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Investigation of a thiazolidinone derivative as an allosteric modulator of follicle stimulating hormone receptor: Evidence for its ability to support follicular development and ovulation

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ABSTRACT

FSH signalling through its cognate receptor is critical for follicular development and ovulation. An earlier study had documented thiazolidinone derivatives to activate FSH receptor expressed in CHO cells and rat granulosa cells; however development of this compound for clinical use was halted for unobvious reasons. The objective of the current study is to extend the previous investigations in detail on the ability of thiazolidinone derivative (henceforth referred to as Compound 5) to activate FSH signalling and learn the barriers that preclude development of this derivative for clinical purposes. Our results demonstrate that the Compound 5 in a dose-dependent manner stimulated cAMP production, activated AKT and ERK signalling pathways and induced estradiol production in cultured rat granulosa cells. Compound 5 also caused dose-dependent increase in estradiol production from human granulosa cells. In increasingly more complex in vitro systems, Compound 5 was able to induce the expansion of mouse cumulus-oocyte-complex and support in vitro development of mouse preantral follicle to preovulatory stage and release of oocyte from the follicle. In vivo, the compound stimulated preovulatory follicular development and ovulation in immature rats. Pharmacokinetic and safety investigations reveal poor oral availability and genotoxcity. Together, our results document Compound 5 to act as a FSHR allosteric modulator but have poor pharmacological properties for development of an oral FSH receptor modulator.

1. Introduction

Infertility affects 1 in 8 women globally during their child bearing years [1]. Follicle stimulating hormone (FSH) is a therapeutic agent used for treatment in assisted reproductive technologies (ART) [1,2]. The clinical efficacy of FSH for this indication is well validated by over 50 years of use. At low doses (usually <75 IU daily dose) FSH stimulates one or two follicles to develop to a stage when ovulation can be induced naturally or by a luteinizing hormone (LH) or human chorionic gonadotropin (hCG) injection, followed by natural insemination or intra-uterine insemination (IUI). At higher doses, (≥75 IU daily dose) FSH stimulates multiple follicles to develop that are often collected for in vitro fertilization (IVF).

In the 1960s FSH purified from post-menopausal women urine was employed for ovarian stimulation in fertility treatments and such purified preparations are still in use clinically [2,3]. Advances in recombinant technologies enabled development and commercialization of recombinant FSH, LH and hCG to be produced in mammalian cell lines in the late 1990s and the recombinant proteins have been proven to be clinically effective in fertility treatments [1–3]. The hormones are administered subcutaneously and require repeated injections for controlled ovarian hyperstimulation [4]. Including the injections of associated therapeutics, a woman could anticipate over 30 injections during a single IVF treatment cycle, lasting 12 days. Improvements in the convenience of FSH injections have been achieved with long lasting versions of recombinant FSH [5–8]. Despite significant improvements in the drug-delivery of FSH, administration by injections is stressful for patients going through the treatment. As a result, patient enrolment and compliance with the injectable treatment schedule as well as the costs of biotherapeutics are major issues for

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clinicians and patients facing in infertility treatments [9]. An orally bioavailable FSH mimetics is therefore desired for the unmet medical need of patient-centred care.

The glycoprotein hormones (FSH, LH, hCG and TSH) are heterodimers with a common α-subunit and distinct β-subunit that confer specificity to their receptors [10,11]. These ligands bind to large receptor proteins that share a high level of identity. The receptors are members of the family A G-protein coupled receptor (GPCR) superfamily that transduce extracellular signals into G protein activation through their seven-helical transmembrane domains. The recent crystal structure reveals that the FSHR ectodomain form a trimer in its basal state and recruitment of FSH to the leucine-rich-repeats of the quiescent FSHR is the first step that changes the conformation of the hormone to form a nascent sulfated tyrosine (sTyr) binding site. In the second step, the FSHR docks sTyr to the binding site and lifts the inhibitory hinge loop to activate the receptor [11,12]. The activated receptor directly interacts with three families of proteins: the G-proteins, the GPCR kinases (GRK) and the arrestins [13,14], resulting in activation of G-protein and/or arrestins [12,15] and down-stream signalling cascades. This cascade include production of cAMP [16], EGF receptor activation, phosphorylation of AKT and ERK and β-arrestin recruitment at the receptor [17–19]. Ultimately this leads to the transcriptional activation of several FSH dependent genes including Cyclin D2 and aromatase resulting in granulosa cell proliferation and estradiol production [16,17,19]. These events along with local factors promote follicular maturation, cumulus expansion and predispose the follicle to respond to ovulatory LH surge.

