existing agonists, like $Zn^{2+}$, should be used more broadly as an aid in the search for 'other agonists' or allosteric modulators. The point is that allosteric modulators very often act as agonists in their own right, and vice versa. Such modulators are generally referred to as PAM-agonists. It is becoming increasingly clear that even rather large agonists may act as positive allosteric modulators for other ligands, including even those with a very similar structure[47–50,55]. In this context it is interesting to note that the endogenous partial agonist $Zn^{2+}$ binds differently compared to the more potent synthetic agonists since elimination of the N-terminal zinc site (His17 and His19) does not affect the potency of the zinc-dependent compounds, although a studie by Cohen et al. indicates that other residues in the extracellular domain, such as e.g. Asp313, are able to modulate $Zn^{2+}$ binding[19].

The main purpose of generating synthetic agonists for GPR39 was to obtain pharmacological tool compounds that could be used to uncover physiological functions of the GPR39 receptor, which analogous to orphan receptors in general, have otherwise been exclusively based on genetic data. Studies in GPR39-deficient mice had also indicated that the receptor may be important for insulin secretion[9,34]. However, this hypothesis could not be confirmed using the developed GPR39 agonists. Lack of acute insulinotropic effects in rodents has also been observed by Fjellström and co-workers[22]. Although we did observe effects on insulin secretion in some studies, this response was not consistent and not dependent on GPR39. In this connection, it should be noted that the effects of other GPR39 agonists on insulin and GLP-1 secretion discovered by Peukert and coworkers at Novartis when searching for inhibitors of Hedgehog[20,21], have not yet been shown to be dependent on GPR39 in control experiments performed using knockout mice[21,45].

Importantly, novel physiological functions of GPR39 could be revealed using the new GPR39 agonists. Almost 10 years ago, it was shown that GPR39 is highly expressed in the stomach[8], but no function had been connected to this expression prior to this study. In the present study, we found that GPR39 stimulates gastric somatostatin secretion in a GPR39-dependent manner and thereby indirectly inhibits ghrelin secretion. This modulation of the ghrelin secretion observed in primary gastric mucosal cells cultures was supported by data from the GPR39 KO mice where plasma ghrelin was increased and under standard condition also food intake was increased. Because somatostatin in the stomach dampens or inhibits both endocrine and exocrine secretion in general, it will be interesting to study the potential indirect effects of GPR39 agonists on, for example, secretion of gastrin, gastric acid and pepsinogen. Notably, a massive accumulation of gastric juice was originally described in GPR39-knockout mice[12], which may now be connected to a lack of somatostatin stimulation in GPR39-deficient mice. The discovered compounds are all zinc-dependent and display at least 10-fold higher potency in the presence of $Zn^{2+}$ than without. It could be argued that the $Zn^{2+}$ dependency is a favorable property if we aim at functionality in tissues where the $Zn^{2+}$ concentration is high such as in the pancreas. However, as described in the present manuscript, the most important functions are related to adipocyte function and gastric hormone secretion. Neither of these tissues has been characterized with high $Zn^{2+}$ concentrations.

**Conclusion** – The present study demonstrates that it is possible to generate novel pharmacological tool compounds that can be used to elucidate the physiological importance of a GPCR based on a combination of structure-based computational chemistry and the use of a low-potency ago-allosteric enhancer. GPR39 is an example of a receptor, where the knockout models have not revealed the physiological role and where the therapeutic potential remains unknown. This applied approach complements the use of high-throughput physical screening of large libraries of chemical compounds as it instead exploits the equally large collective pool of commercially available compounds but, as exemplified in the present case, only

leads to physical testing of a small number of compounds. However, as also indicated, a limited synthesis step may be used to further improve, for example, the compound's ADME properties when the commercial libraries are exhausted.

## Methods

**Homology models.** A pair wise sequence alignment between the human GPR39 receptor (accession number Q43194) and the sequence of bovine rhodopsin was constructed using ClustalW[56]. The alignment was manually refined to ensure proper alignment of conserved class A GPCR motifs. An ensemble of 50 three-dimensional comparative GPR39 models was constructed with MODELLER[57] using bovine rhodopsin (PDB ID 1GZM, 2.65 Å resolution) as a structural template[58]. The N-terminal domain, the extra- and intracellular loops, and the C-terminal domain were excluded. Using a computational protocol as described previously[23,42], we initially applied a physicogenetic methodology to identify binding pocket-related GPCRs and associated ligands to construct a five-feature pocket-based GPR39 pharmacophore search query (further details are presented in Suppl. methods). The pharmacophore model was initially used for the virtual screening of our in-house GPCR-enriched chemical library to generate library 1 using UNITY and associated dbtools in the Tripos software package[59]. Prior to the selection of compounds for experimental testing, the hit list was filtered using automated scripts to remove accepted compounds in high-internal-energy conformations and compounds violating a number of physiochemical properties.

**Design of iterative focused chemical mini-libraries (Library 2 - 4) using virtual ligand screening** - GPR39 agonists identified in the first-generation library were subsequently used as seed structures to search the combined collection of commercial chemical vendor libraries (**Table S1**) stored in UNITY databases and prepared for virtual screening using the Tripos software package[59] to generate three focused mini-libraries

