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Targeting the Relaxin/Insulin-like Family Peptide Receptor 1 and 2 with Small Molecule Compounds

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Abstract

The peptide hormone relaxin has beneficial roles in several organs through its action on its cognate G protein-coupled receptor, RXFP1. Relaxin administration is limited to intravenous, subcutaneous, intramuscular, or spinal injection. Another drawback of peptide-based therapy is the short half-life, which requires continuous delivery of the drug to achieve efficient concentration in target organs. The discovery of a non-peptide small molecule agonist of RXFP1, ML290, provides an alternative to the natural ligand. This review summarizes the development of ML290 and its potential future therapeutic applications in various diseases, including liver fibrosis and cardiovascular diseases. We also discuss the development of small molecule agonists targeting the insulin-like 3 receptor, RXFP2, and propose the potential use of these small molecules in the context of bone and muscle remodeling.

Keywords

RXFP1; RXFP2; G protein-coupled receptor; ML290; relaxin; small molecule agonist

1. Introduction

Relaxin is a 6 kDa peptide hormone that consists of a two-chain structure linked by disulfide bridges. The peptide is initially synthesized as pre-prorelaxin and cleavage of the signal peptide yields pro-relaxin. Subsequent cleavage of the C-peptide by exopeptidases produces mature hormone (Bathgate, Ivell, Sanborn, Sherwood, & Summers, 2006). Over the past few decades, relaxin has been shown to exert significant roles in maternal adaptations to pregnancy through elongation of the pubic ligament, ripening of the cervix and nipple development at the end of pregnancy in rodents to prepare for parturition (Sherwood, 2004). Research in the relaxin field has since advanced into the non-reproductive system, following the detection of relaxin G protein-coupled receptor RXFP1, Relaxin Family Peptide

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Receptor 1, expression in the human and rodent heart, kidney, liver, pancreas, brain, lung, skin, and other organs (Halls, Bathgate, Sutton, Dschietzig, & Summers, 2015; Hsu et al., 2000; Jelinic et al., 2014; Kamat et al., 2004; Ng, Jelinic, Parry, & Leo, 2015; Sherwood, 2004). Recently, the recombinant form of human relaxin-2 (serelaxin) has been used in clinical trials for acute heart failure. The initial trial showed promising results in improving dyspnea and reducing all-cause mortality at 180 days in serelaxin-treated patients when compared to placebo-treated groups (Teerlink et al., 2013). Although this trial reported reduction in several markers of myocardial function such as natriuretic peptide type B, aspartate aminotransferase, troponin T, alanine aminotransferase and cystatin C levels after serelaxin infusion in heart failure patients (Metra et al., 2013), a subsequent expanded trial revealed that serelaxin treatment in heart failure patients did not meet primary and secondary endpoints of the study. Significantly, administration of serelaxin in patients did not show any adverse events, suggesting that the peptide is safe and well-tolerated in humans (Dahlke et al., 2015).

The other member of relaxin peptide family, insulin-like 3 peptide (INSL3), is mainly expressed in male and female gonads and signals through Relaxin Family Peptide Receptor 2, RXFP2 (Bogatcheva & AgoulNIK, 2005; Ivell & Anand-Ivell, 2018). RXFP2 shows a more restricted pattern of expression and apart from its role in testis descent during prenatal development, the potential role of the INSL3/RXFP2 signaling has just recently been reported in muscle and bone metabolism in adult mice and humans (Ferlin et al., 2018; Ferlin, Selice, Carraro, & Foresta, 2013). It is however worth noting that peptide-based therapy poses several disadvantages such as short half-life *in vivo* and the high synthesis cost. Hence, it will be useful to design small molecules that target the relaxin receptor, RXFP1 or RXFP2 for clinical applications in various diseases.

2. Small molecule agonists of RXFP1

2.1 Identification of RXFP1 small molecule agonists

The complex binding mode of endogenous peptide ligands to both large N-terminal ectodomain and extracellular loops of RXFP1 and RXFP2 (Hossain & Bathgate, 2018) makes it difficult to design small molecules replicating such complex interactions in orthosteric sites. As the high-resolution structure of the seven transmembrane domain (7TM) of these receptors transmitting ligand-induced conformational changes into activation of G proteins remains unresolved, the structure mapping design of synthetic allosteric agonists is only possible using modeling based on homology with better characterized GPCRs. Thus, the non-biased high throughput screening of a large number of compounds is utilized in two reported and several unpublished attempts to identify small molecule agonists for RXFP1 (Chen et al., 2013; McBride et al., 2017; Xiao et al., 2013).

