Discovery of a Positive Allosteric Modulator of the TSH Receptor: Potentiation of TSH-mediated Preosteoblast Differentiation In Vitro

Susanne Neumann, Elena Eliseeva, Alisa Boutin, Elena Barnaeva, Marc Ferrer, Noel Southall, David Kim, Xin Hu, Sarah J. Morgan, Juan J. Marugan, and Marvin C. Gershengorn

Primary laboratory of origin: Laboratory of Endocrinology and Receptor Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 50 South Drive, Bethesda, MD 20892, USA

Affiliations:
Laboratory of Endocrinology and Receptor Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 50 South Drive, Bethesda, MD 20892, USA: SN, EE, AB, SJM, MCG

Division of Pre-Clinical Innovations, National Center for Advancing Translational Sciences, National Institutes of Health, 9800 Medical Center Drive, Rockville, MD 20850, USA: EB, MF, NS, DK, XH, JJM
RUNNING TITLE PAGE

**Running Title:** Positive Allosteric Modulator of TSHR Signaling

**Corresponding Author:**

Susanne Neumann, PhD

National Institute of Diabetes and Digestive and Kidney Diseases, 50 South Dr., Building 50, Rm 4130, Bethesda, MD 20892, Phone: 301-451-6307, Fax: 301-480-4214

e-mail: susannen@intra.niddk.nih.gov

Number of text pages: 34

Number of tables: 0

Number of figures: 6

Number of references: 39

Number of words in the abstract: 247

Number of words in the introduction: 749

Number of words in the discussion: 962

**Nonstandard Abbreviations**

ALPL, Alkaline Phosphatase; DMEM, Dulbecco’s modified Eagle’s Medium; ELISA, Enzyme-linked Immunosorbent Assay; EMEM, Eagle's Minimum Essential Medium; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; HBSS, Hank's Balanced Salt Solution; IBMX, 3-Isobutyl-1-Methylxanthine; OPN, Osteopontin; PAM, Positive Allosteric Modulator; PBS, Phosphate Buffered Saline; PTH, Parathyroid Hormone; PTH1R, Parathyroid
Hormone 1 Receptor; qHTS, quantitative High-Throughput Screening; siRNA, small interfering RNA; TSH, Thyrotropin; bTSH, bovine TSH; TSHR, TSH Receptor; U2OS cells, Human Osteosarcoma cells

Section Assignment: Drug Discovery and Translational Medicine
Abstract

Recently, we showed that TSH-enhanced differentiation of a human preosteoblast-like cell model involved a β-arrestin 1 (β-Arr 1) mediated pathway. To study this pathway in more detail, we sought to discover a small molecule ligand that was functionally selective toward human TSH receptor (TSHR) activation of β-Arr 1. High-throughput screening using a cell line stably expressing mutated TSHRs and mutated β-Arr 1 (DiscoverX1 cells) led to the discovery of agonists that stimulated translocation of β-Arr 1 to the TSHR, but did not activate Gs-mediated signaling pathways, i.e. cAMP production. D3-βArr (NCGC00379308) was selected. In DiscoverX1 cells, D3-βArr stimulated β-Arr 1 translocation with a 5.1-fold greater efficacy than TSH and therefore, potentiated the effect of TSH in stimulating β-Arr 1 translocation. In U2OS-TSHR cells expressing wild type TSHRs, which is a model of human preosteoblast-like cells, TSH upregulated the osteoblast-specific genes osteopontin (OPN) and alkaline phosphatase (ALPL). D3-βArr alone had only a weak effect to upregulate these bone markers, but D3-βArr potentiated TSH-induced upregulation of ALPL and OPN mRNA levels 1.6-fold and 5.5-fold, respectively, at the maximum dose of ligands. Furthermore, the PAM effect of D3-βArr resulted in an increase of TSH-induced secretion of OPN protein. In summary, we have discovered the first small molecule positive allosteric modulator of TSHR. As D3-βArr potentiates the effect of TSH to enhance differentiation of a human preosteoblast in an in vitro model, it will allow a novel experimental approach for probing the role of TSH-induced β-Arr 1 signaling in osteoblast differentiation.
Introduction

