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ORIGINAL ARTICLE

The chemically synthesized human relaxin-2 analog, B-R13/17K H2, is an RXFP1 antagonist

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Abstract Relaxin is a pleiotropic hormone which exerts its biological functions through its G-protein coupled receptor, RXFP1. While relaxin is well known for its reproductive and antifibrotic roles, recent studies suggest that it is produced by cancer cells and acts on RXFP1 to induce growth and metastasis. Furthermore, more recently Silvertown et al. demonstrated that lentiviral production of a human gene-2 (H2) relaxin analog reduced the growth of prostate xenograft tumors. The authors proposed that the lentivirally produced peptide was an RXFP1 antagonist; however, the processed form of the peptide produced was not demonstrated. In this study, we have chemically synthesized the H2 relaxin analog, B-R13/17K H2 relaxin, and subjected it to detailed chemical characterization by HPLC, MALDI-TOF mass spectrometry, and amino acid analysis. The biological activity of the synthetic peptide was then tested in three different cell lines. It was found to bind with 500-fold lower affinity than H2 relaxin to RXFP1 receptors over-expressed in HEK-293T cells where it acted as a partial agonist. However, in cells which natively express the RXFP1 receptor, rat renal myofibroblasts and MCF-7 cancer cells, it acted as a full antagonist. Importantly, it was able to significantly inhibit cell invasion induced by H2 relaxin in MCF-7 cells consistent with the results of the lentiviral-driven expression in prostate cancer cells. The relaxin analog, B-R13/17K H2, can now be used as a tool to further understand RXFP1 function, and serve as a template for drug design for a therapeutic to treat prostate and other cancers.

Keywords Relaxin · H2 relaxin · RXFP1 antagonist · B-R13/17K H2 relaxin · GPCR

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Introduction

The relaxin family of peptides in human consists of seven members: relaxin-1 (H1 relaxin), relaxin-2 (H2 relaxin), relaxin-3 (H3 relaxin), insulin-like peptide 3 (INSL3), INSL4, INSL5, and INSL6. Mammalian non-primate species have only two relaxin peptides, relaxin-1 and relaxin-3, together with INSL3, INSL5, and INSL6 (Wilkinson et al. 2005). H2 relaxin is the equivalent of the relaxin-1 peptide in non-primate species which is referred to as "relaxin." Relaxin is produced in the corpus luteum and/or placenta during pregnancy in mammals and is secreted into the blood where it has numerous essential endocrine

functions (Bathgate et al. 2006c). It has an important role as a cardiovascular hormone during pregnancy with potent vasodilatory and renal actions (Conrad and Novak 2004; Samuel et al. 2006). These actions have led to the successful use of H2 relaxin in Phase II clinical trials for the treatment of acute heart failure (Teerlink et al. 2009). Additionally, relaxin is a potent regulator of collagen turnover both during pregnancy and as a locally produced paracrine factor and it has great clinical potential as an anti-fibrotic agent (Samuel et al. 2007). The receptor for relaxin is the leucine-rich repeat-containing GPCR LGR7 (Hsu et al. 2002) which is now referred to as relaxin family peptide receptor 1 (RXFP1) (Bathgate et al. 2006a). There are numerous potential applications for relaxin mimetics that activate RXFP1; however, until recently there has been limited utility for an RXFP1 antagonist and subsequently none has been developed.

There is now increasing evidence that relaxin is produced by cancer cells and can act in an autocrine manner on RXFP1 receptors expressed on these cells. Hence, relaxin is expressed by endometrial (Kamat et al. 2006), mammary (Tashima et al. 1994), thyroid (Hombach-Klonisch et al. 2006), and prostate (Feng et al. 2007; Thompson et al. 2006) tumors. Elevated serum relaxin levels have been reported in breast cancer patients and levels were higher in patients with metastases (Binder et al. 2004). Relaxin has been shown to promote the growth (Bani et al. 1999; Hombach-Klonisch et al. 2006; Sacchi et al. 1994) and invasiveness (Binder et al. 2002; Hombach-Klonisch et al. 2006; Kamat et al. 2006) of numerous types of cancer cells.

More recently, a potential role for relaxin in prostate cancer has been highlighted. H2 relaxin mRNA expression was shown to be significantly higher in recurrent prostate cancer biopsies (Feng et al. 2007, 2009). Relaxin levels increase in androgen-independent (AI) prostate tumors and are particularly high in bone metastases (Thompson et al. 2006). H2 relaxin causes an increase in cell proliferation, invasiveness, and adhesion of prostate cancer cells in vitro (Feng et al. 2007; Vinall et al. 2006). Suppression of relaxin or RXFP1 expression via short interfering RNAs decreased prostate cancer cell invasiveness by 90–95% and growth by 10–25% (Feng et al. 2007). Lentiviral expression of H2 relaxin in PC-3 prostate cancer cells in vivo increased xenograft tumor growth (Silvertown et al. 2006) and transgenic mice over-expressing relaxin showed a decreased survival time in an in vivo model of prostate adenocarcinoma (Feng et al. 2007). siRNA targeting of RXFP1 expression in this in vivo prostate cancer xenograft model resulted in a significant decrease in tumor size and fewer metastases (Feng et al. 2009). These results suggest that an RXFP1 antagonist could be a potential therapeutic to treat prostate cancer.

