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Discovery of substituted benzamides as follicle stimulating hormone receptor allosteric modulators

Henry N. Yu [a,b,*], Thomas E. Richardson [c], Selva Nataraja [b], David J. Fischer [a], Venkataraman Sriraman [a], Xuliang Jiang [a], Pandi Bharathi [c], Robert J. Foglesong [c], Thomas F. N. Haxell [c], Brian H. Heasley [c], Mathew Jenks [c], Jane Li [a], Melanie S. Dugas [a], Regina Collis [a], Hui Tian [a], Stephen Palmer [b], Andreas Goutopoulos [a]

[a] EMD Serono Research Institute, 45A Middlesex Turnpike, Billerica, MA 01821, United States
[b] TocopheRx, Inc., Suite 1077, 15 New England Executive Park, Burlington, MA 01803, United States
[c] Scynexis, Inc., 3501 C Tricenter Boulevard, Durham, NC 27713, United States

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ABSTRACT

Follicle-stimulating hormone (FSH), acting on its receptor (FSHR), plays a pivotal role in the stimulation of follicular development and maturation. Multiple injections of protein formulations are used during clinical protocols for ovulation induction and for in vitro fertilization that are followed by a selection of assisted reproductive technologies. In order to increase patient convenience and compliance several research groups have searched for orally bioavailable FSH mimetics for innovative fertility medicines. We report here the discovery of a series of substituted benzamides as positive allosteric modulators (PAM) targeting FSHR. Optimization of this series has led to enhanced activity in primary rat granulosa cells, as well as remarkable selectivity against the closely related luteinizing hormone receptor (LHR) and thyroid stimulating hormone receptor (TSHR). Two modulators, **9j** and **9k**, showed promising in vitro and pharmacokinetic profiles.

Gonadotropins are a family of structurally-related heterodimer glycoprotein hormones that serve important functions in the reproductive process. This family consists of three members: two pituitary and one placenta hormones. Follicle stimulating hormone (FSH) and luteinizing hormone (LH), are released from the anterior pituitary under the control of gonadrotropin-releasing hormone from the hypothalamus, and chorionic gonadotropin (CG) is produced in the placenta. These three gonadotropins are also structurally related to another glycoprotein hormone, thyroid stimulating hormone (TSH).[1] All four glycoprotein hormones are marketed therapeutic drugs. FSH is used clinically in women for the treatment of anovulatory infertility and for controlled ovarian stimulation in in vitro fertilization (IVF) and in men for hypogonadotropic hypogonadism.[2]

FSH exercises its physiological function by acting on its receptor (FSHR) on specific gonadal somatic cells that leads to initiation of ovarian and testicular differentiation and steroidogenesis. FSHR, together with the other glycoprotein hormone receptors (LHR and TSHR), are members of the G-protein coupled receptor (GPCR) superfamily. The superfamily transduces extracellular signals into G protein activation via their seven-helical transmembrane (7TM) domain.[3] Ligand-stimulated FSHR leads to the activation of a cascade of kinases in a cAMP-dependent manner. Glycoprotein hormone receptors are unique among 7TM GPCRs for their large ectodomains at the N-terminus and short cytoplasmic tails at the C-terminus. While the nature of hormone–receptor interaction has been mapped and the structure of FSH binding to the ectodomain of FSHR has been solved,[4–6] the crystal structure of the full-length FSHR, however, has not been available. Consequently, atomic details about how the protein hormone activates the 7TM domain remain unknown.

Although FSH is highly effective and has been heralded as a useful drug for couples who undergo fertility treatment,[7] more than two-thirds of infertile women discontinue infertility treatments before FSH therapy is initiated. The number of patients dropping out of fertility treatment can be as high as 70%.[8] FSH, a protein mol-

* Corresponding author. Tel.: +1 978 294 1422.
E-mail address: henry.yu@emdserono.com (H.N. Yu).

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0960-894X/

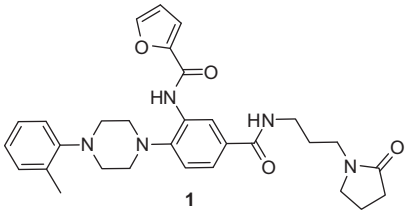


Figure 1. Chemical structure of compound **1**.