(Libraries 2 – 4) acquired from five vendors (ChemDiv, Enamine, InterBioScreen, ChemBridge and Life-Chemical). To improve the potency, efficacy and chemical properties of the novel GPR39 agonists, each iteration/library used structural information obtained from positive as well as negative results to guide the selection of compounds for the next-generation libraries. In this process, compounds were further prioritized based on their predicted "drug-like" properties and novelty (details are provided in Suppl. methods). **Library 2 -** The zinc-dependent agonists **4** (TM-N279) and **5** (Library 1) was used as seed structures for ligand-based virtual screening using *Pharmacophore Multiplets* that have been demonstrated to be effective for lead and scaffold-hopping [60,61]. *Pharmacophore multiplets*, produced from the steric features, hydrophobic centroids, aromatic rings, hydrogen acceptors, hydrogen donors, cations and anions features, were decomposed into its constituent three feature pharmacophore distance multiplets and encoded into binary fingerprints (so called Triplets), to search the pre-computed chemical vendor libraries (Table S1). The high-ranking chemical hit lists were subsequently filtered. Several classes of accepted molecules were rejected based on the criteria described in the supplementary information. Finally, the compound was manually inspected which is a common accepted practice for cherry-picking compounds for experimental testing. **Library 3** was based on similarity (using 2D UNITY fingerprints) and substructure searches around the zinc-independent agonists **6** (Library 2), while **Library 4** was based on similarity searches of the purine-substituted zinc-independent agonists **7**. A Tanimoto Coefficient, TC > 0.70, was applied in the search for similar vendor compounds. In cases where only few compounds were identified, a lower similarity threshold was used. The compound selection criteria for compounds in Libraries 3 and 4 were similar to the criteria used for Library 2. All of the computations were performed on 40-core 3.00-GHz Linux workstations.

**Chemical synthesis**. InterBioScreen Ltd (http://www.ibscreen.com/) synthesized the compounds in Library 5 (**Table 1**) under an agreement with the University of Copenhagen according to Synthesis Schemes 1 to 3

(Suppl. methods). It is important to mention that all chemical structures were confirmed using proton (1H) NMR spectra at 300/400 MHz and liquid chromatography–mass spectrometry (LC-MS) analysis confirmed a minimum purity of 95% for all compounds (spectra of compounds are listed in Supporting data).

***In vitro* ADME and chemical receptor profiling of key compounds.** The solubility (Suppl. data 4), Caco-2 membrane permeability (Suppl. data 5) and liver microsomal stability (Suppl. data 6) of two key compounds **7** and **8** were assessed in mouse and human using Cyprotex Discovery Ltd., a fully integrated R&D CRO. To evaluate the potential off-targets effects, FLIPR assays were conducted to profile the agonist and antagonist activities of **8** and **26** with a panel of 165 GPCRs using the Millipore GPCR profiler service (Suppl. data 2). We also tested the activity of **8** on a series of structurally and functionally related receptors (CCR1, CCR5, GhrR, NMUR2, MtlR, GPBA, NK1R, LPA2, ETB, and D2R) (Suppl. data 3).

***In vitro* Screening of the compound mini-libraries.** The activities of the focused chemical libraries were tested through an inositol phosphate (IP) accumulation assay. HEK293 cells were transiently transfected with GPR39 cDNA in the pCMV-Tag 2B vector via the calcium phosphate method and then frozen at -150°C. Transfected frozen HEK293 cells were rapidly thawed and transferred to cell culture medium containing 5 µCi/ml *myo*-[2-$^{3}$H]inositol. A total of 40,000 cells per well were seeded in poly-D-lysine-coated 96-well plates. After 24 h incubation, the IP accumulation assay was performed as decribed in the Suppl. methods. The $EC_{50}$ values were determined by nonlinear regression using GraphPad Prism v6.03 for Windows, GraphPad Software, La Jolla California USA.

**Physiological analyses.**

*Ex vivo ghrelin and somatostatin secretion studies.* The day after the preparation of gastric mucosal cells, cells were incubated with the ligands in triplicates for 4 h, and the supernatant was collected. Acyl-ghrelin

was measured using a "Rat/Mouse Ghrelin (active) ELISA" (Millipore #EZRGRA-90K). Somatostatin was measured using an in house somatostatin RIA. The response of the cells in terms of ghrelin/somatostatin secretion to the administered ligands was expressed as a percentage of the secreted ghrelin/somatostatin from cells exposed to medium alone.

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**Competing financial interests**

The authors declare no competing financial interests.

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Supporting Information Available: Including description of molecular modeling, preparation of chemical databases, virtual screening, chemical synthesis, in vitro assays, counter screens and ex vivo experimental and analytical procedures.

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**Tables**

**Table 1.** Chemical structures and summary of potencies of 30 synthesized compounds (library 5) in the presence and absence of $Zn^{2+}$.

**Figures**

**Fig. (1). Strategy and results of the initial virtual screening for novel molecules with GPR39 activity.** (**a**) Step 1: The corresponding residue positions in the highly ranked "pocket-related" receptors $NTS_1$, $NTS_2$, MtlR, GhrR, CCR1, CCR3 and CCR8 are shown below. (**b**) Step 2: Three-dimensional pharmacophore model based on GPR39 receptor models and predicted binding poses consistent with active analogs that satisfy many of the mutational hits of ligands that binds to models of pocket-related receptors (See supplemental information for details). The features of the pharmacophore model are: a hydrogen-donor-sites-and-spatial constraint for ligand acceptor interactions with $Arg^{303}$(VI:20, 6.55) (green spheres), a toroidal constraint for a basic or hydrogen donor ligand atom interacting with the carboxyl side chain of $Glu^{330}$(VII:06, 7.39) (red), a hydrogen bond donor interaction with the carboxyl side chain of $Glu^{90}$(II:20, 2.60) and $Glu^{116}$(III:09, 3.33) (magenta spatial cap), a hydrophobic/aromatic feature (yellow sphere) and a receptor pocket surface constraint (grey surface). Steps 3 – 5: Virtual screening of our in-house chemical library, compound property and diversity selection and subsequent *in vitro* testing of the target-focused mini-library using zinc as a positive ago-allosteric modulator. (**c**) Chemical structures of zinc-dependent GPR39 agonists **1** to **5** identified in the first-generation library. (**d**) Dose-response curves of $Zn^{2+}$ and the $Zn^{2+}$-dependent agonist **5** in the presence of different zinc concentrations.