However, there has been no report of an RXFP1 antagonist until Silvertown et al. (2007) demonstrated that lentiviral expression of a H2 relaxin analog caused decreased prostate cancer xenograft growth. The authors engineered a lentivirus to express a H2 pro-relaxin peptide with the arginine residues at positions 13 and 17 on the B-chain mutated to lysine. These arginine residues had previously been demonstrated to be essential for relaxin activity (Büllesbach et al. 1992). Media from cells infected with the lentivirus, and hence producing B-R13/17K H2 pro-relaxin, demonstrated H2 relaxin immunoreactivity and were able to weakly antagonize H2 relaxin-induced cAMP production in THP-1 cells natively expressing RXFP1. Importantly, prostate xenograft tumors infected with lentiviruses expressing this peptide showed markedly reduced tumor cell growth. The authors suggested that the lentiviruses were producing B-R13/17K H2 pro-relaxin and that this peptide was acting as an RXFP1 antagonist. However, the processed form of the peptide produced by these lentiviruses was not demonstrated either in vitro or in vivo.

Consequently, we have chemically synthesized B-R13/17K H2 relaxin as a heterodimeric peptide like native H2 relaxin and subjected it to comprehensive chemical characterization. The peptide was then tested on cells stably over-expressing RXFP1 as well as two cell lines which endogenously express RXFP1, renal myofibroblasts, and MCF-7 breast cancer cells. Synthetic B-R13/17K H2 relaxin was demonstrated to be a RXFP1 antagonist in endogenously expressing cells and was able to inhibit breast cancer cell invasion induced by H2 relaxin.

Materials and methods

Solid phase peptide synthesis

Each of the A- and B-chains of B-R13/17K H2 relaxin was prepared as their C-terminal amide forms using continuous flow solid phase methodology on an automated PerSeptive Biosystems peptide synthesizer. After TFA cleavage and purification of the individual chains, use of a previously reported regioselective disulfide bond formation strategy (Hossain et al. 2008a, b, 2009) led to the production of the target peptide, B-R13/17K H2 relaxin.

Peptide characterization

The purity of the synthetic peptide was assessed by RP-HPLC on analytical Phenomenex C18 column (pore size 300 Å; particle size 5 μm, 4.6 × 250 mm) using a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The product was confirmed by MALDI-TOF mass spectrometry

using a Bruker Autoflex II instrument (Bremen, Germany) in the linear mode at 19.5 kV. The peptide was quantitated by amino acid analysis of a 24 h acid hydrolysate using a Shimadzu microbore RP-HPLC system.

Binding assays

Whole cell binding assays using human embryonic kidney (HEK)-293T cells stably transfected with RXFP1 were performed as described previously (Bathgate et al. 2006b). Competition binding experiments were performed with 100 pM ^{125}I-labeled H2 relaxin in the absence or presence of increasing concentrations of unlabeled hormones. Non-specific binding was determined by the addition of 500 nM H2 relaxin. All data are presented as the mean ± SE of the percentage of specific binding of triplicate wells, repeated in at least three separate experiments, and curves were fitted using one site binding curves in Graphpad Prism 4 (Graphpad Software). Statistical differences in pIC_{50} values were analyzed using Student's *t* test in Graphpad Prism 4.

cAMP activity assays

The influence of the peptides on cAMP signaling was tested in cells stably expressing RXFP1 and a pCRE-β-galactosidase reporter plasmid as described previously (Scott et al. 2006). Cells were stimulated with increasing concentrations of peptides in triplicate within each assay and each experiment was repeated at least three times. Additionally, the ability of B-R13/17K H2 relaxin to antagonize H2 relaxin signaling was tested in cells stably expressing RXFP1 using two different paradigms. First, increasing concentrations of B-R13/17K H2 relaxin were tested in the presence of a dose of H2 relaxin (1 nM) which gives approximately 70–80% of the maximum response. Second, increasing concentrations of H2 relaxin were tested in the presence of a high concentration (1 μM) of B-R13/17K H2 relaxin. Ligand-induced stimulation of cAMP was expressed as a percentage of the maximum H2 relaxin response. Concentration response curves were plotted, and statistical differences in pEC_{50} values were analyzed, using Graphpad Prism 4.

MCF-7 breast cancer cell invasion assay

The activity of B-R13/17K H2 relaxin was tested in MCF7 breast cancer cells which have been previously shown to respond to relaxin (Binder et al. 2002). Hence the effect of 1 μM B-R13/17K H2 relaxin alone or in combination with 16 nM H2 relaxin was tested in an in vitro MCF7 cell migration assay which has been described previously (Binder et al. 2002). Briefly, cells were seeded in the upper well of a Boyden chamber, while the lower well was filled with RPMI + 10% FCS as a chemoattractant. H2 relaxin alone (100 ng/ml; 16.8 nM), the B-R13/17K H2 relaxin alone (1 μM), or 1 μM B-R13/17K H2+ 16.8 nM H2 relaxin was added to cells 2 h after they had been plated, and renewed every 24 h up to 96 h of culture in total. Untreated cells were used as an appropriate control. After 96 h, the floating and adherent cells in the lower chamber were removed and pelleted by centrifugation, then treated and analyzed as described previously (Binder et al. 2002). The data are presented as the number of invaded cells per large corner square of a Neubauer haemocytometer and are representative of the average of the three experiments performed in quadruplicate.