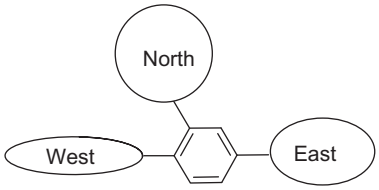


Figure 2. Schematic illustration of the SAR exploration of different regions of the compound series.

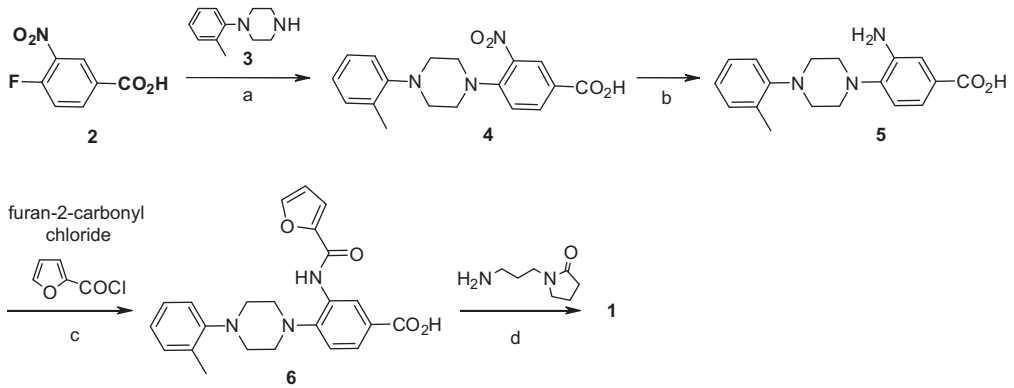
ecule, must be administrated daily or twice daily by injections for two weeks. Although the convenience of FSH injections have been improved with long lasting versions of FSH,[9–12] women report the daily injections are unbearable, and are one of the main reasons that patients who seek fertility treatment decide to drop out.[13] Small molecule drugs, on the other hand, are advantageous, as they can be taken orally.[14] Thus, there is an unmet medical need for bioavailable FSH mimetics that would be more convenient for the patients and potentially improve their treatment compliance. To date, several chemical series of low-molecule-weight (LMW) FSH mimetics have been reported.[15–21] Two have been demonstrated by mutagenesis studies to bind the 7TM domain of FSHR,[19,22] but a defined domain of the receptor where the small molecules binds is unknown and awaits structural studies of the small-molecule/FSHR complex. In addition to being developed for drug candidates, LMW ligands have been useful tools for obtaining GPCR crystals[23] and deciphering the target disulfide pairs.[24] As biased signaling is a general phenomenon in GPCR functions, including FSHR actions,[25] it is advantageous to have several chemical series available for elucidation of the FSHR mechanism and for the 'holy grail' of drug development targeting FSHR.[26] Here we report another chemical series of small molecules targeting FSHR.

The substituted benzamide **1** (Fig. 1) was identified as a promising starting point following screening of a small molecule library. High-throughput screening was performed using a Chinese hamster ovarian (CHO) cell line expressing the recombinant human FSH receptor (CHO-hFSHR) in the presence of an EC_{20} concentration of FSH. This concentration of FSH corresponds to the basal levels of hormone present in the circulation of a great majority of women seeking infertility treatments.[27] Cyclic adenosine monophosphase (cAMP), the second messenger stimulated by FSH, was measured as the readout in this assay.[28] These compounds were also counter-screened in CHO cells expressing human TSHR (hTSHR), human LHR (hLHR) and parental CHO cells. Compounds specific for hFSHR were selected. The EC_{50} of compound **1** in the CHO-hFSHR assay is 164 nM.

Synthesis of **1**, depicted in Scheme 1, is the general synthetic scheme used for derivatives.[29] Displacement of the aryl fluoride of **2** with piperazine **3** in an SNAr reaction followed by hydrogenation of the nitro group of **4** rendered aniline **5** in an overall yield of 35%. Acylation of **5** with furan-2-carbonyl chloride followed by amide coupling of **6** afforded **1** in 50% yield.