**Fig. (2). Mutational mapping of 5 (TM-N973) supported the presumed receptor-ligand-interaction mode**. (**a**) $Zn^{2+}$ (circles) and **5** in the presence of 10 µM $Zn^{2+}$ (squares) were tested for their ability to stimulate Inositol phosphate accumulation in HEK293 cells transiently transfected with human GPR39 wild-type (WT) or mutant receptor. Mock transfected cells are indicated in grey. The positions and the effect of the alanine

mutations are marked in the helical wheel diagram (green: increased potency of compound **5**; red: decreased potency of **5**; yellow: decreased potency of $Zn^{2+}$ compared to WT). Consistent with the presumed binding pocket, $Glu^{90}$(II:20, 2.60), $Phe^{115}$(III:08, 3.32), $Glu^{116}$(III:09, 3.33) and $Glu^{330}$(VII:06, 7.39) are essential for compound **5** function. Alanine substitution of the 2 N-terminally located Histidines His17 and His19, on the other hand, had little effect on compound function. Data points are mean ± SEM of at least three independent experiments performed in duplicate ($n \geq 3$).

**Fig. (3)**. **Illustration of the knowledge-based process used to develop iterative libraries of chemical compounds (steps 1 – 4)**. (**a**) Each iteration/library used structural information from positive as well as negative results to mine the collection of worldwide vendor libraries (**Table S1**) to improve the potency, efficacy and physiochemical chemical properties of novel GPR39 agonists. (**b**) Scatterplot of the *in vitro* potencies of the compounds of three iteratively designed libraries in the presence and absence of $Zn^{2+}$. The compounds in the scatterplots are colored green (drug-like) to red (less drug-like) to represent their predicted drug-like properties. The $Zn^{2+}$-independent agonist activation as well as the physiochemical properties of the compounds were improved through the optimization process from Library 2 to 4. The structures of the best compounds from each library, namely **6** (Library 2, Table S2), **7** (Library 3, Table S3) and **8** (Library 4, table S4), are shown with their computed physiochemical properties. IP accumulation curves of the compounds tested on HEK293 cells transiently transfected with hGPR39 are shown in the lower panel. Potencies of all the compounds were increased by the addition of increasing $Zn^{2+}$ concentrations. Grey data points are compounds plus 10 µM $Zn^{2+}$ tested on mock-transfected cells.

**Fig. (4). Summary of structure activity relationships for compounds in library 2 – 4.** (**a**) Structure activity of **6**, **9** (library 2, Table S2) and **10** (library 3, Table S4). (**b**) Potency of synthesized compounds in library 5 with and without zinc. (**c**) Chemical profiling against 1.25 million annotated chemicals (using Open Babel FP2 fingerprints) reveals that compound **8** and **26** are chemical distinct ($T_C < 0.7$) from any compound in the

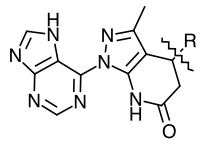
ChEMBL_19 database representing all major target classes. (d) Comparison of IP accumulation (upper panels) and cAMP accumulation (lower panels) induced by compound **8** (left panels) and **26** (right panels). Both compounds induce IP accumulation even in the absence on $Zn^{2+}$. The production of cAMP induced by the compounds, on the other hand, requires the presence of $Zn^{2+}$. Grey data points are compounds plus 10 µM $Zn^{2+}$ tested on mock-transfected cells.

**Fig. (5). Insulin secretion from isolated islets**. **(a, b)** Induced insulin secretion by vehicle (empty symbol) and compound **7** (library 3, Table S3) at 1 µM (filled symbol). **(c, d)** Insulin secretion induced by vehicle (empty symbol) and compound **8** at 1 µM (filled symbol). Islets were isolated on five independent days. Each day is depicted by one symbol. All experiments were performed both in GPR39 deficient mice (square) and wild type littermate controls (circles).

**Fig. (6). Physiological importance of GPR39 elucidated by small-molecule GPR39 agonist.** (**a**) Secretion of ghrelin from primary gastric mucosa cell cultures induced by 10 µM isoproterenol (grey column, n=5), 10 µM $Zn^{2+}$ (dark grey column, n=7), 1 µM **8** plus 10 µM $Zn^{2+}$ (green column, n=5) and 1 µM **26** alone (blue column, n=5) and with 10 µM $Zn^{2+}$ (dark blue column, n=7). Addition of a mixture of somatostatin receptor antagonists to both **8** and **26** plus $Zn^{2+}$ (columns with grids, n=3) resulted in a loss of the inhibitory effect of the compounds on ghrelin secretion. (**b**) Secretion of ghrelin in cells isolated from GPR39-KO mice was induced by 10 µM isoproterenol (grey hatched column, n=2). However, neither 10 µM $Zn^{2+}$ (dark grey hatched column, n=2), 1 µM **26** (blue hatched column, n=2), nor 1 µM **26** together with 10 µM $Zn^{2+}$ (dark-blue hatched column, n=2) had any effect on GPR39-KO cells. (**c**) Plasma ghrelin levels are significantly increased in GPR39-KO mice (black column, n=8) compared to WT mice (white column, n=8) after 4 h in dark phase. (**d, e**) Secretion of somatostatin induced by 10 nM CGRP (grey column, n=2), 10 µM $Zn^{2+}$ (dark grey column, n=7), 1 µM **8** plus 10 µM $Zn^{2+}$ (green column, n=5), 1 µM **26** (blue column, n=5) and 1 µM **26** plus 10 µM $Zn^{2+}$ (dark blue column, n=7) in cells isolated from WT mice (d) and GPR39-KO mice (e, hatched

columns, n=2). Stars above the columns represent significance from an unpaired T-test with Welch's correction compared to the vehicle control. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001. (f) Both compound **8** (left panel) and compound **26** (right panel) stimulate IP turnover in primary murine gastric mucosa cells in the presence of 10 μM $Zn^{2+}$. (**g**) As a mode of action, we propose that the agonists lead to the release of somatostatin from the projection of D-cells to inhibit ghrelin secretion from ghrelin-expressing cells in the gastric mucosa.

