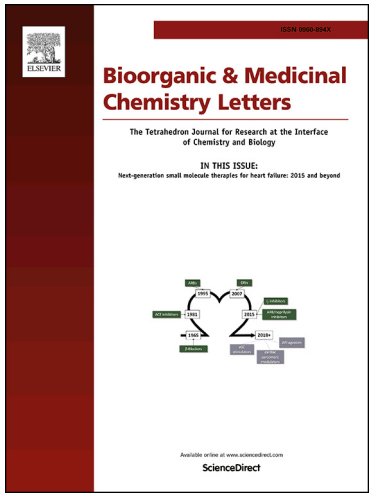


Discovery of a Small Molecule RXFP3/4 Agonist That Increases Food Intake in Rats Upon Acute Central Administration

Brian DeChristopher, Soo-Hee Park, Linh Vong, Derek Bamford, Hyun-Hee Cho, Rohit Duvadie, Allison Fedolak, Christopher Hogan, Toshiyuki Honda, Pramod Pandey, Olga Rozhitskaya, Liansheng Su, Elizabeth Tomlinson, Iain Wallace

PII: S0960-894X(19)30086-1
DOI: https://doi.org/10.1016/j.bmcl.2019.02.013
Reference: BMCL 26293

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 21 September 2018
Revised Date: 4 January 2019
Accepted Date: 10 February 2019

Please cite this article as: DeChristopher, B., Park, S-H., Vong, L., Bamford, D., Cho, H-H., Duvadie, R., Fedolak, A., Hogan, C., Honda, T., Pandey, P., Rozhitskaya, O., Su, L., Tomlinson, E., Wallace, I., Discovery of a Small Molecule RXFP3/4 Agonist That Increases Food Intake in Rats Upon Acute Central Administration, *Bioorganic & Medicinal Chemistry Letters* (2019), doi: https://doi.org/10.1016/j.bmcl.2019.02.013

Discovery of a Small Molecule RXFP3/4 Agonist That Increases Food Intake in Rats Upon Acute Central Administration

Brian DeChristopher,* Soo-Hee Park, Linh Vong, Derek Bamford, Hyun-Hee Cho, Rohit Duvadie, Allison Fedolak, Christopher Hogan, Toshiyuki Honda, Pramod Pandey, Olga Rozhitskaya, Liansheng Su, Elizabeth Tomlinson, and Iain Wallace

Novartis Institutes for BioMedical Research, 250 Massachusetts Avenue, Cambridge, MA 02139.

KEYWORDS: RXFP3, RXFP4, Relaxin-3, Insulin-like peptide

ABSTRACT: The relaxin family peptide receptors have been implicated in numerous physiological processes including energy homeostasis, cardiac function, wound healing, and reproductive function. Two family members, RXFP3 and RXFP4, are class A GPCRs with endogenous peptide ligands (relaxin-3 and insulin-like peptide 5 (INSL5), respectively). Polymorphisms in relaxin-3 and RXFP3 have been associated with obesity, diabetes, and hypercholesterolemia. Moreover, central administration of relaxin-3 in rats has been shown to increase food intake, leading to body weight gain. Reported RXFP3 and RXFP4 ligands have been restricted to peptides (both endogenous and synthetic) as well as a low molecular weight positive allosteric modulator requiring a non-endogenous orthosteric ligand. Described here is the discovery of the first potent low molecular weight dual agonists of RXFP3/4. The scaffold identified is competitive with a chimeric relaxin-3/INSL5 peptide for RXFP3 binding, elicits similar downstream signaling as relaxin-3, and increases food intake in rats following acute central administration. This is the first report of small molecule RXFP3/4 agonism.

The relaxin family peptide receptors (RXFPs) are G protein-coupled receptors with peptide ligands that play roles in diverse physiological processes, including those controlled by central nervous, cardiovascular, reproductive, and metabolic systems.[1] This array of associated activities makes the RXFPs potential therapeutic targets for neuroscience, cardiovascular, and metabolic indications. Among these receptors, RXFP3 has been linked to appetite regulation,[2,3] arousal, stress responses, anxiety,[4] memory,[5] and depression.[6] RXFP4, the most closely related family member to RXFP3, has been linked to insulin secretion and appetite.[7,8] The endogenous peptide ligands of RXFP3 and RXFP4 are relaxin-3 and insulin-like peptide 5 (INSL5), respectively. These complex peptides, which contain two amino acid chains linked by both inter- and intra-chain disulfide bridges, have been used extensively to establish the involvement of RXFP3 and RXFP4 in the various physiological processes detailed above.[9,10]