In order to investigate the structure-activity relationship (SAR) around the central aromatic ring, analogs were prepared in a fashion similar to Scheme 1. Efficacy of these derivatives to stimulate cAMP in CHO-hFSHR cells was monitored. We first explored the SAR in the northern part (Fig. 2) of the molecule (Table 1). The furan regio-isomer (**7a**) was less potent than compound **1**. Blocking the N–H with a methyl group rendered ~8.8 μM potency in analog **7b**, which indicates that the hydrogen donor is critical for the potency. Sulfonamide replacement of the amide gave little agonist activity (**7c**). 5-Methyl furan analog (**7d**) retained potency, but its isomer **7e** was reduced in potency by about three fold. Replacement of the furan with other five-member rings (**7f**, **7g**, **7h**, **7i**) gave a range of results, while 2-cyclopropyloxazole (**7h)** provided the most promising compound (EC_{50} = 99 nM). Interestingly, the simple cyclopropane analog (**7j)** maintained potency of 199 nM. The phenyl analog (**7k**) showed a reduced potency (554 nM). Replacement of the aryl ring in **7k** with a 2-pyridine (**7i**) retained most of the potency (184 nM). The importance of the N position in the pyridine ring is shown by little agonist activity shown in the two pyridine isomers (**7m** and **7n**).

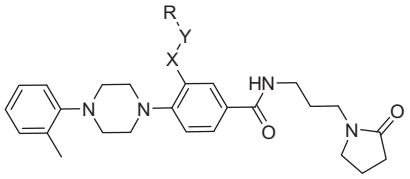
The SAR in the western region of compound **1**, maintaining either a furan or a 2-cyclopropyloxazole in the northern section, quickly demonstrated a steep SAR around the piperazine phenyl moiety (Table 2). A minor change of the 2-methyl group resulted in a loss of potency. For instance, substitution of the 2-methyl with an electron withdrawing group 2-CF_3 (**8a**) leads to a 6-fold decrease in EC_{50}. The electron donating group 2-OMe (**8b**) analog displayed little agonist activity. Isomers of *o*-tolyl showed reduced potencies as well, with an EC_{50} of 1.4 μM for the *m*-tolyl analog (**8c**). The *p*-tolyl analog (**8d)** loses most of its agonist activity, and replacement of the *o*-tolyl group with 2-pyridine (**8f**) or H

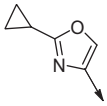
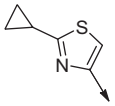


Scheme 1. Reagents and conditions: (a) K_2CO_3, DMF, 36%; (b) Pd/C, H_2, EtOH/MeOH, 94%; (c) Et_3N, DMAP, DCM, 56%; (d) HOBt, EDC, DCM, 84%.

(**8e**) showed a similar result. Replacement of the piperazine with piperidine (**8g**) resulted in a loss of activity. Due to either steric or electronic perturbation within the binding pocket, we were unable to improve on the piperidine phenyl moiety despite over one hundred analogs that were synthesized and screened. It is interesting to note that the 2-methyl piperidine (**8h**) analog showed an antagonistic activity (−36%) at a concentration of 50 μM, suggesting a switch from an agonist to an antagonist with a small structure change. Although the assay format is not designed to identify antagonists, it is not an uncommon finding that GPCR allosteric modulators can switch from agonists to antagonists through substitution.[30–32]

Table 1



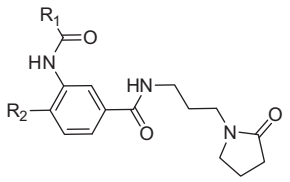
Compound	X	Y	R	EC_{50}[a] (nM)
7a	NH	C(O)		494
7b	NMe	C(O)		8820
7c	NH	$S(O)_2$		10%@12[b] μM
7d	NH	C(O)		129
7e	NH	C(O)		508
7f	NH	C(O)		4900
7g	NH	C(O)		250
7h	NH	C(O)		99
7i	NH	C(O)		184
7j	NH	C(O)		199
7k	NH	C(O)		554
7l	NH	C(O)		185
7m	NH	C(O)		37%@12[b] μM
7n	NH	C(O)		34%@12[b] μM