Bone remodeling is essential for sustaining bone mass and systemic mineral homeostasis, and requires a balance between bone formation by osteoblasts and bone resorption by osteoclasts (Martin et al., 2009; Siddiqui and Partridge, 2016). Parathyroid hormone (PTH) is the major regulator of bone homeostasis (Qin et al., 2004), and acts by activating the PTH 1 receptor (PTH1R), which is a G protein-coupled receptor (GPCR). The anabolic effects of PTH appear to be mediated in large part via G protein-independent, β-arrestin 2 (β-Arr 2) signaling (Gesty-Palmer and Luttrell, 2011). The TSH receptor (TSHR), like PTH1R, is a GPCR (Kleinau et al., 2011) that is expressed primarily in thyroid follicular cells. The physiologic actions of TSH have been thought to be mediated by heterotrimeric G protein signaling pathways, and Gs-mediated cAMP signaling has been considered the primary pathway in the thyroid (Latif et al., 2009). However, TSHR expression has also been reported in bone (Williams, 2011). TSHR expression in rodent osteoblasts and osteoclasts has been demonstrated, effects of TSH on bone homeostasis have been studied, and the results suggested that TSH is a fine-tuning regulator of bone homeostasis (Abe et al., 2003; Abe et al., 2007). Specific effects observed in rodents include: TSHR knockout causes osteoporosis and focal osteosclerosis (Abe et al., 2003); TSH stimulates osteoblastogenesis in embryonic stem cells from mice (Baliram et al., 2011), and TSH administration prevents bone loss in adult, ovariectomized rats and mice (Sun et al., 2008). However, the role of TSH in human bone homeostasis is still unclear (Giusti et al., 2007; Karga, et al. 2010; Leader et al., 2014; Mazziotti et al., 2005). To gain insight into the effects of TSH in human cells, we have used a preosteoblast-like cell line derived from a human osteosarcoma made to stably express wild type TSHR, U2OS-TSHR cells (Boutin et al., 2014; Boutin et al., 2016). U2OS cells have been shown to differentiate into osteoblasts (Salvatori et al., 2009). We
have demonstrated that TSH enhances differentiation of U2OS-TSHR cells to osteoblasts, and that this effect is partly mediated by β-arrestin 1 (β-Arr 1) (Boutin et al., 2014).

Biased agonism, which is selective activation of one signaling pathway by a receptor that exhibits signal transduction via several pathways, has been shown for many GPCRs (Rankovic et al., 2016; Rominger et al., 2014; Wisler et al., 2014). The discovery that β-arrestins play a role in GPCR signaling has generated new strategies for drug therapies. Of note, a peptidic β-Arr 2 biased agonist for PTH1R, PTH-βarr, has been used in elucidating the role of β-Arr signaling in PTH1R biology (Gesty-Palmer and Luttrell, 2011). The physiologic response induced by PTH-βarr in bone is distinct from that of the conventional PTH1R agonist, PTH(1-34), a peptide fragment that contains the 34 N-terminal amino acid residues of the naturally occurring human PTH (hPTH), and that has the same biological activity as hPTH in stimulating bone formation (Mosekilde et al., 1991). Intermittent administration of PTH(1-34) increases bone formation, but PTH(1-34) administration also causes increased bone resorption that can lead to hypercalcemia and hypercalcuria. The β-arrestin selective PTH-βarr induces anabolic bone formation but does not increase markers of bone resorption (Gesty-Palmer et al., 2009). This example underlines that functionally selective ligands could have beneficial effects compared to the native agonists for a GPCR and, therefore, these ligands hold promise for more effective treatments of human diseases.

Modulators of GPCR signaling exhibit little functional activity by themselves but modify the signaling by the cognate ligand. Allosteric modulators bind to a receptor at a site distinct from that of the cognate ligand (orthosteric binding site), in many cases displaying pharmacological
characteristics that are different from orthosteric ligands. Therefore, allosteric modulators offer an alternate approach to gain potential therapeutic benefits (Gentry et al., 2015). Allosteric modulators may agonize (positive allosteric modulator, PAM) or antagonize (negative allosteric modulator, NAM) the activity (potency/efficacy) of orthosteric endogenous ligands (Gentry et al., 2015). PAMs, which potentiate agonist activity, have been developed for a number of receptors and ion channels (Burford et al., 2011; Conn et al., 2009; Wootten et al., 2013) including GPCRs with known ligands (Coopman et al., 2010; Harrington and Fotsch, 2007; Langmead et al., 2008; O'Brien et al., 2003; Wood, et al., 2016) and for two orphan receptors (Huang et al., 2015).

In light of the role of TSHR - β-Arr 1-mediated signaling in osteoblast differentiation (Boutin et al., 2014), we screened for small molecule β-Arr 1 signaling-selective agonists for TSHR that could be used to enhance TSH-induced osteoblast differentiation.
Materials and Methods

Culture of U2OS-TSHR cells, DiscoverX1 and DiscoverX2 cells

The generation of a U2OS (human osteosarcoma) cell line stably expressing wild type TSHRs (U2OS-TSHR) using the expression vector for native human TSHR was described previously (Boutin et al., 2014). DiscoverX1 and DiscoverX2 cells, U2OS cells expressing functional tagged TSHR and functional tagged β-Arr 1 or β-Arr 2, respectively, were purchased from DiscoverX (Fremont, CA, USA). U2OS-TSHR cells were cultured in Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA, USA). EMEM was supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO, USA), 50 units/ml penicillin and 50 µg/ml streptomycin (Life Technologies Inc., Carlsbad, CA, USA). Hygromycin B (250 µg/ml) (ThermoFisher Scientific, Waltham, MA, USA) was used as a selection marker. All experiments for the measurement of osteoblast markers were performed in the medium described above, but with 0.1% bovine serum albumin (BSA) instead of 10% FBS. For DiscoverX1 and DiscoverX2 cells, EMEM was also supplemented with 2 mM glutamine and 500 µg/ml geneticin (Mediatech, Manassas, VA, USA). Cells were cultured at 37°C in a humidified 5% CO2 incubator.

