Original Article

Kisspeptin receptor agonist has therapeutic potential for female reproductive disorders.

Short title: Kisspeptin receptor agonist in women.

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Conflict of interest statement

This was an investigator led study.

AA & WSD have conducted consulting work for Myovant Sciences Ltd.

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Key words: Kisspeptin receptor agonist, fertility, polycystic ovary syndrome (PCOS), hypothalamic amenorrhea (HA).

Abstract

Background:

Kisspeptin is a key regulator of hypothalamic gonadotropin-releasing hormone (GnRH) neurons and is essential for reproductive health. A specific kisspeptin receptor (KISS1R) agonist could significantly expand the potential clinical utility of therapeutics targeting the kisspeptin pathway. Herein, we investigate the effects of the KISS1R-agonist (MVT-602) in healthy women, and in women with reproductive disorders.

Methods:

We conducted *in vivo* and *in vitro* studies to characterize the action of MVT-602 in comparison to native kisspeptin-54 (KP54). We determined the pharmacokinetic and pharmacodynamic properties of MVT-602 (doses 0.01 and 0.03nmol/kg) versus KP54 (9.6 nmol/kg) in the follicular phase of healthy women (n=9), and in women with polycystic ovary syndrome (PCOS; n=6), or hypothalamic amenorrhea (HA; n=6). Further, we investigated their effects on KISS1R-mediated inositol monophosphate (IP_1) and Ca^{2+} signalling in cell lines and on action potential firing of GnRH neurons in brain slices.

Results:

In healthy women, the amplitude of luteinizing hormone (LH) rise was similar to that after KP54, but peaked later (21.4 vs 4.7 h; P=0.0002), with correspondingly increased AUC of LH-exposure (169.0 vs 38.5 h.iU/L; P=0.0058). LH-increases following MVT-602 were similar in PCOS and healthy women, but advanced in HA (P=0.004). In keeping with the clinical data, MVT-602 induced more potent signaling of KISS1R-mediated IP_1 accumulation and a longer duration of GnRH neuron firing than KP54 (115 vs 55 min; P=0.0012).

Conclusions:

Taken together, these clinical and mechanistic data identify MVT-602 as having considerable therapeutic potential for the treatment of female reproductive disorders.

Trial registration: ISRCTN21681316.

Introduction

Kisspeptin is recognized to play a pivotal role in the regulation of reproductive hormone secretion following two landmark reports in 2003, demonstrating that decreased signaling of its G-protein-coupled receptor (KISS1R) resulted in failure of the hypothalamic-pituitary-gonadal (HPG) axis (1,2). Thereafter, it was established that kisspeptin activates gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus, thereby stimulating the downstream HPG axis (3,4). Current evidence suggests that kisspeptin neurons are key integrators of peripheral signals including sex steroids and metabolic cues (5,6).

The kisspeptins are a family of peptides encoded by the *KISS1* gene (7); the 145-amino acid precursor protein is proteolytically cleaved to shorter peptides and the number of remaining amino acids is indicated by their suffix e.g., KP54. Exogenous kisspeptin-54 (KP54) administration robustly stimulates gonadotropin secretion in both healthy men (8) and women (9,10). Due to its fundamental role in regulating physiological reproductive hormone secretion, there has been tremendous interest in targeting the kisspeptin pathway to treat reproductive disorders in humans (11). It has been shown that a single subcutaneous (SC) bolus of KP54 induces an luteinizing hormone (LH) rise that safely matures oocytes in women undergoing *in vitro* fertilization (IVF) therapy (12,13). Additionally, KP54 administration can restore physiological reproductive hormone secretion in patients with functional hypogonadism associated with deficient GnRH secretion such as in hypothalamic amenorrhea (HA) (14). However, therapeutic use of native KP54 using frequent high-dose administration risks tachyphylaxis and resultant insufficient stimulation of the reproductive axis (15,16). Consequently, the development of KISS1R agonists with a longer duration of action could allow for less frequent dosing than native KP54 and could capitalize on the potential clinical utility of kisspeptin-based therapeutics.

All kisspeptin peptides share a common C-terminal decapeptide sequence, equivalent to KP10, and activate the Gαq/11-coupled KISS1R, leading to stimulation of phospholipase C and increases in the intracellular second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), and release of Ca^{2+} from intracellular stores (17). KP54 is the major circulating form of kisspeptin in humans and has

a terminal half-life of 27.6 min (8). Kisspeptin-10 (KP10) has also been widely studied in humans (10,18), but has a shorter terminal half-life of 4 min (8,19).

Recently, KISS1R agonists have been developed through modification of KP10 to have increased potency and stability in order to advance kisspeptin-targeted therapeutics through the translational pathway (20–22). MVT-602 (previously known as TAK-448) is a KISS1R agonist with a longer duration of action than native KP54 (8,23). In healthy men, a single dose of MVT-602 induced sustained stimulation with peak gonadotropin levels occurring between 6-12 h and levels remaining elevated for 48-72 h (23).

MVT-602 is expected to also have substantial translational potential for the treatment of female reproductive disorders, but its effects in women have not been investigated. We therefore aimed to comprehensively determine the pharmacokinetic (PK) and pharmacodynamic (PD) profiles of the KISS1R agonist, MVT-602, in healthy women and in the two commonest forms of oligo/anovulatory subfertility, namely polycystic ovary syndrome (PCOS) and HA (24–26). Additionally, we investigated the effects of MVT-602 and KP54 on intracellular signaling following activation of the G-protein coupled KISS1R, and on action potential firing of GnRH neurons in brain slices. Furthermore, as sex steroid milieu at the time of administration is known to influence the response to kisspeptin (9), we also sought to determine the impact of estrogen supplementation on the gonadotropin response to MVT-602 in women.

Results

Baseline characteristics of participants are summarized in **Table 1**. Age, body mass, and body mass index (BMI) did not differ between healthy women, women with PCOS and women with HA. All healthy women had regular menstrual cycles with an average cycle length of 28.1 ± 1.2 days, whereas all women with PCOS or HA were oligo/amenorrheic. Women with PCOS had higher serum anti-Müllerian hormone levels (AMH) and lower sex hormone binding globulin levels (SHBG) as expected. No serious adverse effects were identified after injection of either MVT-602 or KP54.

Preliminary dose-finding study of MVT-602 in healthy women

As MVT-602 had not been previously administered to women, a broad range of doses between 0.003 nmol/kg to 1.0 nmol/kg of MVT-602 administered as a single SC bolus were evaluated in the early follicular phase (day 1-4 of the menstrual cycle) of healthy women (n = 3) during a preliminary dose-finding study. Highest LH levels occurred 24 h following administration and no greater increases in serum gonadotropins or estradiol levels were observed using doses higher than 0.03 nmol/kg. Therefore, detailed endocrine profiles were evaluated in subsequent phases of the study using MVT-602 doses of 0.01 and 0.03 nmol/kg (**Figure 1** - study protocol).

Comparison of MVT-602 with KP54 in the follicular phase of healthy women

We compared MVT-602 to a dose of KP54 (9.6 nmol/kg) that is known to induce a near maximal response on LH in women (9,12,13). Plasma MVT-602 and KP54 levels peaked at 0.4 - 1 h following both peptides (**Figure 2A** & **Table 2**). Peak MVT-602 levels were dose-proportionately higher after 0.03 nmol/kg than after 0.01 nmol/kg (C_{max} 29.5 vs 8.1 pmol/L; **Table 2)**. Despite PK parameters being similar for both ligands ($t_{1/2}$ 1.68 - 2.02 h; **Table 2**), the time course of LH release was markedly different (**Figure 2B**). The effect on LH was modelled using a pharmacodynamic biphasic model providing parameters for both the primary episode of in LH-response commencing within the first 10 h after administration, and where present, the secondary episode of LH-response commencing after 10 h

(**Table 2**). Although the amplitude of LH response was similar after KP54 and MVT-602, the timing of peak LH was much later after MVT-602 than KP54 (21.4 h vs 4.7 h; P = 0.0005) **(Table 2)**. Consequently, the area under the curve of LH was increased at least 4-fold after MVT-602 0.03 nmol/kg in comparison to KP54 (169.0 vs 38.5 iU.h/L, P = 0.01; **Figure 2C & Table 2**). FSH levels followed a similar trajectory to LH in response to both peptides (**Figure 2D**). Elevated serum estradiol was maintained for at least 48 h after a single SC injection of 0.01 or 0.03 nmol/kg dose of MVT-602 (**Figure 2E**).

Effect of MVT-602 and KP54 on human kisspeptin receptor (KISS1R) signaling

We next examined if the prolonged effects of MVT-602 on serum LH, FSH and estradiol levels could be explained by differences in signaling at a cellular level. KISS1R signals via the Gαq/11-pathway (27) leading to stimulation of phospholipase C and increases in the intracellular second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), and release of Ca^{2+} from intracellular stores [17]. We thus compared KISS1R-mediated Gαq/11 signaling following treatment with either KP54 or MVT-602. HEK 293 cells expressing human KISS1R were treated with peptide, and intracellular levels of IP_1, a downstream metabolite of IP_3, and intracellular Ca^{2+} were measured (**Supplemental Figure 1**). No signal response was observed from either peptide in cells not transfected with the receptor.