Renal myofibroblast differentiation assay

Renal myofibroblasts isolated from the obstructed kidneys of male Sprague–Dawley rats following unilateral ureteric obstruction express RXFP1 and respond to H2 relaxin by dedifferentiation into fibroblasts, as measured by decreases in α-smooth muscle actin (α-SMA) production (Masterson et al. 2004; Mookerjee et al. 2009). The effects of a high concentration of B-R13/17K H2 relaxin (1 μM) alone or in combination with doses of H2 relaxin which have previously been shown to maximally stimulate renal myofibroblasts (1.68, 16.8 nM) were tested in these cells. Cultures were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) (DMEM-FBS); and used between passages 13–18 for experimentation. All experiments were performed three separate times in triplicate. Cells were seeded into six-well plates at an equal density of 1–2 × 10^5 cells per well, on cover slips and immediately treated with H2 relaxin alone (1.68 or 16.8 nM), B-R13/17K H2 alone (1 μM) or with 1.68 nM H2 relaxin+ 1 μM B-R13/17K H2 or with 16.8 nM H2 relaxin+ 1 μM B-R13/17K H2 for 72 h in a final volume of 1 ml DMEM-FBS. Untreated cells cultured for 72 h in 1 ml DMEM-FBS acted as appropriate controls. Following the 72 h culture period, all cover slips were removed, fixed, and immunostained for α-SMA, as described previously (Mookerjee et al. 2009). Six random fields per cover-slip were photographed and analyzed by quantitating the proportion of cells that were α-SMA positive, as described previously (Mookerjee et al. 2009). The proportion of α-SMA positive cells counted in treated groups was then expressed as a relative ratio of positive cells counted in untreated control groups, which was expressed as 1.

Results

Synthesis of the H2 relaxin analog

The H2 relaxin analog, B-R13/17K H2 relaxin, was chemically synthesized for functional assessment (Fig. 1a). A highly efficient solid phase peptide synthesis strategy

using regioselectively S-protected A- and B-chains followed by sequential disulfide bond formation was utilized (Hossain et al. 2008a, b, 2009). The purified final product, B-R13/17K H2 relaxin, obtained from this chemical synthesis was 1.2 mg with a good overall yield (~10% starting from the B-chain) given the complexity of this peptide. The peptide was comprehensively chemically characterized by analytical RP-HPLC and MALDI-TOF mass spectrometry. The single peak in the HPLC profile and MALDI-TOF mass spectrometry confirmed the high purity of the compound (Fig. 1b, c). The observed mass, $[M + H]^+_{obs} = 5908.34$ matched with the theoretical mass, $[M + H]^+_{calcld} = 5907.11$ (Fig. 1c). The content of the peptide, which was found to be 69.8%, and the amino acid composition were determined by amino acid analysis.

Activity of B-R13/17K H2 relaxin in cells over-expressing RXFP1

The B-R13/17K H2 relaxin analog was tested in HEK-293T cells stably over-expressing the RXFP1 receptor (Fig. 2). The data from the competition binding assay showed that the modified H2 relaxin bound to RXFP1 with a significantly lower affinity ($P < 0.001$) than H2 relaxin (B-R13/17K H2: $pIC_{50} = 6.29 \pm 0.28$, $n = 4$; H2 relaxin: $pIC_{50} = 8.92 \pm 0.05$, $n = 4$; Fig. 2a). Additionally, the modified peptide was able to stimulate cAMP activity in RXFP1-expressing cells with activity similar to its binding affinity (B-R13/17K H2: $pEC_{50} = 6.67 \pm 0.31$, $n = 4$; H2 relaxin: $pEC_{50} = 10.34 \pm 0.04$, $n = 5$; Fig. 2a, b). However, the maximum activity of the peptide was only approximately 70% of that of H2 relaxin suggesting that it was acting as a partial agonist. The peptide B-R13/17K H2 was then tested for its ability to antagonize H2 relaxin-induced cAMP signaling (Fig. 3). The peptide did not decrease cAMP activity induced by 1 nM of H2 relaxin but was able to augment cAMP signaling at the highest dose of 10 μM (Fig. 3a). Additionally, when the peptide was added at 1 μM together with increasing amounts of H2 relaxin, it demonstrated no antagonist activity. It did, however, demonstrate the expected partial agonist activity at low concentrations of H2 relaxin without affecting the top half of the H2 relaxin-induced cAMP dose–response curve (Fig. 3b).

B-R13/17K H2 relaxin antagonizes H2 relaxin-induced invasiveness of MCF-7 breast cancer cells in vitro

We tested the effects of H2 relaxin in the absence or presence of B-13/17K H2 relaxin on MCF-7 cell invasiveness. H2 relaxin (16.8 nM) was able to significantly stimulate cell invasion (by two- to threefold) when added to MCF-7 breast cancer cells over 94 h in culture ($P < 0.001$ vs. untreated control group; Fig. 4) in a similar

