9th International Conference on Relaxin and Related Peptides

Coast Canmore Hotel and Conference Centre

Program and Abstracts

Phoenix Pharmaceuticals, Inc.

FVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN

CαK-peptide (Insulin isoform with Cα-peptide) Cat#036-44

Endogenous

Relaxin 2 (Human)	#035-62
Relaxin 3 (INSL7) (Human)	#035-36
Insulin (Human)	#035-06
INSL3 (Human)	#035-27
INSL5 (Human)	#035-70
INSL5 (Mouse)	#035-40

Predicted

Harpegnathos-Sal Insulin (39-96) amide	#036-45
Harpegnathos-Sal Insulin (31-107) amide	#036-47
LCDV-1/ sc LCDV1-VILP	#036-61
Des-C-peptide LCDV-1	#036-58

Custom, Synthetic Analogue

h Relaxin 2A / hINSL5B	#036-22
h Relaxin 3A / h Relaxin 2B	#036-23
h Relaxin 3A / hINSL5B	#036-24
hINSL5A / h Relaxin 2B	#036-25
INSL5A &7B Chains	#035-71
INSL5 (Human)-A13NR / RXFP4 Antagonist (Human)	#036-72

Antibodies, custom synthesis &assay kits are available!

DSWMEEVIKLCGRELVRAQIAICGMSTWS

<ELYSALANKCCHVGCTKRSLARFC

Relaxin-2 (Human) Cat#035-62

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Welcome to Relaxin 2023!

Relaxin, Related Peptides, and Receptors continue to generate great excitement in the scientific community. The **9th International Conference on Relaxin, Related Peptides, and Receptors** is a 3-day event held in the beautiful setting of the Canadian Rockies and builds on >40 years of international relaxin meetings. The first meeting of relaxinologists comprised a small group of scientists attending the 15th Midwest Conference on Endocrinology and Metabolism, in Columbia, MO in 1979. The first international forum on relaxin was held in Hawaii in 1980, followed by The Kroc Foundation conference on *"Relaxin: Structure, Function, and Evolution"* in Santa Ynes, California in 1981. The 1st International Relaxin Conference was organized by Dr. Mario Bigazzi in Florence, Italy in 1982. Seven additional International Conferences on Relaxin, Related Peptides, and Receptors have followed at exquisite venues, including Adelaide, 1994, Broome, 2000, Grand Tetons, 2004, Maui-Hawaii, 2008, Florence, 2013, Borneo-Malaysia, 2015, and Mexico, 2018. These meetings have witnessed the discovery of several relaxin-like peptides, the emergence of RXFP receptors, and clinical trials. The key objectives of all Relaxin conferences has been to (i) foster scientific exchange and enhance collaborations, (ii) promote excellence in research quality, (iii) drive innovation in fundamental and translational research in the relaxin field, (iv) empower EDI by promoting female scientists, investigators from nations around the world, and young investigators to continue their pursuit of relaxin research, and (v) continue supporting the global relaxin research community.

The current scientific program reflects exciting ongoing Relaxin research by international research teams and pharmaceutical companies to better understand the biology and function of relaxin, relaxin-like members, and receptors and identify clinical applications. The 9th International Conference on Relaxin, Related Peptides, and Receptors promotes inclusivity. All scientific sessions are chaired by a junior and senior scientist. Trainees and world experts on Relaxin and Receptors will present their work in oral presentations and in a dedicated poster session. We hope to engage early career scientists (trainees, post-doctoral fellows, early career researchers) and convey the exciting opportunities Relaxin research offers in the basic sciences and in translational/ clinical research. Join us in welcoming young investigator scientist Samantha Pauls and relaxinologist and global citizen, Alastair Summerlee, for their highly anticipated presentations that highlight "*Professional development and mentorship*" and offer insights into mysterious collectables like *"string of beads, a handful of dust and an iron fish"* one may encounter during Relaxin research. The closing award ceremony will announce winners of poster prizes and trainee travel awards.

We believe this RELAXIN 2023 conference meeting will be another excellent opportunity for academic, clinical and industry researchers to share recent discoveries and to forge new collaborations. We hope that you, your colleagues, and companions enjoy the conference and have a chance to experience the stunning landscapes of the Canadian Rockies!

The organizers of Relaxin 2023 acknowledge that the meeting venue is located within the Treaty 7 region of Southern Alberta. We recognize that the conference will be held in the traditional and ancestral territory of the Blackfoot Confederacy: Kainai, Piikani and Siksika people as well as the Tsuu T'ina Nation and Stoney Nakoda First Nations. In addition, this territory is home to the Métis Nation of Alberta, Region 3. We are grateful to these First Nations for their generosity in sharing their homeland with us.

Once again, welcome!

Enjoy RELAXIN 2023!

Sabine, Tom, and Brian
RELAXIN 2023 Local Organizing Committee

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The 9th International Conference on Relaxin and Related Peptides, Canmore 2023 would like to thank the following organizations for their support:

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Conference Committees

Local Organizing Committee

- Sabine Hombach-Klonisch (Canada)
- Thomas Klonisch (Canada)
- Brian Wilson (Canada)
- Jacki Armstrong (Canada)

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- Alexander Agoulnik (USA)
- Ravinder Anand-Ivell (UK)
- Carol Bagnell (USA)
- Ross Bathgate (Australia)
- Bob Bennett (USA)
- Gavin Dawe (Singapore)
- Thomas Dschietzig (Germany)
- Laura Goldsmith (USA)
- Akhter Hossain (Australia)
- Tharindunee Jayakody (Sri Lanka)
- Derek Lobb (Canada)

Registration, conference funding assistance

- Joy Thrun, ClassicTravel
- Melissa Wesner, ClassicTravel

PROGRAM AT A GLANCE

SUNDAY, SEPTEMBER 17, 2023

5:00 PM – 8:00 PM	Registration (Hotel Lobby)
6:00 PM – 9:00 PM	Welcome Reception: Drinks and Hors d'oeuvres