## Tabel 1



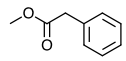
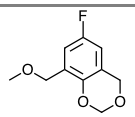
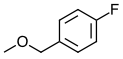
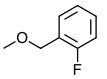
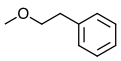
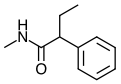
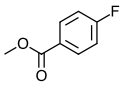
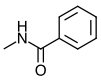
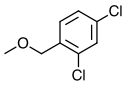
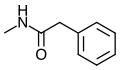
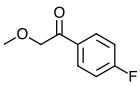
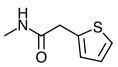
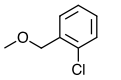
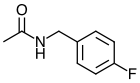
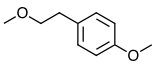
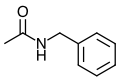
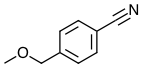
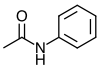
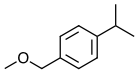
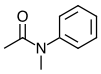
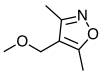
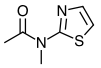
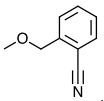
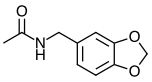
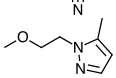
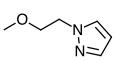
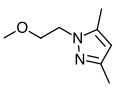
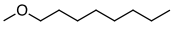
| # | Id | R | Comp. + 10 uM Zn EC50 (nM) Mean ± SEM | *n* | Comp. alone EC50 (nM) Mean ± SEM | *n* | # | Id | R | Comp. + 10 uM Zn EC50 (nM) Mean ± SEM | *n* | Comp. alone EC50 (nM) Mean ± SEM | *n* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **11** | TM-N1547 |  | 6 ± 1.9 | 2 | 381 | 1 | **26** | TM-N1562 |  | 1.5 ± 0.4 | 5 | 30 ± 2 | 5 |
| **12** | TM-N1548 |  | 1 ± 0.3 | 2 | 126 | 1 | **27** | TM-N1563 |  | 4 ± 0.1 | 2 | 78 | 1 |
| **13** | TM-N1549 |  | 3 ± 0.5 | 2 | 40 | 1 | **28** | TM-N1564 |  | 5 ± 0.0 | 2 | 79 | 1 |
| **14** | TM-N1550 |  | 4 ± 1.9 | 2 | 245 | 1 | **29** | TM-N1565 |  | 3 ± 1.0 | 2 | 158 | 1 |
| **15** | TM-N1551 |  | 4 ± 0.0 | 2 | 243 | 1 | **30** | TM-N1566 |  | 3 ± 0.2 | 2 | 418 | 1 |
| **16** | TM-N1552 |  | 3 ± 1.6 | 2 | 973 | 1 | **31** | TM-N1567 |  | 3 ± 1.0 | 2 | 244 | 1 |
| **17** | TM-N1553 |  | 2 ± 0.5 | 2 | 47 | 1 | **32** | TM-N1568 |  | 4 ± 0.9 | 2 | 1216 | 1 |
| **18** | TM-N1554 |  | 2 ± 0.1 | 2 | 70 | 1 | **33** | TM-N1569 |  | 5 ± 1.5 | 2 | 2931 | 1 |
| **19** | TM-N1555 |  | 4 ± 1.2 | 2 | 60 | 1 | **34** | TM-N1570 |  | 10 ± 0.6 | 2 | _ | 1 |
| **20** | TM-N1556 |  | 5 ± 0.8 | 2 | 1690 | 1 | **35** | TM-N1571 |  | 32 ± 5.1 | 2 | 5224 | 1 |
| **21** | TM-N1557 |  | 5 ± 1.4 | 2 | _ | 1 | **36** | TM-N1572 |  | 18 ± 7.4 | 2 | 3304 | 1 |
| **22** | TM-N1558 |  | 8 ± 2.3 | 2 | 127 | 1 | **37** | TM-N1573 |  | 7 ± 3.2 | 2 | 791 | 1 |
| **23** | TM-N1559 |  | 10 ± 2.3 | 2 | 1622 | 1 | **38** | TM-N1574 |  | 4 ± 0.5 | 2 | 111 | 1 |
| **24** | TM-N1560 |  | 16 ± 8.3 | 2 | 2265 | 1 | **39** | TM-N1575 |  | 2 ± 0.3 | 2 | 62 | 1 |
| **25** | TM-N1561 |  | 16 ± 3.2 | 2 | 2004 | 1 | **40** | TM-N1576 |  | 8 ± 0.3 | 2 | 388 | 1 |