The development of a small molecule FSH agonist for infertility therapy is a challenging task. Most orally bioavailable small molecules have a mass less than 500 Da compared to the protein ligand with a mass of 33,000 Da. Unlike FSH that binds to the extracellular domain of FSHR, two small molecules have been reported to bind to the 7TM domain [20,21]. However, there has not been subsequent development of these agonists, nor any of the thienopyrimidines, hexahydroquinolines and isooxazolylythiazolesm carabzoloe and pyrazole derivates (reviewed by Heitman and Ijzerman [22]). We have used a thiazolidinone derivative "Compound 5" [(3-((2*S*,5*R*)-5-(2-((3-ethoxy-4-methoxyphenethyl)amino)-2-oxoethyl)-4-oxo-2-(4-(phenylethynyl)phenyl)thiazolidin-3-yl)benzamide] [20] as a research tool to understand the pharmacokinetic and pharmacodynamic challenges, as well as the safety and toxicology barriers that may impede development of small molecule FSH agonists. Thiazolidinone class of FSHR agonists were identified through screening of combinatorial chemical scaffolds and were further optimized for potency and selectivity versus the other glycoprotein hormone receptors in contrast to the other known FSHR agonists in the literature [20,23,24]. In addition to its utility to understand pharmacological suitability, it would be important to be aware of any dissimilarities in the cellular signalling pathways of a novel small molecule agonist, such as a thiazolidinone derivative from an approved protein therapeutic. It is uncertain if the proposed positive allosteric modulators of the FSH receptor cause complementation of multiple FSH signalling pathways despite their independent site of action on the receptor.

The cellular potency of Compound 5 appeared sufficient to stimulate the desired response in rat granulosa cells in terms of estradiol production [20], however, it was unclear if the molecule exhibited parallel potency in vivo and human granulosa cells for development as a clinical candidate molecule. We chose to investigate the effect of Compound 5 in complex in vitro and in vivo systems and to understand if it lacks essential properties for development as a clinical candidate that could explain why it has not been developed further. These investigations are intended to identify shortcomings of thiazolidinones in order to improve the criteria needed to be in place for subsequent chemical series, and increase the likelihood of a success in developing an orally active allosteric agonist of the FSH receptor. The results obtained provide insight on the pharmacokinetic profile and safety concerns that discourage further development of Compound 5 as commercial clinical candidate.

2. Materials and methods

2.1. Reagents

DMEM: F-12, foetal bovine serum (FBS) and all other cell culture solutions were procured from Invitrogen (Carslabad, CA). Compound 5 (Fig. 1A) was synthesized at EMD Serono and confirmed for its structure and purity by NMR, LC/MS and HPLC. The purity of the compound employed for the study ranged from 95 to 97%. Recombinant FSH and hCG were obtained internally that are the commercial products of EMD Serono (Rockland, MA). PMSG (pregnant mare serum gonadotropin) and other fine chemicals were procured from Sigma-Aldrich (St. Louis, MO, USA). Cell culture plates and dishes were procured from Corning Inc (Oneonta, Corning, NY). Diethylstilbesterol (DES) time release pellets and trochar were obtained from Innovative Research of America, Sarasota, FL. Alzet pumps and surgical accessories were procured from Durect Corporation, Cupertino, CA.

2.2. Animals

Immature CD rats (18–21 day old; females) and B62DF1 mice were procured from Charles-River laboratories (Wilmington, MA). All animals were treated in accordance to EMD-SRI approved

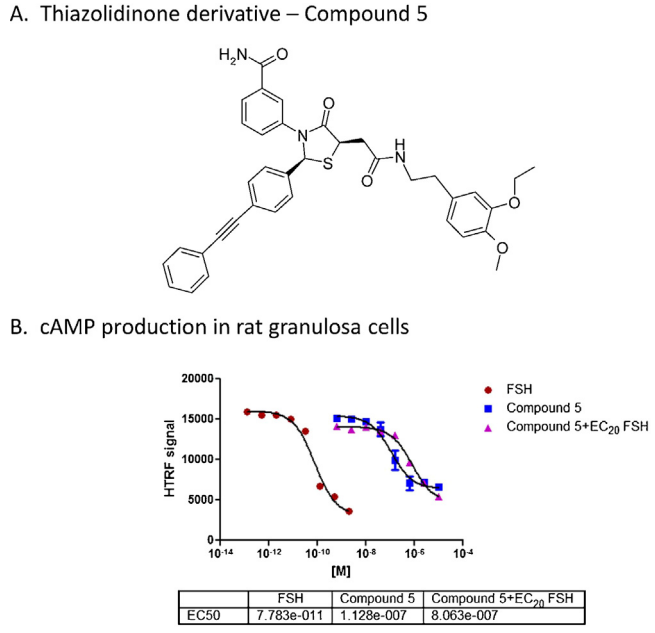

Fig. 1. *Structure and Compound 5 (A) and its effect on cAMP production in granulosa cells (B).* Cultured granulosa cells were treated with serially diluted FSH or small molecule with an initial concentration of 2 nM or 10 μM in DMEM:F12 (supplemented with 0.1% BSA and IBMX) to determine their dose-dependent effect on cAMP production. The effect of small molecule cAMP production in the presence of EC_{20} FSH was also assessed. After 1 h incubation at 37 °C the cells were lysed and incubated with antibody to determine the total cAMP synthesis by HTRF assay (Cisbio, Bedford, MA) as described in Section 2. The final values were plotted in the form of a graph and analyzed employing Graphpad Prism software. The observed difference in EC_{50} is statistically significant [*** $p < 0.001$ by t test; FSH vs Compound 5].