# Electronic Supplementary Information (ESI)

## A single-chain derivative of the relaxin hormone is a functionally selective agonist of the G protein-coupled receptor, RXFP1

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**Figure S1**

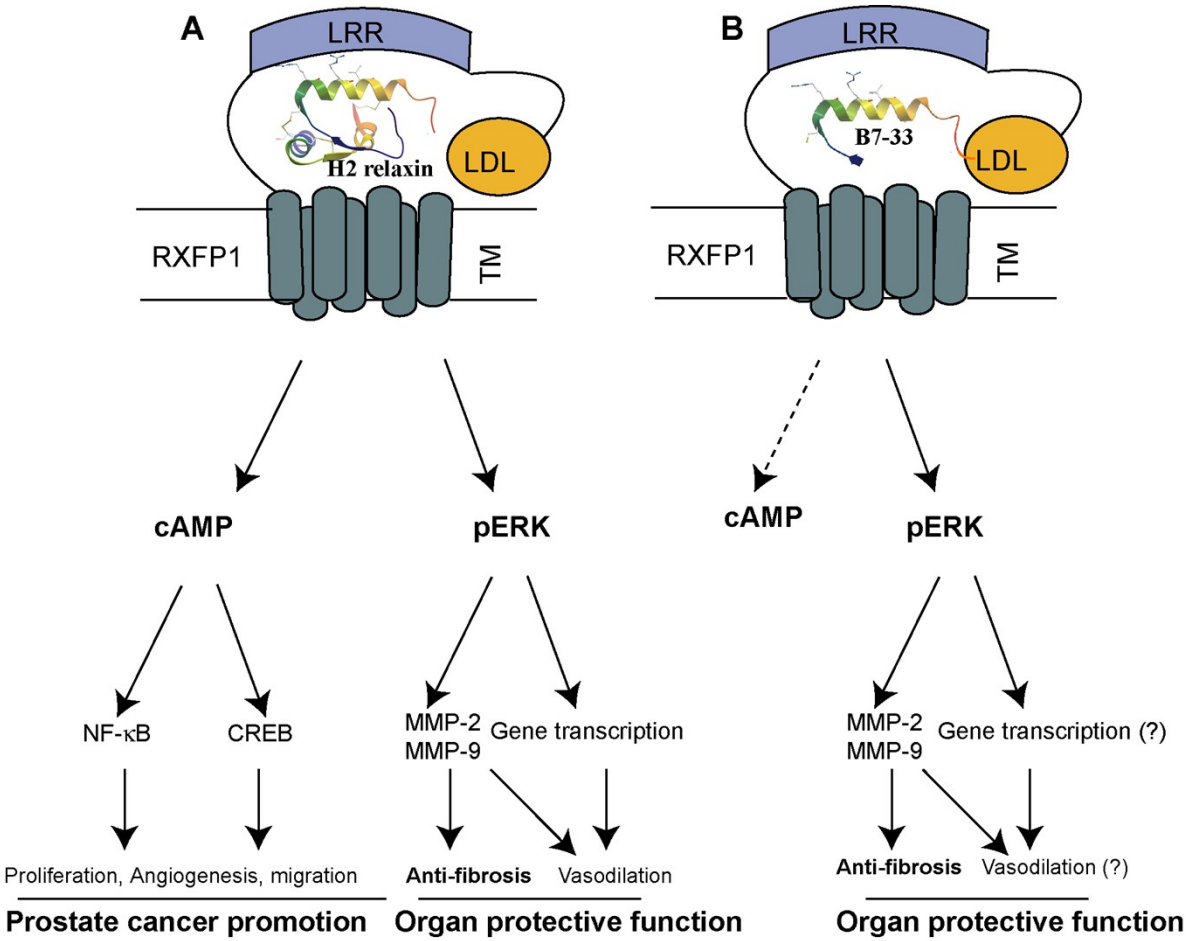



**Fig. S1** Schematic representation of the mechanism of RXFP1 activation by H2 relaxin and B7-33. While (A) H2 relaxin was shown to stimulate both cAMP and ERK pathways, (B) B7-33 selectively activated ERK pathway in naturally expresing RXFP1 cells leading to its strong H2-relaxin-like anti-fibrotic action *in vitro* ((myo)fibroblasts) and *in vivo*. Accumalation of cAMP is believed to be related to prostate cancer promotion and activation of ERK and subsequent promotion of MMPs is related to benificial effects (e.g. anti-fibrosis and vasodilation) of relaxin. B7-33 is thus a novel functionally selective RXFP1 agonist.

**Figure S2**

**A**

| | 13 17 20 |
|---|---|
| AcB7-33 | Ac-VIKLSGRELVRAQ I AISGMSTWSKRSL-NH2 |
| R13A(AcB7-33) | Ac-VIKLSGAELVRAQ I AISGMSTWSKRSL-NH2 |
| R17A(AcB7-33) | Ac-VIKLSGRELVAAQ I AISGMSTWSKRSL-NH2 |
| I20A(AcB7-33) | Ac-VIKLSGRELVRAQAAISGMSTWSKRSL-NH2 |
| R13/17A. I20A(AcB7-33) | Ac-VIKLSGAELVAAQA AISGMSTWSKRSL-NH2 |

**B**

| | Human RXFP1 (HEK-293T) | |
|---|---|---|
| Peptides | Eu-H2 pKi | cAMP pEC50 |
| H2 relaxin | 8.96 ± 0.03 (5) | 10.49 ± 0.13 (4) |
| B7-33 | 5.54 ± 0.13 (5)# | 5.12 ± 0.06 (3)# |
| AcB7-33 | 6.00 ± 0.0 (1)# | 5.40 ± 0.04 (3)# |
| R13A(AcB7-33) | No binding | No activity |
| R17A(AcB7-33) | No binding | No activity |
| I20A(AcB7-33) | No binding | No activity |
| R13/17A. I20A(AcB7-33) | No binding | No activity |

#P<0.001 vs H2 relaxin

**Fig. S2** (A) Amino acid sequences of B7-33 variants with key residues mutations. (**B**) Pooled binding affinity (pKi) and cAMP activity (pEC50) data. The dramatic reduction in the binding affinity and cAMP activity of mutated analogues for RXFP1 indicated that B7-33 used the same residues as H2 relaxin to interact with the LRRs of RXFP1.