The only known successful screening campaign is described by the National Center for Advancing Translational Sciences (NCATS) and our laboratory resulted in an identification of only two small molecules of similar structure among over 350K compounds screened (Chen et al., 2013), suggesting limited chemical space for RXFP1 drug discovery. The screening is based on the well-characterized ligand-induced activation of downstream cAMP production in transfected HEK293-RXFP1 cells. A cell-based homogenous time resolved

fluorescence (HTRF) cAMP technology that assured robust assay compatible with the high-throughput robotics and direct measuring of cAMP in cell lysates is used as the screening strategy. Importantly, a number of well-designed counterscreen steps confirmed that the identified compounds were indeed specific activators of RXFP1. First, the primary screening of compounds is performed at several different concentrations that allow us to generate concentration-response curves to monitor the potencies of each compound. Second, the counterscreen of parental cell line HEK293T is followed by analysis of HEK293-RXFP2, HEK293 cells transfected with unrelated vasopressin 1b GPCR, the THP1 cells expressing endogenous RXFP1, and the cytotoxicity evaluation of the compounds. Finally, the cAMP response is verified by independent methods which include ELISA-based cAMP assay and CRE-luciferase reporter assay (Chen et al., 2013; Huang et al., 2015; Xiao et al., 2013). Collectively, these strategies led to the identification and discovery of highly specific small molecule agonists of RXFP1, which precluded the potential issue seen with the natural relaxin peptide that is able to activate RXFP1, RXFP2 and RXFP3 in *in vitro* assays.

2.2 Structure-activity relationship studies

Two primary hits from the screening campaign displayed EC₅₀ values of 6.2 μ M and 2.9 μ M, and efficacies of 60% and 80% in HEK293-RXFP1 cells (Chen et al., 2013; Wilson et al., 2018). Extensive structure-activity relationship (SAR) studies were carried out to further improve the potency of the compounds. This effort resulted in at least 10-fold improvement of activity and similar efficacy to the natural hormone (Wilson et al., 2018; Xiao et al., 2013). Further testing of the best compounds using well-established relaxin/RXFP1-mediated biological assays such as the activation of VEGF gene expression in THP1 cells or cell impedance led to the identification of a lead compound, ML290 (Xiao et al., 2013). ML290 is easy to synthesize when compared to relaxin, as the chemical structure of this small molecule allows a simple two-step synthesis protocol for compound production.

Interestingly, none of the tested agonists in this series activates cAMP in HEK293 cells transfected with mouse RXFP1 receptor (Xiao et al., 2013). This surprising finding provided us with an insight into the exact activation site of ML290 as the use of chimeric human-mouse receptors combining different parts of the two receptors led to an identification of extracellular loop 3 (ECL3) in 7TM as the critical region for ML290 interaction with the receptor. Extensive analysis of ML290 response in site-specific RXFP1 mutants combined with computational modeling showed that the agonist binds to an allosteric site of RXFP1. ML290 appears to bind inside the 7TM and interacts with ECL3, where the G659/T660 motif within ECL3 is crucial to the observed selectivity of agonists for human RXFP1. Further analysis showed that ML290 did not activate guinea pig and rabbit RXFP1, but was efficient in activating pig or macaque RXFP1 (Huang et al., 2015). Notably, ML290 did not directly compete with endogenous relaxin binding to the receptor, nor affect its binding kinetics, but rather this small molecule enhances relaxin binding to RXFP1 (Kocan et al., 2017).