Analysis with varying doses of each ligand (10 pM – 1 μM) revealed that the two ligands did not significantly differ in efficacy (R_{max} KP54 = 96.05 ± 3.96 and MVT-602 = 89.14 ± 6.25; mean ± SEM; where data is normalized as a percentage of 1 μM KP54). However, MVT-602 was significantly ($P < 0.0001$) more potent (pEC50 10.71 ± 0.12) than KP54 (pEC50 8.04 ± 0.06), as evidenced by the leftward shifted dose-response curve in **Figure 3A**.

Activation of KISS1R with KP10 induces both a transient and a more sustained intracellular Ca^{2+} response (28,29). Thus, to determine if MVT-602 and KP54 resulted in differences in the kinetics of KISS1R-Gαq/11 signaling, real time Ca^{2+} mobilization was monitored. Cells were treated with 10 nM of either ligand, which is a saturating dose for MVT-602, and a dose of KP54 corresponding to its EC50

in terms of IP_1 levels (**Figure 3A**). Stimulating cells at higher doses of KP54 did not significantly alter intracellular Ca^{2+} signal responses (**Supplemental Figure 2**) suggesting that maximal responses were achieved with 10nM of either MVT-602 or KP54. Treatment of cells with either KP54 or MVT-602 produced a rapid rise in intracellular Ca^{2+} (with maximal intensity reached within ~30-60 sec) and was sustained over time (**Figure 3B, Supplemental videos S1-2**), similar to Ca^{2+} signal profiles reported for KP10 (28,29). The acute maximal response was not different following MVT-602 treatment in comparison to KP54 (**Figure 3C**). Over the 1 h stimulation, both KP54 and MVT-602 displayed a sustained response over time as quantitated by the area under the curve and at each time frame. However, there was no difference in the sustained signal profiles between KP54 and MVT-602 (**Figure 3D** and **Supplemental videos S1-2**). Overall, this suggests that whilst MVT-602 may exhibit a much higher potency at the KISS1R, it does not differ in its ability to alter the acute nor persistent Ca^{2+} signal profile over time when compared to KP54.

MVT-602 increases firing rate in GnRH neurons for a more prolonged duration than KP54

Targeted extracellular recordings of GFP-identified GnRH neurons were used to evaluate the effects of MVT-602 and KP54 on action potential firing rate. Following either peptide, 7 of 8 neurons responded with an at least 30% increase in firing. Individual recordings of neurons exposed to KP54 and MVT-602 are presented in **Figure 4A** & **Figure 4B**, respectively, and the (mean ± SEM) firing frequency over time after each peptide is presented in **Figure 4C**. Although the onset of effect was similar after each peptide, typically commencing within 1-2 min of treatment, the duration of the effect was markedly increased to a median of 115 min after MVT-602 as compared to 55 min after KP54 ($P = 0.0012$) (**Figure 4D**). The firing frequency of all GnRH neurons had returned to baseline firing activity by 3 h. This is unlikely to represent a loss of viability of these cells as recordings lasting as long as 6 h have been reported (30,31). The peak firing frequency was higher after MVT-602 than KP54 ($P = 0.007$) (**Figure 4E**). These data are in keeping with our human clinical data, which show that MVT-602 induces a significantly prolonged duration of LH release compared to KP54.

MVT-602 in the healthy follicular phase, PCOS and HA

Plasma MVT-602 levels were similar in both healthy and oligo/anovulatory women after receiving a SC bolus of 0.03 nmol/kg of MVT-602 (P = 0.76; **Figure 5A**). The rise in LH levels after MVT-602 was similar in women with PCOS to those in the healthy follicular phase (**Figure 5B** & **Table 3**). However, in women with HA, LH levels increased sooner after MVT-602 (time of first peak: 6.2 h in HA vs 15.1 h in the healthy follicular phase; P = 0.008, **Figure 5B** & **Table 3**). The total area under the curve of LH increase did not differ between these groups (**Figure 5C**). Furthermore, the increase in FSH levels following MVT-602 was particularly marked in women with HA (P = 0.0011) (**Figure 5D**) with a corresponding greater increase in estradiol (P = 0.023) (**Figure 5E**). Raw values for serum hormone levels after MVT-602 in the healthy follicular phase, PCOS and HA are presented in **Supplemental Figure 3**.

Effect of estrogen supplementation on the response to MVT-602 in the healthy follicular phase

As sex steroid milieu at the time of administration is known to influence the response to kisspeptin [8], we sought to determine the impact of estrogen supplementation on the response to MVT-602 in women. To do this, we examined the impact of increasing estradiol levels at the time of MVT-602 injection in the healthy follicular phase by applying a 200 μg per day estradiol patch from 24 h prior to administration of MVT-602 to achieve levels similar to those observed in the pre-ovulatory phase of the menstrual cycle. The peak rise in LH after MVT-602 was increased from 7.5 iU/L to 24.4 iU/L following estradiol pre-treatment (P < 0.0001) (**Figure 6A & 6C**). The peak in FSH was also greater, increasing from 1.7 iU/L to 7.6 iU/L (P < 0.0001) (**Figure 6B & 6D**). However, the time of peak gonadotropin rise was not altered by estradiol supplementation. Individual and grouped hormonal profiles after MVT-602 with and without estradiol pretreatment are presented in **Supplemental Figure 4**.

Discussion

MVT-602 is a nano-peptide KISS1R agonist developed to have increased stability, potency and water solubility (20). This is the first study to determine its PD and PK profiles in healthy women, and to directly compare these properties to those of native KP54, and to those in women with the two commonest causes of oligo/anovulatory infertility, namely PCOS and HA. Both MVT-602 and KP54 induced a similar peak LH amplitude, consistent with their analogous mechanism of action via stimulation of KISS1R on hypothalamic GnRH neurons (4,32). However, although PK properties were similar following SC bolus administration, MVT-602 produced a markedly prolonged PD effect than KP54 (time of peak LH: 21-22 h vs 4.7 h). Accordingly, the area under the curve of LH was increased more than four-fold after MVT-602 in comparison to KP54 (33), identifying MVT-602 as a novel therapeutic for the treatment of female reproductive disorders using KISS1R-targeted drugs.

Due to kisspeptin's action to directly stimulate hypothalamic GnRH neurons there has been tremendous interest in the development of KISS1R agonists for the treatment of reproductive disorders (11). KP54 can be used to mature oocytes safely for IVF treatment (12), without causing the dangerous complication of 'ovarian hyperstimulation syndrome' (OHSS), even in women at increased *a priori* risk of OHSS (13). Moreover, extending the duration of the LH response with a second dose of KP54 further improves oocyte maturation without increasing the occurrence of OHSS (34). Hypothalamic kisspeptin signaling is known to be obligatory for the occurrence of preovulatory gonadotropin surge (35,36). The natural mid-cycle gonadotropin surge in women is triphasic comprising a rapidly ascending limb lasting 14 h, followed by a plateau lasting for 14 h, and then a descending limb lasting for 20 h (33). The most commonly used current trigger of oocyte maturation during IVF treatment is hCG, which has an excessive duration of action lasting more than one week and consequently is the major cause of OHSS (37). HCG provides only LH-like activity without the accompanying FSH rise that accompanies the natural mid-cycle LH surge (33,38,39). Furthermore, activation of LH receptor by hCG results in diverse intracellular signaling than by LH, reflecting the physiological role of hCG in the maintenance of pregnancy rather than in the induction of oocyte maturation / ovulation (37). Whilst GnRH agonists

induce rises in both LH and FSH, the amplitude of LH reached is supraphysiological (~150 iU/L), and the duration of the LH surge is shorter (peak LH at 4-6 h) (12,33,38–40). Thus, the endocrine profile of MVT-602 is distinct and is likely to be more similar to that of the natural mid-cycle gonadotropin surge than that induced by currently available triggers of oocyte maturation. Further studies assessing whether MVT-602' more physiological endocrine profile translates to improved clinical outcomes during IVF treatment would be of great interest. Kisspeptin also offers potential for the treatment of functional hypogonadal disorders associated with hypothalamic dysfunction such as diabetes-induced secondary hypogonadism (41) and age related hypogonadism (42). Whilst the treatment of functional hypogonadal disorders using kisspeptin could thus represent an attractive therapeutic intervention, these would require chronic stimulation protocols. However, chronic administration of KP54 can result in tachyphylaxis (43); which is most commonly encountered in the context of frequent high-dose administration (44). A continuous SC infusion of MVT-602 in men induced tachyphylaxis at a minimum steady state concentration of at least 228 pg/mL (186 pmol/L) (23). It is notable that the peak circulating MVT-602 level in the present study in women was ~6-fold lower (<30 pmol/L) after a SC bolus of MVT-602 (0.03 nmol/kg) (**Table 2**). Furthermore, sensitivity to kisspeptin can modulate the susceptibility to tachyphylaxis; i.e., the same dose of KP54 administered twice daily induced tachyphylaxis in women with HA (15), but not when administered in the healthy follicular phase (45). As women in the follicular phase are less sensitive to MVT-602, one could speculate that higher circulating levels may be needed to induce tachyphylaxis in comparison to men, whereas even lower doses could be chosen to achieve continued gonadotropin stimulation whilst avoiding tachyphylaxis in most women with HA. Indeed, whilst MVT-602 can also induce tachyphylaxis if given continuously as a high-dose SC infusion (23), its ability to induce a prolonged duration of gonadotropin elevation lasting ~48 h after a single bolus injection could facilitate chronic stimulation protocols that use infrequent (e.g., every 2-3 days) low-dose bolus administration that could mitigate against tachyphylaxis (16). Conversely, the prolonged duration of action of MVT-602 on GnRH neuronal firing *in vitro* and on LH release in women, coupled with its increased potency, could indicate that tachyphylaxis may yet challenge continued stimulation with the use of MVT-602 in a chronic protocol. Consequently, further

studies investigating chronic administration protocols of MVT-602 are indicated to assess whether persistent stimulation can be achieved with low-dose intermittent administration.