Monday, SEPTEMBER 18, 2023

7:00 AM – 8:15 AM	Breakfast
8:15 AM – 8:30 AM	Welcome, Introductory Remarks and Meeting Logistics
8:30 AM – 9:30 AM	**Keynote Speaker: Dr. Andrew Kruse, Harvard Medical School – "Activation mechanism of the RXFP1 relaxin receptor"**
9:30 AM – 10:40 PM	**Session 1:** Receptor Mechanisms and Signaling I
10:40 AM – 11:15 AM	Coffee Break
11:15 AM – 12:15 PM	**Session 2:** Receptor mechanisms and signaling II
12:15 PM – 1:15 PM	Lunch Buffet
1:15 PM – 2:15 PM	**Keynote Speaker: Dr. Juan Marugan, NCATS, NIH – "Optimization of Relaxin receptor agonists based on functional assays. Why signal is not good enough."**
2:15 PM – 3:15 PM	**Session 3:** Relaxin receptors: small molecule development
3:15 PM – 3:45 PM	Coffee Break
3:45 PM – 4:45 PM	**Session 4:** Neurobiology
5:00 PM – 6:00 PM	Dinner
6:30 PM – 7:30 PM	**Trainee-Investigator exchange and a Professional Development Session by Dr. Samantha Pauls, College of Pharmacy, RFHS, University of Manitoba**
7:30 PM – 9:00 PM	Networking

Tuesday, SEPTEMBER 19, 2023

7:00 AM – 8:15 AM	Breakfast
8:15 AM – 9:15 AM	**Keynote Speaker: Drs. Menying Hu & Leaf Huang, UNC, Chapel Hill – "Relaxin gene therapy against liver fibrosis: inspiration from spontaneous regression of fibrosis"**
9:15 AM – 10:20 AM	**Session 5:** Inflammation and cancer
10:20 AM – 10:45 PM	Coffee Break

10:45 AM – 11:45 PM	**Session 6:** Frontiers in the Biology of Relaxin and Related Peptides
12:00 PM – 12:55 PM	Lunch Buffet
1:00 PM – 1:15 PM	Group Photograph
1:30 PM – 5:30 PM	Lake Louise Excursion
6:00 PM	Dinner on your own
7:30 PM	Taproom Social – Tank 310 (Grizzly Paw Brewing Company)

Wednesday, SEPTEMBER 20, 2023

7:00 AM – 8:30 AM	Breakfast
8:30 AM – 9:45 AM	**Session 7:** Anti-fibrotic and Matrix Remodeling Actions
9:45 AM – 10:15 AM	Coffee Break
10:15 AM – 11:40 AM	**Session 8:** Cardiovascular Actions – Part I
11:40 AM – 12:30 PM	Lunch buffet
12:30 PM – 1:40 PM	**Session 9:** Cardiovascular Actions – Part II
1:45 PM – 3:00 PM	**Poster Session and Discussion**
2:30 PM – 3:00 PM	Coffee and snacks
3:00 PM – 5:30 PM	**Business Meeting (discussion of future relaxin meetings)**
6:00 PM – 7:30 PM	Farewell Dinner (Arnica Room)
7:30 PM – 8:30 PM	**Dr. Alastair Summerlee, Sprott School of Business, Carleton University – Researcher and Global Citizen**
8:30 PM	Poster Prizes & Trainee Travel Awards (to be announced at the Farewell Dinner), Closing remarks

Thursday, SEPTEMBER 21, 2023

7:30 AM – 9:00 AM	Breakfast on your own

KEYNOTE SPEAKERS

Dr. Andrew Kruse

Dr. Kruse is a Professor of Biological Chemistry and Molecular Pharmacology at Harvard Medical School. His research focuses on the structure and function of transmembrane receptors, using a combination of biophysical and cell biological approaches. Research in the Kruse lab also makes extensive use of combinatorial protein engineering methods such as yeast surface display of single-domain antibody fragments. Dr. Kruse began his independent career as an Assistant Professor at Harvard Medical School in 2014. Key research accomplishments include defining the structural basis for agonist action at the angiotensin II type 1 receptor and other G protein-protein coupled receptors (GPCRs), cloning the sigma-2 receptor, and determining the first structure of a tetraspanin protein and showing how it regulates B cell activation. The Kruse lab also developed a single-domain antibody fragment discovery platform. Dr. Kruse is a co-founder of Tectonic Therapeutic, a biotechnology company, and the Institute for Protein Innovation, a non-profit research organization. He has received awards including an Amgen Young Investigator Award (2019), an Alfred P. Sloan Research Fellowship (2017), a Vallee Scholars Award (2016), and an NIH Director’s Early Independence Award (2015). He received B.S. degrees in Mathematics and Biochemistry from the University of Minnesota in 2009 and completed a Ph.D. in Structural Biology at Stanford University in 2014, where he trained with Dr. Brian Kobilka.

KEYNOTE SPEAKERS

Dr. Juan Marugan

Since 2008, Dr. Marugan has held a Leader position within the Early Translation Branch of the National Center for Advancing Translation Sciences (NCATS). His team focuses on validating new therapeutic approaches in areas of medical need and on improving the translational process. A graduate from Universidad de Salamanca in Spain, he graduated as a Ph.D. in Organic Chemistry at Universidad Complutense de Madrid, prior to complete two medicinal chemistry postdoctoral fellowships with Dr. Andrew Hamilton at University of Pittsburgh and with Dr. Stuart Levy at Tuft University. As investigator Dr. Marugan has more than twenty five years of experience in pharmaceutical industry (Pharmamar S.A, 3-Dimentional Pharmaceuticals, Johnson & Johnson, Mithridion Inc.) and at NIH working in translational research, drug discovery and development. He has been personally involved in the investigation of multiple aspects of the translational process, with extensive hands-on experience on medicinal chemistry and preclinical research including assay development, library screening and hit selection, structure activity relationship optimization studies, in vivo pharmacological, tolerability and efficacy evaluation, preclinical development, and IND filing. Part of his job responsibilities include lead discovery and optimization with early ADME and PK profiling, managing internal and external resources, scale-up, SAR optimization, determination of the mechanism of action and in-vivo proof of principle studies. During his career, he held positions of increasing responsibility from Research Scientist to Head of Preclinical Drug Research with extensive experience as team leader in preclinical lead optimization programs, producing multiple series entering human clinical trials. Part of his scientific contributions can be followed up by reading his public disclosures (>150 peer-reviewed publications, 52 filled patents, and 123 scientific public presentations).