**Figure S3**

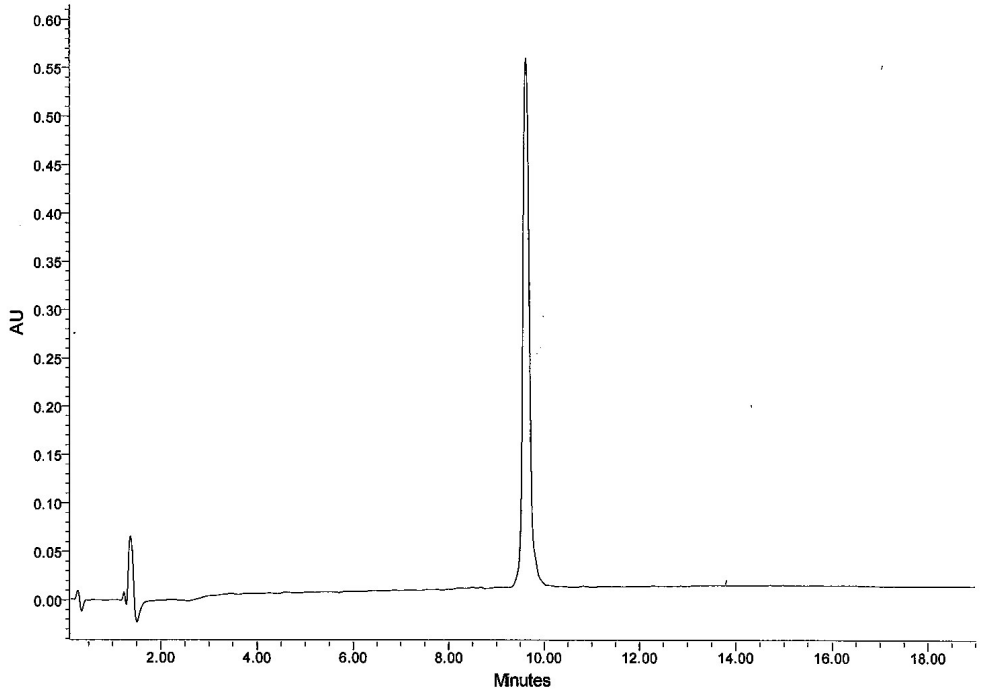
| Peptides | Purity (%) |
|---|---|
| B7-33 | 97 |
| AcB7-33 | 95 |
| R13A(AcB7-33) | 95 |
| R17A(AcB7-33) | 94 |
| I20A(AcB7-33) | 98 |
| R13/R17A.I20A(AcB7-33) | 97 |

**Fig. S3** Purity of B7-33 and its variants

**Figure S4**

*1a. HPLC profile of B7-33*

Analytical RP-HPLC
Sample : 2533613F
Result Id : 10213




| | RT | PeakArea | PeakHeight | % Area |
|---|---|---|---|---|
| 1 | 9.40 | 15925 | 7206 | 0.30 |
| 2 | 9.61 | 5134976 | 546357 | 97.15 |
| 3 | 9.81 | 119433 | 31742 | 2.26 |
| 4 | 9.95 | 15094 | 5453 | 0.29 |

Column : 150 x 4.6 mm, Monitor C18, Column Engineering
Solvent A : 0.1% trifluoroacetic acid in 100% water
Solvent B : 0.1% trifluoroacetic acid in 90% acetonitrile (aqueous)
Gradient : 10%B for 1.0 min; 10-66.6%B (Linear) over 15.0 min; then column reequilibration
Flow rate : 1.5 mL/minute
Wavelength : PDA 214.0 nm

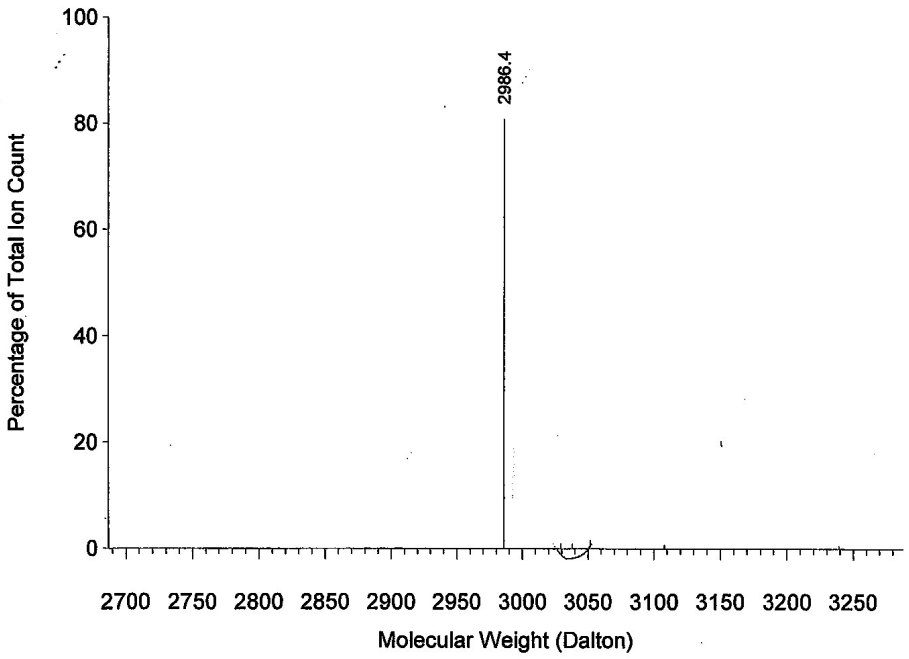
Date Acquired: 27-Sep-2014 4:11:21 EST
Acq Method Set S6_15cm_25min__TFA
Analysis using Empower 2 Software Build 2154