Continuous exposure of the KISS1R to KP10 elicits a biphasic increase in intracellular Ca^{2+} signaling, with an acute first-phase response lasting 5 min, followed by a more sustained second-phase response lasting more than 30 min (46). KISS1R is a Gαq/11-coupled receptor (27), however it is also recognized that mechanisms in addition to Gαq/11-signaling can induce LH/GnRH secretion, such as via the adaptor protein arrestin to activate ERK signaling (47,48). In addition to its key role as an adaptor protein, arrestin also mediates GPCR internalization and, notably, the persistent second wave in Ca^{2+} signaling after KP10 is dependent on KISS1R internalization (46). Here, we report for the first time that continuous exposure to KP54, and MVT-602, also produced prolonged Ca^{2+} signaling responses, akin to KP10, which may suggest that receptor trafficking may also be involved in regulating signal kinetics from KP54 and MVT-602.

We compared MVT-602 to a dose of KP54 (9.6 nmol/kg) that is likely to induce a near maximal response on LH during clinical studies (9,12,13). The 500-fold increased potency of MVT-602 at the human KISS1R to induce IP_1 signaling is consistent with the clinical data, in which a 300-fold lower dose of MVT-602 produced similar elevations in gonadotropins as KP54. PK parameters for KP54 and MVT-602 were surprisingly similar. The half-life of KP54 reported in the present study (1.7 h) is longer than that previously reported (~0.5 h) (8), which is likely attributable to the mode of administration in the present study (SC rather than intravenous). MVT-602 has been reported to have a similar half-life (1.5-3.5 h) in men using the same SC route of administration (23).

C6 is a Kiss1r agonist synthesized through modification of KP10, by addition of a serum albumin-binding motif and ω-methylation of arginine to resist trypsin-like proteases (49). Thus, C6 was confirmed to have increased potency compared to KP10 in a calcium mobilization assay (EC50 0.33 vs 2.6 nM) (49). C6 has been studied in preclinical models but not in humans; intramuscular injection of 15 nmol of C6 induced an LH-rise that lasted ~12 h in the ewe in both breeding and non-breeding seasons (49). C6 also increased gonadotropin release and triggered ovulation in alpine goats, suggesting

that C6 could offer an alternative to pregnant mare serum gonadotropin (PMSG) in animal husbandry (50). Application of C6 at doses 0.01 to 40 nM stimulated action potential firing in approximately half of murine GnRH neurons tested (49). Once daily intraperitoneal administration of C6 for 5 days advanced puberty in mice (49). One could hypothesize that the reduced potency of C6 could theoretically reduce the chance of tachyphylaxis, however the rise in LH was reduced after 5 days of daily administration, suggesting that C6 may also be susceptible to tachyphylaxis despite its reduced potency (49). C6 has yet to be tested in humans and thus further studies would be needed to compare the endocrine profile of C6 with that of KP54 or MVT-602 in women.

Of note, there may be ligand-dependent differences in receptor regulation that cannot be recapitulated in a HEK 293 cell model, thus it was also important to compare the effects of MVT-602 versus KP54 on GnRH neurons. Kisspeptin is a strong activator of action potential firing in GnRH neurons (30) and GnRH release (51,52), with the latter dependent upon both intra and extracellular Ca^{2+} sources (52). Exposure of GnRH neurons in brain slices to only a short exposure of MVT-602 (5 min) elevated firing for a longer duration (115 vs 55 min) and to a greater peak firing rate than an equimolar amount of KP54. These findings are consistent with the prolonged elevation of gonadotropin levels following MVT-602 compared to KP54 we observed in women. There could be multiple mechanisms underlying the distinct kinetic profiles in firing rate between KP54 and MVT-602 following a brief exposure to GnRH neurons. Each ligand could have differential binding kinetics, ability to activate distinct signaling pathways, including signal crosstalk with other neuronal GPCR's, or altered ability for Kiss1r to be desensitized. In addition, it would be interesting to determine how these distinct firing rates, and signaling mechanisms, impact GnRH secretion and pulsatility.

It is interesting to consider the distinct durations of ligand exposure required for Kiss1r action during electrophysiological studies in GnRH neurons versus intracellular Ca^{2+} signaling studies in HEK 293 cells. Brief exposure to a Kiss1r ligand can induce persistent action potential firing in GnRH neurons, whereas sustained exposure of Kiss1r to the ligand is required to induce Ca^{2+} signaling in HEK 293 cells. No previous study has investigated the effects of KP54 or MVT-602 on either the action potential

firing in GnRH neurons, or on intracellular Ca^{2+} signaling in HEK 293 cells. Thus, there is no direct comparison to our data available in the literature. In response to KP10 (10-100nM), however, phospholipase C (PLC) appears to be the major pathway for intracellular signaling in GnRH neurons (53); exposure of GnRH neurons to KP10 (10-100 nM) for 1-3 min elicits depolarization that lasts for at least 20 min (4), however the number of GnRH neurons that responded to KP10, was reduced from 80% to 15% with pharmacological PLC antagonism and to 7% with IP_3 antagonism (53). Intracellular Ca^{2+} studies in GnRH neurons find that exposure to KP10 (100 nM) for ~3 min evoked an immediate rise in intracellular Ca^{2+} that lasted for 2-3 min before a sudden drop and a gradual return to basal concentrations (53). These data are congruous with our own findings in suggesting that the mechanisms contributing to the prolonged increase in firing rate of GnRH neurons may be independent of both persistent activation of Kiss1r and of continued elevation of intracellular Ca^{2+}. In summary, whilst experimental models evaluating both GnRH neuronal firing and intracellular Ca^{2+} / IP_1 signaling provide valuable insight into the differential effects of MVT-602 and KP54 at Kiss1r, there may be additional differences in downstream signaling molecules that may not have been captured. For example, it is possible that phosphorylation of a downstream molecule after Kiss1r activation with a much longer duration of action could enable GnRH neurons to continue to fire without requiring ongoing presence of the ligand at the receptor. By comparison, IP_1 / Ca^{2+} intracellular signaling experiments may provide a more direct portrayal of ongoing Kiss1r activation.

Interestingly, the rise in LH following MVT-602 during clinical studies was delayed for ~6 h when studied in the healthy follicular phase. Similarly, KP54 also has a similar, albeit briefer, plateau in LH-rise between 0.5-1.5 h before a further LH-rise to its peak at 4.9 h, suggesting that the observed delay in LH-rise could be a KISS1R-mediated effect. However, the onset of the firing response following both ligands was similarly rapid during electrophysiological GnRH neuron studies, indicating that factors preceding receptor occupancy, such as the time needed for gaining access across the blood brain barrier (BBB), are more likely to underlie this delay than deferred activation after binding to KISS1R. Indeed, MVT-602 was selected for its hydrophilic properties (20), which could conceivably alter its ability to cross the BBB, whereas KP54 is considered to cross the BBB (54,55). Interestingly, the delay

in LH-rise was not apparent in women with HA, nor in men (23), suggesting that such a factor would have to plausibly differ between these physiological states.

Another possible contributor to the differential onset of LH-increase is differences in the sex hormone milieu at the time of administration. The LH-rise following KP54 is recognized to be modified by the sex hormone milieu at the time of administration, being markedly increased in the pre-ovulatory phase of the menstrual cycle when estradiol levels are highest (9). Correspondingly, estradiol enhances GnRH neuron firing response to kisspeptin in a model of the preovulatory phase (30) and increases expression of *Kiss1r* in GT1-7 GnRH neurons (56). Thus, we examined the impact of artificially increasing ambient estradiol levels in the healthy follicular phase from 24 h prior to the time of administration of MVT-602 using estradiol patches to achieve estradiol levels analogous to those encountered during the preovulatory phase. We observed that peak gonadotropin-rises were markedly increased by more than 3-fold after estradiol pretreatment, however the time-course of LH-increase did not differ from that in the control healthy follicular phase. This observation was consistent with the over 4-fold increased LH-response to a SC bolus of KP54 (6.4-12.8 nmol/kg) after controlled ovarian stimulation, in which estradiol levels are markedly elevated, compared to the healthy follicular phase (9,12,13). Conceivably, administration of the estradiol patch 24 h before MVT-602 administration could allow sufficient time for a switch from negative to positive feedback at GnRH neurons and/or the pituitary and thus augment the LH-response (57).