KEYNOTE SPEAKERS

Drs. Mengying Hu and Leaf Huang

Dr. Hu received her PhD degree in pharmaceutical sciences from the University of North Carolina at Chapel Hill under the guidance of Dr. Leaf Huang, working on liposomal delivery of nucleic acid-based therapies to modulate the stromal and immune microenvironment in advanced liver diseases. She is now a senior postdoc associate in Weill Cornell Medical College focusing on biology and translational value of immune cell-derived extracellular vesicles.

Professional Development

Dr. Samantha Pauls

Dr. Samantha Pauls is an Assistant Professor in the College of Pharmacy at the University of Manitoba. She completed a PhD in Biochemistry and Medical Genetics at the University of Manitoba in 2016 followed by a postdoctoral fellowship at the Canadian Centre for Agri-Food Research in Health and Medicine (CCARM) from 2016-2020. Although her academic research is in the field of inflammation and immunometabolism, she also serves all graduate student and postdoctoral researchers at the Rady Faculty of Health Sciences as programming lead for professional development. She seeks to inspire and equip the next generation of health scientists as they find their niche in the ever-changing workforce.

Dr. Alastair Summerlee

Dr. Alastair Summerlee is an innovative leader with significant experience in the post-secondary system including serving as president and vice-chancellor of the University of Guelph and Carleton University in Canada. He is an award-winning teacher and an internationally renowned researcher, including in the field of relaxinology, and holds and held significant research funding. Summerlee is also a passionate humanitarian and community leader, recognized at the UN for his work with refugees, disease prevention and hunger alleviation. He is a tireless advocate for education and for the role of the postsecondary system as the social and moral conscience of society.

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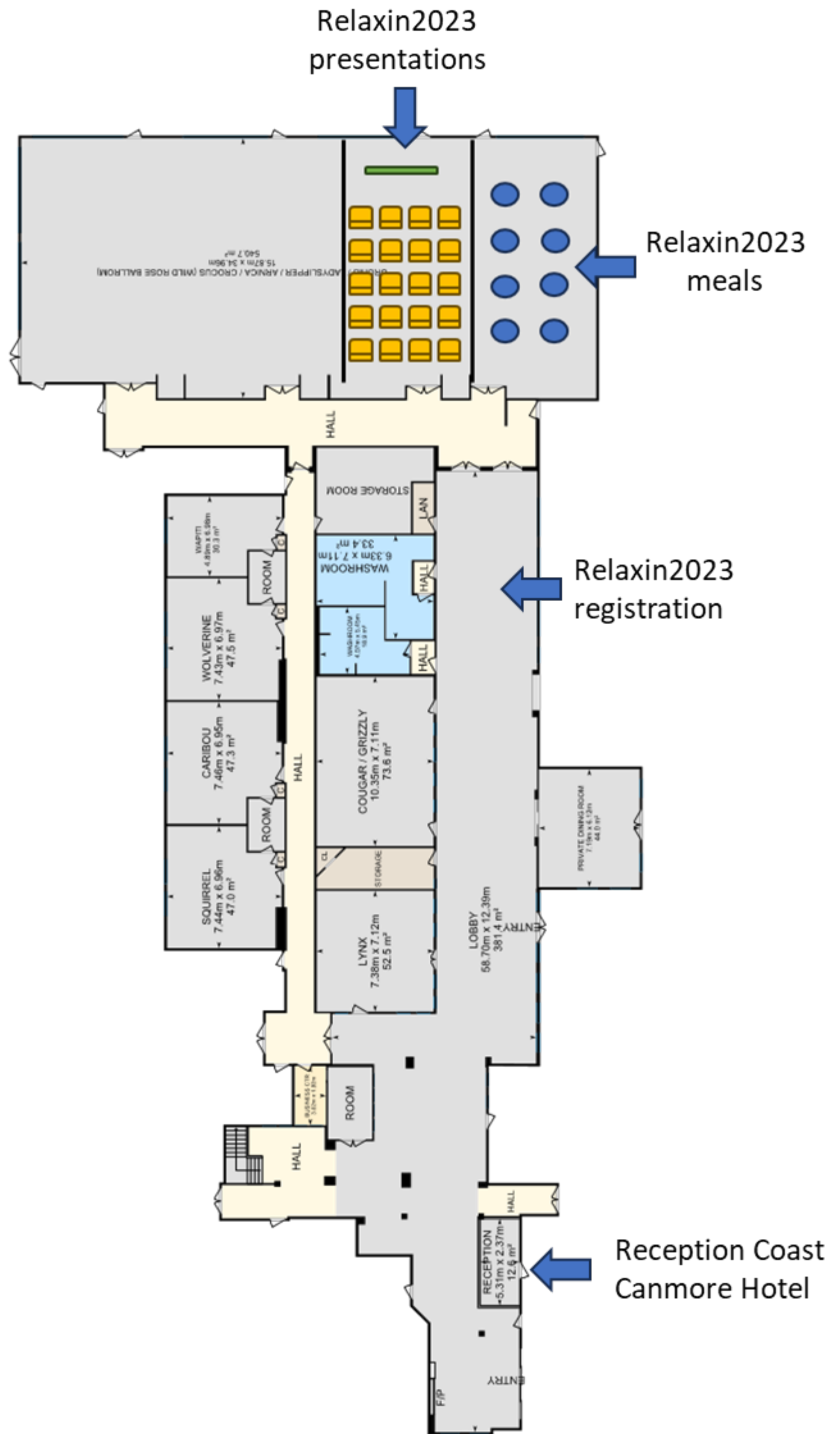
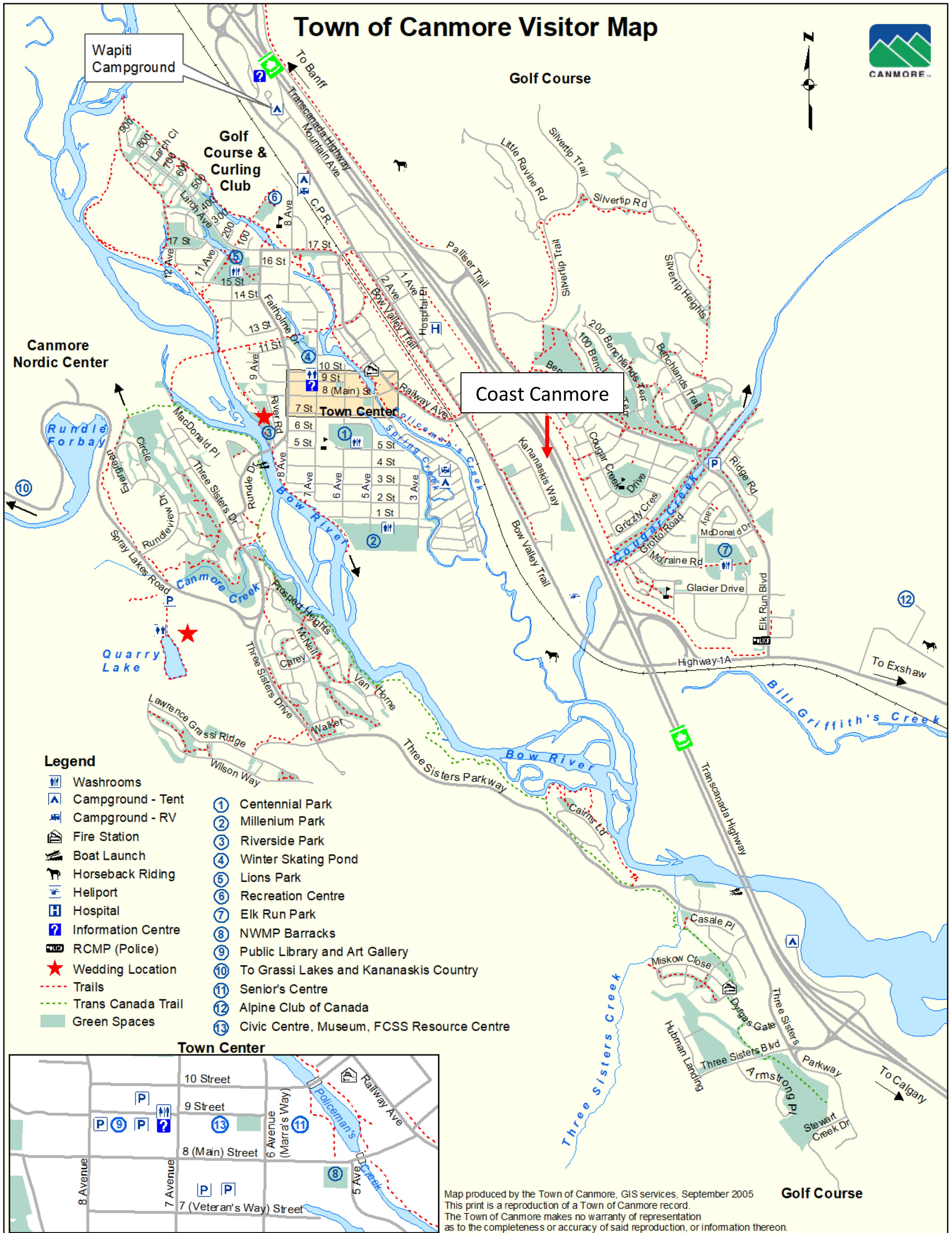
HOTEL FLOOR PLAN AND MAP OF CANMORE