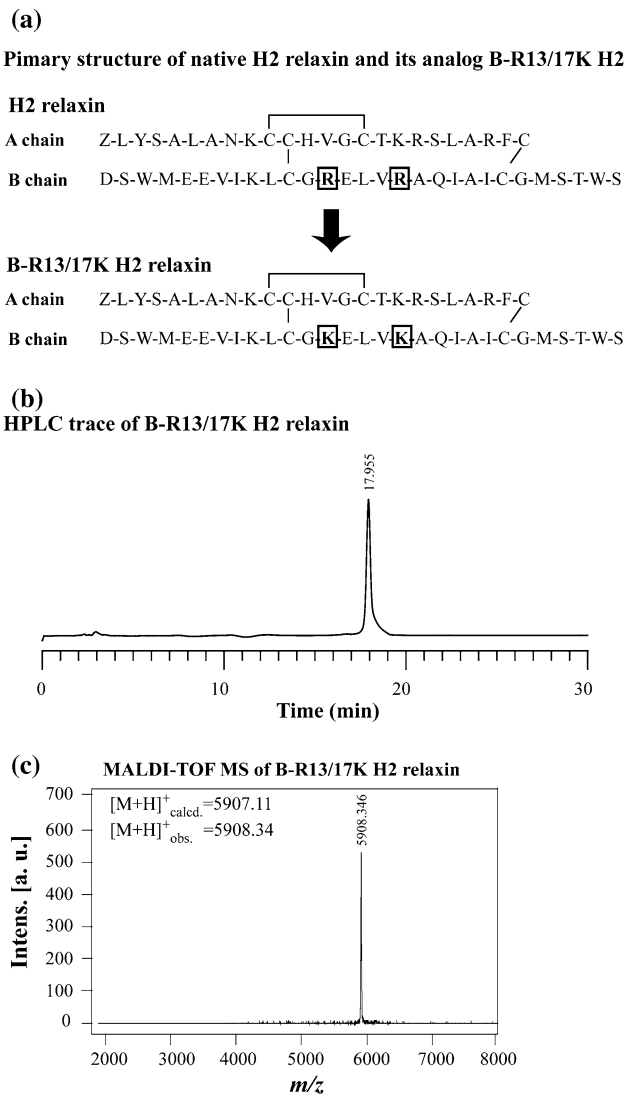

Fig. 1 **a** The primary structure of native H2 relaxin and its analog B-R13/17K H2 relaxin. The modified residues are marked in *bold* and *boxed*; Z-pyroglutamic acid. **b** RP-HPLC profile of B-R13/17K H2 relaxin; eluant A, 0.1% aqueous TFA; eluant B, 0.1% TFA in acetonitrile; gradient = 20–50% B over 30 min; Phenomenex C18 column, pore size 300 Å, particle size 5 μm, 4.6 × 250 mm. **c** MALDI-TOF mass spectrum of B-R13/17K H2 relaxin with the correct monoisotopic mass; ($[M + H]^+_{calcd} = 5907.11$; $[M + H]^+_{obs} = 5908.34$)

manner to porcine relaxin as reported previously (Binder et al. 2002). B-R13/17K H2 (1 μM) alone did not have any effect on cell invasion, but was able to significantly antagonize the H2 relaxin-induced cell invasion over 94 h in vitro ($P < 0.001$ vs. H2 relaxin alone; Fig. 4).

B-R13/17K H2 antagonizes the inhibitory effects of H2 relaxin on renal myofibroblast differentiation in vitro

H2 relaxin administration to renal myofibroblasts significantly decreased α-SMA staining over 72 h in culture, at

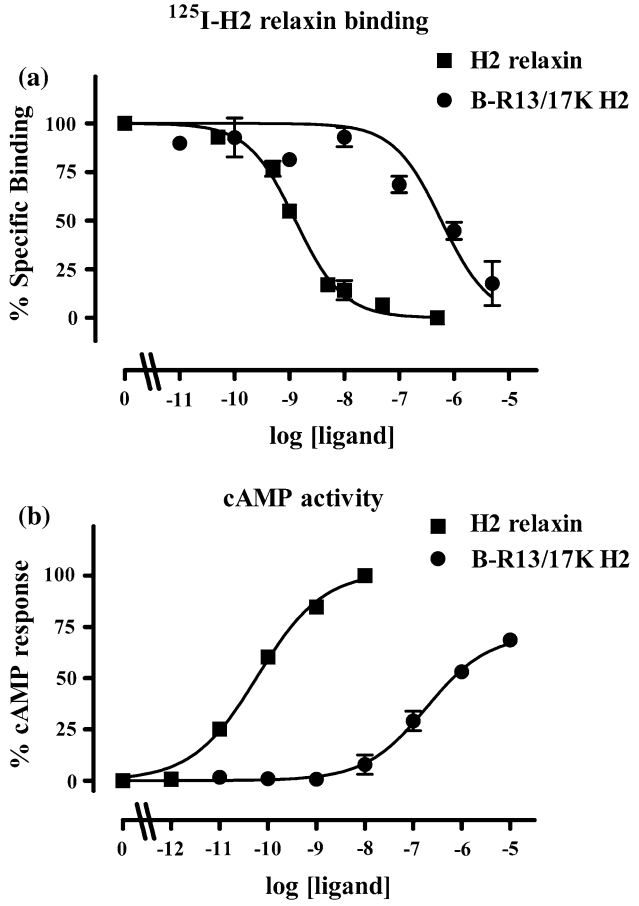

Fig. 2 Activity of B-R13/17K H2 relaxin in comparison to H2 relaxin in HEK-293T cells over-expressing RXFP1 **a** whole cell competition binding using ^{125}I-relaxin. **b** cAMP responses expressed as the percent maximum H2 relaxin response and represent the mean ± SEM of at least three experiments performed in triplicate