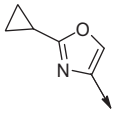
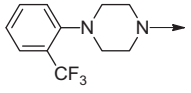
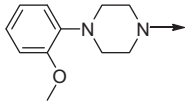
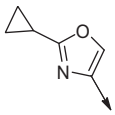
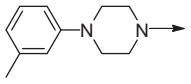
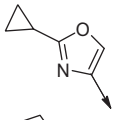
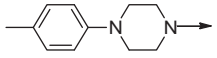
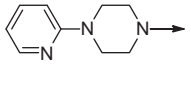
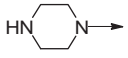
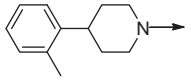
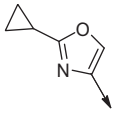
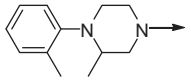
[a] Values are means of at least two experiments.
[b] % Response of the compounds compared to maximal FSH response.

To explore the SAR at the eastern region systematically, we synthesized and tested additional compounds (Table 3). A constraint approach to the propyl linker (**9a**) led to loss of activity; however, shorter linkers, such as **9b**, **9d**, **9e**, **9k**, proved to be more tolerated, and so did the replacement of the pyrrolidin-2-one with other five- (**9e**, **9f**, **9h**, **9i**, **9j**) or six-member rings (**9g** and **9k**). This indicates that this region of the protein binding pocket accommodates different fragments although it is still critical for potency, as the complete removal of the pyrrolidin-2-one (**9c**) shifted the EC_{50} to 1.8 μM. More interestingly, when the carbonyl was replaced with methylene, **9l** switches to a potential antagonist, with -91% activity at 50 μM. Similar to **8h** above, a proper assay format is needed to validate the antagonist property of the compound. An FSHR antagonist has the potential to be a novel, non-steroidal contraception treatment as well as a useful tool for characterization of the full-length crystal structure of FSHR. We are currently carrying out more research on the antagonist mode with **9l**, **8h** and their related analogs. These results will be disclosed in future publications.

Compound **1** was tested in in vitro rat microsomal stability assays[15] and it showed high rates of clearance in human and rat microsomes at 312 μl/min/mg and 233 μl/min/mg, respectively. Likewise, an in vitro metabolic liability was observed in **7h**, with Clint values in human and rat microsomes of 260 μl/min/mg and

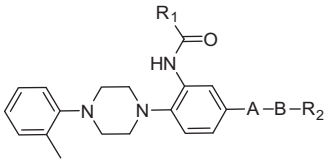
Table 2

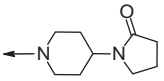
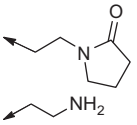
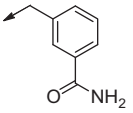
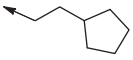
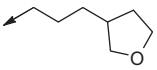
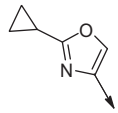
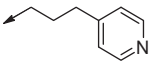
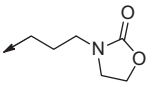
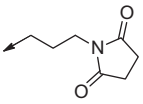
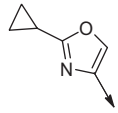
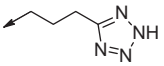
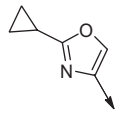
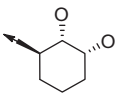
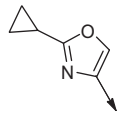
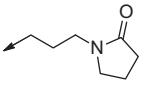


Compound	R_1	R_2	EC_{50}[a] (nM)
8a			1001
8b			20%@50[b] μM
8c			1440
8d			13%@50[b] μM
8f			20%@50[b] μM
8e			1%@50[b] μM
8g			1%@50[b] μM
8h			−36%@50[b] μM

[a] Values are means of at least two experiments.
[b] % Response of the compounds compared to maximal FSH response.