We also determined the endocrine profile induced by MVT-602 in the two commonest oligo/anovulatory disorders causing subfertility, namely PCOS and HA (24–26). The MVT-602 induced increase in LH level occurred sooner in women with HA in comparison to the healthy follicular phase. Levels of MVT-602 itself did not differ, suggesting that the advanced LH response could not be attributed to a difference in PK parameters, at least in the circulation. Notably, in order to ensure that women with PCOS had a more uniform endocrine milieu, most women with PCOS received a seven-day course of progesterone to induce a withdrawal bleed prior to each study visit, whereas healthy women and women with HA did not. Consequently, it is possible that exposure to administered

progesterone in the previous cycle could have negatively impacted reproductive hormone levels in women with PCOS (58). Disturbance of LH pulsatility in HA is associated with reduced energy availability (< 30 kcal per kg of lean body-mass) (59). Kisspeptin expression is reduced and *Kiss1r* expression is increased in the hypothalamus of a rodent model deprived of nutrition for 72 h (3). Thus, our findings could putatively be explained by a greater abundance of KISS1R in HA leading to an exaggerated gonadotropin rise. Indeed, the LH-rise after exogenous KP54 is increased in women with HA in comparison to healthy women in the follicular phase (15). MVT-602 induced an exaggerated and early rise in LH in women with HA and some women also appeared to have a distinct secondary rise in LH. Notably, the rise in FSH was particularly pronounced in women with HA, and it is possible that the resultant enhanced estradiol level following MVT-602 administration to women with HA (**Figure 5E**) could exceed the threshold to induce a switch from negative to positive estradiol feedback and thus prompt a secondary LH-rise in these women. Moreover, the marked FSH response coupled with the prolonged duration of action raises the prospect of using MVT-602 as a sole ovulation induction agent in women with HA.

In summary, we delineate the endocrine profile of the novel KISS1R agonist, MVT-602, in healthy women and those with PCOS and HA for the first time. MVT-602 induced more prolonged stimulation of the reproductive axis via hypothalamic GnRH neurons than is possible with native KP54. Our *in vitro* data suggests that this prolonged action of MVT-602 compared to KP54 is due to its increased potency on intracellular Ca^{2+} release and extended duration of stimulation of GnRH neurons. Taken together, our clinical and mechanistic data identify MVT-602 as a novel therapeutic agent for the treatment of female reproductive disorders.

Material and methods

Peptides:

KP54 was synthesized and purified by Bachem (Bachem Holding AG). Sterile vials of kisspeptin-54 were produced by Bachem (Clinalfa; Bachem Distribution Services GmbH). Both products were prepared according to good manufacturing practice (GMP). Vials of freeze-dried kisspeptin-54 were stored at –20 °C and reconstituted in 0.9% saline as described previously (8,9).

MVT-602 (GMP grade) was kindly provided by Myovant Sciences Ltd as a clear sterile solution (3.5 ml) vials containing 0.2 mg (163.2 nmol) per 2 ml (100 ppm) for use in the study. For lower doses, vials were diluted using 5% dextrose for injection.

Clinical Studies:

Study Protocol: Ethical approval for this study was granted by the West London Research Ethics committee, London, UK (reference: 12/LO/0507) and participants provided written informed consent. The study was conducted in accordance with Declaration of Helsinki. Twelve healthy women, six women with HA and six women with PCOS were recruited via newspaper advertisements.

Healthy ovulatory women aged 18-35 years, with a menstrual cycle length <35 days, BMI 18-30 kg/m_2, not taking any medications or hormonal contraception, were invited to take part in the study. PCOS was defined according to the 2018 international PCOS guidelines (24) and women were diagnosed with HA in accordance with Endocrine Society guidelines (25). All women underwent detailed medical history, medication history, menstrual history and clinical examination. The following blood tests were assessed during the screening visit: full blood count, renal function, liver function, bone profile, thyroid hormone profile, luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol, progesterone, androstenedione, dehydroepiandrosterone, testosterone, sex hormone binding globulin (SHBG), prolactin, and anti-Müllerian hormone (AMH).

Study Protocol: All study visits were carried out at the Clinical Research Unit, Imperial College Healthcare NHS Trust. The study was single-blinded and the order of receiving the intervention was decided using an online algorithm (www.random.org). All study visits were commenced in the morning (~8am) and participants could eat and drink non-alcoholic beverages *ad libitum* before and during each study visit. Pregnancy was excluded using a urine pregnancy test at the beginning of each study visit. Blood pressure and pulse rate were recorded throughout each study visit and occurrence of any symptoms recorded for the week following each intervention. Details of preliminary dose-finding study for MVT-602 is presented in supplementary methods.

Comparison of MVT-602 with KP54 in the healthy women: Following analysis of the preliminary dose-finding study, two doses of MVT-602, 0.03nmol/kg, 0.01 nmol/kg, were selected for comparison with kisspeptin-54 (9.6 nmol/kg), or 0.9% saline vehicle in the early follicular phase of a further nine healthy women. This dose of KP54 (9.6 nmol/kg) was selected as it induces a near maximal gonadotropin rise for KP54 and safely induces oocyte maturation during IVF treatment (9,12,13). Each woman received the following four interventions in random order (MVT-602 0.01 nmol/kg, MVT-602 0.03 nmol/kg, KP54 9.6 nmol/kg and 0.9% saline) during the early follicular phase (days 1-4) in four different menstrual cycles (with only one intervention administered per menstrual cycle). Following KP54 and 0.9% saline, serum reproductive hormone levels (LH, FSH, estradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 10 h, and additionally at 24 h. Following MVT-602, the duration of 30-minutely blood-sampling for measurement of reproductive hormones was extended to 24 h, and with additional samples at 28, 32 and 48 h (see **Figure 1** for study protocol).

Comparison of the gonadotropin response to MVT-602 in healthy women in the follicular phase and women with PCOS and women with HA: Six women with HA and six women with PCOS received a single SC dose of either MVT-602 0.03 nmol/kg, KP54 at 9.6 nmol/kg, or 0.9% saline vehicle in each study visit. All women in this study followed the same protocol as described above. Anovulatory women with PCOS had menses induced with an oral course of progesterone (10mg of medroxyprogesterone twice daily for 1 week) and had their study visits on days 1 to 4 following their

withdrawal bleed. One woman with PCOS had spontaneous menstrual bleeding every 6 weeks and thus did not require progesterone induction. Women with HA did not have progesterone induction as this was unlikely to result in a withdrawal bleed but had study visits scheduled to occur once per month to ensure consistent treatment wash-out times.

The effect of estrogen supplementation on the response to KISS1R agonist, MVT-602: To investigate the effect of sex-steroid milieu on the response to MVT-602, five healthy women in the early follicular phase had a transdermal estradiol patch (200 μg/day) applied 24 h prior to the start of each study visit and continued for 72 h (60). A single SC bolus of MVT-602 0.03 nmol/kg was administered 24 h after the initiation of estradiol treatment. Serum reproductive hormone levels (LH, FSH, estradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 24 h, with additional samples at 28, 32, 48 h and 72 h post MVT-602. Details of hormone assays are presented in supplementary methods.

Statistical methods: Statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software, La Jolla, CA). Parametrically-distributed continuous variables were reported as mean ± standard deviation (SD), whereas skewed continuous variables were summarized using median (interquartile range; IQR). Parametrically-distributed variables were compared using unpaired two-tailed Student's t-test (two groups) or one-way ANOVA (multiple groups) with *post hoc* Tukey's. Non-parametrically distributed variables were compared using Mann Whitney U test (two groups) or Kruskal-Wallis test with *post hoc* Dunn's test (multiple groups), as appropriate. Changes in gonadotropin levels over time were compared by two-way repeated-measures ANOVA with post hoc Tukey's test.