*1b. ESI trace of B7-33*

Mass Spectrometry

Sample : 2533613.F

H-VIKLSGRELVRAQIAISGMSTWSKRSL-NH2

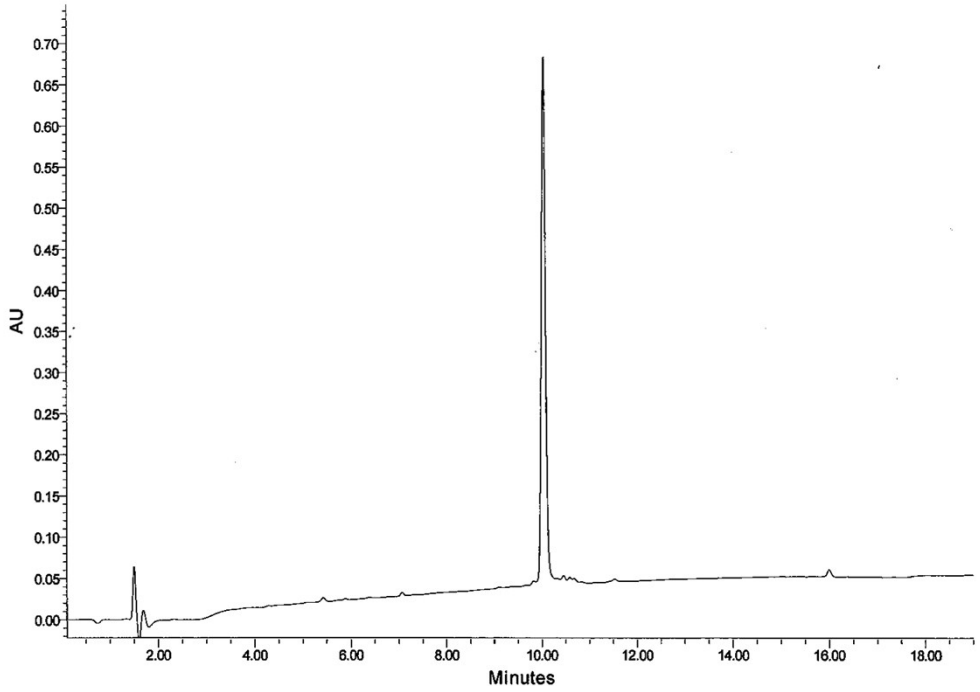



Theoretical MW of parent is 2986.6 dalton
3.9% of ion count is below range in figure
MS operated in the positive ion mode

| | | |
|---|---|---|
| Instrument | : | Perkin-Elmer Sciex API 100 |
| Eluent | : | 0.1% Acetic acid in 60% acetonitrile |
| Ion Source | : | Ionspray |
| Detection | : | Ion counting |

*2a. HPLC profile of AcB7-33*

Analytical RP-HPLC
Sample : 2533622F
Result Id : 6700




| | RT | PeakArea | PeakHeight | % Area |
|---|---|---|---|---|
| 1 | 9.83 | 28321 | 4988 | 0.68 |
| 2 | 10.04 | 3974667 | 641073 | 95.12 |
| 3 | 10.30 | 28379 | 6077 | 0.68 |
| 4 | 10.45 | 54337 | 9022 | 1.30 |
| 5 | 10.58 | 35937 | 6504 | 0.86 |
| 6 | 10.68 | 24185 | 4744 | 0.58 |
| 7 | 15.99 | 32648 | 6932 | 0.78 |

Column : 150 x 4.6 mm, Monitor C18, Column Engineering
Solvent A : 0.1% trifluoroacetic acid in 100% water
Solvent B : 0.1% trifluoroacetic acid in 90% acetonitrile (aqueous)
Gradient : 10%B for 1.0 min; 10-66.6%B (Linear) over 15.0 min; then column reequilibration
Flow rate : 1.5 mL/minute
Wavelength : PDA 214.0 nm

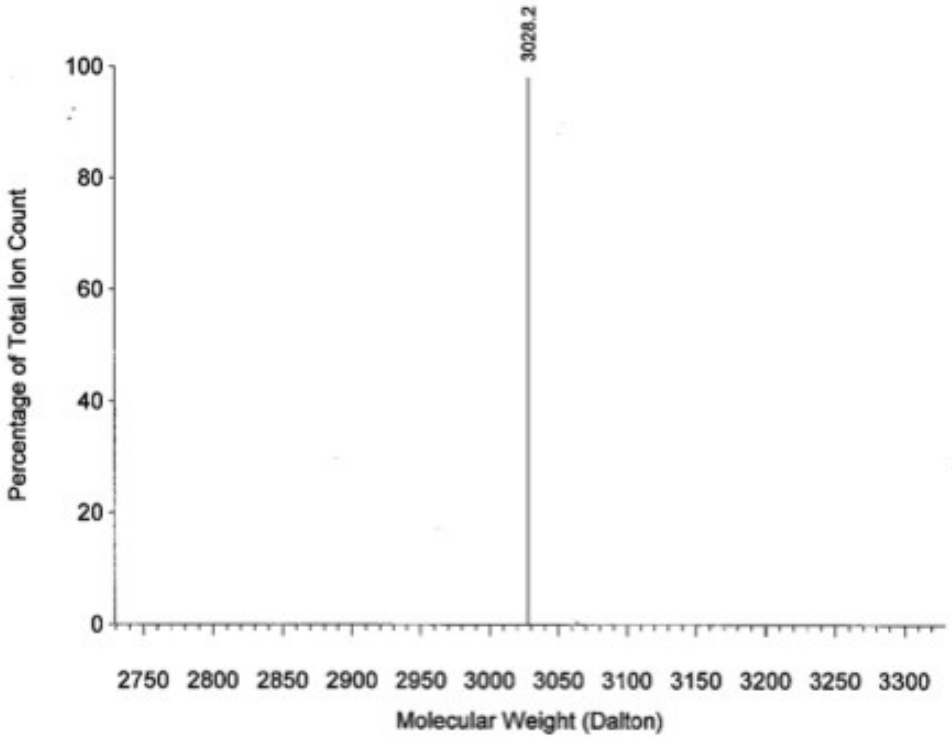
Date Acquired: 13-Oct-2014 18:03:38 EST
Acq Method Set S7_15cm_25min_TFA
Analysis using Empower 2 Software Build 2154