Despite the attractiveness of RXFP3 and RXFP4 as therapeutic targets, ligands of these receptors have been limited to the endogenous peptides or their analogs.[11,12,13] In the case of RXFP3, a positive allosteric modulator requiring a non-endogenous relaxin-3 analog for function has also been reported.[14] Whereas significant progress has been made in the understanding of structure activity relationships of relaxin-3 and INSL5 as well as the design of simplified agonist and antagonist peptide analogs (with varying degrees of RXFP3/4 selectivity), there remains a need to identify small molecule ligands for these targets. Such chemical matter could potentially improve access to specific tissues of interest, diversify routes of administration, improve pharmacokinetic properties, and simplify synthetic access relative to peptide ligands. Toward this end, a high throughput screen was initiated to identify low molecular weight compounds capable of phenocopying relaxin-3 both *in vitro* and *in vivo*. Detailed herein is the identification of a hit scaffold capable of agonizing both RXFP3 and RXFP4 *in vitro* as well as the discovery of a potent compound showing a relaxin-3-like signaling profile *in vitro* that also increases food intake in rats following acute intracerebroventricular (ICV) administration.

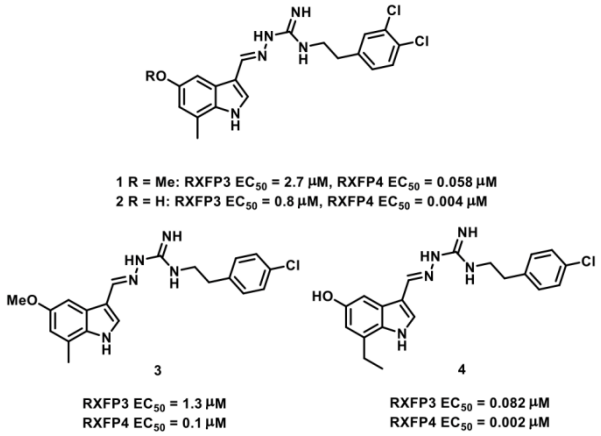


Figure 1. Low molecular weight RXFP3/4 ligands with their corresponding EC_{50} values for cAMP assays in RXFP3 and RXFP4-overexpressing cells, respectively. For the RXFP3 assay, n = 26 (compound **1**), n = 3 (compound **2**), n = 2 (compound **3**), and n = 11 (compound **4**). For the RXFP4 assay, n = 10 (compound **1**), n = 2 (compound **2**), n = 1 (compound **3**), and n = 2 (compound **4**). EC_{50} values represent an average of separate experiments when n > 1.

Compound **1** (figure 1)[15] was identified from a 70 thousand compound screen using a homogeneous time resolved fluorescence (HTRF) assay of inhibition of forskolin-stimulated cAMP accumulation in RXFP3-overexpressing CHO-K1 cells (for **1**, RXFP3 EC_{50} = 2.7 µM, E_{max} = 97% of relaxin-3). Subsequently, compound **1** was found to bind competitively with [^{125}I]-R3/I5 (a chimeric peptide consisting of the relaxin-3 B-chain and the INSL5 A-chain)[16] to RXFP3 with a K_i value of 1.7 µM (figure 2). This ability to compete with [^{125}I]-R3/I5 for RXFP3 binding is shared by relaxin-3 (figure 2). Additional testing revealed that **1** was capable of inhibiting cAMP accumulation in RXFP4-overexpressing CHO-K1 cells (EC_{50} = 58 nM, E_{max} = 99% of relaxin-3), indicating that this scaffold is capable of RXFP3/4 dual agonism. RXFP3- and RXFP4-overexpressing cells were shown to have comparable receptor expression levels (see supplemental table 1 in the supporting information).