Table 3



Compound	R_1	A	B	R_2	EC_{50}[a] (nM)	Microsomal stability (human, rat) (μl/min/mg protein)
9a		C(O)	B + R_2 =		40%@12[b] μM	
9b		C(O)	NH		30	
9c		C(O)	NH		1850	
9d		C(O)	NH		46	54, 31
9e		C(O)	NH		129	
9f		C(O)	NH		88	
9g		C(O)	NH		153	
9h		C(O)	NH		28	59, 25
9i		C(O)	NH		184	33, 38
9j		C(O)	NH		89	20, 18
9k		C(O)	NH		53	72, 13
9l		CH_2	NH		4.5%@12[b] μM	

[a] Values are means of at least two experiments.
[b] % Response of the compounds compared to the maximal FSH response.

225 μl/min/mg, respectively. Improved in vitro metabolic stability was achieved with substitution of the pyrrolidin-2-one moiety (Table 2, **9d**, **9h**–**9k**).

Compound **9j** and **9k** were chosen to be further profiled in a rat granulosa cell assay[33] and evaluated for in vivo pharmacokinetics (Table 4). Both compounds induced estradiol (E2) production in the rat granulosa cells with EC_{50} values of 170 nM and 5 nM, respectively. Both compounds achieved an E_{max} ~80% as compared to the maximum normalized FSH response of 100%. These partial-agonist modulators could be used to selectively target specific signaling pathway of FSHR and have the potential for better control and prevention of unwanted effects like ovarian hyperstimulation in IVF treatment.[30] Interestingly, the compounds required EC_{20} FSH concentration for stimulating the primary granulosa cells, thus behaving as PAM. Thus, women undergoing infertility treatment in the clinic have basal levels of FSH,[27] so the compounds can potentiate this low level of FSH to achieve the desired clinical effect. Both compounds were advanced into

Table 4
In vitro and in vivo data for selected compounds

Compound	Rat granulosa cell EC_{50}, (% efficacy compared to FSH)[a]	Immature rat pharmacokinetic parameters[b]			
		F%	CL (mL/min/kg)	$T_{1/2}$	V_{dss} (L/Kg)
9j	170 nM (78%)	21	13.2	1.95	5.46
9k	5 nM (81%)	23	3.41	4.4	1.65

[a] Values are the means from at least two experiments.
[b] Clearance (CL), and volume of distribution were generated following a 0.2 mg/kg iv dose in immature Sprague-Dawley rats. Oral bioavailability (%*F*) and half-life ($T_{1/2}$) were determined following a 0.5 mg/kg po dose in immature Sprague-Dawley rats.

PK studies. The in vivo PK study showed that both compounds have a distinct PK profile; compound **9j** exhibited high clearance and a short half-life, while **9k** had medium clearance and a longer half-life. The additional advantage of **9j** was the aqueous solubility (160 μM at pH 7.4). In addition, both compounds showed significant in vitro selectivity over the related luteinizing hormone receptor (LHR) (>1000-fold selectivity) and thyrotropin receptor (TSHR) (>1000-fold selectivity). Short acting FSHR compounds with surge-like PK properties have been reported to have an inhibitory effect in an animal model;[34] thus molecules with distinct PK profiles make both compounds valuable research tools in animal model studies. Both compounds are being advanced into PK/PD studies via various routes of administration. The complicated in vivo biological result will be reported in due course.[35]

In summary, we have identified a series of benzamides from high-throughput screening, represented by compound **1**, as FSHR PAMs. Development of these novel FSHR PAMs was prepared using compound **1** as a starting point for SAR development. Although the western region had a particularly steep SAR, the western side chains were useful in modulating both potency and microsomal stability. Compounds **9j** and **9k** displayed good potency on CHO cells expressed with human FSHR and also in primary granulosa cell assays. These partial agonist modulators have the potential for better control and prevention of ovarian hyper-stimulation in IVF treatment. Compounds **9j** and **9k** had acceptable PK properties and are candidates for future PK/PD studies.