*2b. ESI trace of AcB7-33*

Mass Spectrometry

Sample : 2533622.F

Ac-VIKLSGRELVRAQIAISGMSTWSKRSL-NH2

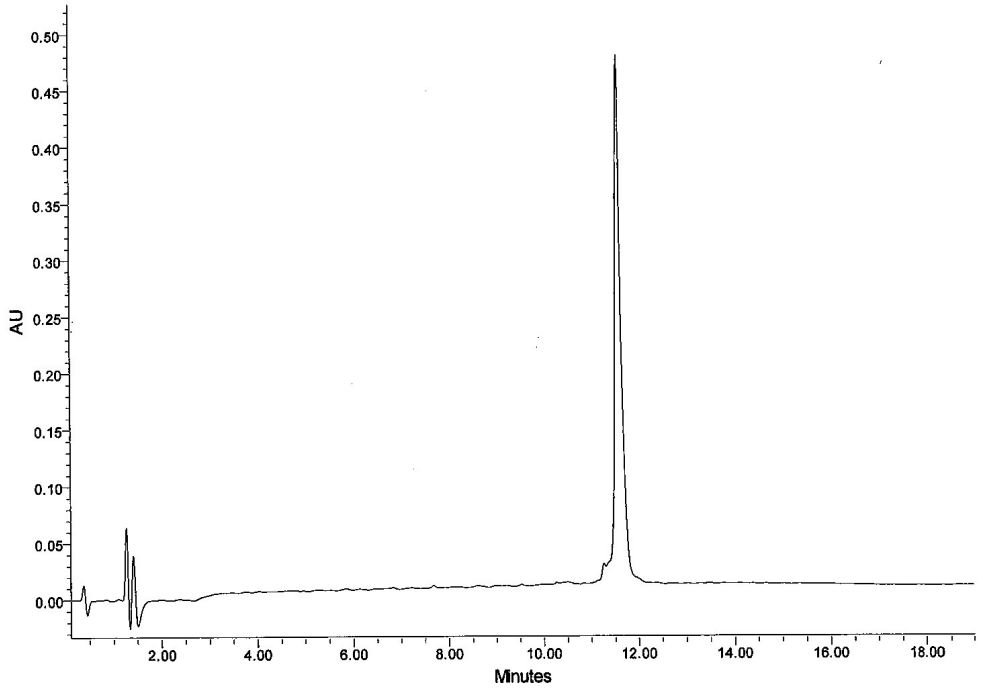



Theoretical MW of parent is 3028.6 dalton
0.5% of ion count is below range in figure
0.1% of ion count is above range in figure
MS operated in the positive ion mode

| | | |
|---|---|---|
| Instrument | : | Perkin-Elmer Sciex API 100 |
| Eluent | : | 0.1% Acetic acid in 60% acetonitrile |
| Ion Source | : | Ionspray |
| Detection | : | Ion counting |

*3a. HPLC profile of R13A(AcB7-33)*

Analytical RP-HPLC
Sample : 2533617F
Result Id : 9664




| | RT | PeakArea | PeakHeight | % Area |
|---|---|---|---|---|
| 1 | 10.27 | 6762 | 1336 | 0.15 |
| 2 | 10.49 | 19420 | 1583 | 0.43 |
| 3 | 11.15 | 12202 | 2637 | 0.27 |
| 4 | 11.27 | 98987 | 17621 | 2.21 |
| 5 | 11.37 | 56644 | 18589 | 1.27 |
| 6 | 11.57 | 4255815 | 467032 | 95.10 |
| 7 | 11.95 | 25420 | 4709 | 0.57 |

Column : 150 x 4.6 mm, Monitor C18, Column Engineering
Solvent A : 0.1% trifluoroacetic acid in 100% water
Solvent B : 0.1% trifluoroacetic acid in 90% acetonitrile (aqueous)
Gradient : 10%B for 1.0 min; 10-66.6%B (Linear) over 15.0 min; then column reequilibration
Flow rate : 1.5 mL/minute
Wavelength : PDA 214.0 nm

Date Acquired: 25-Sep-2014 20:44:33 EST
Acq Method Set S6_15cm_25min__TFA
Analysis using Empower 2 Software Build 2154