Medicinal chemistry optimization was undertaken to improve the potency of **1** for expanded *in vitro* and *in vivo* validation using relaxin-3 as a positive control. This optimization identified the indole 5 position as tunable for both RXFP3 and RXFP4 potency, as evidenced by 5-hydroxy indole-containing compound **2** (figure 1). Replacement of the dichlorophenethyl amine of **1** with a 4-chlorophenethyl amine (compound **3**, figure 1) was also well tolerated. Finally, installation of an ethyl group at the indole 7 position in combination with the modifications shown in compounds **2** and **3** provided the potent compound **4**, which had average cAMP inhibition EC_{50} values of 82 nM for RXFP3 (E_{max} = 105% of relaxin-3) and 2 nM for RXFP4 (E_{max} = 100% of relaxin-3).

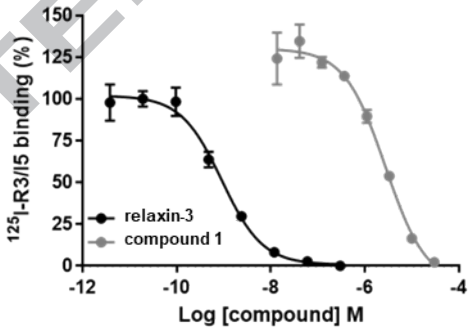


Figure 2. Dose responsive competitive displacement of [^{125}I]-R3/I5 from RXFP3-expressing cell membranes by both relaxin-3 and compound **1**.

Given that the RXFP3 and RXFP4 cAMP EC_{50} values shown in figure 1 represent averages of separate cellular experiments (n values shown in the figure 1 caption), the potencies of compound **1**, compound **4**, and relaxin-3 were compared directly in the same experiment. In the case of RXFP3 (figure 3A), compound **4** (EC_{50} = 12.8 nM) was 273-fold more potent than compound **1** (EC_{50} = 3.5 µM). These values are compared to relaxin-3, which had an EC_{50} = 0.2 nM. For RXFP4 (figure 3B), compound **4** (EC_{50} = 1.6 nM) was 9-fold more potent than compound **1** (EC_{50} = 14.6 nM). These values are compared to relaxin-3, which had an RXFP4 EC_{50} = 0.7 nM. This data further supports the improved cAMP potency of compound **4** relative to compound **1** that was observed in the aggregate data shown in figure 1 (on average, ~30-fold potency improvement was observed for compound **4** versus compound **1** in both the RXFP3 and RXFP4 cAMP assays).

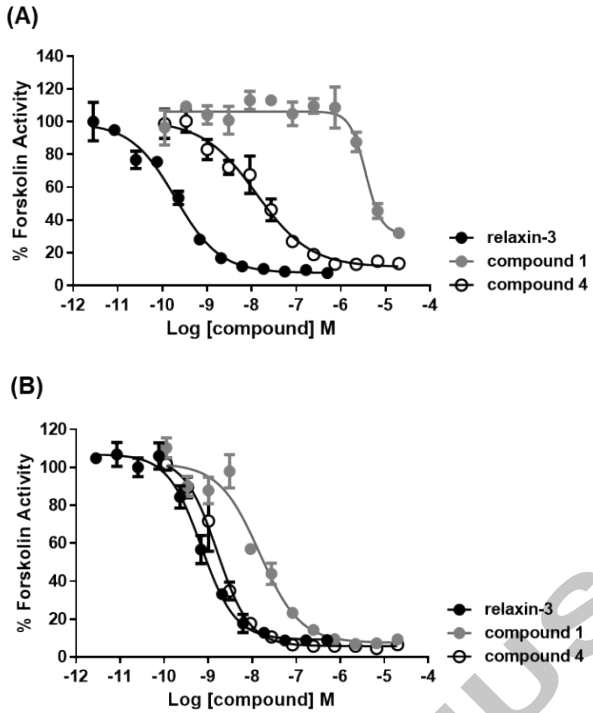


Figure 3. Direct comparison of inhibition of forskolin-stimulated cAMP accumulation by relaxin-3, compound **1**, and compound **4** in CHO-K1 cells stably over-expressing RXFP3 (A) and RXFP4 (B).

The observed selectivity of **4** favoring RXFP4 over RXFP3 was confirmed by [^{35}S]GTPγS binding experiments using membranes from CHO-K1 cells overexpressing either RXFP3 or RXFP4 (figure 4). GTPγS binding EC_{50} values were 119 nM for RXFP3 (figure 4A) and 11 nM for RXFP4 (figure 4B), consistent with the trend observed in the cAMP EC_{50} values. **4** also induced β-arrestin recruitment (EC_{50} = 4.9 μM relative to an EC_{50} of 83 nM for relaxin-3) in an RXFP3 overexpressing U2OS cell line (figure 5A). These results indicate that **4** engages both G protein-mediated cAMP signaling and G protein-independent β-arrestin recruitment, a profile shared by relaxin-3.