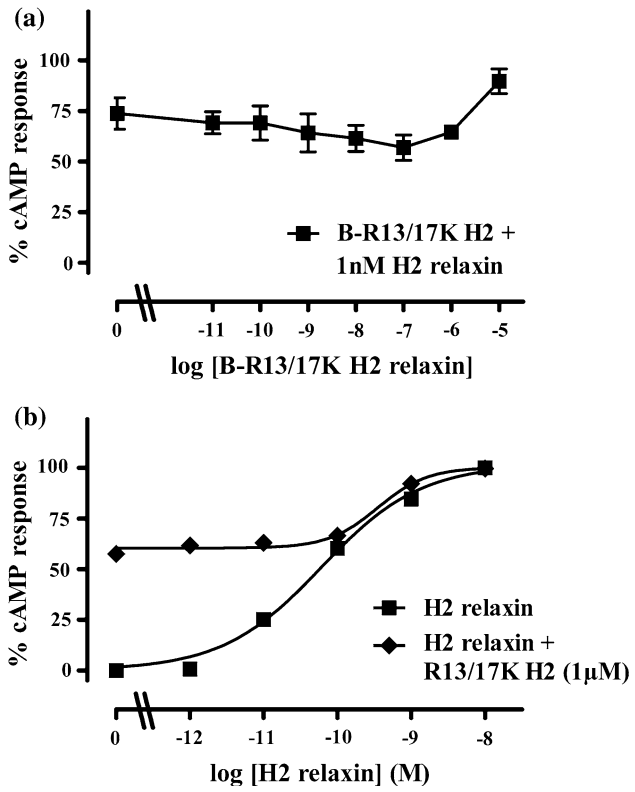

Fig. 3 **a** Ability of increasing concentrations of B-R13/17K H2 relaxin to influence cAMP activity in response to 1 nM H2 relaxin. **b** Ability of increasing concentrations of H2 relaxin to influence cAMP activity in response to 1 μM B-R13/17K H2 relaxin compared to H2 relaxin stimulation alone. cAMP responses are expressed as the percent maximum H2 relaxin response and represent the mean ± SEM of at least three experiments performed in triplicate

concentrations of 1.68 and 16.8 nM (by ~10 and ~20%, respectively, of that measured in untreated control cultures; both $P < 0.05$ vs. untreated cultures; Fig. 5). B-R13/17K H2 (1 μM) alone did not have any effect on basal α-SMA staining. However, when it was added together with H2 relaxin, it significantly blocked the H2 relaxin-induced inhibition of α-SMA staining over 72 h in vitro ($P < 0.05$) at both 1.68 and 16.8 nM such that the levels of α-SMA were equivalent to that measured in the untreated control group (Fig. 5).

Discussion

Until the recent discovery of the potential role of relaxin in prostate cancer growth and invasiveness, there was limited utility for a RXFP1 antagonist. Therefore, there are very few attempts found in the literature concerning the generation of an RXFP1 antagonist. Studies by Büllesbach et al. (1992) first reported the importance of Arg13 and Arg17

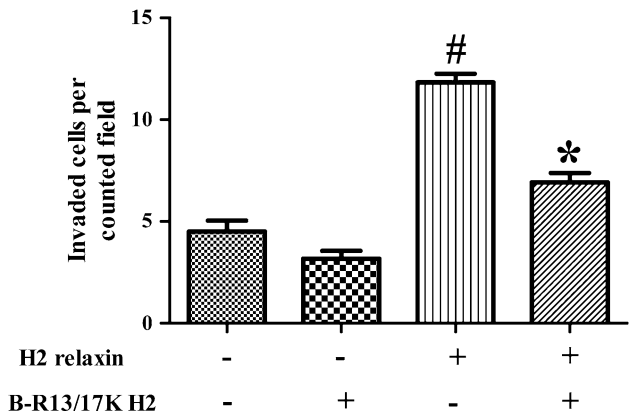

Fig. 4 Effects of B-R13/17K H2 relaxin (1 μM) on MCF-7 cell invasion in the presence or absence of H2 relaxin (100 ng/ml; 16.8 nM). The data are presented as the number of invaded cells per large corner square of a Neubauer hemocytometer and are representative of the average of three experiments performed in quadruplicate. #$P < 0.001$ versus control; *$P < 0.001$ versus H2 relaxin

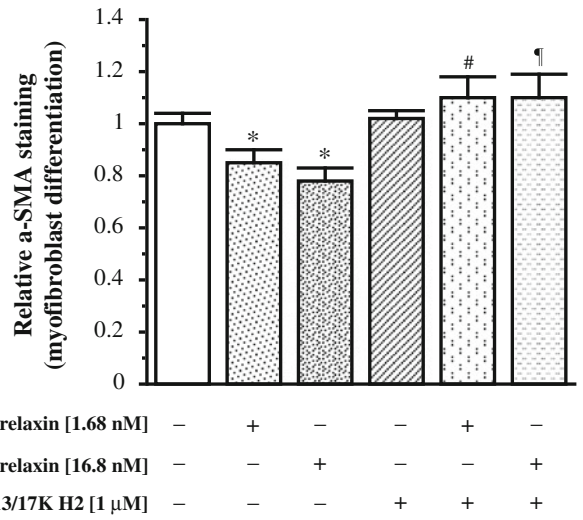

Fig. 5 The relative mean ± SE α-SMA staining from untreated rat renal myofibroblasts versus cells treated with H2 relaxin (1.68 or 16.8 nM) alone, B-R13/17K H2 relaxin (1 μM) alone or with H2 relaxin (1.68 or 16.8 nM) and B-R13/17K H2 relaxin (1 μM), after 72 h in culture. All data were generated from three separate experiments, conducted in triplicate and expressed as the relative ratio of the α-SMA-stained positive cells counted in the untreated (control) group, which was expressed as 1. *$P < 0.05$ versus control group; #$P < 0.05$ versus 1.68 nM H2 relaxin alone treated group; ¶$P < 0.05$ versus 16.8 nM H2 relaxin alone treated group