Quantitative high-throughput screening (qHTS)

qHTS was performed against 368,816 compounds from the Molecular Libraries Small Molecule Repository (MLSMR) at the National Center for Advancing Translational Sciences (NCATS) using the DiscoverX1 cells and the PathHunter® β-Arr 1 or β-Arr 2 translocate to the TSHR the
active β-galactosidase enzyme is created that can cleave a substrate to generate a chemiluminescent signal (DiscoverX). Two doses of the compounds - 11 μM and 57 μM - were used to measure TSHR activation using a fully automated robotic screening system (Kalypsys, San Diego, CA, USA) (Inglese et al., 2006). Briefly, 4.8x10³ DiscoverX1 cells were seeded with a MultiDrop Combi dispenser (ThermoScientific, Logan, UT) onto white solid bottom tissue culture treated 1536-well plates (Aurora Microplates, Inc., Whitefish, MT, USA) in 3 μL of AssayComplete™ Cell Plating 5 Reagent (DiscoverX) and cultured overnight. Then compounds (as DMSO solutions) were added with a Pintool (Perkin Elmer, Waltham, MA, USA) to the assay plates at 23 nL/well. The plates were incubated for 6 h at ambient temperature followed by addition of 1.5 μL PathHunter™ Detection Reagent per well per the manufacturer’s instructions. After 60 min incubation at ambient temperature the luminescence signal was measured in a ViewLux® uHTS Microplate Imager (Perkin Elmer) with 1 min exposure.

2,190 active compounds from the primary screen were selected based on their activity >30% of basal signal at the lower dose and >50% activity of the higher dose. The compounds were retested in the PathHunter® β-arrestin 1 Assay platform described above using a 7-concentration setting in the range of 49 nM to 76 μM. A parallel counter-screening for cAMP production was performed to select for compounds that activate the β-arrestin 1-mediated pathway only, and did not induce Gs-mediated cAMP production. cAMP production was measured in HEK-TSHR cells in the presence of 50 μM Ro-20-1724 (a phosphodiesterase inhibitor) (Sigma-Aldrich). Briefly, 2x10³ HEK-TSHR cells/well were seeded onto 1536-well white solid bottom plates in HBSS with 10 mM HEPES and 50 μM Ro-20-1724 followed by immediate addition of selected compounds to the cells at 23 nL/well and incubation at 37°C for 120 min. Subsequently,
detection reagents of Homogeneous-Time-Resolved-Fluorescence (HTRF®) cAMP Assay (Cisbio, Bedford, MA, USA) were prepared per the manufacturer’s instructions, added as a pre-mix in equal volumes of Europium³⁺-Cryptate-labeled anti-cAMP antibody and d² dye-labeled cAMP at 4µL/well, and incubated for 30 min at ambient temperature. The signal was measured by EnVision™ Multilabel Plate Reader (PerkinElmer) with excitation at 330 nm, emission at 620 nm (donor) and 665nm (acceptor). Results were analyzed as the ratio of 665nm/620nm multiplied by 10⁴.

High-throughput screening data from each assay plate were normalized plate-wise to corresponding intraplate controls, and corrected for systematic deviations in activity values using a combination of control wells within and between the screening plates (Inglese et al., 2006; Southall et al., 2009). Compound concentration-response curves were analyzed using in-house software, to the standard Hill equation and classified as described previously (Southall et al., 2009). The EC₅₀ values of compounds (concentration at 50% efficacy) were calculated by nonlinear regression analysis using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

**DiscoverX PathHunter® β-arrestin Assay for characterization of hit compounds**

β-Arr 1 and -2 translocation to the TSHR after receptor activation with the small molecule ligand D3-βArr or bovine TSH (bTSH, Sigma Aldrich) was measured with the PathHunter® β-arrestin Assay (DiscoverX). Briefly, 1.5 x 10⁴ cells per well were seeded into 96-well plates (Corning costar # 3610; Sigma Aldrich) in AssayComplete™ Cell Plating 5 Reagent (DiscoverX) 24 h before the experiment. DiscoverX1 cells and DiscoverX2 cells were exposed to TSH
concentrations between 0 and 10 µM and to D3-βArr concentrations between 0 and 100 µM for 4 h at room temperature in AssayComplete™ Cell Plating 5 Reagent and afterwards the signal (β-arrestin 1 or -2 translocation to the TSHR) was detected using the PathHunter® Detection Kit per the manufacturer’s instructions. The PathHunter® CHO-K1 GLP1R β-Arrestin-1 cells (DiscoverX) were used to test the specificity of the D3-βArr signal at the TSHR.

**Measurement of cAMP production in DiscoverX1 cells**

5 x 10^4 cells per well were seeded into 48-well plates. After 24 h the cells were assayed for activation (agonist) or inhibition (antagonist) of TSHR signaling in response to added ligands. Measurement of agonistic response: The cells were incubated for 60 min in HBSS/10 mM HEPES with 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich) and 0.18 µM TSH or 10 µM D3-βArr in a humidified 5% CO2 incubator at 37°C. Measurement of antagonistic response: The cells were incubated with 10 µM D3-βArr for 20 min in HBSS/10 mM HEPES. After pre-incubation, the medium was replaced with HBSS/10 mM HEPES containing 1 mM IBMX and 18 nM TSH and 10 µM D3-βArr in a humidified 5% CO2 incubator at 37°C. After 60 min, the incubation was terminated by aspiration of the incubation medium and addition of lysis buffer of the cAMP-Screen Direct™ System (Life Technologies Corporation, Grand Island, NY, USA). cAMP content was determined as described by the manufacturer. The chemiluminescence signal was measured in a VICTOR3 V 1420 Multilabel Counter (PerkinElmer, Shelton, CT, USA).
Measurement of cAMP production in primary cultures of human thyrocytes