*3b. ESI trace of R13A(AcB7-33)*

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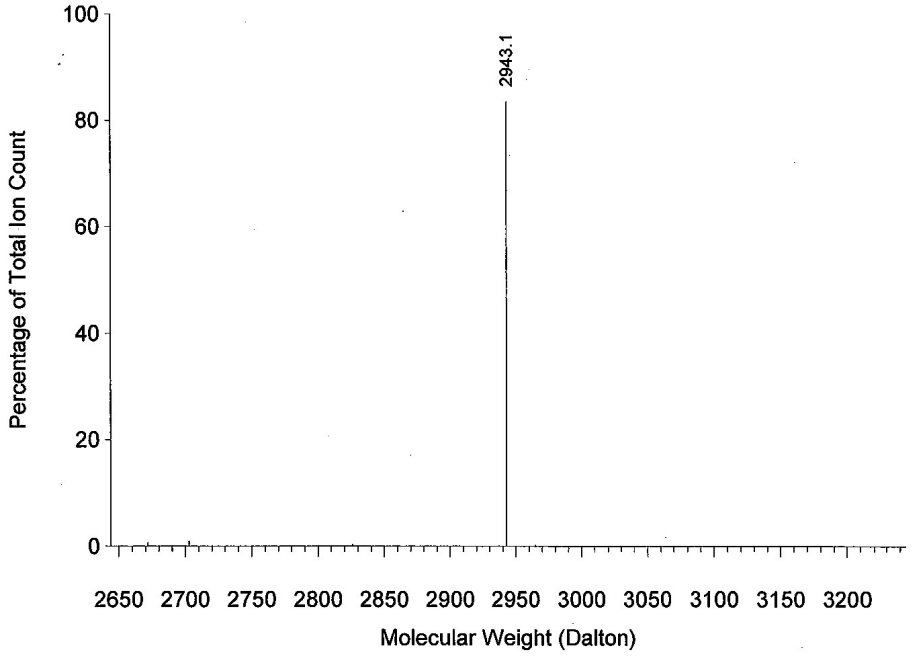
Mass Spectrometry

Sample : 2533617.F

Ac-VIKLSGAELVRAQIAISGMSTWSKRSL-NH2

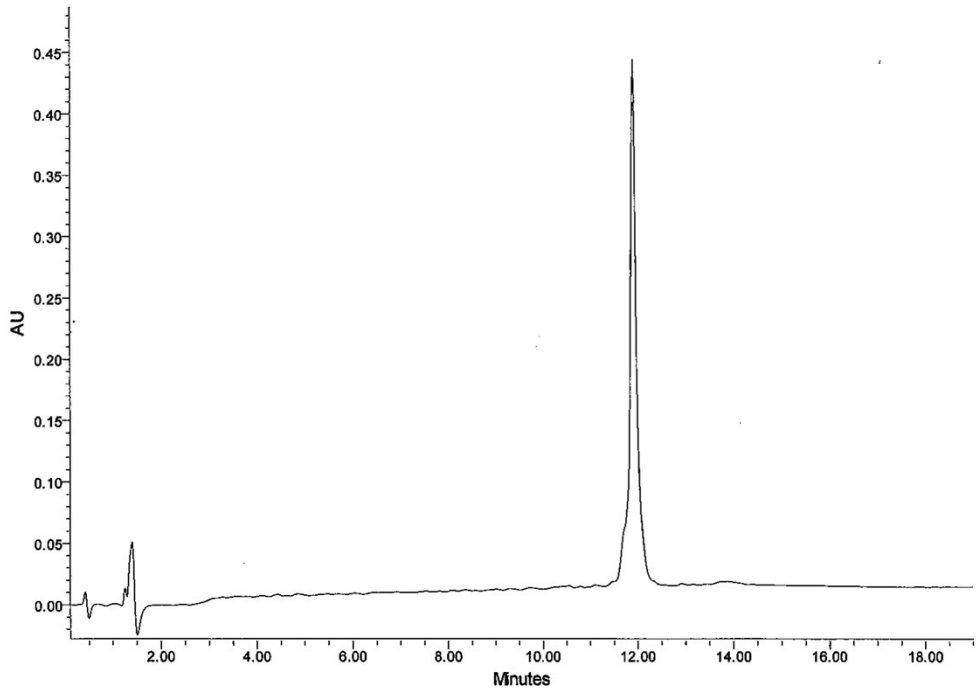



Theoretical MW of parent is 2943.5 dalton
13.0% of ion count is below range in figure
0.5% of ion count is above range in figure
MS operated in the positive ion mode

| | | |
|---|---|---|
| Instrument | : | Perkin-Elmer Sciex API 100 |
| Eluent | : | 0.1% Acetic acid in 60% acetonitrile |
| Ion Source | : | Ionspray |
| Detection | : | Ion counting |

*4a. HPLC profile of R17A(AcB7-33)*

Analytical RP-HPLC
Sample : 2533616J
Result Id : 10200




| | RT | PeakArea | PeakHeight | % Area |
|---|---|---|---|---|
| 1 | 11.52 | 33657 | 4383 | 0.77 |
| 2 | 11.71 | 225174 | 46444 | 5.14 |
| 3 | 11.89 | 4095868 | 428875 | 93.58 |
| 4 | 12.29 | 22126 | 4036 | 0.51 |

Column : 150 x 4.6 mm, Monitor C18, Column Engineering
Solvent A : 0.1% trifluoroacetic acid in 100% water
Solvent B : 0.1% trifluoroacetic acid in 90% acetonitrile (aqueous)
Gradient : 10%B for 1.0 min; 10-66.6%B (Linear) over 15.0 min; then column reequilibration
Flow rate : 1.5 mL/minute
Wavelength : PDA 214.0 nm