2.3 Cellular signaling of ML290

The primary selection of small molecule agonists is based on the cAMP response in cells expressing RXFP1 (Kocan et al., 2017). However, the full spectrum of cellular responses

generated by relaxin/RXFP1 is cell type-dependent and involves multiple signaling pathways. For example, in vascular cells, relaxin stimulates cGMP production, p-ERK1/2 and p38MAPK phosphorylation (p-p38MAPK), as well as increases neuronal NOS, ET_B, and VEGFA production, in addition to activating cAMP (Halls et al., 2015; Sarwar, Samuel, Bathgate, Stewart, & Summers, 2015). In HEK293-RXFP1 cells, ML290 stimulates cAMP accumulation and p38MAPK phosphorylation, but not cGMP accumulation or ERK1/2 phosphorylation (Kocan et al., 2017). In primary human vascular endothelial and smooth muscle cells, ML290 increases both cAMP and cGMP accumulation, but not p-ERK1/2 (Kocan et al., 2017). It is however worth noting that ML290 has higher potency for cGMP accumulation and p-p38MAPK than for cAMP accumulation in vascular cells (Kocan et al., 2017). Biased signaling of ML290 might be related to differences in strong coupling of RXFP1 to G_{αs} and G_{αoB}, but weak coupling to G_{αi3}, whereas all three of these G proteins are coupled to RXFP1 after relaxin stimulation (Halls, Bathgate, & Summers, 2006; Halls et al., 2009; Halls, van der Westhuizen, Bathgate, & Summers, 2007; Nguyen & Dessauer, 2005; Nguyen, Yang, Sanborn, & Dessauer, 2003).

Very little work to date has investigated the effects of ML290 under diseased conditions *in vitro*. In human cardiac fibroblasts, ML290 increases cGMP accumulation but did not affect p-ERK1/2, and it potentially inhibits TGFβ-1-induced Smad2 and Smad3 phosphorylation, as well as it increases MMP2 expression (Kocan et al., 2017). In activated primary human hepatic stellate cells or LX-2 cell line, ML290 treatment significantly downregulated fibrotic genes and upregulated MMP1 and MMP3 expression (McBride et al., 2017; Wilson et al., 2018).

2.4 Pharmacological properties of ML290

Relaxin peptide has relatively low plasma stability and short half-life *in vivo*, thus requiring continuous infusion of the drug to achieve optimal effects in target organs. Compounds screened from the first series demonstrated favorable absorption, distribution, metabolism and excretion (ADME) properties, which are one of the key criteria for further preclinical evaluation. Hence, the discovery of ML290 would serve as an alternative to other peptide ligands for RXFP1. This small molecule (MW=506.5 Da) has low cytotoxicity and high stability *in vivo* and *in vitro*, with reported half-life of approximately eight hours in the plasma and heart of mice injected with 30 mg/kg of ML290 intraperitoneally (Agoulnik, Agoulnik, Hu, & Marugan, 2017; Wilson et al., 2018). Importantly, ML290 administration did not induce any signs of toxicity, abnormal behavior, or serum marker changes in animals even after multiple injections for several weeks (Wilson et al., 2018).

2.5 Humanized mouse model to study the therapeutic effects of ML290

The vast majority of studies evaluating therapeutic effects of relaxin are done in rodent models of human diseases. ML290 does not activate mouse RXFP1, and it behaves as an antagonist when cAMP activation is tested in combination with relaxin (Hu et al., 2016). It remains unclear whether ML290 can activate additional signaling pathways apart from cAMP after stimulation of the mouse receptor. In any case, to reproduce the full spectrum of ML290 actions on human RXFP1 *in vivo*, we recently produced a transgenic strain of mice with human *RXFP1* (*hRXFP1*) instead of mouse *Rxfp1* (*mRxfp1*) (Kaftanovskaya et al.,

2017). A knock-in/knock-out of *hRXFP1* cDNA is introduced into the *mRxfp1* gene. Insertion of the targeting vector into the *mRxfp1* locus caused disruption of *mRxfp1* and expression of *hRXFP1*. The transcriptional expression pattern of the *hRXFP1* allele driven by endogenous mouse promoter was similar to *mRxfp1*. Female mice homozygous for *hRXFP1* showed relaxation of the pubic symphysis at parturition and normal development of mammary nipples and vaginal epithelium, indicating full complementation of *mRxfp1* gene ablation. We have demonstrated that similar to relaxin, intravenous injection of ML290 led to an increase in heart rate in humanized but not in wild-type females, suggesting specific target engagement by ML290 *in vivo* (Kaftanovskaya et al., 2017). Thus, this unique humanized RXFP1 mouse serves as an invaluable tool for testing of ML290 in various preclinical studies, together with readily available *Rxfp1*-deficient and conventional wild-type mice of the same background to test the specificity of the agonist on human RXFP1 *in vivo*.