residues in the B-chain for H2 relaxin activity. In the mouse pubic symphysis bioassay, synthetic H2 relaxin was shown to be as potent as native porcine relaxin, whereas the Arg13 and/or Arg17 mutated H2 relaxin derivatives had no significant activity at doses up to 40-fold higher than the effective H2 relaxin standard. In fact, some of the chemically modified H2 analogs, B-R13/17K H2 relaxin, B-R17A H2 relaxin, and B-R13/17Citr H2 relaxin, were completely inactive. However, the analog B-R13/17K H2 exhibited very weak affinity (~2,200-fold lower affinity than that of native H2 relaxin) in a receptor binding assay using rat cortical membranes which express the native RXFP1 receptor (Büllesbach et al. 1992). Since B-R13/17K H2 relaxin was completely inactive in the pubic symphysis bioassay while retaining partial affinity for the receptor, Silvertown et al. (2007) first hypothesized that B-R13/17K H2 relaxin might act as an antagonist.

Subsequently, the authors demonstrated that a lentivirus engineered to produce pro-B-R13/17K H2 relaxin exhibited antagonistic properties both in vitro and in vivo by interfering with H2-relaxin-induced signaling and impairing prostate tumor growth. Although the authors showed clear antagonistic activity from the viral transduction, they did not determine the processed form of the peptide produced by the viral infection. The lentiviral construct encoded the prohormone form of B-R13/17K H2 relaxin including the C-chain sequence and would likely have been produced as a pro-hormone with the C-chain still present. It has been previously demonstrated that relaxin peptides with the C-chain intact retain full activity (Bathgate et al. 2006c, 2008). It is also possible that this virally produced pro-hormone undergoes proteolytic trimming or post-translational modification in vitro or in vivo, hence the precise form of the peptide that produced the antagonistic effect is unknown. Therefore in this study we have synthesized the B-R13/17K H2 relaxin based on the native heterodimeric structure of H2 relaxin and tested its activity.

The peptide was first tested in HEK-293T cells which are stably transfected to over-express RXFP1. In these cells, B-R13/17K H2 relaxin bound with an affinity 500 times lower than H2 relaxin. This is consistent with the affinity difference of 2,200-fold reported previously (Büllesbach et al. 1992). However, in contrast to the studies using the pubic symphysis bioassay where the peptide was inactive (Büllesbach et al. 1992), B-R13/17K H2 relaxin was able to stimulate cAMP activity in HEK-293T cells over-expressing RXFP1. The activity of the peptide was 5,000-fold lower than H2 relaxin and importantly was only able to produce a maximal cAMP level which was 70% of the H2 relaxin-stimulated level suggesting that it was acting as a weak partial agonist. Further studies where the peptide was tested in the presence of H2 relaxin highlighted that the peptide demonstrated no antagonist activity but displayed classic partial agonist activity.

We then evaluated the activity of the B-R13/17K H2 relaxin peptide in two cell systems which express native RXFP1 receptors at levels much lower than in the HEK-293T cells. The human breast cancer cell line MCF-7 has been previously been shown to respond to porcine relaxin in vitro (Binder et al. 2002; Sacchi et al. 1994). Specifically the ability of B-R13/17K H2 relaxin to stimulate cell invasion alone, or inhibit H2 relaxin-induced cell invasion, was tested. B-R13/17K H2 relaxin was not able to stimulate cell invasion at 1 μM, a concentration at which it was clearly active in HEK-293T cells. Importantly, it was able to significantly inhibit cell invasion induced by 16.8 nM H2 relaxin. Therefore in MCF-7 cells, B-R13/17K H2 relaxin is acting as a full antagonist consistent with the results of the lentiviral-driven expression in prostate cancer cells.

Finally, B-R13/17K H2 relaxin was tested in a completely different primary cell system which also responds to H2 relaxin, renal myofibroblasts. H2 relaxin has been clearly shown to inhibit renal myofibroblast differentiation by interfering with TGF-beta1/Smad2 signaling through RXFP1 and a nNOS-NO-cGMP-dependent pathway (Masterson et al. 2004; Mookerjee et al. 2009). In a similar manner to the MCF-7 cells, B-R13/17K H2 relaxin at 1 μM had no effect on renal myofibroblast differentiation alone, but was able to completely inhibit the reduction in renal

myofibroblast differentiation induced by both 1.68 and 16.8 nM H2 relaxin. Hence in this rat primary cell system, B-R13/17K H2 relaxin also acted as a full antagonist.

Therefore, we have been able to confirm that B-R13/17K H2 relaxin is a RXFP1 antagonist in native cell systems, and it is likely that a pro-hormone form of this peptide is being produced in the lentiviral-driven expression studies in vivo or in vitro (Silvertown et al. 2007). It remains to be clarified why the peptide acts as a partial agonist in RXFP1 over-expressing cells. Importantly, in the in vitro studies on lentiviral-driven expression of B-R13/17K H2 relaxin, the authors also showed a small stimulatory effect on cAMP activity in HEK-293T cells over-expressing RXFP1 (Silvertown et al. 2007). It is important to note that the HEK-293T cells which over-express RXFP1 have approximately 50,000–100,000 receptors per cell (Callander et al. 2009; Sudo et al. 2003) levels which are 25–250 times higher than native expressing cells such as fibroblasts (~3,000 receptors/cell; (Palejwala et al. 1998) and THP-1 cells (~275 receptors/cell; Parsell et al. 1996). It is well established that compounds acting as GPCRs can demonstrate partial agonism when the receptor levels are high but demonstrate antagonism when receptor concentrations are low (Hoyer and Boddeke 1993). Additionally, now it is also known that the same GPCR can couple to different signaling pathways in different cells and that binding of the same ligand in different cells can demonstrate signal pathway-dependent pharmacology (Baker and Hill 2007). Indeed it has been demonstrated that the upregulation of matrix metalloproteases by relaxin in fibroblasts is not associated with cAMP signaling (Palejwala et al. 2001). The increased invasiveness of MCF-7 cells induced by relaxin is due to up-regulation of matrix metalloproteases; however, the signaling pathways responsible for this effect are unknown. Renal fibroblast differentiation induced by H2 relaxin does not likely involve cAMP (Mookerjee et al., 2009). Hence it is possible that the partial agonistic effect that is observed with B-R13/17K H2 relaxin is due to differential signaling in the different cell systems but is more likely due to the much higher receptor levels in stably transfected HEK-293T cells.