References and notes

1. Jiang, X.; Dias, J. A.; He, X. *Mol. Cell. Endocrinol.* **2014**, *382*, 424.
2. Chappel, S. C.; Howles, C. *Hum. Reprod.* **1991**, *6*, 1206.
3. Pierce, K. L.; Premont, R. T.; Lefkowitz, R. J. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 639.
4. Jiang, X.; Dreano, M.; Buckler, D. R.; Cheng, S.; Ythier, A.; Wu, H.; Hendrickson, W. A.; el Tayar, N. *Structure* **1995**, *3*, 1341.
5. Fan, Q. R.; Hendrickson, W. A. *Nature* **2005**, *433*, 269.
6. Jiang, X.; Liu, H.; Chen, X.; Chen, P. H.; Fischer, D.; Sriraman, V.; Yu, H. N.; Arkinstall, S.; He, X. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 12491.
7. Quints, L., Ed.Life; Henry Luce: New York, NY, 1956; Vol. 59, p 24.
8. Huppelschoten, A. G.; van Dongen, A. J. C. M.; Philipse, I. C. P.; Hamilton, C. J. C. M.; Verhaak, C. M.; Nelen, W. L. D. M.; Kremer, J. A. M. *Hum. Reprod.* **2013**, *28*, 2177.
9. Garone, L. M.; Arkinstall, S. J.; Brondyk, W. H.; Campbell, R. K.; Jiang, X.; McKenna, S. D.; Tepper, M.; US 7700112, 2010.
10. Brondyk, W. H.; Jiang, X.; Campbell, R.; US 7317095, 2008.
11. Loutradis, D.; Drakakis, P.; Vlismas, A.; Antsaklis, A. *Curr. Opin. Investig. Drugs* **2009**, *10*, 372.
12. Muda, M.; Jiang, X.; McKenna, S. D.; US 7956034, 2011.
13. Gameiro, S.; Boivin, J.; Peronace, L.; Verhaak, C. *Hum. Reprod. Update* **2012**, *18*, 652.
14. McGregor, M. J.; Luo, Z.; Jiang, X. In *Drug Discovery Research. New Frontiers in the Post-Genomic Era*; Huang, Z., Ed.; Wiley-VCH: Weinheim, Germany, 2007; p 63.
15. Guo, T.; Adang, A. E.; Dolle, R. E.; Dong, G.; Fitzpatrick, D.; Geng, P.; Ho, K.-K.; Kultgen, S. G.; Liu, R.; McDonald, E. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1713.
16. Guo, T.; Adang, A. E.; Dong, G.; Fitzpatrick, D.; Geng, P.; Ho, K.-K.; Jibilian, C. H.; Kultgen, S. G.; Liu, R.; McDonald, E. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1717.
17. Pelletier, J. C.; Rogers, J.; Wrobel, J.; Perez, M. C.; Shen, E. S. *Bioorg. Med. Chem.* **2005**, *13*, 5986.
18. Wrobel, J.; Jetter, J.; Kao, W.; Rogers, J.; Di, L.; Chi, J.; Perez, M. C.; Chen, G. C.; Shen, E. S. *Bioorg. Med. Chem.* **2006**, *14*, 5729.
19. Yanofsky, S. D.; Shen, E. S.; Holden, F.; Whitehorn, E.; Aguilar, B.; Tate, E.; Holmes, C. P.; Scheuerman, R.; MacLean, D.; Wu, M. M. *J. Biol. Chem.* **2006**, *281*, 13226.
20. van Straten, N.; Timmers, M. *Annu. Rep. Med. Chem.* **2009**, *44*, 171.
21. Jorand-Lebrun, C.; Brondyk, B.; Lin, J.; Magar, S.; Murray, R.; Reddy, A.; Shroff, H.; Wands, G.; Weiser, W.; Xu, Q. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2080.
22. van Koppen, C. J.; Verbost, P. M.; van de Lagemaat, R.; Karstens, W. J.; Loozen, H. J.; van Achterberg, T. A.; van Amstel, M. G.; Brands, J. H.; van Doornmalen, E. J.; Wat, J.; Mulder, S. J.; Raafs, B. C.; Verkaik, S.; Hanssen, R. G.; Timmers, C. M. *Biochem. Pharmacol.* **2013**, *85*, 1162.
23. Rosenbaum, D. M.; Rasmussen, S. G.; Kobilka, B. K. *Nature* **2009**, *459*, 356.
24. Bruysters, M.; Verhoef-Post, M.; Themmen, A. P. *J. Biol. Chem.* **2008**, *283*, 25821.
25. Landomiel, F.; Gallay, N.; Jégot, G.; Tranchant, T.; Durand, G.; Bourquard, T.; Crépieux, P.; Poupon, A.; Reiter, E. *Mol. Cell. Endocrinol.* **2014**, *382*, 452.