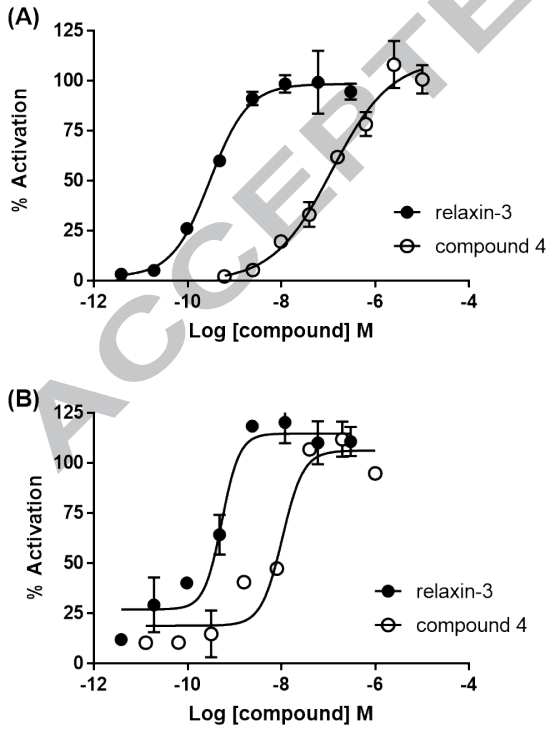


Figure 4. Dose-responsive GTPγS binding induced by both relaxin-3 and compound **4** to CHO-K1 membranes overexpressing RXFP3 (A) or RXFP4 (B).

The activation of MAPKs (ERK1/2, JNK1, and p38-MAPK) was examined following addition of relaxin-3 (100 nM) and **4** (5 μM) to assess functional selectivity of **4**. Relaxin-3 and **4** activated ERK1/2 and JNK (figure 5B), but not p38MAPK (data not shown) in RXFP3-overexpressing CHO-K1 cells. Maximal activation of ERK1/2 and JNK with relaxin-3 and **4** occurred at 2 minutes after addition. No ERK1/2 or JNK1/2 responses were detected in parental CHO-K1 cells treated with relaxin-3 or **4** (data not shown). Western blots confirmed that the total JNK or ERK1/2 in treated and untreated RXFP3-overexpressing CHO-K1 cells was not altered. Based on these results, **4** displays a similar signaling fingerprint compared to relaxin-3.

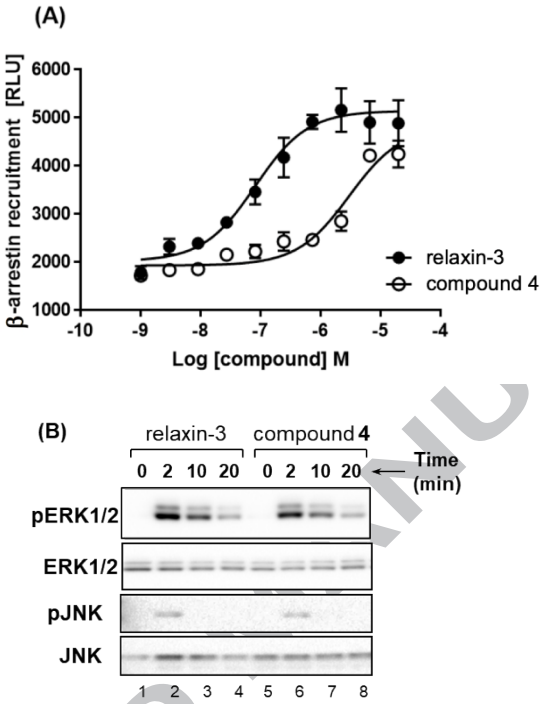


Figure 5. (A) Comparison of the ability of relaxin-3 and compound **4** to induce β-arrestin recruitment activity in U2OS cells stably expressing human RXFP3. (B) Comparison of the time-course of ERK and JNK phosphorylation following treatment of CHO-K1 cells expressing human RXFP3 with relaxin-3 (100 nM) or compound **4** (5 μM).