Figure 1

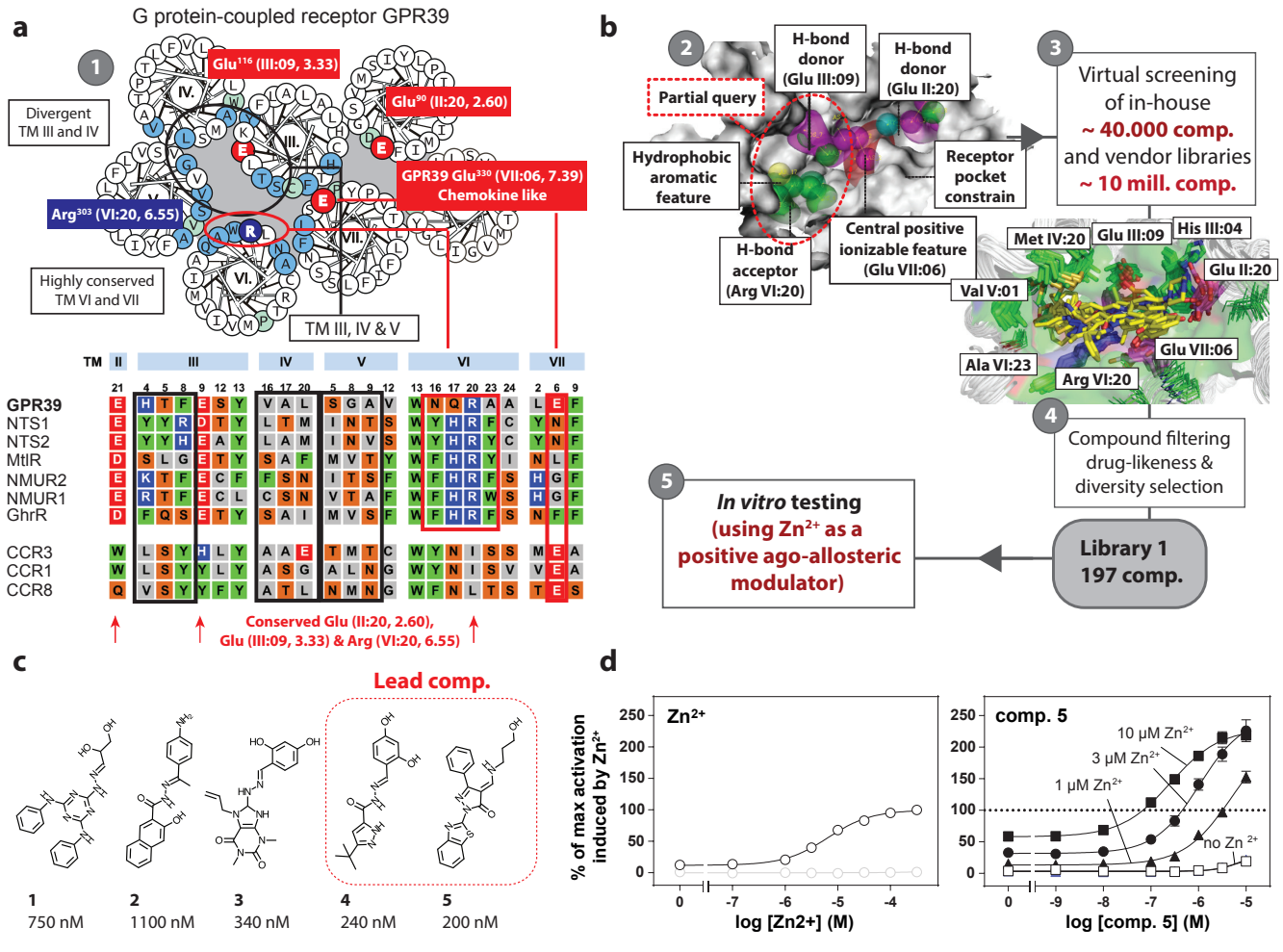

a
G protein-coupled receptor GPR39
Glu116 (III:09, 3.33)
Glu90 (II:20, 2.60)
GPR39 Glu330 (VII:06, 7.39) Chemokine like
Arg303 (VI:20, 6.55)
Divergent TM III and IV
Highly conserved TM VI and VII
TM III, IV & V
GPR39
NTS1
NTS2
MtlR
NMUR2
NMUR1
GhrR
CCR3
CCR1
CCR8
Conserved Glu (II:20, 2.60), Glu (III:09, 3.33) & Arg (VI:20, 6.55)
b
H-bond donor (Glu III:09)
H-bond donor (Glu II:20)
Partial query
Hydrophobic aromatic feature
Receptor pocket constrain
H-bond acceptor (Arg VI:20)
Central positive ionizable feature (Glu VII:06)
Virtual screening of in-house ~ 40.000 comp. and vendor libraries ~ 10 mill. comp.
Met IV:20
Glu III:09
His III:04
Glu II:20
Val V:01
Glu VII:06
Ala VI:23
Arg VI:20
Compound filtering drug-likeness & diversity selection
Library 1 197 comp.
In vitro testing (using Zn2+ as a positive ago-allosteric modulator)
c
Lead comp.
1 750 nM
2 1100 nM
3 340 nM
4 240 nM
5 200 nM
d
Zn2+
% of max activation induced by Zn2+
log [Zn2+] (M)
comp. 5
10 µM Zn2+
3 µM Zn2+
1 µM Zn2+
no Zn 2+
log [comp. 5] (M)