Pharmacokinetic (PK) model to characterize PK parameters

To extract pharmacokinetic parameters for KP54 and MVT-602, we used a PK model incorporating drug absorption and elimination (61). This allowed capture of any delayed absorption as reflected in the measured plasma concentrations of MVT-602 and KP54 after SC administration of the peptides. The model describes drug transfer from the site of administration to central circulation as a first order

process with rate constant $1/\tau_a$. Clearance of the drug from central circulation is also modeled as a first order process with rate $1/\tau_c$. The equation describing peptide plasma concentration, $C(t)$, as a function of time is:

$$C(t) = \frac{C_{max}\left(e^{-t/\tau_a} - e^{-t/\tau_c}\right)}{\left(e^{-T_{peak}/\tau_a} - e^{-T_{peak}/\tau_c}\right)}$$

where C_{max} is the maximum peptide plasma concertation and $T_{peak} = \frac{\tau_a \tau_c}{\tau_a - \tau_c} \log(\tau_a/\tau_c)$ is the time the maximum is attained. To infer parameter estimates, we assumed that each model parameter θ (C_{max}, τ_c, τ_a) has a Gaussian distribution within different groups (healthy, PCOS, HA) i.e., $N(\mu_{\theta,group}, \sigma_{\theta,group})$. Additive Gaussian error was used with standard deviation informed from the CV of the assays. As the pair of parameters τ_c and τ_a cannot be uniquely identified from our data (ie, exchanging the values of the two parameters has no effect on the predicted plasma concentration), we report a quantity that is invariant to exchanges of these two parameters, namely $\log(2) * (\tau_a + \tau_c)$, corresponding to the half-life of the peptide in the system.

Pharmacodynamic (PD) model to characterize LH secretion

To extract information regarding LH dynamics in response to administration of kisspeptin-54 (KP54) and MVT-602, we used a parametric model describing a superposition of two episodes of LH-rise. Details of the mathematical form of the model used is presented in supplementary methods.

Effect of MVT-602 and KP54 on human kisspeptin receptor (KISS1R) signaling

Details of reagents and DNA constructs and cell maintenance and transfection are presented in supplementary methods.

IP_1 accumulation: HEK 293 cells (add source) were plated into clear 96-well plates (Costar; ThermoFisher) 24 h post transfection. Then, 48 h after plating, cells were stimulated at the indicated concentrations and time points in the presence of 50 mM LiCl in DMEM. The levels of IP_1 were then determined on cell lysates using an IP-One Gq HTRF kit (Cishio, Perkin Elmer) according to

manufacturer instructions. Protein concentration of lysates was then determined by Bradford assay and IP_1 levels were normalized to protein concentration.

Ca^{2+} mobilization: HEK 293 cells were plated into 35 mm imaging dishes (No.1.5 coverslip; MatTek) 24 h post transfection. Cells were incubated with Fluo-4AM for 30 min at 37 °C, 5 % CO2, moved to the dark and incubation continued a further 30 min at room temperature. Cells were imaged live using a Leica SP5 confocal microscope fitted with a 20x dry objective. Movies were recorded at 1 frame/1.29 sec for 60 frames before ligand addition and imaged for a further 60 min after ligand addition. The intensity of each responding cell was measured using the ImageJ plugin, Time Series Analyzer. The fluorescence intensity was obtained by subtracting the average background intensity for each cell calculated from the average intensity across the first 60 frames (before ligand addition). Changes in fluorescence across the time course i.e., bleaching or stress induced fluorescence, was then accounted for across each frame by subtracting the average background fluorescence from 30 non-responding cells within the same field of view, allowing normalization of each cell to its own background fluorescence and normalizing background fluorescence across all frames.

Statistical Analysis: Data are expressed as mean ± SEM, unless otherwise stated. Unpaired two tailed Student's t-tests and ANOVA tests were performed using GraphPad Prism 8.

Effects of MVT-602 and KP54 on Electrophysiological Recordings in GnRH neurons

Details of animals, brain slice preparation, electrophysiological recordings and statistical analysis of electrophysiological studies are presented in supplementary methods.

Experimental design: To study the effects of MVT-602 on GnRH neurons compared to KP54, we executed extracellular recordings as a blind experiment. All drug stocks were reconstituted in water and diluted to a final concentration of 10 nM in ACSF for treatment. Once the extracellular recording configuration was established, recordings were stabilized for 5 to 10 min, and then spontaneous basal (control) activity was recorded for 10 min. Either 10 nM KP54 or 10 nM MVT-602 was then bath-applied for 5 min, followed by a wash period of at least 40 min to determine if effects were reversible.

No more than two cells per mouse were recorded with only one cell per brain slice. The range of firing rate observed within an animal was similar to that among animals within a group. All recorded neurons were mapped to a brain atlas (65) to determine the relation between anatomical location and response to treatment. No correlation between the location of cells and response was observed in this study.

Action currents during targeted-extracellular recording reflect action potential firing. Action currents were detected using custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR). Data were binned at 5-min intervals and mean firing rate (number of action potentials/5-min bin) was calculated for control (last 5 min of control period), treatment (last 3 min of treatment and first 2 min of wash out period; the first 2 min of the treatment are skipped to allow for solution exchange), and wash out periods (sequential 5-min bins). Cells were defined as responding if firing frequency during treatment changed by ≥30%. One cell from each group did not respond to treatment and was thus tested with first kisspeptin-10 (Phoenix Pharmaceuticals, Burlingame, CA) and then 20 mM KCl. Neither cell responded to kisspeptin-10 but both responded to KCl, indicating technically acceptable recordings but lack of response to kisspeptin. Because these recordings were of short duration and included the additional quality tests, they were excluded from the analysis and are reported as non-responding cells.

Author contributions

All authors provided contributions to study conception and design, acquisition of data or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content, and final approval of the version to be published. Here are the most important contributions of each author: AA, PCE, MP, SAC, RR, CMS, CP, SM, AH, WSD designed the study; AA, PCE, MP, SAC, RR, CMS, CP, EM, MM, CI-E, DP, ANC conducted experiments and acquired the data; AA, PCE, MP, SAC, DP, KP, CNJ, LW, RS, ANC recruited patients for the clinical studies; AA, PCE, MP, SAC, RR, CMS, CP, BO, CM, MV, KTA, SM, AH, WSD analyzed the data; AA, PCE, MP, EM, CI-E, PB, ANC conducted assays for biochemical analytes; all authors contributed to writing and revising the manuscript. WSD takes final responsibility for this article.

Conflict of interest statement

AA and WSD have undertaken consultancy work for Myovant Sciences Ltd.

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Figure Legends

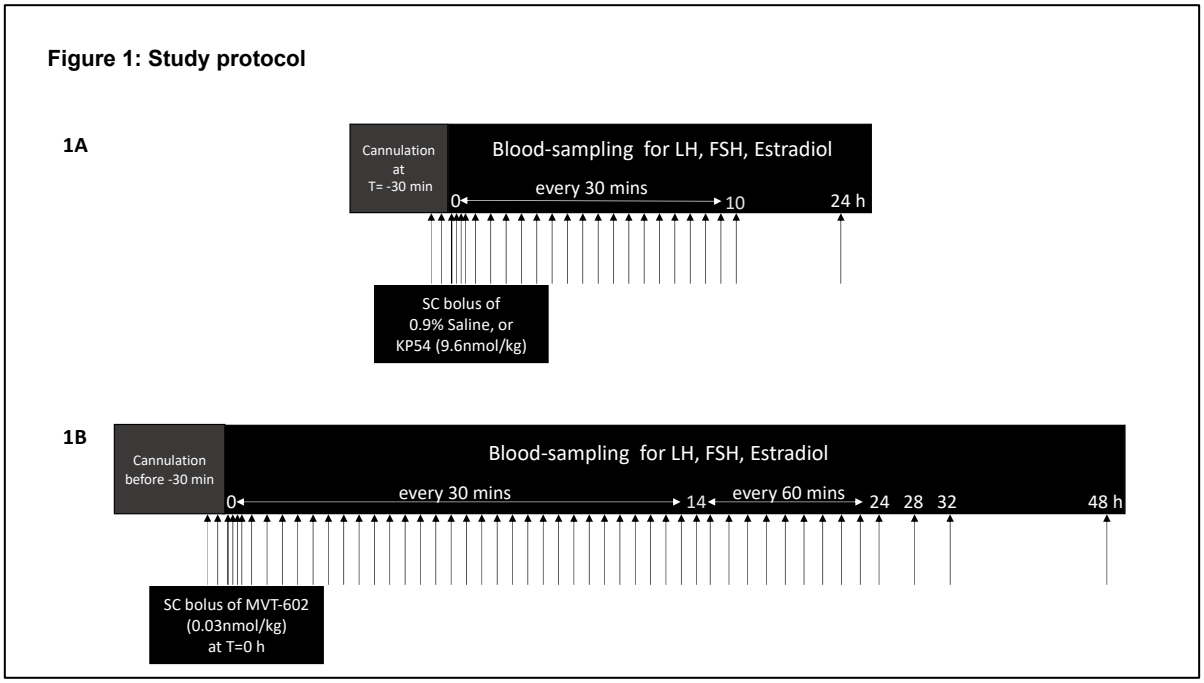

Figure 1- Study protocol. Study participants were admitted to the Clinical Research Facility at 8 am on the morning of each study visit. An intravenous cannula was inserted into one antecubital fossa and blood was sampled at T -30 min, T -15 min and T = 0 h prior to administration of each intervention to determine the basal hormonal values. A subcutaneous (SC) bolus of kisspeptin-54 (KP54) or 0.9% saline (Figure 1A), or of MVT-602 (Figure 1B) was administered at T = 0 h.