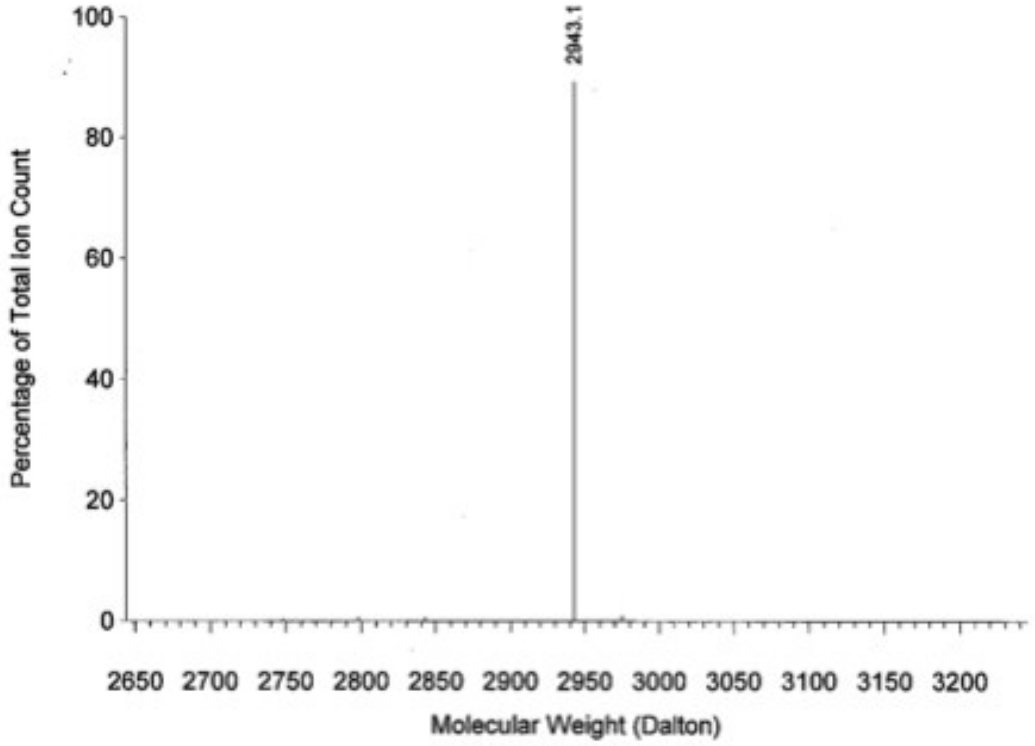
Date Acquired: 27-Sep-2014 2:27:02 EST
Acq Method Set S6_15cm_25min__TFA
Analysis using Empower 2 Software Build 2154

*4b. ESI trace of R17A(AcB7-33)*

Mass Spectrometry

Sample : 2533616.J

Ac-VIKLSGRELVAAQIAISGMSTWSKRSL-NH2

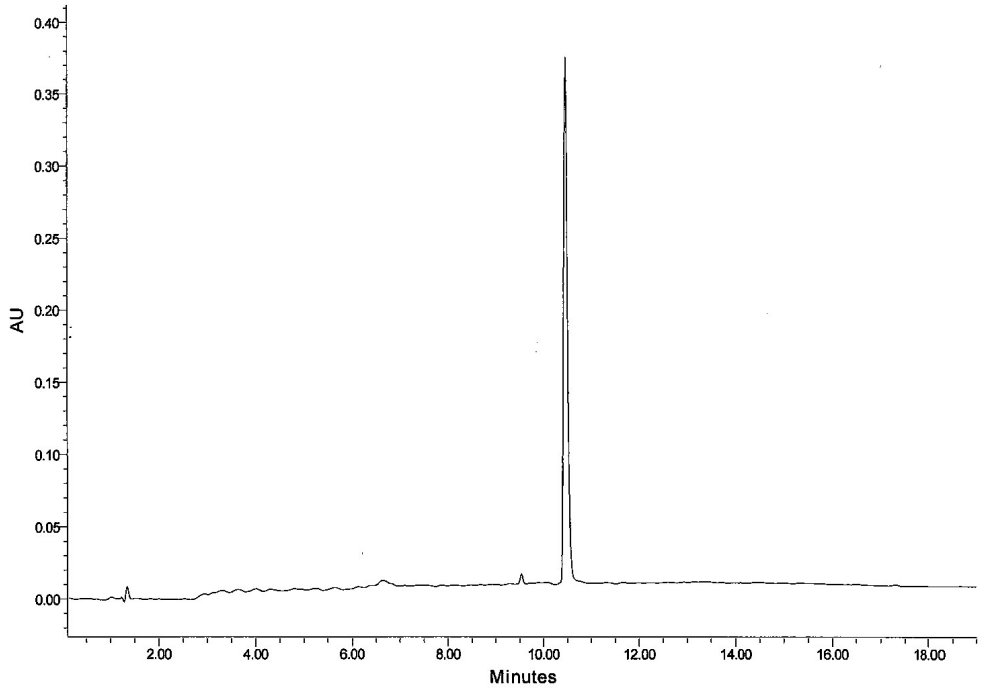



Theoretical MW of parent is 2943.5 dalton
7.1% of ion count is below range in figure
MS operated in the positive ion mode

| | | |
|---|---|---|
| Instrument | : | Perkin-Elmer Sciex API 100 |
| Eluent | : | 0.1% Acetic acid in 60% acetonitrile |
| Ion Source | : | Ionspray |
| Detection | : | Ion counting |

*5a. HPLC profile of I20A(AcB7-33)*

Analytical RP-HPLC
Sample : 2533618Fr1
Result Id : 10150




| | RT | PeakArea | PeakHeight | % Area |
|---|---|---|---|---|
| 1 | 9.54 | 22668 | 6232 | 1.09 |
| 2 | 10.36 | 3659 | 1479 | 0.18 |
| 3 | 10.46 | 2035561 | 365152 | 98.17 |
| 4 | 10.71 | 11582 | 1986 | 0.56 |

Column : 150 x 4.6 mm, Monitor C18, Column Engineering
Solvent A : 0.1% trifluoroacetic acid in 100% water
Solvent B : 0.1% trifluoroacetic acid in 90% acetonitrile (aqueous)
Gradient : 10%B for 1.0 min; 10-66.6%B (Linear) over 15.0 min; then column reequilibration
Flow rate : 1.5 mL/minute
Wavelength : PDA 214.0 nm