2.6 Preclinical development of ML290

As mentioned in section 2.3, ML290 treatment effectively suppresses fibrotic phenotypes in human cardiac fibroblasts and hepatic stellate cells. Based on these findings, current preclinical work assessing the therapeutic effects of ML290 in a mouse model of liver fibrosis now warrants such translation of the beneficial role of this small molecule in this setting. Moreover, ML290 not only activates production of cAMP, but also strongly induces cGMP accumulation in vascular cells, an important indicator of nitric oxide production in the vasculature. The higher potency for cGMP accumulation indicates that ML290 may be a direct vasodilator, a well-reported characteristic of relaxin.

3. Small molecule agonists of RXFP2

3.1 INSL3/RXFP2 signaling

INSL3 is the only known physiological ligand for RXFP2. RXFP2 is structurally similar to RXFP1, sharing homology in both the extracellular leucine-rich repeat domain and seven transmembrane domain. It is the only other GPCR that has the low-density lipoprotein A module (Bruell et al., 2013; Overbeek et al., 2001). RXFP2, as well as RXFP1, modulates adenylyl cyclase activity and increases or decreases cAMP levels by activating either the $G_{\alpha s}$ - or $G_{\alpha o}$ - coupled pathways. The $G_{\alpha i}$ pathway is unique to RXFP1 (Halls et al., 2006).

3.2 Screening strategy

Following the success of the screening campaign for RXFP1 agonists indicates that a similar approach may be used to identify RXFP2-specific small molecule agonists. Treatment of RXFP2 transfected HEK293 cells with high concentrations of relaxin causes activation of the canonical cAMP pathway (Hsu et al., 2002), although interaction sites of relaxin and INSL3 with RXFP2 are different (Scott, Rosengren, & Bathgate, 2012). The structural similarity between RXFP1 and RXFP2 suggests that such small molecule agonists could utilize the same binding sites as ML290 in RXFP1, and can be derived from the latter chemotype. However, testing of molecules from ML290 SAR optimization studies did not reveal any compounds with RXFP2 agonistic effects. Thus, the non-biased high-throughput screening can be used again to identify small molecule agonists specific for RXFP2. The

counterscreen with HEK293-RXFP1 cells will be a powerful method to eliminate not only cross-activating compounds, but also non-specific RXFP compounds affecting all downstream signaling of cAMP induction pathway.

3.3 Potential therapeutic targets of RXFP2 signaling

Therapeutic applications targeting the RXFP2 signaling are not as widely studied as compared to RXFP1. The first identified biological role of INSL3 and RXFP2 was their involvement in testicular descent, a developmental process when differentiating testes descend to the scrotum. Male mice deficient for either INSL3 or RXFP2 develop cryptorchidism (Adham, Emmen, & Engel, 2000; Gorlov et al., 2002; Nef & Parada, 1999; Overbeek et al., 2001). If left untreated, cryptorchidism causes a failure of spermatogenesis and germ cell apoptosis resulting in infertility as well as an increased risk of testicular cancer in adulthood (Barthold & Gonzalez, 2003). Analysis using transgenic mouse models renders two main conclusions: 1) INSL3 controls the outgrowth and differentiation of gubernacular ligaments, and the formation of *processus vaginalis* (scrotal sac) during prenatal development, and 2) INSL3 peptide is a paracrine anti-apoptotic factor for male and female germ cells in adult gonads (Adham & Agoulunik, 2004; Bogatcheva & Agoulunik, 2005; Bogatcheva et al., 2003; Feng et al., 2007; Gorlov et al., 2002; Huang, Rivas, & Agoulunik, 2012; Kaftanovskaya et al., 2011; Overbeek et al., 2001; Tomiyama, Hutson, Truong, & Agoulunik, 2003). Treatment with INSL3 initiates meiotic progression of arrested oocytes in preovulatory follicles *in vitro* and *in vivo*, and suppresses gonadotrophin-releasing hormone (GnRH) antagonist-induced male germ cell apoptosis *in vivo*, indicating importance of the INSL3 paracrine system in mediating gonadotropin actions in adult gonads (Kawamura et al., 2004). As INSL3/RXFP2 signaling plays a major role during prenatal development, therapeutic application of RXFP2 agonists is highly unlikely. It is however possible that such compounds might be used in newborn males with cryptorchidism to stimulate testicular descent. Further preclinical studies on better animal models will be required.