26. Lunenfeld, B. *Hum. Reprod. Update* **2004**, *10*, 453.
27. de Ziegler, D.; Fraisse, T.; de Candolle, G.; Vulliemoz, N.; Bellavia, M.; Colamaria, S. *Reprod. Biomed. Online* **2007**, *15*, 507.
28. In Cho-hFSHR-cells (2500 cells/well) were plated in 5 μl of phenol red free DMEM/F12 + 1% FBS. Cells were plated in 384 well, solid white low volume plates (Greiner 784075) by Multidrop. Varying concentrations of stimulants (FSH and compounds with 200 μM IBMX) was added in 5 μl of media. Compounds were generally stimulated in the presence of EC_{20} FSH (0.265 pM). The plates were incubated at 37 °C for 1 h. Following stimulation, cAMP produced was measured using HTRF reagents (CisBio #62AM4PEC), as described by the manufacturer. In brief, 10 μl of mixed HTRF reagents were added per well and incubated at room temperature for 1 h. The plates were read on EnVisionmultilabel plate reader (Perkin Elmer, CA). The readout was the calculated fluorescence ratio (665 nm/620 nm). Dose response curves for FSH and compounds were generated using Genedata (Genedata, Basel, Switzerland). EC_{50} value and % response of the compounds compared to maximal FSH were calculated.
29. Yu, H.; Li, J.; Richardson, T. E.; Bharathi, P.; Heasley, B.; Goutopoulos, A. US 20130023522, 2013.
30. Arey, B. J. *Endocrine* **2008**, *34*, 1.
31. Wood, M. R.; Hopkins, C. R.; Brogan, J. T.; Conn, P. J.; Lindsley, C. W. *Biochemistry* **2011**, *50*, 2403.
32. Dias, J. A.; Bonnet, B.; Weaver, B. A.; Watts, J.; Kluetzman, K.; Thomas, R. M.; Poli, S.; Mutel, V.; Campo, B. *Mol. Cell. Endocrinol.* **2011**, *333*, 143.
33. Primary rat granulosa cells were isolated from rats implanted with diethylstilbesterol [DES] pellets (1 mg/day; 7-day release). The pellets were implanted subcutaneously on 22-day old rats with trochar employing aseptic techniques after isoflurane anaesthesia. After 3 days animals were euthanized, ovaries excised, cleaned and granulosa cells isolated by repeated disruption of the follicle as described earlier.[18] Granulosa cells isolated from 4–5 rats were used to culture one plate and equal volume of cells was plated in each well. The cells were cultured overnight in 5% serum containing FBS at 37 °C. Granulosa cells after overnight culture were replaced with serum free medium containing 0.1% BSA and 100 nM androstenedione The cells were treated with FSH or the compounds in the presence or absence of low dose of FSH (EC_{20}). A dose response curve with 4-fold dilution of FSH starting from 5 nM and 2-fold dilution of compound starting 10 μM at 8 different concentrations was tested. At 24 h after treatment supernatant was collected and the estradiol concentration was determined by ELISA (DRG, Mountainside, NJ). The optical densities of the ELISA were measured by Spectramax, (Sunnyvale, CA). The values were analyzed in Microsoft Excel and Graphpad Prism.
34. van de Lagemaat, R.; van Koppen, C. J.; Krajnc-Franken, M. A.; Folmer, B. J.; van Diepen, H. A.; Mulders, S. M.; Timmers, C. M. *Reproduction* **2011**, *142*, 893.
35. Sriraman, V.; Denisa, D.; de Matos, D.; Yu, H.; Palmer, S.; Nataraja, S. *Biochem. Pharmacol.* **2014**. http://dx.doi.org/10.1016/j.bcp.2014.02.023.