In conclusion, we have successfully synthesized and characterized the relaxin analog, B-R13/17K H2 relaxin and confirmed that is a RXFP1 antagonist. The outcome of this study will open a new era in the field of relaxin research where the relaxin analog, B-R13/17K H2, can be used as a key tool to further understand H2 relaxin/RXFP1 signaling and the resulting downstream physiological actions. Most importantly, this peptide has potential as a therapeutic to treat prostate and other cancers and as a template for further drug design and development.

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References

Baker JG, Hill SJ (2007) Multiple GPCR conformations and signalling pathways: implications for antagonist affinity estimates. Trends Pharmacol Sci 28:374–381

Bani D, Flagiello D, Poupon MF, Nistri S, Poirson-Bichat F, Bigazzi M, Bani Sacchi T (1999) Relaxin promotes differentiation of human breast cancer cells MCF-7 transplanted into nude mice. Virchows Arch 435:509–519

Bathgate RA, Ivell R, Sanborn BM, Sherwood OD, Summers RJ (2006a) International Union of Pharmacology LVII: recommendations for the nomenclature of receptors for relaxin family peptides. Pharmacol Rev 58:7–31

Bathgate RA, Lin F, Hanson NF, Otvos L Jr, Guidolin A, Giannakis C, Bastiras S, Layfield SL, Ferraro T, Ma S, Zhao C, Gundlach AL, Samuel CS, Tregear GW, Wade JD (2006b) Relaxin-3: improved synthesis strategy and demonstration of its high-affinity interaction with the relaxin receptor LGR7 both in vitro and in vivo. Biochemistry 45:1043–1053

Bathgate RAD, Hsueh AJ, Sherwood OD (2006c) Physiology and molecular biology of the relaxin peptide family. In: Neill JD (ed) Physiology of reproduction. Elsevier, San Diego, pp 679–770

Bathgate RA, Lekgabe ED, McGuane JT, Su Y, Pham T, Ferraro T, Layfield S, Hannan RD, Thomas WG, Samuel CS, Du XJ (2008) Adenovirus-mediated delivery of relaxin reverses cardiac fibrosis. Mol Cell Endocrinol 280:30–38

Binder C, Hagemann T, Husen B, Schulz M, Einspanier A (2002) Relaxin enhances in vitro invasiveness of breast cancer cell lines by up-regulation of matrix metalloproteases. Mol Hum Reprod 8:789–796

Binder C, Simon A, Binder L, Hagemann T, Schulz M, Emons G, Trumper L, Einspanier A (2004) Elevated concentrations of serum relaxin are associated with metastatic disease in breast cancer patients. Breast Cancer Res Treat 87:157–166

Büllesbach EE, Yang S, Schwabe C (1992) The receptor-binding site of human relaxin II. A dual prong-binding mechanism. J Biol Chem 267:22957–22960

Callander GE, Thomas WG, Bathgate RA (2009) Prolonged RXFP1 and RXFP2 signaling can be explained by poor internalization and a lack of beta-arrestin recruitment. Am J Physiol Cell Physiol 296:C1058–C1066

Conrad KP, Novak J (2004) Emerging role of relaxin in renal and cardiovascular function. Am J Physiol Regul Integr Comp Physiol 287:R250–R261

Feng S, Agoulnik IU, Bogatcheva NV, Kamat AA, Kwabi-Addo B, Li R, Ayala G, Ittmann MM, Agoulnik AI (2007) Relaxin promotes prostate cancer progression. Clin Cancer Res 13:1695–1702

Feng S, Agoulnik IU, Li Z, Han HD, Lopez-Berestein G, Sood A, Ittmann MM, Agoulnik AI (2009) Relaxin/RXFP1 signaling in prostate cancer progression. Ann N Y Acad Sci 1160:379–380

Hombach-Klonisch S, Bialek J, Trojanowicz B, Weber E, Holzhausen HJ, Silvertown JD, Summerlee AJ, Dralle H, Hoang-Vu C, Klonisch T (2006) Relaxin enhances the oncogenic potential of human thyroid carcinoma cells. Am J Pathol 169:617–632

Hossain MA, Bathgate RA, Kong CK, Shabanpoor F, Zhang S, Haugaard-Jonsson LM, Rosengren KJ, Tregear GW, Wade JD (2008a) Synthesis, conformation, and activity of human insulin-like peptide 5 (INSL5). Chembiochem 9:1816–1822

Hossain MA, Rosengren KJ, Haugaard-Jonsson LM, Zhang S, Layfield S, Ferraro T, Daly NL, Tregear GW, Wade JD, Bathgate RA (2008b) The A-chain of human relaxin family peptides has distinct roles in the binding and activation of the different relaxin family peptide receptors. J Biol Chem 283:17287–17297