In addition to the role of RXFP2 in testicular descent, the INSL3/RXFP2 signaling axis has also been demonstrated in the context of bone and muscle metabolism. Men with mutations of the RXFP2 receptor and *Rxfp2*-deficient mice have reduced bone mass and develop osteoporosis (Ferlin et al., 2008). Expression of functional RXFP2 is shown in human and mouse osteoblasts and osteocytes (Ferlin et al., 2008). INSL3 treatment in human osteoblasts stimulates cell differentiation and proliferation, with simultaneous increase in the expression of osteoblast markers (Ferlin, Perilli, Giansello, Tagliavoro, & Foresta, 2011). Moreover, INSL3 treatment also led to the mineralization of extracellular matrix in osteoblasts, suggesting the pivotal role of INSL3/RXFP2 signaling to maintain physiological bone remodeling (Ferlin et al., 2011).

Studies on C2C12 mouse myoblast cell line showed that INSL3 exerts a trophic effect on differentiated myotubes, stimulating cell size increase and protein synthesis (Ferlin et al., 2018). In fact, *Rxfp2*-deficient mice have exacerbated muscle loss after denervation, indicating a vital role of the receptor in mediating protein turnover in beta oxidative stress fibers (Ferlin et al., 2018). These findings suggest that targeting the INSL3/RXFP2 signaling

axis displays an overall anabolic role on skeletal muscle, and may have a potential therapeutic effect for the treatment of hypogonadism (Ferlin et al., 2018).

Although RXFP2 is expressed in several other organs, the specific signaling mechanisms mediated by this receptor have yet to be fully characterized. RXFP2 is expressed in human and rat brain, and it is particularly enriched in the thalamus, suggesting the potential involvement of the INSL3/RXFP2 system in neurodegenerative and movement disorders (Sedaghat, Shen, Finkelstein, Henderson, & Gundlach, 2008). In addition, INSL3 and RXFP2 are also expressed in the human ocular surface and tears. Topical application of INSL3 improves corneal wound healing and ocular surface homeostasis in a corneal defect mouse model (Hampel et al., 2013). Furthermore, INSL3/RXFP2 has been described in maintaining the uterine structural integrity (Li et al., 2011), and potentially in other female reproductive abnormalities (Ivell & Anand-Ivell, 2018). Availability of specific small molecule agonists of RXFP2 undoubtedly will help to identify new roles of RXFP2 cellular signaling, as well as characterization of the potential pharmacological applications of such compounds.

4. Future directions

We now present a concise development of a small molecule agonist of RXFP1, ML290, from library screening to the advancement of generating a unique strain of mouse line for preclinical testing of the compound. With the availability of the humanized mice, future studies on the potential therapeutic applications of ML290 in chronic diseases such diabetes, hypertension and heart disease can be fully elucidated, in addition to utilizing cell-based assays. Extensive efforts are currently underway to improve the activity and solubility of ML290 for *in vivo* administration, with the goal of achieving higher oral bioavailability that may be more applicable for future translational studies. Similar strategies are employed for screening of small molecule agonists of RXFP2, as there is no known non-peptide agonist of this receptor at present. The outcomes from this extensive screening now warrants such compounds for their potential therapeutic role in various diseases, in particular those related to bone and muscle remodeling.

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Abbreviations

7TM	seven transmembrane
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CRE	cAMP response element

ECL	extracellular loop
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinase 1/2
ET_B	endothelin receptor type B
GPCR	G protein-coupled receptor
HEK	293 human embryonic kidney cells
INSL	3 insulin-like 3
MMP	metalloproteinase
NOS	nitric oxide synthase
p38 MAPK	p38 mitogen-activated protein kinases
RXFP	relaxin/insulin-like family peptide receptor
SAR	structure-activity relationship
THP1	human leukemia monocytic cell line
VEGF	vascular endothelial growth factor

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Highlights:

- Availability of a unique human *RXFP1* mouse strain for preclinical studies.
- Distinct cellular signaling pathways mediated by a biased allosteric agonist of RXFP1, ML290.
- Screening efforts for small molecule compounds targeting RXFP2.