Town of Canmore Visitor Map
Wapiti Campground
Golf Course
Golf Course & Curling Club
Canmore Nordic Center
Coast Canmore
Town Center
Rundle Forebay
Quarry Lake
Bow River
Legend
Washrooms
Campground - Tent
Campground - RV
Fire Station
Boat Launch
Horseback Riding
Heliport
Hospital
Information Centre
RCMP (Police)
Wedding Location
Trails
Trans Canada Trail
Green Spaces
1 Centennial Park
2 Millenium Park
3 Riverside Park
4 Winter Skating Pond
5 Lions Park
6 Recreation Centre
7 Elk Run Park
8 NWMP Barracks
9 Public Library and Art Gallery
10 To Grassi Lakes and Kananaskis Country
11 Senior's Centre
12 Alpine Club of Canada
13 Civic Centre, Museum, FCSS Resource Centre
Town Center
10 Street
9 Street
8 (Main) Street
7 (Veteran's Way) Street
Railway Ave
To Banff
To Exshaw
To Calgary
Transcanada Highway
Highway 1A
Three Sisters Parkway
Golf Course
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FULL PROGRAM

All events will take place in the Arnica and Crocus meeting rooms unless otherwise noted.

Sunday, September 17

Arrival of participants

5 pm – 8 pm	Registration open
6 pm until 9 pm	Welcome Reception - Meet and Greet

Monday, September 18

7:00 – 8:15 am	Breakfast
8:15 – 8:30 am	Welcome, introductory remarks and meeting logistics
8:30 – 9:30 am	**Keynote speaker presentation: Dr. Andrew Kruse, *Harvard Medical School* Structural basis for RXFP1 activation**
9:30 – 10:40 am	**Session 1: Receptor mechanisms and signaling-I**
	Session chairs: Dr. R. Bathgate and Dr. Craig Smith
9:30 – 9:45 am	**Ross Bathgate.** *The Florey, University of Melbourne:* Overview Of Relaxin Receptor Family Therapeutic Targeting.
9:45 – 10:05 am	**Monika Papworth.** *AstraZeneca*: AZD3427- engineering and characterisation of a long-acting relaxin fusion.
10:05 – 10:25 am	**Elisabetta Bianchi.** *Senior Director Peptide Chemistry, IRBM:* From the synthesis of Long-Acting Single Chain Peptides Agonist of RXFP1 to therapeutic candidates for renal and CV diseases”.
10:25 – 10:40 am	**Brad Hoare**. *The Florey, University of Melbourne*: Ongoing Attempts To Understand the Molecular Mechanisms By Which B7-33 And H2-relaxin Differently Bind And Activate RXFP1.
10:40 – 11:15 am	Coffee break
11:15 – 12:15 pm	**Session 2: Receptor mechanisms and signaling-II**
	Session chairs: Dr. A. Hossain and Dr. B. Hoare
11:15 – 11:30 am	**Akhter Hossain.** *The Florey, University of Melbourne*: Developing agonist/antagonist peptides for insulin/relaxin family peptide receptors.
11:30 – 11:45 am	**Johan Rosengren.** *University of Queensland* **–** Addressing receptor selectivity towards RXFP3 and RXFP4 using disulfide stabilised peptides mimicking relaxin-3.
11:45 – 12:00 pm	**Mark Grinstaff.** *Boston University***:** Minimally Invasive, Sustained-release Relaxin-2 Microparticles Reverse Arthrofibrosis.

12:00 – 12:15 pm	**Thomas Handley,** *The Florey, University of Melbourne:* Developing Single-B-Chain Specific Biologically Active Insulin-Like Peptide 5 (INSL5) analogues.
12:15 – 1:15 pm	Lunch buffet
1:15 – 2:15 pm	**Keynote speaker presentation: Dr. Juan Marugan, *NCATS, NIH* Optimization of RXFP1 agonists based in functional assays. Why signal is not good enough?**
2:15 – 3:15 pm	**Session 3: Relaxin receptors: Small molecule development**
	Session chairs: Dr. A. Agoulnik and Dr. T. Handley
2:15 – 2:35 pm	**Kenneth Granberg.** *AstraZeneca:* Discovery of AZD5462, The First Oral Small Molecule RXFP1 Agonist To Enter Clinical Trials.
2:35 – 2:55 pm	**Mark Henderson.** *NCATS, NIH, Bethesda:* Discovery of Selective Small Molecule Agonists of the Relaxin Family Receptor 2: An Emerging Therapeutic Target for the Treatment of Bone Loss.
2:55 – 3:15 pm	**Ross Bathgate.** *The Florey, University of Melbourne:* Cryo-EM structure of RXFP4 and development of small molecule RXFP3 and RXFP4 agonists.
3:15 – 3:45 pm	Coffee break
3:45 – 4:45 pm	**Session 4: Neurobiology**
	Session chairs: Dr. G. Dawe, Dr. T. Jayakody
3:45 – 4:00 pm	**Craig Smith.** *Deakin University:* Characterizing RXFP3-Expressing Neurons Using Transgenic RXFP3-Cre/Fluorophore Mice, And The Effects Of Blood-Brain Barrier Penetrating RXFP3-Acting Drugs On Rodent Brain And Behaviour.
4:00 – 4:15 pm	**Tharindunee Jayakody.** *National University of Singapore:* Biased RXFP3 Ligands and Receptor Dynamics: Tools to understand the neurobiology of RXFP3
4:15 – 4:30 pm	**Gavin Dawe.** *National University of Singapore, Singapore:* Exploring the Role of the Relaxin-3/RXFP3 System in Neuropsychiatric Disorders: A Genetic Risk Variant Study Using the UK Biobank
4:30 – 4:45 pm	**Monica Navarro-Sánchez.** *Universitat Jaume I:* Impact of Relaxin-3/ RXFP3 Signalling on Retrosplenial Cortex Processing of Contextual Fear Conditioning Acquisition and Extinction in Rats
5:00 – 6:00 pm	Dinner
6:30 – 7:30 pm	**Professional development session by Dr. Samantha Pauls, *College of Pharmacy, RFHS, University of Manitoba*: Forging your path: Lessons learned from careers in academia.**
7:30 – 9:00 pm	Networking

Tuesday, September 19

7:00 – 8:15 am	Breakfast
8:15 – 9:15 am	**Keynote speaker presentation: Drs. Leaf Huang & Menying Hu. *UNC, Chapel Hill*: Relaxin gene therapy against liver fibrosis: inspiration from spontaneous regression of fibrosis.**
9:15 – 10:20 am	**Session 5: Inflammation and cancer**
	Session chairs: Dr. S. Hombach-Klonisch and Dr. C. Burke
9:15 – 9:30 am	**Sabine Hombach-Klonisch.** *University of Manitoba:* C1q Tumor Necrosis Factor-Related Protein 8 (CTRP8) is a novel marker of myeloid-derived innate immune cells.
9:30 – 9:45 am	**Robert Rottapel.** *Princess Margaret Cancer Center, Toronto*: Inhibition of relaxin autocrine signaling confers therapeutic vulnerability in ovarian cancer.
9:45 – 10:00 am	**Anupam Kotwal.** *University of Nebraska Medical Center:* RLN2 Is A Novel Biomarker For Differentiated Thyroid Carcinoma In Humans.
10:00 – 10:20 am	**Thomas Klonisch.** *University of Manitoba:* C1qTNF related protein 8 (CTRP8) and RXFP1 promote distinct glioma pathologies in mice.
10:20 – 10:45 am	Coffee break
10:45 – 11:45 pm	**Session 6: Frontiers in the Biology of Relaxin and Related Peptides**
	Session chairs: Dr. C. Bagnell and Dr. T. Thanasupawat
10:45 – 11:00 am	**John Furness.** *University of Melbourne:* Role of INSL5 and RXFP4 In the control of colorectal function: Implications for Constipation and Diarrhea Treatment
11:00 – 11:15 am	**Caitlyn Burke.** *MS State Univ:* Synthetic mRNA-induced Expression of H2 Relaxin by Bovine Vaginal and Cervical Tissues
11:15 – 11:30 pm	**Hagen Nicolaus.** *Friedrich-Alexander-Universität Erlangen-Nürnberg:* Relaxin Receptor RXFP1 Is Targeted By Novel Interaction Partners CTRP1, CTRP6 And CTRP8 In Ocular Surface Wound Closure
11:30 – 11:45 pm	**Brian Wilson.** *Acadia University:* Anti-inflammatory and Neurogenic Actions Of Relaxin-3 In Zebrafish Following Traumatic Brain Injury
12:00 – 1:00 pm	Lunch buffet
1:00 – 1:15 pm	**Group photograph**
1:30 – 5:30 pm	**Lake Louise excursion**
6:00 pm	Dinner on your own
7:30 pm	**Tap room get-together**