Thyroid tissue samples were collected from normal thyroid tissue from patients undergoing total thyroidectomy for thyroid cancer at the National Institutes of Health Clinical Center. Patients provided informed consent on an Institutional Review Board-approved protocol, and materials were received anonymously via approval of research activity through the Office of Human Subjects Research. Primary cultures of human thyrocytes were established from 5 donors as described previously (Neumann et al., 2011). Thyrocytes (0.8×10^5 cells/well) were seeded into 24-well plates in DMEM containing 10% FBS. 24 h prior to the experiment, media was changed to 0.1% BSA-containing DMEM. Cells were equilibrated for 30 min in HBSS/10 mM HEPES, pH 7.4, prior to stimulation. Cells were pretreated with DMSO (control) or 10 µM D3-βArr in HBSS/10 mM HEPES for 1 h. Cells were then stimulated with vehicle or 90 nM TSH (EC50 for cAMP in human thyrocytes) for 2 h at 37°C in a humidified incubator in the presence of 1 mM IBMX. Incubations were stopped, and the cells were lysed by adding 125 μL lysis buffer from the cAMP-Screen Direct™ System. The cAMP content of the cell lysate was determined using the method described in the manufacturer's protocol. The chemiluminescence signal was measured in a VICTOR3™ V 1420 Multilabel Counter (PerkinElmer).

Quantitative Real Time PCR

Total RNA was purified using RNeasy Mini Kits (Qiagen Inc, Valencia, CA, USA). First strand cDNA was prepared using a High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation). RT-PCR was performed in 25 µl reactions using cDNA prepared from 100 ng or less of total RNA and TaqMan Universal PCR Master Mix (Life Technologies Corporation). The mRNA expression of osteoblast-specific genes alkaline phosphatase (ALPL) and osteopontin
(OPN, gene name SSP1 (secreted phosphoprotein 1)) was measured using primers and probes from Life Technologies Corporation. Quantitative RT-PCR results were normalized to GAPDH to correct for differences in RNA input.

**Osteopontin (OPN) secretion measurement by ELISA**

U2OS-TSHR cells were seeded into 24-well plates at 7 x 10^4 cells per well. The cells were serum starved in EMEM with 0.1% BSA 24 h before addition of TSHR ligands. Thereafter, the cells were incubated with D3-βArr or TSH alone or in combination for 7 days in 0.1% BSA-containing EMEM. Cell culture supernatants were used to determine OPN secretion levels by ELISA (Human OPN Quantikine ELISA, R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

**Transfection of U2OS-TSHR cells with siRNA**

U2OS-TSHR cells were seeded in EMEM with 10% FBS into 100 mm dishes at 1.75 x 10^6 cells per dish. After 24 h, the cells were transfected with ON-TARGETplus SMART pool human β-arrestin 1 or β-arrestin 2 siRNA or ON-TARGET plus non-targeting pool siRNA using DharmaFECT 1 transfection reagent per the manufacturer’s instructions (Thermo Fisher Scientific Inc., Waltham, MA, USA). 48 h after transfection, cells were seeded into 24-well plates at 7 x 10^4 cells per well and incubated overnight. 24 h later the cells were treated with TSH with and without D3-βArr in EMEM containing 0.1% BSA. After 5 days at 37°C in a humidified incubator, measurement of OPN secretion was performed as described above.
Data and statistical analysis

Data analysis was performed with GraphPad Prism Version 7 for Windows (GraphPad Software). Concentration response data in figures 1, 3, and 5 were analyzed with nonlinear regression curve fit, dose-response - stimulation - log(agonist) vs. response - variable slope (four parameter) in GraphPad Prism 7. All experimental data are presented as mean ± S.D. The data were analyzed by unpaired two-tailed t-test; *P<0.05 was considered significant.
Results

Discovery of a functionally selective TSHR agonist for β-Arr 1-mediated signaling by qHTS

A HTS assay using DiscoverX1 cells allowed us to measure β-arrestin translocation stimulated by TSHR activation. A total of 368,816 compounds were screened using a fully automated screening system in the qHTS format (Inglese et al., 2006). We identified 975 hit compounds that translocated β-Arr 1 to the TSHR and that were inactive regarding stimulating TSHR-mediated cAMP production. 106 out of these 975 hit compounds were selected based on potency and efficacy after testing in a 9-point dose response assay in DiscoverX1 cells. We finally selected D3-βArr (Fig. 1A) as the lead compound to study further based on its signaling activity and selectivity for TSHR. The glucagon-like peptide 1 receptor (GLP1R) has been shown to signal through β-Arr 1 as well (Quoyer et al., 2010). We used an engineered cell line, CHO-K1-GLP1R-β Arrestin 1 (DiscoverX), which allows measurement of the translocation of β-Arr 1 to the GLP1R with the PathHunter®β-Arrestin Assay, as control to test the specificity of D3- βArr signaling. In contrast to GLP1, D3-βArr did not activate β-Arr 1 translocation to the GLP1R (Supplemental Fig. 1), a result that is consistent with the idea that D3-βArr initiates translocation of β-Arr 1 by direct TSHR activation.