Figure 2

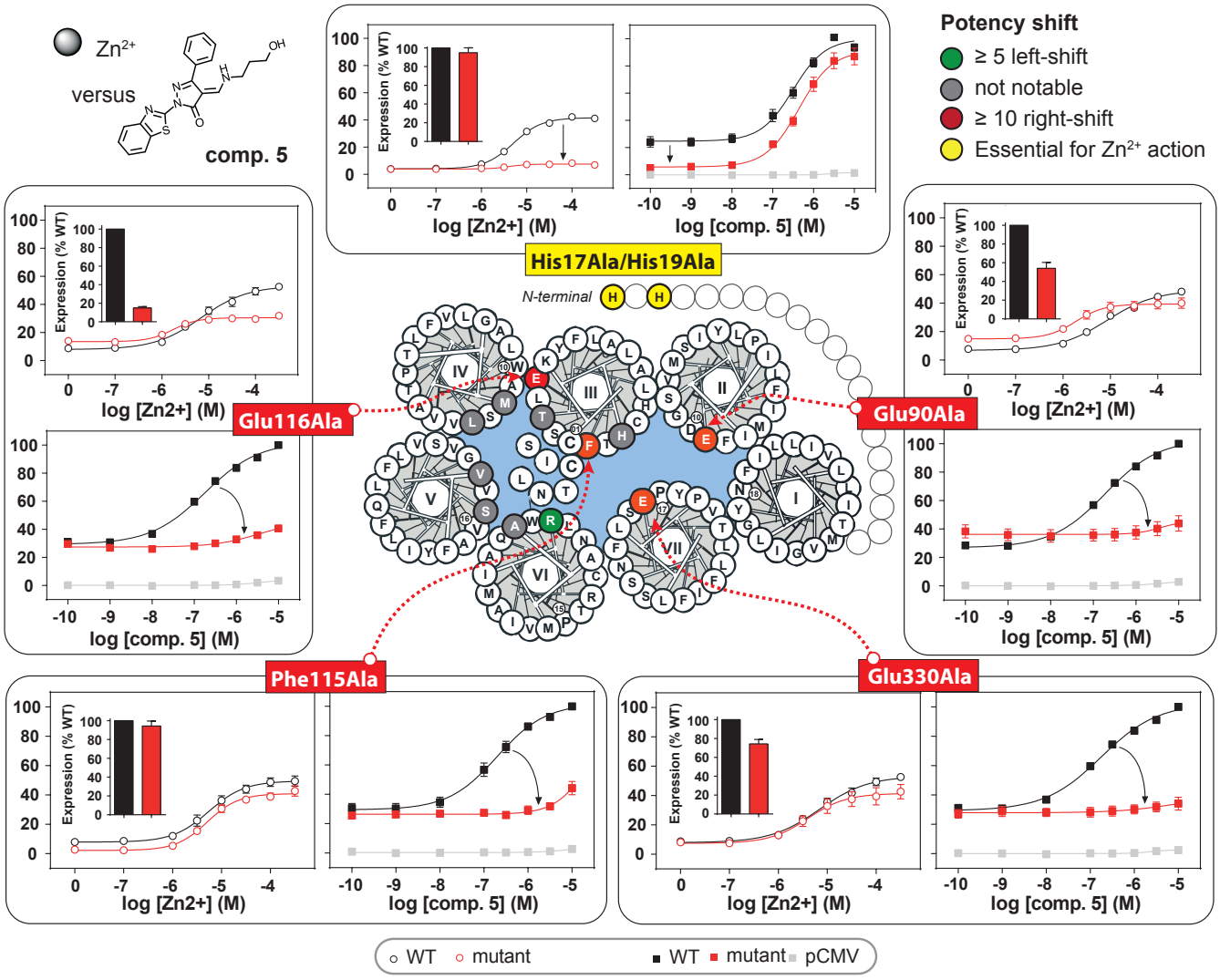

Zn2+
versus
comp. 5
Potency shift
≥ 5 left-shift
not notable
≥ 10 right-shift
Essential for Zn2+ action
His17Ala/His19Ala
N-terminal
Glu116Ala
Glu90Ala
Phe115Ala
Glu330Ala
Expression (% WT)
log [Zn2+] (M)
log [comp. 5] (M)
WT
mutant
pCMV