Hossain MA, Rosengren KJ, Zhang S, Bathgate RA, Tregear GW, van Lierop BJ, Robinson AJ, Wade JD (2009) Solid phase synthesis and structural analysis of novel A-chain dicarba analogs of human relaxin-3 (INSL7) that exhibit full biological activity. Org Biomol Chem 7:1547–1553

Hoyer D, Boddeke HW (1993) Partial agonists, full agonists, antagonists: dilemmas of definition. Trends Pharmacol Sci 14:270–275

Hsu SY, Nakabayashi K, Nishi S, Kumagai J, Kudo M, Sherwood OD, Hsueh AJ (2002) Activation of orphan receptors by the hormone relaxin. Science 295:671–674

Kamat AA, Feng S, Agoulnik IU, Kheradmand F, Bogatcheva NV, Coffey D, Sood AK, Agoulnik AI (2006) The role of relaxin in endometrial cancer. Cancer Biol Ther 5:71–77

Masterson R, Hewitson TD, Kelynack K, Martic M, Parry L, Bathgate R, Darby I, Becker G (2004) Relaxin down-regulates renal fibroblast function and promotes matrix remodelling in vitro. Nephrol Dial Transplant 19:544–552

Mookerjee I, Hewitson TD, Halls ML, Summers RJ, Mathai ML, Bathgate RA, Tregear GW, Samuel CS (2009) Relaxin inhibits renal myofibroblast differentiation via RXFP1, the nitric oxide pathway, and Smad2. FASEB J 23:1219–1229

Palejwala S, Stein D, Wojtczuk A, Weiss G, Goldsmith LT (1998) Demonstration of a relaxin receptor and relaxin-stimulated tyrosine phosphorylation in human lower uterine segment fibroblasts. Endocrinology 139:1208–1212

Palejwala S, Stein DE, Weiss G, Monia BP, Tortoriello D, Goldsmith LT (2001) Relaxin positively regulates matrix metalloproteinase expression in human lower uterine segment fibroblasts using a tyrosine kinase signaling pathway. Endocrinology 142:3405–3413

Parsell DA, Mak JY, Amento EP, Unemori EN (1996) Relaxin binds to and elicits a response from cells of the human monocytic cell line, THP-1. J Biol Chem 271:27936–27941

Sacchi TB, Bani D, Brandi ML, Falchetti A, Bigazzi M (1994) Relaxin influences growth, differentiation and cell–cell adhesion of human breast-cancer cells in culture. Int J Cancer 57:129–134

Samuel CS, Du XJ, Bathgate RA, Summers RJ (2006) 'Relaxin' the stiffened heart and arteries: the therapeutic potential for relaxin in the treatment of cardiovascular disease. Pharmacol Ther 112:529–552

Samuel CS, Hewitson TD, Unemori EN, Tang ML (2007) Drugs of the future: the hormone relaxin. Cell Mol Life Sci 64:1539–1557

Scott DJ, Layfield S, Yan Y, Sudo S, Hsueh AJ, Tregear GW, Bathgate RA (2006) Characterization of novel splice variants of LGR7 and LGR8 reveals that receptor signaling is mediated by their unique low density lipoprotein class A modules. J Biol Chem 281:34942–34954

Silvertown JD, Ng J, Sato T, Summerlee AJ, Medin JA (2006) H2 relaxin overexpression increases in vivo prostate xenograft tumor growth and angiogenesis. Int J Cancer 118:62–73

Silvertown JD, Symes JC, Neschadim A, Nonaka T, Kao JC, Summerlee AJ, Medin JA (2007) Analog of H2 relaxin exhibits antagonistic properties and impairs prostate tumor growth. Faseb J 21:754–765

Sudo S, Kumagai J, Nishi S, Layfield S, Ferraro T, Bathgate RA, Hsueh AJ (2003) H3 relaxin is a specific ligand for LGR7 and activates the receptor by interacting with both the ectodomain and the exoloop 2. J Biol Chem 278:7855–7862

Tashima LS, Mazoujian G, Bryant-Greenwood GD (1994) Human relaxins in normal, benign and neoplastic breast tissue. J Mol Endocrinol 12:351–364

Teerlink JR, Metra M, Felker GM, Ponikowski P, Voors AA, Weatherley BD, Marmor A, Katz A, Grzybowski J, Unemori E, Teichman SL, Cotter G (2009) Relaxin for the treatment of patients with acute heart failure (Pre-RELAX-AHF): a multicentre, randomised, placebo-controlled, parallel-group, dose-finding phase IIb study. Lancet 373:1429–1439

Thompson VC, Morris TG, Cochrane DR, Cavanagh J, Wafa LA, Hamilton T, Wang S, Fazli L, Gleave ME, Nelson CC (2006) Relaxin becomes upregulated during prostate cancer progression to androgen independence and is negatively regulated by androgens. Prostate 66:1698–1709

Vinall RL, Tepper CG, Shi XB, Xue LA, Gandour-Edwards R, de Vere White RW (2006) The R273H p53 mutation can facilitate the androgen-independent growth of LNCaP by a mechanism that involves H2 relaxin and its cognate receptor LGR7. Oncogene 25:2082–2093

Wilkinson TN, Speed TP, Tregear GW, Bathgate RA (2005) Evolution of the relaxin-like peptide family. BMC Evol Biol 5:14