The concentration-dependence of D3-βArr-stimulated translocation of β-Arr 1 and -2 compared to their translocation by TSH is illustrated in Figure 1B. D3-βArr stimulated β-Arr 1 translocation with an efficacy 3-fold greater than TSH (Fig. 1B). While D3-βArr was more effective in translocating β-Arr 1 than TSH, it was 1.9-fold less effective in translocating β-Arr 2. The EC50 for β-Arr 1 translocation by TSH was 0.08 µM (Confidence Interval (CI) 0.05 to 0.1 µM). The EC50 for β-Arr 1 translocation by D3-βArr was 11.6 µM (CI 9.5 to 14.2 µM). The
translocation assay clearly demonstrated that D3-βArr binds and activates the TSHR. However, D3-βArr had no agonistic effect on cAMP production nor did it inhibit TSH-induced cAMP production in DiscoverX1 cells (Fig. 2A, B). Noteworthy, D3-βArr did not inhibit TSH-induced cAMP production in primary cultures of human thyrocytes from 5 donors in which TSHRs are expressed at physiological levels (Fig. 2C). Therefore, D3-βArr is a functionally selective TSHR agonist toward β-Arr 1 translocation.

**D3-βarr is a positive allosteric modulator for TSH-induced translocation of β-Arr 1**

We found that D3-βArr potentiates the ability of TSH to translocate β-Arr 1 to the TSHR in DiscoverX1 cells. When we added increasing concentrations of TSH with a constant dose of D3-βArr (10 µM), we found that D3-βArr increased the efficacy of TSH in the translocation of β-Arr 1 at a maximum of 5.1±0.1-fold over TSH alone (Fig. 3). Thus, D3-βArr is a positive allosteric modulator (PAM) for TSHR. There was no effect of D3-βArr on TSH potency. The EC50s were 64 nM for TSH alone, and 62 nM in combination with D3-βArr.

**D3-βarr is a positive allosteric modulator for TSH-enhanced osteoblast differentiation**

We have demonstrated previously that β-Arr 1 plays a role in TSH-mediated upregulation of osteoblast marker genes in vitro (Boutin, et al., 2014). Therefore, we next determined if the PAM effect of D3-βArr on β-Arr 1 translocation to the TSHR will translate into potentiation of TSH-induced upregulation of osteoblast-specific genes osteopontin (OPN) and alkaline phosphatase (ALPL). For these experiments, we used U2OS-TSHR cells that are derived from a human osteosarcoma and are a model of osteoblast precursors that we used previously (Boutin et al., 2014; Boutin et al., 2016). U2OS cells are frequently used to study the regulation of osteoblastic
genes. U2OS-TSHR cells were made to stably express TSHRs as the parental line has only a very low endogenous expression level of TSHR. TSHR mRNAs in parental U2OS cells were 1/10,000 of the level in U2OS-TSHR cells. Treatment of parental U2OS cells with TSH did not result in a physiological response like cAMP production or increase in osteoblast gene expression. Therefore, the changes in gene expression in U2OS-TSHR cells described below are specific for the TSHR.

Figure 4 illustrates the effects of D3-βArr, TSH or the combination of D3-βArr and TSH on the mRNA levels of these genes in U2OS-TSHR cells. D3-βArr alone did not significantly increase OPN mRNA, but induced a 4±0.6-fold higher ALPL mRNA level. TSH, as shown previously (Boutin et al., 2014), increased OPN and ALPL mRNA levels in a dose-dependent manner. Importantly, D3-βArr potentiated the TSH-mediated increase of OPN and ALPL mRNA. At 0.3 µM TSH, D3-βArr (10 µM) increased OPN mRNA levels from 6±1-fold to 325±37-fold, and ALPL mRNA levels from 21±1-fold to 33±1.4-fold above control. At 1 µM TSH, D3-βArr (10 µM) increased OPN mRNA levels from 184±20-fold to 1010±111-fold, and ALPL mRNA levels from 44±3.8-fold to 69±5.4-fold above control.

Furthermore, we confirmed the potentiating effect of D3-βArr on the level of OPN protein secretion (Fig. 5). U2OS-TSHR cells were treated with increasing doses of TSH (0 to 10 µM) alone or in combination with 1µM or 5 µM D3-βArr. Importantly, D3-βArr alone had no agonistic effect on OPN secretion. OPN secretion was only increased in the presence of TSH, and D3-βArr acted as a potentiator of the TSH-induced effect on OPN secretion in a dose-dependent manner. A low dose of TSH (0.5 µM) increased OPN secretion 1.7±0.1-fold over
control, and 1 µM and 5 µM D3-βArr potentiated the TSH effect to increase OPN secretion to 2.5±0.2-fold and 3.2±0.2-fold above control, respectively. A higher dose of TSH (5 µM) increased OPN 21.7±0.9-fold, and 1 µM and 5 µM D3-βArr increased OPN secretion further to 31.5±1.9-fold and 41.0±1.7-fold above control, respectively. Therefore, D3-βArr is a PAM of TSH-enhanced differentiation of osteoblast markers. Noteworthy, D3-βArr does not alter the potency of TSH for OPN secretion. The EC50s were 1.2 µM for TSH alone, and 1.2 µM and 1.1 µM for TSH in combination with 1 µM and 5 µM D3-βArr, respectively.