Figure 3

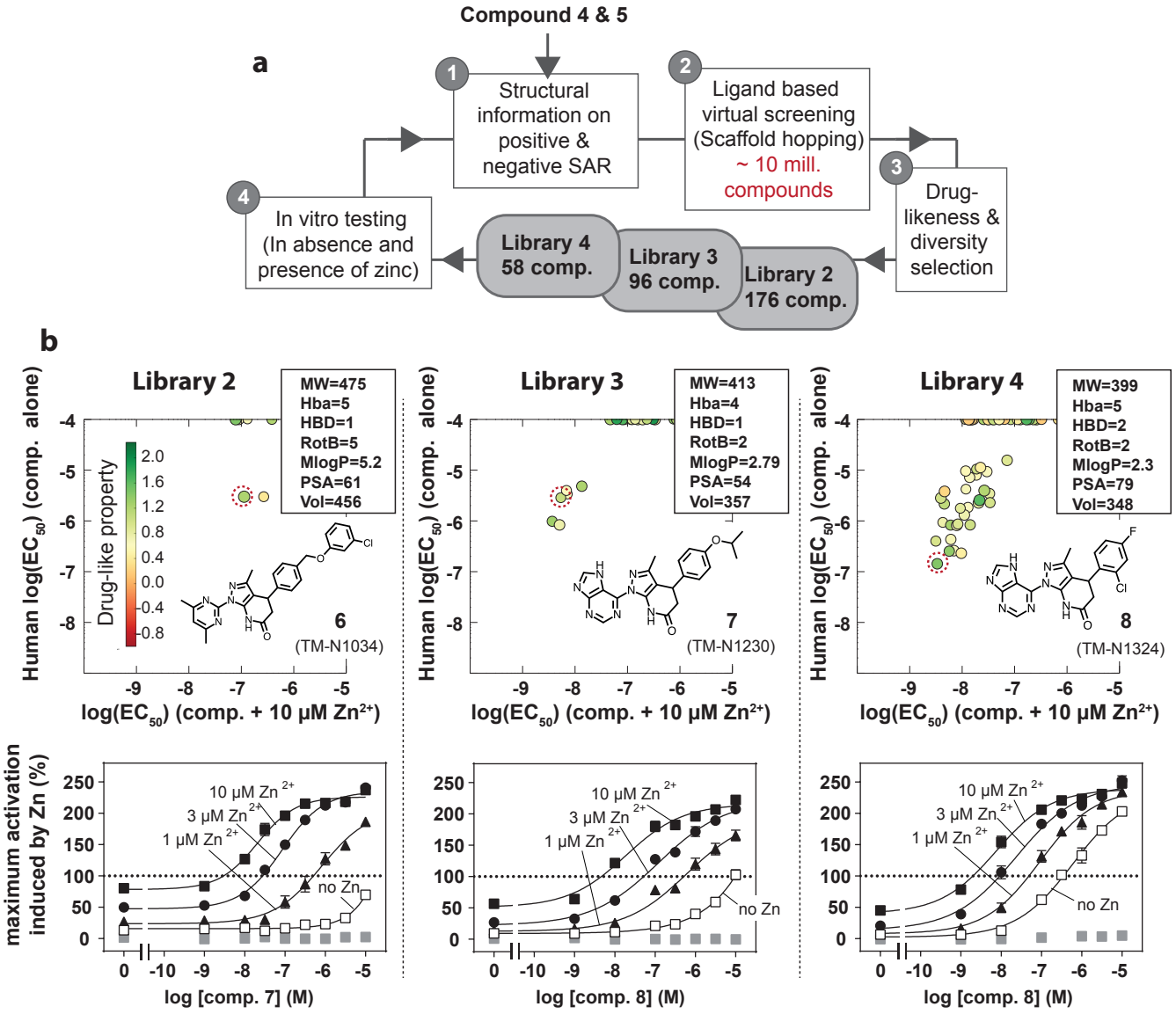

a
Compound 4 & 5
1
Structural information on positive & negative SAR
2
Ligand based virtual screening (Scaffold hopping)
~ 10 mill. compounds
3
Drug-likeness & diversity selection
4
In vitro testing (In absence and presence of zinc)
Library 4 58 comp.
Library 3 96 comp.
Library 2 176 comp.
b
Library 2
MW=475
Hba=5
HBD=1
RotB=5
MlogP=5.2
PSA=61
Vol=456
Drug-like property
6
(TM-N1034)
Library 3
MW=413
Hba=4
HBD=1
RotB=2
MlogP=2.79
PSA=54
Vol=357
7
(TM-N1230)
Library 4
MW=399
Hba=5
HBD=2
RotB=2
MlogP=2.3
PSA=79
Vol=348
8
(TM-N1324)
Human log(EC50) (comp. alone)
log(EC50) (comp. + 10 µM Zn2+)
maximum activation induced by Zn (%)
10 µM Zn 2+
3 µM Zn 2+
1 µM Zn 2+
no Zn
log [comp. 7] (M)
log [comp. 8] (M)

Figure 4

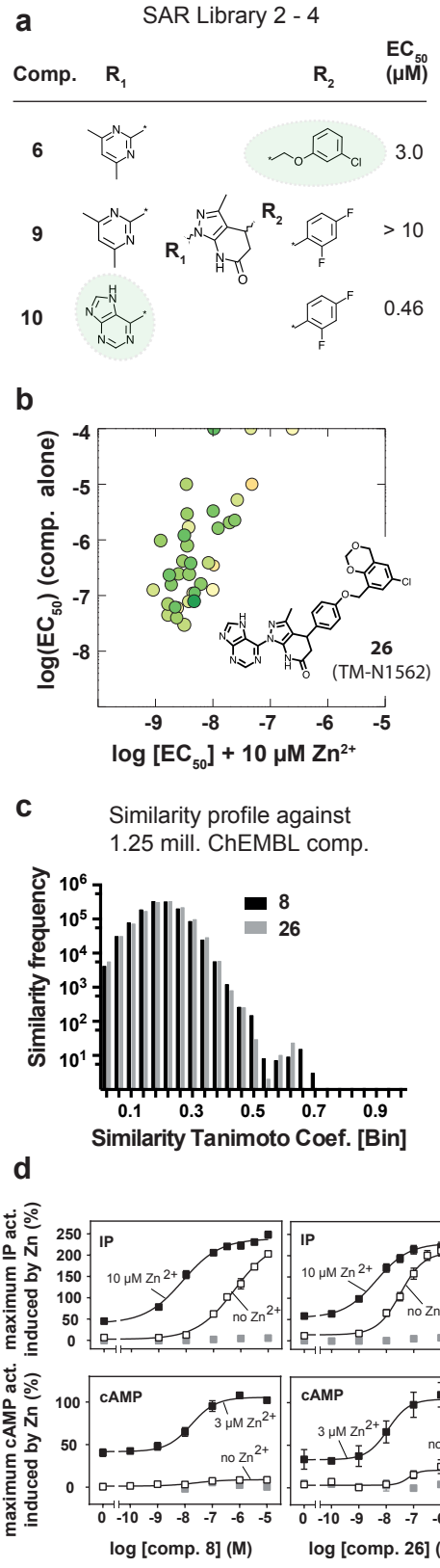

a
SAR Library 2 - 4
Comp.
R1
R2
EC50 (μM)
6
3.0
9
> 10
10
0.46
b
log(EC50) (comp. alone)
log [EC50] + 10 μM Zn2+
26
(TM-N1562)
c
Similarity profile against
1.25 mill. ChEMBL comp.
Similarity frequency
8
26
Similarity Tanimoto Coef. [Bin]
d
maximum IP act. induced by Zn (%)
maximum cAMP act. induced by Zn (%)
IP
cAMP
10 μM Zn2+
3 μM Zn2+
no Zn2+
log [comp. 8] (M)
log [comp. 26] (M)