<u>Wednesday, September 20</u>

7:00 – 8:30 am	Breakfast
8:30 – 9:45 am	**<u>Session 7</u>: Anti-fibrotic and matrix remodeling actions**
	Session chairs: Dr. R. Bennett and Dr. H. Ng
8:30 – 8:45 am	**Yifang Li.** *Monash University, Melbourne:* Engineering Bone Marrow-derived Stromal Cells To Deliver Anti-fibrotic Cargo As A Novel Treatment Option For Hypertensive Kidney Disease
8:45 – 9:00 am	**Chrishan Samuel.** *Monash University Melbourne:* Enhancing the therapeutic applicability of relaxin and related peptides
9:00 – 9:15 am	**Edward Rodriguez.** *Harvard Medical School, Boston*: Arthrofibrosis: Prevalence and Clinical Shortcomings
9:15 – 9:30 am	**Yingze Zhang.** *University of Pittsburgh School of Medicine:* Histone modification and transcriptional regulation of RXFP1 gene expression in lung fibroblasts
9:30 – 9:45 am	**Amanda Williamson.** *Boston University:* Investigation of Relaxin-2 As A Treatment for Hypertrophic And Keloid Scars.
9:45 – 10:15 am	Coffee break
10:15 – 11:40 pm	**<u>Session 8</u>: Cardiovascular Actions – Part 1**
	Session chairs: Dr. T. Dschietzig and Dr. A. Valentin
10:15 – 11:00 am	**Alana Aragón-Herrera**. *Health Research Institute of Santiago de Compostela (IDIS):* 'Metabolic Effects of Relaxin-2 At Cardiac Level And Its Potential As A Biomarker For Atrial Fibrillation
11:00 – 11:20 am	**Guillermo Romero.** *University of Pittsburgh School of Medicine:* Relaxin reduces inflammation, reverses fibrosis, and suppresses atrial fibrillation in aged hearts
11:20 – 11:40 am	**Xiaojun Wang**: *Lilly Research Laboratories Indianapolis:* Long-acting relaxin (LY3540378) demonstrated improved renal hemodynamics response in preclinical and clinical studies
11:40 – 12:30 pm	Lunch buffet
12:30 – 1:40 pm	**<u>Session 9</u>: Cardiovascular Actions – Part 2**
	Session chairs: Dr. G. Salama and Dr. A. Aragón-Herrera
12:30 – 12:50 pm	**Philipp Schlegel.** *University of Heidelberg:* RXFP1 Gene Therapy for Treatment of Pressure Overload Induced Chronic Heart Failure
12:50 – 1:05 pm	**Ana Valentin.** *Florida International University:* Therapeutic effects of relaxin receptor agonist ML290 vascular calcification in combination with lifestyle intervention in a model of atherosclerosis

1:05 – 1:25 pm	**Kathleen Connolly**. *AstraZeneca:* Phase 1 Safety, Pharmacokinetics, and Pharmacodynamics of the RXPF1 Agonists AZD3427 and AZD5462 in Healthy Volunteers and Heart Failure Patients.
1:25 – 1:40 pm	**Guy Salama.** *University of Pittsburgh:* Relaxin reverses diastolic dysfunction in heart failure with preserved ejection fraction
1.45 pm – 3 pm	**Poster session and Discussion**
2:30 – 3:00 pm	Coffee and nibbles
3:00 – 5:30 pm	**Business meeting:** discussion on future Relaxin meeting
6:00 – 7:30 pm	**Farewell Dinner** (Crocus room)
7:30 – 8:30 pm	**"Researcher and Global Citizen" Dr. Alastair Summerlee, *Sprott School of Business:* Who knows where studying relaxin can take you? Or a string of beads, a handful of dust and an iron fish.**
8:30 pm	**Poster and presentation prizes & trainee travel awards**, closing remarks and conference drinks.

Thursday, September 21

7:30 – 9:00 am	Breakfast on your own

ABSTRACTS: KEYNOTE SPEAKERS

Structural basis for RXFP1 activation

Andrew Kruse Ph.D. *Harvard Medical School, Boston, MA, USA*

The relaxin family peptide receptor 1 (RXFP1) is the receptor for relaxin-2, an important regulator of reproductive and cardiovascular physiology. RXFP1 is a multi-domain G protein-coupled receptor (GPCR) with an ectodomain consisting of a low-density lipoprotein receptor class A (LDLa) module and leucine-rich repeats. The mechanism of RXFP1 signal transduction is clearly distinct from that of other GPCRs, but remains very poorly understood. We determined the cryo-electron microscopy structure of active-state human RXFP1 bound to a single-chain analog of the endogenous agonist relaxin-2 and the heterotrimeric G_s protein. Evolutionary coupling analysis and structure-guided functional experiments reveal that RXFP1 signals through a mechanism of autoinhibition by the ectodomain. Our results explain how this unusual GPCR functions, providing a path to rational drug development targeting the relaxin receptors.

ABSTRACTS: KEYNOTE SPEAKERS

Optimization of RXFP1 agonists based in functional assays. Why signal is not good enough?

Courtney B. Myhr[2]; Kenneth J. Wilson[1]; Noel Southall[1], Phillip Sanderson[1]; Alexander I. Agoulnik[2]; Irina Agoulnik[2]; Paul B. Yu[3]; Mark Henderson[1]; Juan Jose Marugan[1]

1.- National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD.