The potentiating effect of D3-βArr on TSH-induced OPN secretion is mediated by β-Arr 1

Since D3-βArr induces translocation of β-Arr 1 and to a lesser extent of β-Arr 2 to the TSHR, we asked if the effect of this PAM on OPN secretion is mediated by β-Arrestins and if one or both, β-Arr 1 and β-Arr 2, are involved (Fig. 6). β-Arr-1 and -2 were knocked down separately in U2OS-TSHR cells using siRNA. RT-qPCR showed a knockdown efficiency of 74.8±5.2% and 92.4±1.2% for β-Arr 1 and β-Arr 2, respectively (Fig. 6A). In cells treated with scrambled siRNA (control), TSH increased OPN secretion 3.0±0.2-fold, and the potentiating effect of D3-βArr on TSH increased the response to 5.3±0.2-fold over basal of control. Knockdown of β-Arr 1 inhibited TSH-induced OPN secretion to 1.9±0.2-fold over basal of control (36% inhibition), and the synergistic response of TSH and D3-βArr to 2.6±0.2-fold over basal of control (51% inhibition), which is below the level of OPN secretion induced by TSH alone. The silencing of β-Arr 2 had no effect on OPN secretion (TSH: 2.8±0.3-fold, TSH and D3-βArr: 5.8±0.4-fold over basal of control). The β-Arr 1 knockdown showed that TSH-induced OPN secretion is also mediated by β-Arr 1, and the PAM effect of D3-βArr on TSH-induced OPN secretion appears to be solely mediated by β-Arr 1.
Discussion

In vivo studies in rodents have shown that TSH can prevent bone loss and stimulate bone formation (Abe et al., 2007; Sampath et al., 2007; Sun et al., 2008). Based on these studies and others, it has been proposed that these effects may occur by activating TSHRs on preosteoblasts. However, the TSHR-mediated signaling mechanisms in bone are only now being delineated and few studies have used human cells. We have, therefore, performed our studies in human-derived preosteoblast-like U2OS-TSHR cells in which wild type, human TSHRs are stably expressed. It has been known that the TSHR can interact with members of all G protein families (Laugwitz et al., 1996), and we have shown previously (Boutin et al., 2014) that TSHR activation by TSH will lead to β-Arr 1-mediated signaling. Specifically, we showed that TSHR-induced upregulation of osteoblast gene markers in U2OS-TSHR cells, like PTH effects in bone precursors (Gesty-Palmer et al., 2009), is mediated by G protein- and β-Arrin-mediated pathways (Boutin et al., 2014; Boutin et al., 2016) with TSHR acting via β-Arr 1 and PTH1R via β-Arr 2. We demonstrated that TSH-induced upregulation of interleukin 11 is primarily mediated via Gs-cAMP-protein kinase A, ALPL upregulation is predominantly regulated by activation of Gq/11-phosphokinase C-ERK1/2, and OPN expression and secretion is mediated by β-Arr 1 and Gi-p38α mitogen-activated protein kinase cascades (Boutin et al., 2016).