Figure 5

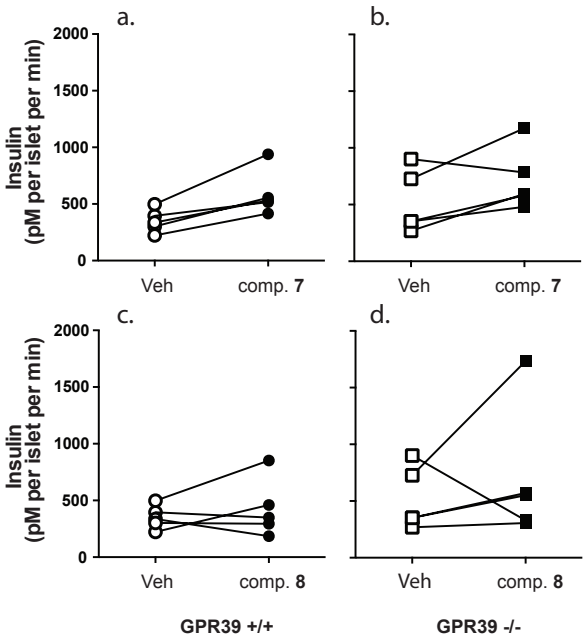
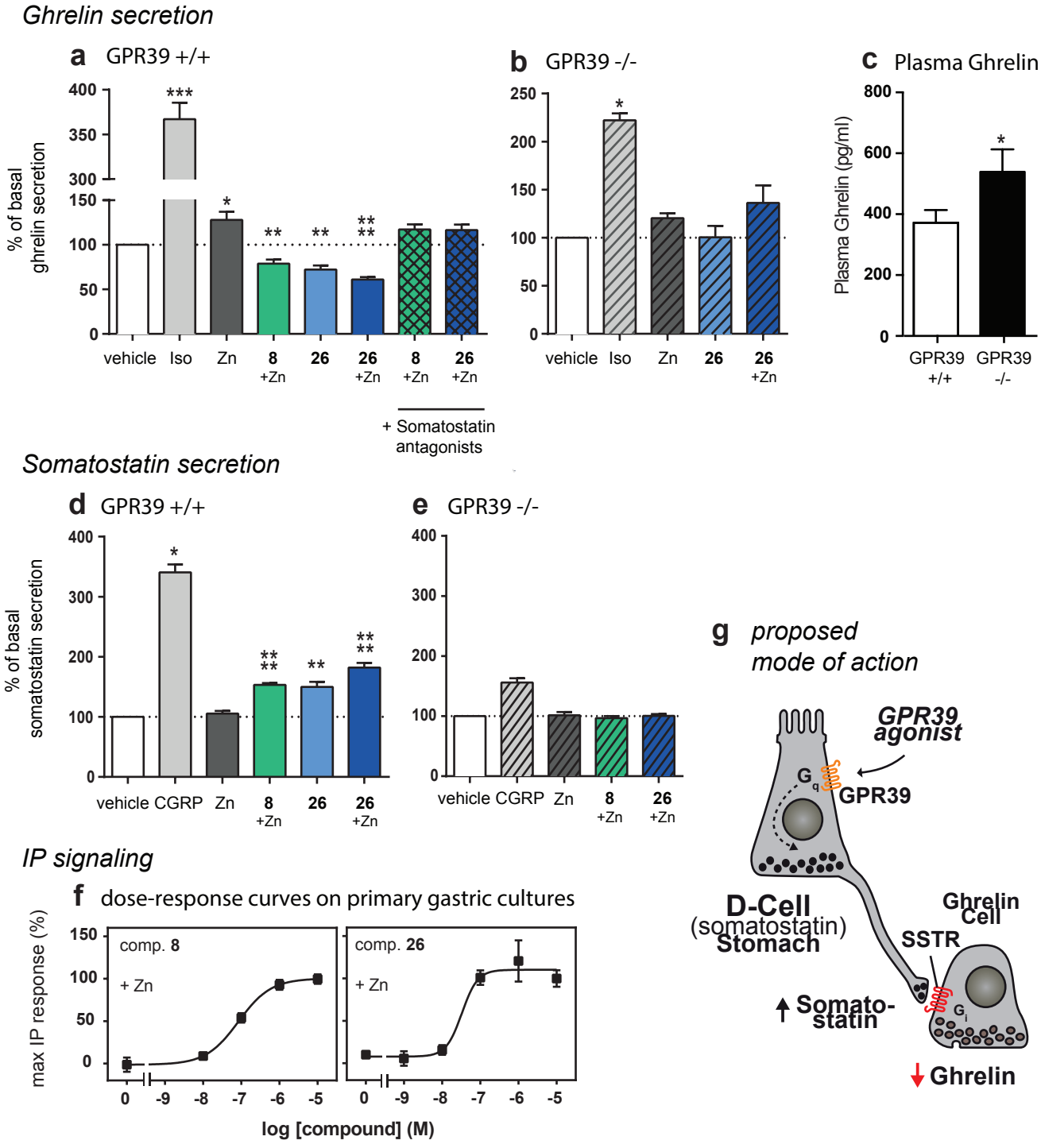

a.
b.
c.
d.
Insulin (pM per islet per min)
2000
1500
1000
500
0
Veh
comp. 7
comp. 8
GPR39 +/+
GPR39 -/-

Figure 6


Ghrelin secretion
a GPR39 +/+
% of basal ghrelin secretion
vehicle Iso Zn 8 +Zn 26 26 +Zn 8 +Zn 26 +Zn
+ Somatostatin antagonists
b GPR39 -/-
vehicle Iso Zn 26 26 +Zn
c Plasma Ghrelin
Plasma Ghrelin (pg/ml)
GPR39 +/+
GPR39 -/-
Somatostatin secretion
d GPR39 +/+
% of basal somatostatin secretion
vehicle CGRP Zn 8 +Zn 26 26 +Zn
e GPR39 -/-
IP signaling
f dose-response curves on primary gastric cultures
max IP response (%)
comp. 8
+ Zn
comp. 26
+ Zn
log [compound] (M)
g proposed mode of action
GPR39 agonist
GPR39
D-Cell (somatostatin) Stomach
Ghrelin Cell
SSTR
Somato-statin
Ghrelin

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