2.- Department of Human and Molecular Genetics, Herbert Wertheim College of Medicine, Florida International University, Miami, FL.

3.- Division of Cardiology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.

Animal efficacy studies using recombinant RNL2 hormone and H2 relaxin analogues have validated RXFP1 agonists as a promising therapeutic modality for multiple diseases including numerous fibrotic disorders, organ transplant, pulmonary arterial hypertension and cancer. Our screen and optimization efforts resulted in the discovery of the first small molecule agonist of RXFP1, known as ML290. However, signaling and pharmacological studies have shown the complexity and peculiarities of RXFP1 activation for both peptides and small molecule agonists. For example, in a cell-type dependent manner, RNL2 activates multiple signaling pathways that can up- or downregulate cAMP levels, while ML290 is a biased agonist. Importantly, while signaling assays enable large HTS campaigns for GPCR's and can facilitate initial structure activity relationship (SAR) studies, usually these types of assays are not adequate for selecting the most pharmacologically promising agonists. Indeed, evaluation of our best RXFP1 cAMP agonists using cellular functional assays and in vivo efficacy studies show a poor correlation between them. To address these hurdles, our team has developed several cellular functional assays adaptable to medium throughput screens, which allows the proper rank ordering of RXFP1 agonists and facilitates the selection of a preclinical development candidate.

ABSTRACTS: KEYNOTE SPEAKERS

Relaxin gene therapy against liver fibrosis: inspiration from spontaneous regression of fibrosis

Mengying Hu Ph.D., **Leaf Huang, Ph.D.**, *Weill Cornell Medicine, New York, New York, United States*

Liver is a vital organ for the sustenance of life due to its major functions of detoxification and protein synthesis. Therefore, liver diseases, mainly including liver fibrosis and malignancy, are the leading health threat throughout the world. Efficient treatments for liver fibrosis and liver metastasis (the most aggressive liver malignancy) are still limited nowadays. Tracing back the root of both diseases has revealed the fundamental role of activated hepatic stellate cells (aHSCs) for the initiation and progression in both diseases. We discovered that spontaneous resolution of liver fibrosis is mediated by endogenously secreted relaxin (RLN), which regulates cooperation between macrophages and aHSCs and leads to aHSC deactivation. Therefore, introduction of exogenous RLN has a great potential to treat both liver fibrosis and liver metastasis. However, RLN has a rather short half-life (~10min) and its receptor is widely expressed throughout the body, which makes systemic application of recombinant RLN inefficient and prone to side effects. Alternatively, targeted delivery of RLN gene by using lipid nanoparticles coupled to the Sigma-1 receptor established a local depot of RLN at the diseased site in the liver. This talk will demonstrate robust stromal and immune microenvironment remodulation effect of targeted RLN gene therapy in liver fibrosis and multiple liver metastasis models, emphasizing the potential of RLN gene therapy to overcome major hurdles of recombinant protein therapy.

ABSTRACTS ORAL PRESENTATIONS

SESSION 1

Overview Of Relaxin Receptor Family Therapeutic Targeting.

Ross A.D. Bathgate[1,2], **Daniel J. Scott**[1,2], **Martina Kocan**[1], **Bradley L. Hoare**[1], **Christopher Draper-Joyce**[1,2], **Paul R. Gooley**[2] **and Mohammed Akhter Hossain**[1,3]. *[1]The Florey, [2]Department of Biochemistry and Pharmacology, [3]School of Chemistry, University of Melbourne, Parkville, Victoria 3010, Australia.*

Relaxin, insulin-like peptide 3 (INSL3), relaxin-3, and INSL5 are the cognate ligands for the relaxin family peptide (RXFP) receptors 1–4, respectively. Each ligand-receptor pairing has evolved distinct physiological actions and there is considerable interest in targeting all four receptors for therapeutic development. This presentation will provide an overview of academic and industry work developing therapeutics to target relaxin family peptide receptors.

AZD3427- Engineering and Characterisation of a Long-acting Relaxin Fusion

Monika A Papworth[1], **Isabelle Sermadiras**[1], **Judy Paterson**[1], **Esther Martin**[1], **Marcin Wolny**[1], **Weidong Hao**[2], **Agnieszka Sadowska**[2], **Ruoyan Chen**[2], **Weike Bao**[2], **Denison Kuruvilla**[3], **Junhui Sun**[4] **and Elizabeth Murphy**[4]
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Introduction: AZD3427 is a novel therapeutic protein fusion consisting of a half-life extending Fc and the Relaxin-2 heterodimer, which is engineered to closely resemble the structure of the natural relaxin hormone. AZD3427 is designed to improve the pharmacokinetics and stability of human Relaxin-2, while maintaining its *in vitro* and *in vivo* pharmacology profile. AZD3427 is currently in clinical development.

From the Synthesis of Long-Acting Single Chain Peptide Agonists of RXFP1 to Therapeutic Candidates for Renal and CV diseases

Elisabetta Bianchi[1], Bruno Poirier[3], Olivier Pasquier[4], Edith Monteagudo[2], Raffaele Ingenito[1], Laurence Riva[3], Xavier Chenede[3], Simone Esposito[2], Laurence Gauzy-Lazo[5], Olivier Duclos[5], Philip Janiak[3], Sergio Mallart[5], and Stephane Illiano[3]. *IRBM, [1]Peptide Chemistry, [2]DMPK/ADME, via Pontina km 30,600, 00071, Pomezia (Roma), Italy; Sanofi R&D, [3]Cardio-Vascular and Metabolism, [4]DMPK, [5]Integrated Drug Discovery, Chilly Mazarin 91385 and Vitry sur Seine 94400, France.*

Introduction: Based on recent clinical trials, Relaxin-2 may offer therapeutic benefits in the treatment of heart failure. However the short half-life and the need for IV administration precludes its use for long-term treatment.

Objective: Identify long-lasting RXFP1 agonists suitable for self-subcutaneous administration in patients with subacute heart failure.