Figure 1A- Study protocol diagram for the KP54 and 0.9% saline visits. After a SC bolus of KP54 (9.6 nmol/kg) or 0.9% saline at T = 0 h, serum hormone levels (LH, FSH, estradiol and progesterone) were measured every 5-15 min for the first 30 min, and then every 30 min until 10 h, and additionally at 24 h.

Figure 1B- Study protocol diagram for MVT-602 visits. After a SC bolus of MVT-602 (0.03 nmol/kg) was administered at T = 0 h, serum hormone levels (LH, FSH, estradiol and progesterone) were measured every 5-15 min for the first 30 min, then every 30 min until 14 h, and then every 60 min until 24 h and additionally at 28, 32 and 48 h. A further blood test at 72 h was carried out in studies using estradiol pre-treatment.

Figure 2:

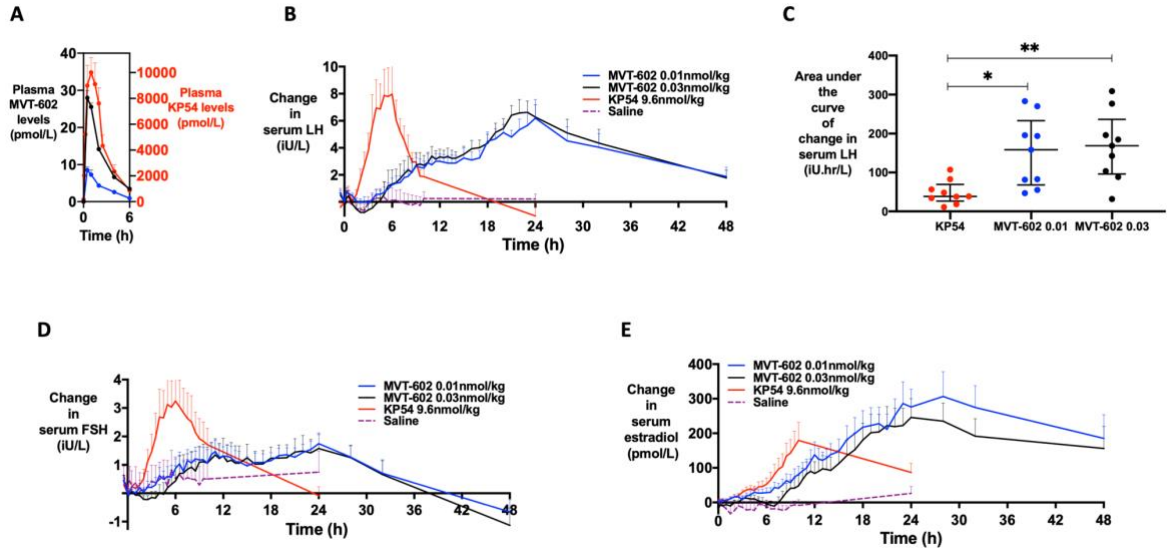

Figure 2- Clinical studies of MVT-602 and kisspeptin-54 (KP54) in healthy women in the follicular phase

Figure 2A: Mean (±SEM) of the plasma concentration of MVT-602 (left hand y axis), or kisspeptin-54 (KP54) in pmol/L (right hand y axis) vs time (h) in healthy women receiving a SC bolus of MVT 602 at 0.03 nmol/kg in blue, 0.01 nmol/kg in black, and KP54 in red in the first 6 h following SC administration at time 0 h of each peptide.

Figure 2B, 2D, & 2E: Mean (±SEM) of change from baseline levels in serum LH (iU/L) (**2B**), serum FSH (iU/L) (**2D**), and serum estradiol (pmol/L) (**2E**) in healthy women during the early follicular phase (n = 9) receiving a SC bolus of MVT-602 at time 0 h. MVT-602 doses of 0.01nmol/kg are presented in blue (over 48hrs), MVT-602 0.03 nmol/kg in black (over 48hrs), KP54 9.6 nmol/kg in red (over 24hrs) and 0.9% saline in purple (over 24 h).

Figure 2C: Median (IQR) of modelled values of AUC of serum LH (iU.h/L) after KP54 (9.6 nmol/kg), MVT-602 (0.01 nmol/kg) and MVT-602 (0.03 nmol/kg). Groups were compared by Kruskal Wallis test with post-hoc Dunn's multiple comparison. The duration of sampling for KP54 was 24 h as LH had returned to baseline within this timeframe, whereas it was 48 h for MVT-602.

Figure 3:

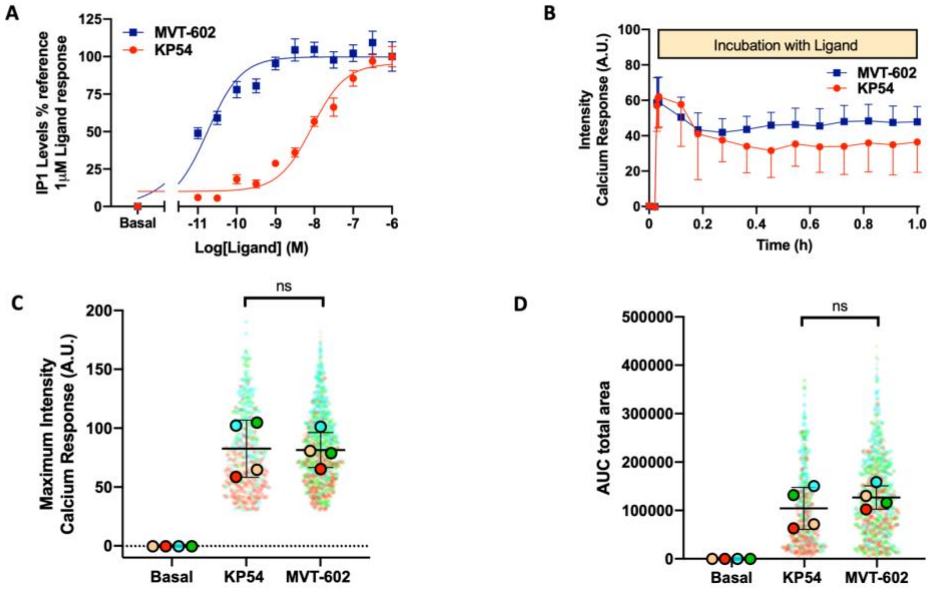

Figure 3- Effect of KP54 and MVT-602 on KISS1R-mediated Gαq/11 signaling via IP_1 and Ca^{2+} activation.

Figure 3A: Intracellular levels of IP_1 accumulation at varying concentrations of KP54 or MVT-602 following 45 min stimulation in HEK 293 cells expressing FLAG-KISS1R. Data represent mean (±SEM) of n = 4 independent experiments conducted in triplicate wells and are normalized as a percentage of ligand response.

Figure 3B: Intracellular Ca^{2+} levels measured by Fluo4-AM Ca^{2+} indicator dye and live confocal microscopy. Intensity profile produced following 10 nM stimulation with KP54 (red) or MVT-602 (blue) over 1 h chronic stimulation in cells transiently transfected with FLAG-KISS1R of n = 4 independent experiments conducted in duplicate wells. Data is shown following subtraction of the average background intensity for each cell as described in methods.

Figure 3C: Maximum intensity and **Figure 3D:** total area under the curve (AUC), calculated from data depicted in **(B)** following ligand treatments over 1 h chronic stimulation. Data shows individual cells analysis (total number of cells; basal n = 1324, KP54 n = 472, MVT-602 n = 852) overlaid with the mean (±SD) values of n = 4 independent experiment conducted in duplicate wells. Cells attributed to each biological repeat are shown in the corresponding color. There were no significant differences between KP54 and MVT-602 following analysis by two-tailed, unpaired Student's t-test.

Figure 4:

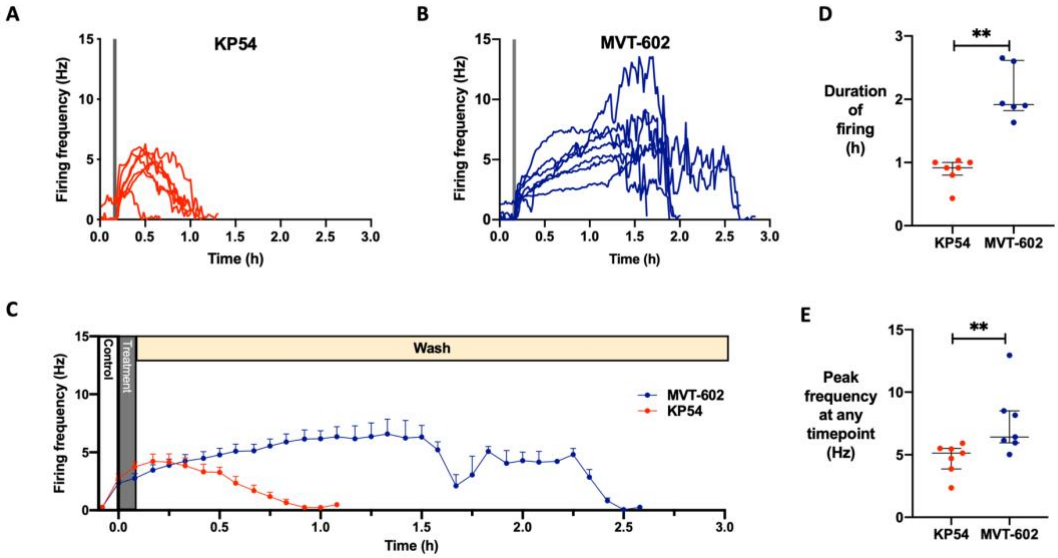

Figure 4: Targeted extracellular recordings of firing rate of GFP-identified GnRH neurons revealed that both KP54 and MVT-602 rapidly increase action potential firing rate, but the response to MVT-602 is more prolonged. Spontaneous basal activity (control) was recorded for 10 min, then either 10 nM KP54 or 10 nM MVT-602 was bath-applied for 5 min, followed by a wash-period of at least 40 min.