Central administration of relaxin-3 has been shown to stimulate food intake in satiated rats.[2] To determine if **4** would elicit a similar effect on food intake, 1 μmol of **4** was administered ICV to satiated rats (figure 6). Between 0 and 2 hours post-administration, relaxin-3 (figure 6A) and **4** (figure 6B) both elicited an increase in food intake (2.9 ± 0.7 grams for relaxin-3 versus 0.8 ± 0.3 grams for artificial cerebrospinal fluid (aCSF)-treated rats; 3.5 ± 0.6 grams for **4** versus 1.7 ± 0.5 grams for 75% DMSO/25% aCSF-treated rats). This increase was still observed at 4 hours post-administration of relaxin-3 (4.6 ± 0.9 grams versus 1.7 ± 0.4 grams for aCSF-treated rats (figure 6A)). For **4**, the increase in food intake was still observed at both 4 (7.1 ± 1.1 grams versus 2.5 ± 0.6 grams for 75% DMSO/25% aCSF-treated rats) and 24 (35.3 ± 2.0 grams versus 24.1 ± 2.7 grams for 75% DMSO/25% aCSF-treated rats) hours post-administration (figure 6B).

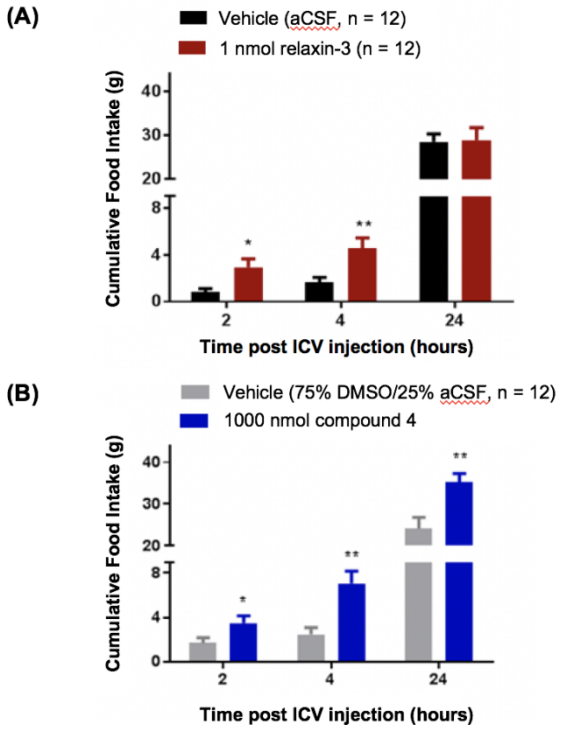
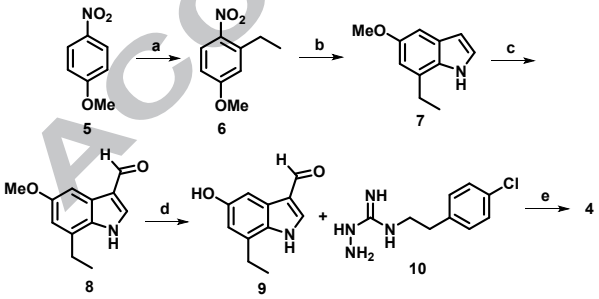


Figure 6. Effects of relaxin-3 (A) and compound **4** (B) on food intake in Sprague-Dawley rats following ICV administration; *p < 0.05, **p < 0.01.

The synthesis of **4** is shown in scheme 1. Installation of the ethyl group onto 1-methoxy-4-nitrobenzene **5** was followed by treatment with vinylmagnesium bromide to form indole **7**. Next, Vilsmeier-Haack chemistry was used for installation of the indole 3-position aldehyde to form **8**. Removal of the methyl group from the 5-methoxy moiety was then followed by installation of aminoguanidine **10** (see supporting information for synthesis details) to complete the synthesis of **4**.

Scheme 1. Synthesis of compound **4**.



Reagents and Conditions: (a) EtMgCl, DDQ, THF, -15 °C, 21%; (b) Vinylmagnesium bromide, THF, -45 °C, 14%; (c) Oxalyl chloride, DMF, 0-100 °C, 55%; (d) BBr_3, DCM, -78 °C to rt; (e) $TsOH.H_2O$, MeOH, rt, 59% (two steps).