Functionally selective GPCR signaling can be caused by ligands that selectively stimulate different signaling pathways leading to distinct physiological responses. Our finding that TSHR-induced differentiation of U2OS-TSHR cells to osteoblasts is mediated in part by β-Arr 1 (Boutin et al., 2014) supports the idea that β-Arr 1 signaling plays a pivotal role in anabolic effects of TSH on bone metabolism. This finding is similar to those induced by the peptidic
biased agonist PTH-βarr, which increases bone formation by activation of PTH1R-mediated β-Arr 2 signaling (Gesty-Palmer et al., 2009). To study the role of the β-Arr 1 mediated signaling pathway in TSHR physiology in bone in more detail, we used a HTS to identify a functionally selective agonist of β-Arr 1 signaling by the human TSHR. We identified the small molecule TSHR agonist D3-βArr (Fig. 1A), and demonstrated that D3-βArr translocates β-Arr 1 to the TSHR, and that it exhibits selective recruitment of β-Arr 1 over that of β-Arr 2 (Fig. 1B). D3-βArr did not translocate β-Arr 1 to the GLP1R in control experiments with CHO-GLP1R-β Arrestin 1 cells (Supplemental Fig. 1), which utilize the same enzyme fragment complementation technology as DiscoverX1 cells, supporting the idea that D3-βArr directly activates the TSHR. Furthermore, D3-βArr is a functionally selective ligand. It does not stimulate Gs-mediated signaling (Fig. 2A) nor does it inhibit TSH-induced cAMP production in DiscoverX1 cells overexpressing the TSHR (Fig. 2B) or in primary cultures of human thyrocytes in which the TSHR is expressed at an endogenous level (Fig. 2C). The functional selectivity of D3-βArr allowed us to accentuate the β-Arr 1-mediated signaling over signaling by G protein-dependent pathways as would have occurred with the native ligand TSH. Indeed, the stronger efficacy for translocation of β-Arr 1 induced by D3-βArr in comparison to TSH (Fig. 1B) might be explained by β-Arr 1 activation in the absence of potential competition with G protein(s) for binding to TSHR. The functional selectivity of D3-βArr toward β-Arr 1 signaling, combined with the fact that D3-βArr does not stimulate nor inhibit TSH-induced cAMP, is promising since Gs-mediated cAMP production is a major player in thyroid hormone synthesis, and therefore, a stimulatory or inhibitory effect of D3-βArr on thyroid metabolism would be unwanted in animals or humans.
As demonstrated in Figure 1B, D3-βArr alone will induce TSHR-mediated translocation of βArr 1 to the receptor in DiscoverX1 cells. This demonstrates that D3-βArr binds to and activates the TSHR without the presence of TSH. Furthermore, a pivotal finding of this study is that D3-βArr potentiated the efficacy of TSH in stimulating β-Arr 1 translocation to the TSHR (Fig. 3). Since we had demonstrated previously the role of β-Arr 1-mediated signaling in osteoblast differentiation in vitro, we opted to evaluate the potentiating effect of D3-βArr on bone marker expression. These experiments were carried out in previously established U2OS-TSHR cells that stably express the wild type, human TSHR and have endogenous wild type β-Arr 1 and β-Arr 2 expression (Boutin et al., 2014). D3-βArr potentiated TSH-mediated upregulation of OPN and ALPL mRNA (Fig. 4), and OPN secretion (Fig. 5). Importantly, D3-βArr alone has no effect on OPN secretion, but it potentiates the efficacy of the orthosteric ligand TSH thus making the overall response dependent on the presence of the natural ligand, and indicating selectivity of D3-βArr towards the TSHR. Therefore, the physiological rhythms of TSH - TSHR signaling in vivo will be preserved, and adverse effects of the small molecule ligand will be limited. The knockdown of β-Arr 1 confirmed our previous finding that β-Arr 1-mediated signaling plays a pivotal role in TSH-induced OPN secretion (Boutin et al., 2014). Recently, we have shown that multiple TSHR-activated signaling pathways lead to OPN secretion (Boutin et al., 2016). More importantly, we demonstrated that the potentiating effect of D3-βArr is solely mediated by β-Arr 1 and not by β-Arr 2 (Fig. 6B).

In conclusion, we have discovered a functionally selective PAM for the TSHR, which to our knowledge, is the first nonpeptidic PAM to affect TSHR-induced upregulation of osteoblast marker genes in a human in vitro cell model. D3-βArr provides a novel experimental approach to
study TSHR-mediated β-Arr 1 signaling in bone and to understand the functional mechanisms underlying the selective signaling induced by this small molecule TSHR agonist. This PAM or an analog of D3-βArr may be a valuable probe to study TSHR physiology in bone *in vivo* in future studies.
Authorship Contributions

**Participated in research design:** Neumann, Eliseeva, Boutin, Ferrer, Southall, Morgan, Marugan, Gershengorn

**Conducted experiments:** Neumann, Eliseeva, Boutin, Barnaeva, Kim, Morgan

**Contributed new reagents or analytic tools:** Kim, Marugan

**Performed data analysis:** Neumann, Eliseeva, Boutin, Barnaeva, Ferrer, Southall, Kim, Hu, Morgan, Marugan, Gershengorn

**Wrote or contributed to the writing of the manuscript:** Neumann, Gershengorn
References


Latif R, Morshed SA, Zaidi M, and Davies TF (2009) The thyroid-stimulating hormone receptor: impact of thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies


Mosekilde L, Sogaard CH, Danielsen CC, and Torring O (1991) The anabolic effects of human parathyroid hormone (hPTH) on rat vertebral body mass are also reflected in the quality of bone, assessed by biomechanical testing: a comparison study between hPTH-(1-34) and hPTH-(1-84). *Endocrinology* **129**:421-428.


Footnotes

* This research was supported by the Intramural Research Program of the National Institutes of Health, the National Institute of Diabetes and Digestive and Kidney Diseases [Z01 DK011006], and the National Center for Advancing Translational Sciences.

Reprint requests should be addressed to:

Susanne Neumann, PhD
National Institute of Diabetes and Digestive and Kidney Diseases, 50 South Dr., Building 50, Rm 4130, Bethesda, MD 20892, Phone: 301-451-6307, Fax: 301-480-4214
e-mail: susannen@intra.niddk.nih.gov
Figures

Figure 1. D3-βArr translocates β-Arrrestins to the TSHR and is functionally selective toward β-Arr 1 A: 2-dimensional structure of D3-βArr (N-(1-phenylethyl)-2-(1-piperazinyl)-4-quinazolinamine; NCGC00379308). B: β-Arrrestin translocation to the TSHR was measured with the PathHunter® β-Arrrestin Assay. DiscoverX1 cells (ARR1) and DiscoverX2 cells (ARR2) were exposed to the indicated concentrations of D3-βArr or TSH for 240 min. The dose-dependencies of TSH-induced translocation are the same in ARR1 and ARR2 cells although the absolute levels of fluorescence are different in the two cell lines. We show the levels in the two cell lines as % of maximum TSH response (TSH Rmax) in each cell line. D3-βArr is more effective in translocating β-Arr 1 than TSH, but it is less effective in translocating β-Arr 2 than TSH. The data are from three experiments with duplicate samples and are presented as mean ± S.D. Statistical significance is determined by unpaired two-tailed t-test (****P<0.0001).