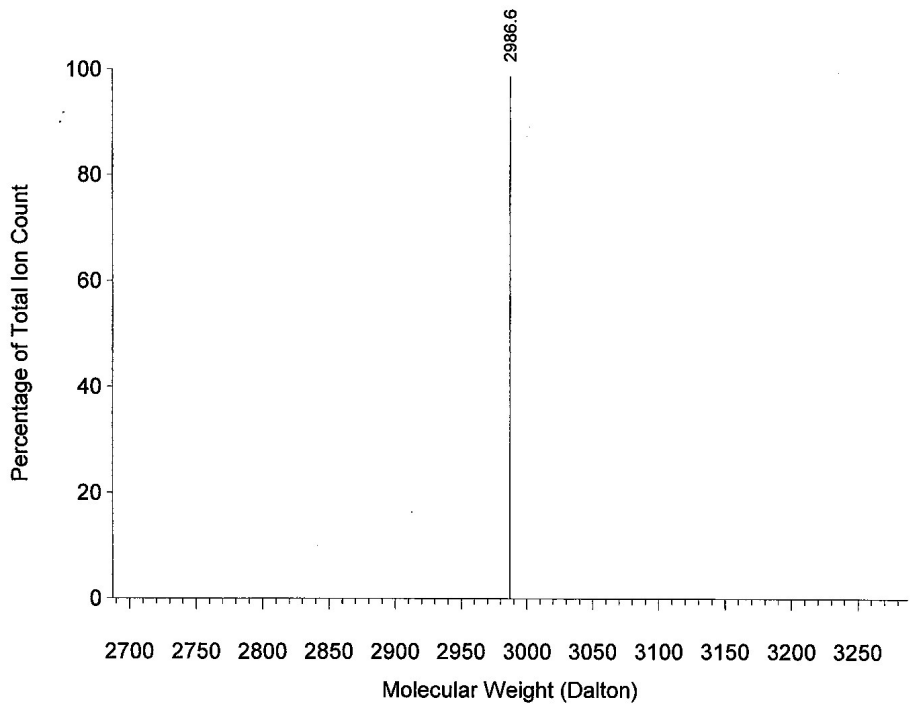
Date Acquired: 26-Sep-2014 11:33:11 EST
Acq Method Set S6_15cm_25min__TFA
Analysis using Empower 2 Software Build 2154

*5b. ESI trace of I20A(AcB7-33)*

Mass Spectrometry

Sample : 2533618.F

Ac-VIKLSGRELVRAQAAISGMSTWSKRSL-NH2

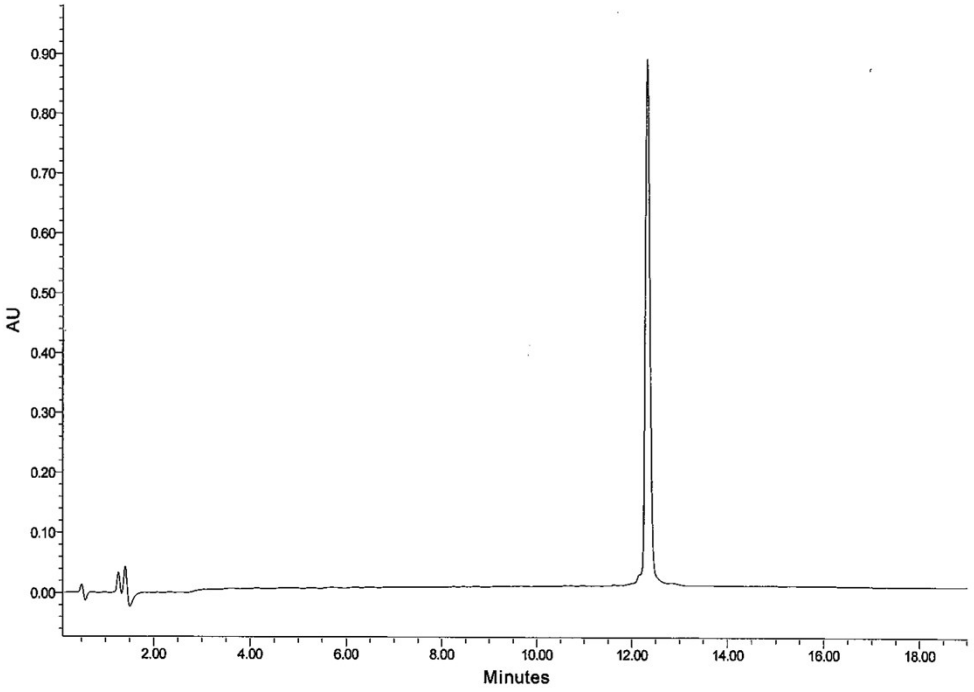



Theoretical MW of parent is 2986.6 dalton
0.8% of ion count is below range in figure
0.1% of ion count is above range in figure
MS operated in the positive ion mode

| | | |
|---|---|---|
| Instrument | : | Perkin-Elmer Sciex API 100 |
| Eluent | : | 0.1% Acetic acid in 60% acetonitrile |
| Ion Source | : | Ionspray |
| Detection | : | Ion counting |

*6a. HPLC profile of R13/17A.I20(AcB7-33)*

Analytical RP-HPLC
Sample : 2533619E
Result Id : 10266




| | RT | PeakArea | PeakHeight | % Area |
|---|---|---|---|---|
| 1 | 11.63 | 7171 | 1710 | 0.12 |
| 2 | 11.95 | 8763 | 2179 | 0.14 |
| 3 | 12.04 | 16285 | 3784 | 0.26 |
| 4 | 12.16 | 84763 | 18021 | 1.37 |
| 5 | 12.31 | 6012586 | 878853 | 97.13 |
| 6 | 12.85 | 53071 | 3922 | 0.86 |
| 7 | 12.96 | 7436 | 2071 | 0.12 |

Column : 150 x 4.6 mm, Monitor C18, Column Engineering
Solvent A : 0.1% trifluoroacetic acid in 100% water
Solvent B : 0.1% trifluoroacetic acid in 90% acetonitrile (aqueous)
Gradient : 10%B for 1.0 min; 10-66.6%B (Linear) over 15.0 min; then column reequilibration
Flow rate : 1.5 mL/minute
Wavelength : PDA 214.0 nm