In summary, high throughput screening efforts identified the first known low molecular weight, R3/I5-competitive RXFP3 agonist scaffold capable of inhibiting forskolin-stimulated cAMP production in both RXFP3- and RXFP4- overexpressing CHO-K1 cells. The potency of this scaffold was optimized to provide a tool compound **4** that had a downstream signaling profile consistent with the endogenous RXFP3 ligand relaxin-3. Moreover, **4** was capable of stimulating food intake following ICV administration in rats. This orexigenic effect is consistent with that observed with relaxin-3 both in this study and previously.[2,3] Compound **4** represents a scaffold that could be used for further mechanistic studies or target validation around RXFP3/4 and as a starting point for additional medicinal chemistry.

Corresponding Author

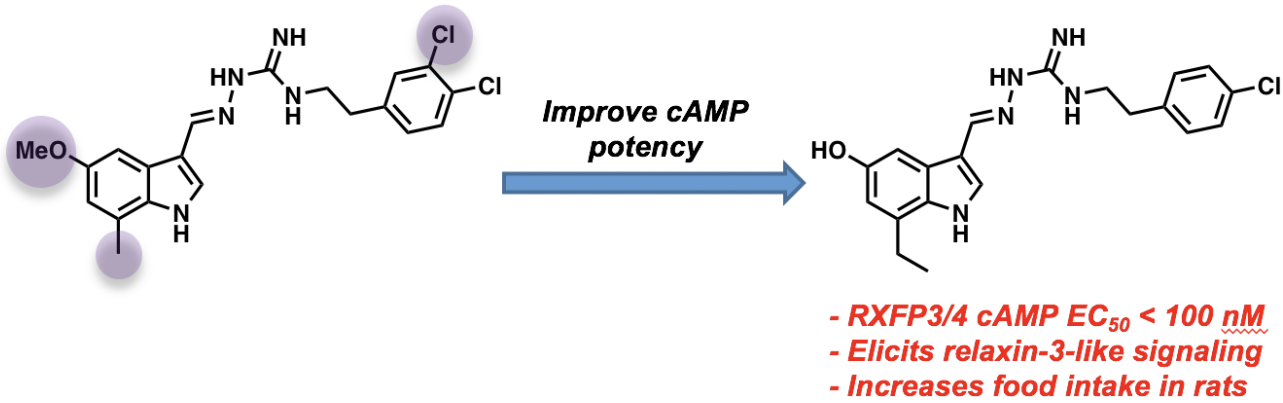
*badechri@gmail.com

Conflicts of Interest

The authors report no conflicts of interest associated with this work.