Figure 4A & 4B: Firing frequency (Hz in 1-min bins) of individual GnRH neurons vs time (h) (n = 7) before, during (grey bar) and after exposure to either 10 nM KP54 (4A) or 10 nM MVT-602 (4B).

Figure 4C: Mean (±SEM) firing frequency of GnRH neurons (n = 7 per group, 5 min bins) over time (h), before, during (grey bar) and after exposure to either 10 nM KP54 in red or 10 nM MVT-602 in blue. Statistical analysis by mixed-effect model (REML) was truncated at 65 min because all KP54 cells had returned to baseline (P = 0.003).

Figure 4D: Median (IQR) of duration of response (h) of GnRH neurons maintaining a firing frequency (h) >1 Hz (n=7 per group) after exposure to 10 nM KP54 (red) or 10 nM MVT-602 (blue). Groups were compared by the Mann-Whitney U test (** P = 0.0012).

Figure 4E: Median (IQR) of the peak firing frequency (Hz) of GnRH neurons (n = 7 per group) at any time-point after exposure to 10 nM KP54 (red) or 10 nM MVT-602 (blue). Groups were compared by the Mann-Whitney U test (** P = 0.007).

Figure 5:

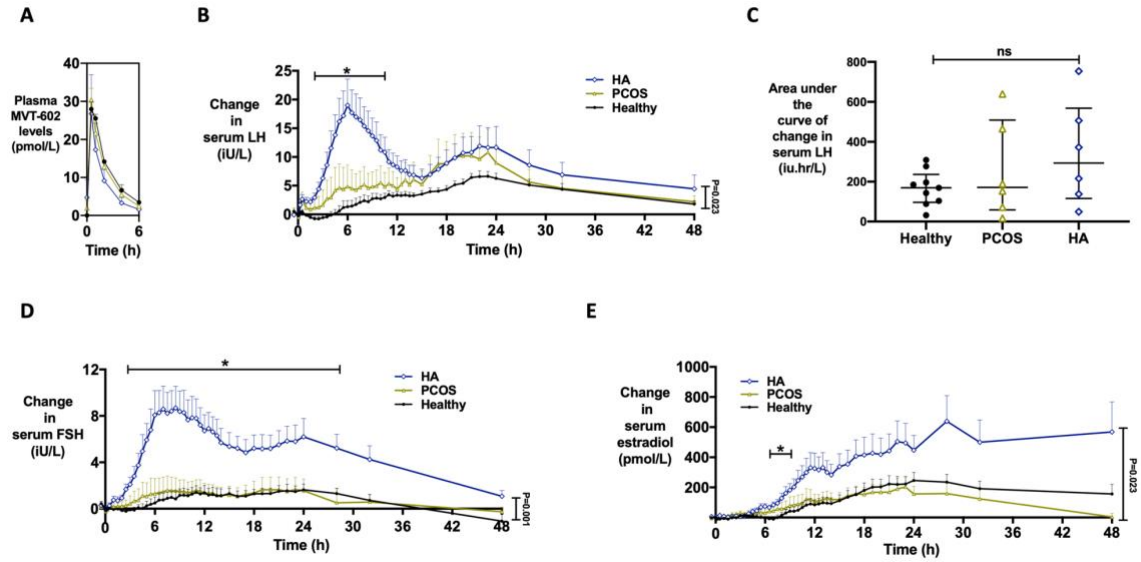

Figure 5- Clinical studies of MVT-602 in women with oligo/anovulatory disorders

Figure 5A: Mean (±SEM) of the plasma concentration of MVT-602 (pmol/L) vs time (h) in women receiving a SC bolus of 0.03 nmol/kg of MVT 602. Healthy women in the follicular phase are presented in black (n = 9), women with PCOS in olive green (n = 6) and women with HA in blue (n = 6). No significant difference was detected by two-way ANOVA.

Figure 5B, 5D, & 5E: Mean (±SEM) of change from baseline levels in serum LH (iU/L) (**5B),** serum FSH (iU/L) **(5D)** and serum estradiol (pmol/L) **(5E)** in healthy women in black (n = 9), women with PCOS in olive green (n = 6) and women with HA in dark blue (n = 6) after receiving a single SC bolus of MVT 602 0.03nmol/kg. Groups were compared by two-way ANOVA. P-value was * P = 0.025 for LH, ** P = 0.0011 for FSH and * P = 0.02 for estradiol.

Figure 5C: Median (IQR) of modelled values of AUC of serum LH (iU.h/L) with MVT 602 0.03 nmol/kg in women in the healthy follicular phase, PCOS and HA. No significant difference was detected by Kruskal Wallis test.

Figure 6:

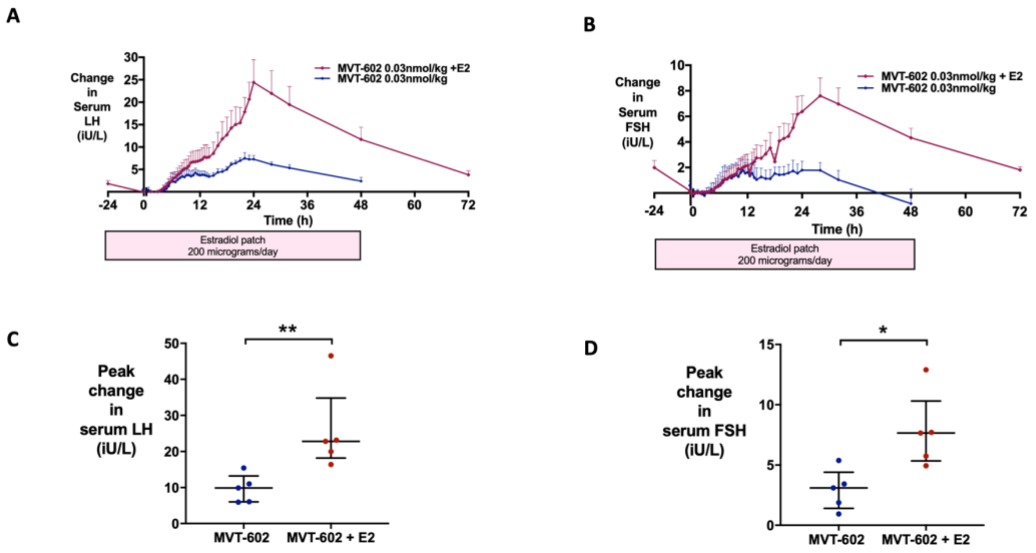

Figure 6- Effect of estradiol supplementation on response to MVT-602 in women during the healthy follicular phase

Figure 6A & 6B: Mean (±SEM) of change from baseline in serum LH (iU/L) **(6A)** and serum FSH (iU/L) **(6B)** in healthy women receiving a single SC bolus of MVT-602 0.03 nmol/kg is presented in blue, and in women with estradiol supplementation (n = 5 per group) via a 200 µg per day transdermal patch applied from 24 h prior to MVT-602 administration at time 0 h and continued until time 48hrs in maroon.

Figure 6C & 6D: Scatter plot of median (IQR) of maximal rise in serum LH (iU/L) **(6C)** and serum FSH (iU/L) **(6D)** in healthy women receiving a single SC bolus of MVT-602 0.03 nmol/kg (blue), and in women with estradiol supplementation (n = 5 per group) via a 200 µg per day transdermal patch applied from 24 h prior to MVT-602 administration at time 0 h and continued until time 48 h (maroon). Groups were compared by Mann Whitney U test (* P = 0.016, ** P = 0.0079).