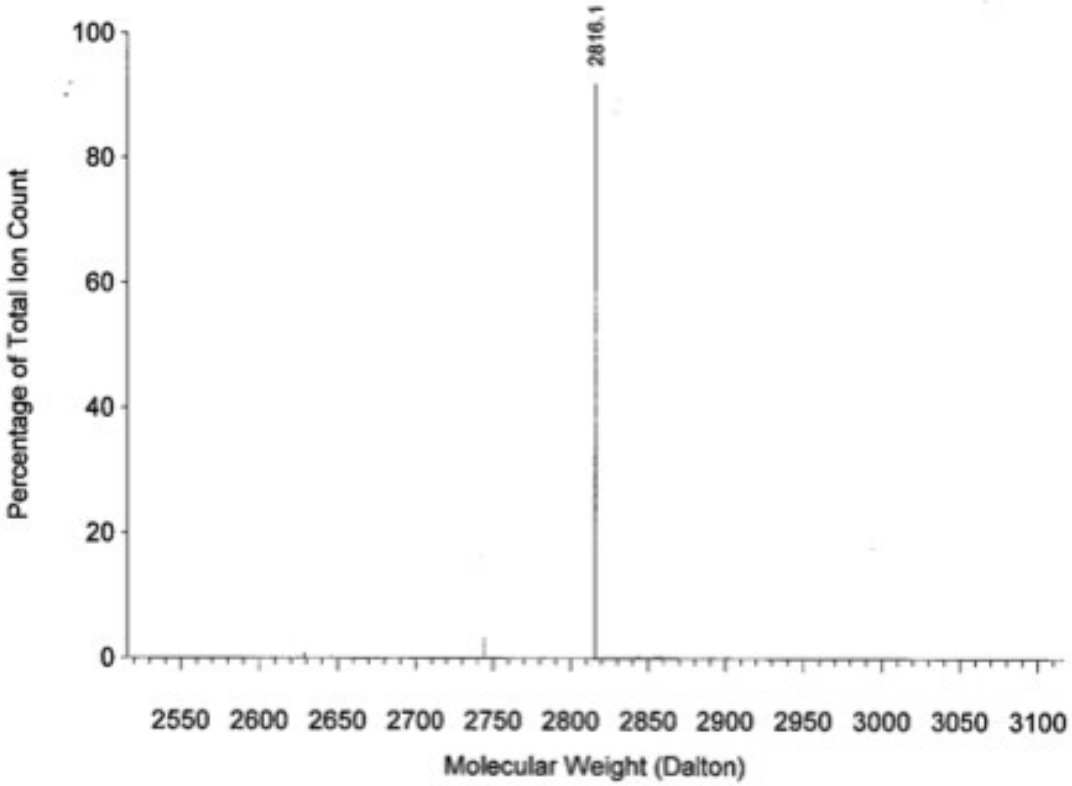
Date Acquired: 25-Sep-2014 21:36:48 EST
Acq Method Set S6_15cm_25min_TFA
Analysis using Empower 2 Software Build 2154

*6b. ESI trace of R13/17A.I20(AcB7-33)*

Mass Spectrometry

Sample : 2533619.E

Ac-VIKLSGAELVAAQAAISGMSTWSKRSL-NH2




Theoretical MW of parent is 2816.3 dalton
2.0% of ion count is below range in figure
0.2% of ion count is above range in figure
MS operated in the positive ion mode

| | | |
|---|---|---|
| Instrument | : | Perkin-Elmer Sciex API 100 |
| Eluent | : | 0.1% Acetic acid in 60% acetonitrile |
| Ion Source | : | Ionspray |
| Detection | : | Ion counting |

**Figure S5**

| H2 relaxin | B7-33 |
|---|---|
| 2 chain peptide, 3 disulfide bridges, 53 amino acids<br>6 HPLC purification steps<br>Manufactured in 12 days | Single chain peptide, no disulfide bridge, 27 amino acids<br>1 HPLC purification step<br>Manufactured in 2 days |

**Fig. S5** Comparison between H2 relaxin and B7-33 and steps involved in their chemical assembly. B7-33 is far easier and cheaper to manufacture by chemical means and thus more cost-effective as a drug.

**Figure S6**

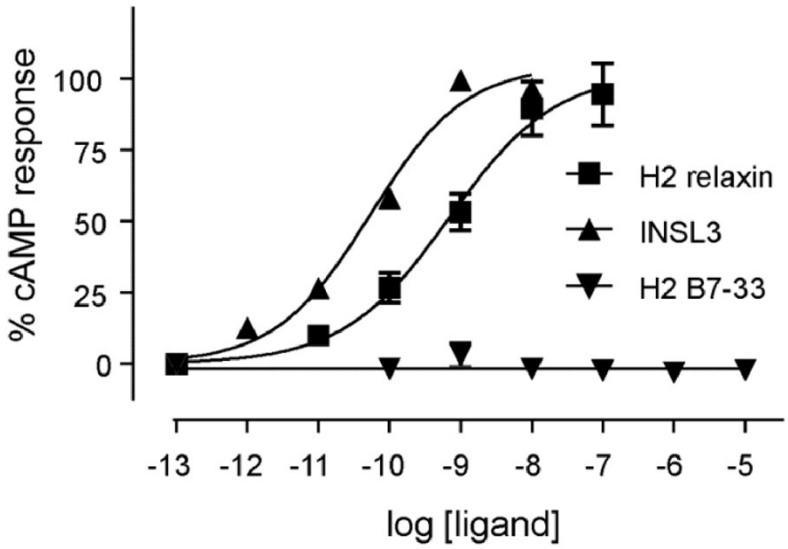



**Fig. S6** cAMP stimulating activity of B7-33 compared with H2 relaxin and the native ligand of RXFP2 (INSL3) in cells over expressing RXFP2. B7-33 did not activate cAMP responses in these cells. The data are expressed as mean ± SEM of n=3 independent experiments.