References

1. Bathgate, R. A. D.; Halls, M. L.; van der Westhuizen, G. E.; Callander, G. E.; Kocan, M.; Summers, R. J. "Relaxin family peptides and their receptors" *Phys. Rev.* **2013**, *93*, 405-480.
2. Ganella, D. E.; Ryan, P. J.; Bathgate, R. A. D.; Gundlach, A. L. "Increased feeding and body weight gain in rats after acute and chronic activation of RXFP3 by relaxin-3 and receptor-selective peptides: functional and therapeutic implications" *Behav. Pharmacol.* **2012**, *23*, 516-525.
3. de Avila, C.; Chometton, S.; Lenglos, C.; Calvez, J. ; Gundlach, A. L.; Timofeeva, E. "Differential effects of relaxin-3 and a selective relaxin-3 receptor agonist on food and water intake and hypothalamic neuronal activity in rats" *Behav. Brain Res.* **2018**, *336*, 135-144.
4. Kumar, J. R.; Rajkumar, R.; Jayakody, T.; Marwari, S.; Hong, J. M.; Ma, S.; Gundlach, A. L.; Lai, M. K. P.; Dawe, G. S. "Relaxin' the brain: a case for targeting the nucleus incertus network and relaxin-3/RXFP3 system in neuropsychiatric disorders" *Br. J. Pharmacol.* **2017**, *174*, 1061-1076.
5. Albert-Gasco, H.; Garcia-Aviles, A.; Moustafa, S.; Sanchez-Sarasua, S.; Gundlach, A. L.; Olucha-Bordonau, F. E.; Sanchez-Perez, A. M. "Central relaxin-3 receptor (RXFP3) activation increases ERK phosphorylation in septal cholinergic neurons and impairs spatial working memory" *Brain Struct. Funct.* **2017**, *222*, 449-463.
6. Smith, C. M.; Walker, A. W.; Hosken, I. T.; Chua, B. E.; Zhang, C.; Haidar, M.; Gundlach, A. L. "Relaxin-3/RXFP3 networks: an emerging target for the treatment of depression and other neuropsychiatric diseases?" *Front. Pharmacol.* **2014**, *5*, 1-17.
7. Luo, X.; Li, T.; Zhu, Y.; Dai, Y.; Zhao, J.; Guo, Z.-Y.; Wang, M.-W. "The insulinotropic effect of insulin-like peptide 5 *in vitro* and *in vivo*" *Biochem. J.* **2015**, *466*, 467-473.
8. Grosse, J.; Heffron, H.; Burling, K.; Hossain, M. A.; Habib, A. M.; Rogers, G. J.; Richards, P.; Larder, R.; Rimmington, D.; Adriaenssens, A. A.; Parton, L.; Powell, J.; Binda, M.; Colledge, W. H.; Doran, J.; Toyoda, Y.; Wade, J. D.; Aparicio, S.; Carlton, M. B. L.; Coll, A. P.; Reimann, F.; O'Rahilly, S.; Gribble, F. M. "Insulin-like peptide 5 is an orexigenic gastrointestinal hormone" *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 11133-11138.
9. Bathgate, R. A. D.; Lin, F.; Hanson, N. F.; Otvos, L.; Guidolin, A.; Giannakis, C.; Bastiras, S.; Layfield, S. L.; Ferraro, T.; Ma, S.; Zhao, C.; Gundlach, A. L.; Samuel, C. S.; Tregear, G. W.; Wade, J. D. "Relaxin-3: Improved synthesis strategy and demonstration of its high-affinity interaction with the relaxin receptor LGR7 both in vitro and in vivo" *Biochemistry* **2006**, *45*, 1043-1053.
10. Hossain, M. A.; Bathgate, R. A. D.; Kong, C. K.; Shabanpoor, F.; Zhang, S.; Haugaard,-Jonsson, L. M.; Rosengren, K. J.; Tregear, G. W.; Wade, J. W. "Synthesis, conformation, and activity of human insulin-like peptide 5 (INSL5)" *ChemBioChem* **2008**, *9*, 1816-1822.
11. Patil, N. A.; Rosengren, K. J.; Separovic, F.; Wade, J. D.; Bathgate, R. A. D.; Hossain, M. A. "Relaxin family peptides: structure-activity relationship studies" *Br. J. Pharmacol.* **2017**, *174*, 950-961.
12. Jayakody, T.; Marwari, S.; Lakshminarayanan, R.; Tan, F. C. K.; Johannes, C. W.; Dymock, B. W.; Poulsen, A.; Herr, D. R.; Dawe, G. S. "Hydrocarbon stapled B chain analogues of relaxin-3 retain biological activity" *Peptides* **2016**, *84*, 44-57.
13. Wei, D.; Hu, M.-J.; Shao, X.-X.; Wang, J.-H.; Nie, W.-H.; Liu, Y.-L.; Xu, Z.-G.; Guo, Z.-Y. "Development of a selective agonist for relaxin family peptide receptor 3" *Sci. Rep.* **2017**, *7*, 3230.
14. Alvarez-Jaimes, L.; Sutton, S. W.; Nepomuceno, D.; Motley, S. T.; Cik, M.; Stocking, E.; Shoblock, J.; Bonaventure, P. "In vitro pharmacological characterization of RXFP3 allosterism: an example of probe dependency" *PLoS One* **2012**, *7*, e30792.
15. Giger, R. K. A.; Mattes, H. "Certain aminoguanidine compounds, pharmaceutical compositions containing them and their use in treating gastrointestinal motility disorders and disorders associated with cephalic pain" US 5510353 A1, 1996.
16. Haugaard-Jonsson, L. M.; Hossain, M. A.; Daly, N. L.; Bathgate, R. A. D.; Wade, J. D.; Craik, D. J.; Rosengren, K. J. "Structure of the R3/I5 chimeric relaxin peptide, a selective GPCR135 and GPCR142 agonist" *J. Biol. Chem.* **2008**, *283*, 23811-23818.



- *RXFP3/4 cAMP EC_{50} < 100 nM*
- *Elicits relaxin-3-like signaling*
- *Increases food intake in rats*