Tables

Table 1 Baseline characteristics

Clinical Characteristics	Healthy women (n = 9)	Women with PCOS (n = 6)	Women with HA (n = 6)	P-value
Age (years)	26.0 (21.0, 32.8)	24.5 (21.0, 26.3)	25.0 (23.0, 30.8)	0.56
Mass (kg)	64.7 (43.4, 72.6)	61.2 (51.9, 72.1)	55.3 (51.5, 61.0)	0.28
Body Mass Index (kg/m_2)	24.5 (20.1, 25.5)	23.2 (18.4, 25.1)	20.7 (19.4, 23.1)	0.11
Serum LH (iU/L)	3.7 (3.0, 4.4)	4.4 (1.9, 9.3)	2.9 (1.4, 3.6)	0.22
Serum FSH (iU/L)	5.1 (2.9, 5.9)	4.3 (2.9, 5.0)	5.2 (4.6, 6.3)	0.22
Serum Estradiol (pmol/L)	93.0 (72.3, 135.3)	81.0 (67.3, 107)	72.5 (50.5, 110.3)	0.47
Sex Hormone Binding Globulin (nmol/L)	69.0 (48.8, 109.5)	32.0 (31.3, 48.3)	65.0 (39.8, 100.3)	0.04
Serum AMH (pmol/L)	23.9 (2.4, 36.8)	70.7 (29.8, 104.3)	20.8 (14.9, 23.2)	0.01

Table 1 Baseline characteristics of healthy women, women with HA and women with PCOS. Median (25_{th} centile, 75_{th} centile) is presented of hormone levels on the morning of first study visit. Comparison is made using Kruskal-Wallis with *post-hoc* Dunn's multiple comparison test. LH = luteinizing hormone, FSH = follicle stimulating hormone, AMH = anti-Müllerian hormone. Women with PCOS had significantly lower SHBG and higher AMH compared to healthy women and women with HA.

1 **Table 2**

Pharmacokinetic response	**KP54 9.6 nmol/kg** (n = 9)	**MVT-602 0.01 nmol/kg** (n = 3)	**MVT-602 0.03 nmol/kg** (n = 3)	**P-value**
$t_{1/2}$ h (range)	1.68 (1.31, 1.78)	2.02 (1.58, 2.26)	1.78 (1.60, 2.11)	0.22
t_{max} h (range)	**1.00 (0.91, 1.22)**	0.42 (0.32, 0.46)	**0.36 (0.34, 0.37)**	0.0004 **(0.02)**
C_{max} pmol/L (range)	**8855 (7207, 14821)**	**8.11 (7.78, 10.17)**	29.49 (28.15, 29.53	<0.0001 **(0.01)**

Pharmacodynamic response on LH	LH-rise Episode 1	LH-rise Episode 2	LH-rise Episode 1	LH-rise Episode 2	LH-rise Episode 1	LH-rise Episode 2	
	(n = 9)		(n = 9)		(n = 9)		
Time of start of episode (h)	**2.44 (1.96-3.32)**	n/a	4.63 (2.93-8.38)	19.33 (17.89-21.26)	**5.85 (3.89-9.38)**	18.34 (17.52-19.84)	0.0036 **(0.0036)**
Duration of ascent of LH (h)	**2.07 (1.63-3.63)**	n/a	4.24 (2.88-10.31)	0.21 (0.08-2.24)	**5.72 (2.63-17.57)**	1.70 (0.27-3.97)	0.0085 **(0.01)**
Duration of decay in LH (h)	**2.36 (2.04-4.14)**	n/a	**23.03*** (10.14-33.29)**	4.24 (2.88-10.31)	**15.98** (6.14-18.21)**	11.66 (4.96-16.2)	0.0002 **(***0.0003, **0.005)**
Time of maximal LH (h)	**4.70 (4.03-5.31)**	n/a	19.69 (11.72-34.81)	**21.86** (18.83-27.37)**	15.14 (10.47-20.73)	**21.42*** (20.27-23.03)**	0.0002 **(**0.0015, ***0.0005)**
Amplitude of LH response (iU/L)	9.91 (5.88, 18.15)	n/a	8.18 (5.13, 11.89)	12.11 (7.74, 15.89)	9.10 (6.60, 9.55)	10.64 (7.81, 12.29)	0.76
AUC of LH (h.iU/L)	**38.52 (26.5-69.7)**		**158.60* (68.3-233.0)**		**169.00** (96.0-236.5)**		0.0058 **(*0.01, **0.029)**

Table 2- Pharmacokinetic properties of and pharmacodynamic response to kisspeptin-54 (KP54) and MVT-602 on serum luteinizing hormone (LH). Median (range) is presented for pharmacokinetic parameters and median (IQR) for pharmacodynamic parameters. To extract pharmacokinetic parameters for KP54 and MVT-602, we used a PK model incorporating drug absorption and elimination [51]. To extract information regarding LH dynamics in response to administration of kisspeptin-54 (KP54) and MVT-602, we used a parametric model describing a superposition of two episodes of LH-rise. As some LH rises had distinct phases of increase in serum LH after MVT-602, the model generated parameters for both a first phase increase commencing within the first 10h of administration (LH-rise episode 1), and a second phase increase commencing after 10 h (LH-rise episode 2). Time of start indicates the time of onset of rise in LH. Amplitude of LH response refers to absolute LH levels. The area under the curve (AUC) of LH incorporates both LH-rise episode 1 and episode 2. Time of start of increase in LH, duration of ascent, duration of decay, and time of maximal LH were compared using LH-rise episode 1, whereas amplitude of LH was compared using the greater of the two LH-amplitudes. Groups were compared by Kruskal Wallis test with post hoc Dunn's. Post-hoc P-value presented in brackets if initial P-value < 0.05 and corresponds to groups in bold (if more than one group then also by stars).

1 **Table 3**

Pharmacokinetic response	**Healthy** (n = 3)		**PCOS** (n = 3)		**HA** (n = 3)		**P-value**
$t_{1/2}$ h (range)	1.78 (1.60, 2.11)		1.55 (1.24, 1.64)		1.71 (1.29, 1.75)		0.25
t_{max} h (range)	**0.36 (0.34, 0.37)**		**0.24 (0.12, 0.24)**		0.27 (0.24, 0.29)		0.01 **(0.03)**
C_{max} pmol/L (range)	29.06 (28.15, 29.53)		21.52 (6.37, 37.41)		27.00 (26.91, 31.69)		0.72
Pharmacodynamic response on LH	LH-rise Episode 1 (n = 9)	LH-rise Episode 2	LH-rise Episode 1 (n = 6)	LH-rise Episode 2	LH-rise Episode 1 (n = 6)	LH-rise Episode 2	
Time of start of episode (h)	**5.85 (3.89-9.38)**	18.34 (17.52-19.84)	3.86 (1.27-11.90)	17.17 (15.32-19.51)	**2.51 (2.14-2.97)**	16.49 (15.46-17.54)	0.033 **(0.033)**
Duration of ascent of LH (h)	5.72 (2.63-17.57)	1.70 (0.27-3.97)	4.16 (2.02-5.96)	1.84 (0.94-2.34)	3.41 (2.67-3.88)	3.66 (2.48-4.27)	0.21
Duration of decay in LH (h)	**15.98 (6.14-18.21)**	11.66 (4.96-16.2)	9.10 (4.37-4.75)	5.19 (3.38-13.06)	**4.00 (3.77-4.75)**	12.73 (6.39-20.25)	0.005 (**0.01**)
Time of maximal LH (h)	**15.14 (10.47-20.73)**	21.42 (20.27-23.03)	13.13 (9.07-24.32)	20.01 (18.82-22.22)	**6.24 (5.77-6.59)**	22.19 (20.99-24.10)	0.004 (**0.008**)
Amplitude of LH response (iU/L)	9.10 (6.60, 9.55)	10.64 (7.81, 12.29)	9.96 (5.43, 25.01)	12.82 (7.19, 26.02)	16.82 (7.61, 27.45)	11.28 (5.10, 20.80)	0.49
AUC of LH (h.iU/L)	169.00 (96.0-236.5)		171.30 (58.4-568.0)		293.70 (115.6-568.0)		0.50

2 **Table 3- Pharmacokinetic properties of and pharmacodynamic response to MVT-602 on serum**

3 **luteinizing hormone (LH) in healthy women, women with PCOS and women with HA.** Median

(range) is presented for pharmacokinetic parameters and median (IQR) for pharmacodynamic parameters. To extract pharmacokinetic parameters for KP54 and MVT-602, we used a PK model incorporating drug absorption and elimination [51]. To extract information regarding LH dynamics in response to administration of kisspeptin-54 (KP54) and MVT-602, we used a parametric model describing a superposition of two episodes of LH-rise. As some LH rises had distinct phases of increase in serum LH after MVT-602, the model generated parameters for both a first phase increase commencing within the first 10h of administration (LH-rise episode 1), and a second phase increase commencing after 10 h (LH-rise episode 2). Time of start indicates the time of onset of rise in LH. Amplitude of LH response refers to absolute LH levels. The area under the curve (AUC) of LH incorporates both LH-rise episode 1 and episode 2. Time of start of increase in LH, duration of ascent, duration of decay, and time of maximal LH were compared using LH-rise episode 1, whereas amplitude of LH was compared using the greater of the two LH-amplitudes. Groups were compared by Kruskal Wallis test with post hoc Dunn's. Post-hoc P-value presented in brackets if initial P-value <0.05 and corresponds to groups in bold.