Figure 2. D3-βArr is a functionally selective TSHR agonist of β-Arr 1 mediated signaling and does not antagonize TSH-induced cAMP production. A: DiscoverX1 cells were exposed to 0.18 μM TSH or 10 μM D3-βArr in HBSS with 1 mM IBMX for 1 h. cAMP levels were measured by ELISA. In contrast to TSH, D3-βArr-induced activation of the TSHR does not lead to cAMP production. The data are from three experiments with duplicate samples. B and C: TSH does not antagonize TSHR stimulation of cAMP production. B: DiscoverX1 cells were pretreated with DMSO (Basal) or 10 μM D3-βArr in HBSS for 30 min. Subsequently, cells were exposed to 18 nM TSH (EC50) with or without 10 μM D3-βArr in HBSS with 1 mM IBMX for 1 h. cAMP levels were measured by ELISA. The data are from three experiments with duplicate
samples. 

C: Primary cultures of human thyrocytes from 5 different donors were studied. Cells were pretreated with DMSO (Basal) or 10 µM D3-βArr in HBSS/HEPES for 1 h. Subsequently, cells were exposed to 90 nM TSH (EC50 for cAMP in human thyrocytes) with or without 10 µM D3-βArr in HBSS with 1 mM IBMX for 2h at 37°C. Incubations were stopped, the cells were lysed, and cAMP levels were measured by ELISA. A-C: The data are presented as mean ± S.D. Statistical significance is determined by unpaired two-tailed t-test (****P<0.0001; N.S., not significant).

Figure 3. D3-βArr is a positive allosteric modulator for TSHR - β-Arr 1 translocation in DiscoverX1 cells. β-Arr 1 translocation to the TSHR was measured with the PathHunter® β-Arrestin Assay. DiscoverX1 cells were exposed to increasing concentrations of TSH with or without 10 µM D3-βArr for 240 min. D3-βArr potentiates the effect of TSH in translocating β-Arr 1 to the TSHR. The data are from four experiments with duplicate samples and are presented as mean ± S.D. Statistical significance is determined by unpaired two-tailed t-test (****P<0.0001).

Figure 4. D3-βArr is a positive allosteric modulator for TSH-induced upregulation of OPN and ALPL mRNAs in U2OS-TSHR cells. Cells were treated with the indicated doses of TSH with and without 10 µM D3-βArr in EMEM with 0.1% BSA. The data represent the gene expression levels of osteoblastic markers OPN and ALPL after 7 days of incubation with ligands. Samples were analyzed by quantitative RT-PCR. The data are from 3 experiments with duplicate samples and are presented as mean ± S.D. Statistical significance is determined by unpaired two-tailed t-test (**P<0.01; ***P<0.001; ****P<0.0001).
**Figure 5. D3-βArr is a positive allosteric modulator for TSH-enhanced OPN secretion in U2OS-TSHR cells.** Cells were treated with increasing doses of TSH with and without 1 µM or 5 µM D3-βArr in EMEM with 0.1% BSA for 7 days. OPN levels in cell culture supernatants were measured by ELISA. The data are from two experiments (1 µM) and three experiments (5 µM) with duplicate samples and are presented as mean ± S.D. Statistical significance is determined by unpaired two-tailed t-test (****P<0.0001).

**Figure 6. The potentiating effect of D3-βArr on TSH-induced OPN secretion is mediated by β-Arr 1 in U2OS-TSHR cells.** Cells were transfected with non-targeting (CONTROL), β-Arr 1 (ARR1) and β-Arr 2 (ARR2) siRNAs, respectively. A: Knockdown of ARR1 and ARR2 is demonstrated by RT-PCR. ARR1 and ARR2 mRNAs were measured 72 hours after transfection. The mRNA level in non-treated control cells was set at 100%. The data are from 4 independent experiments with duplicate samples and are presented as mean ± SE. B: 72 h after transfection with siRNA, the cells were treated with 1.8 µM TSH with and without 10 µM D3-βArr in EMEM with 0.1% BSA for 5 days. OPN secretion was determined by measurement of OPN by ELISA in cell culture medium. The bars represent the mean ± S.D. of four experiments with duplicate samples. Statistical significance is determined by unpaired two-tailed t-test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; N.S., not significant).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Supplemental Figure 1. D3-βArr does not activate β-Arr1 translocation to the glucagon-like peptide 1 receptor (GLP-1R) in CHO-K1-GLP1R-β Arrestin 1 cells. This is consistent with the idea that D3-βArr is a TSH receptor-specific ligand. 10,000 cells per well were seeded in 96-well plates in AssayComplete™ Cell Plating 5 Reagent (DiscoverX) 24 h before the experiment. Cells were stimulated with Exendin (native ligand) or D3-βArr at indicated concentrations for 90 min at 37°C, and subsequently, the signal (β-Arr1 translocation to the GLP-1R) was detected using the PathHunter® Detection Kit (DiscoverX) per the manufacturer’s instructions. The data are presented as mean ± S.D.