Methods: Extensive structural activity relationship studies were accomplished by multiple cycles of design and synthesis of peptide analogues of Relaxin-2 B chain followed by screening for RXFP1 agonistic activity in cellular assays. *In vitro* metabolic stability assessment and *in vivo* PK studies were instrumental to optimize the pharmacokinetic profile and bioavailability as QD candidate. A mast-cell degranulation assay was used to assess potential liabilities for pseudo-allergic reactions induced by subcutaneous administration of peptide candidates.

Results: More than 800 analogues were designed and synthesized to obtain potent and long-lasting RXFP1 agonists. One first peptide preclinical candidate showed extensive unexpected oxidative metabolism in pre-clinical species which supported further modifications to obtain the clinical candidate R2R01. Preliminary phase 1 clinical studies suggested that R2R01 showed the expected PK profile and bioavailability.

Conclusions: All results obtained so far support further transition towards phase 2 clinical studies of peptide R2R01 as RXFP1 peptide agonist for the treatment of renal and HF diseases.

Ongoing Attempts To Understand the Molecular Mechanisms By Which B7-33 And H2-relaxin Differently Bind And Activate RXFP1

Bradley L. Hoare[1], Mohammed A. Hossain[1], Ross A.D. Bathgate[1]
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Introduction: The complex two-chain structure of relaxin family peptides is a significant challenge for their large-scale production for use as therapeutics and as research tools. We have shown that a single chain analogue of the B-chain of H2-relaxin, called B7-33, is an agonist at RXFP1 and can replicate the potent cardioprotective and anti-fibrotic actions of H2-relaxin in various relevant *in vitro* and *in vivo* models. B7-33 shows functional selectivity at RXFP1, meaning that there are differences in the functional outcomes of RXFP1 signalling compared to H2-relaxin stimulation of RXFP1. A notable example is that H2-relaxin enhances prostate tumour growth, whereas B7-33 does not. However, it is unclear how B7-33 can elicit different cellular responses compared to H2-relaxin when activating RXFP1, and how their mode of RXFP1 interaction might differ.
Objective: To investigate whether H2-relaxin and B7-33 stimulation of RXFP1 results in the activation of different G proteins and to use biophysical techniques to understand the differences in how H2-relaxin and B7-33 each bind to the extracellular binding domain of RXFP1.
Methods: HEK293T cells overexpressing RXFP1 were transfected with different biosensors which report on G protein activation, covering 10 different types of G protein (s, i1, i2, i3, oA, oB, z, q, 13, 15), and concentration-response curves were generated for H2-relaxin and B7-33. Additionally, the extracellular domain of RXFP1 (ectoRXFP1) was produced and purified as a soluble protein for downstream biophysical studies to uncover the binding mode of B7-33 compared to H2-relaxin.
Results: RXFP1 was able to activate all 10 different G protein biosensors when stimulated by H2-relaxin or B7-33, however there was no apparent functional selectivity to suggest that B7-33 can activate one particular G protein over another in HEK293T cells. Purification of functional ectoRXFP1 protein was successful and we have used biolayer interferometry (BLI) to characterise its binding to H2-relaxin and B7-33.
Conclusion: We did not find evidence that B7-33 stimulation of RXFP1 differentially activates G proteins in HEK293T cells. Current work is focused on using biophysical techniques such as hydrogen-deuterium exchange (HDX) to model the similarities and differences by which H2-relaxin and B7-33 interact with the extracellular domain of RXFP1.

SESSION 2

Developing Agonist/Antagonist Peptides for Insulin/Relaxin Family Peptide Receptors

Praveen Praveen[1], Mengjie Liu[1], Ruslan V Pustovit[1], Thomas NG Handley[1], Hongkang Wu[1], K. Johan Rosengren[2], Chrishan Samuel[3], John B Furness[1,4], Ross AD Bathgate[1], Mohammed Akhter Hossain[1]
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Introduction: Insulin and insulin-like relaxin peptides, the natural ligands for the insulin receptor (IR) and relaxin family peptide receptors (RXFPs) respectively, have diverse physiological functions.
Objective: The aims of these studies were to develop agonist and antagonist peptides as research tools and drug leads with improved pharmacokinetic properties.
Methods: We rationally designed and chemically synthesized simplified or single-chain agonists and antagonist peptides for the IR and RXFP1, RXFP3, and RXFP4. They have been tested in cells expressing the receptors and in appropriate animal models.
Results: In the past five years, we developed chemical methods to prepare a single-B-chain memetic agonist for (A) RXFP1 (a strong anti-fibrotic agent); (B) RXFP3 (an analgesic); (C) RXFP4 (an anti-constipation agent). We also developed an antagonist for (D) RXFP1 which can treat prostate cancer and (E) RXFP4 which can reverse diarrhea-like symptoms in pre-clinical animal models. We also developed insulin molecules that are long-acting and non-fibrillating.
Conclusions: This presentation will give an overview of the design of peptidomimetics and the development of novel chemical methods for preparing therapeutic insulin and insulin-like peptides.

ADDRESSING RECEPTOR SELECTIVITY TOWARDS RXFP3 AND RXFP4 USING DISULFIDE STABILISED PEPTIDES MIMICKING RELAXIN-3

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Introduction: Data highlighting the involvement of RXFP3/relaxin-3 in the control of stress, feeding, addiction, and neuroendocrine function, and INSL5/RXFP4 in gut function, suggest that these signalling systems are valid targets for treatment of neurological and gut disorders. However, the pharmacological modulation of these receptor is challenging, given their native ligands are two-chain peptide requiring complex synthesis and are poorly selective for their specific receptors. Extensive structure activity data collected over more than a decade have identified key interactions between these ligands and their receptors, and recently the cryo-EM structure of the INSL5/RXFP4 complex was solved. Key residues are all located in the B-chain, and consequently we have focussed on developing single chain variants based on the B-chain.
Objective: We wanted to explore molecular grafting of key residues into the disulfide peptide apamin, which is structurally similar to the relaxin-3 B-chain.
Methods: Apamin grafted variants containing relaxin-3 residues were synthesized using solid phase peptide chemistry and evaluated for binding and activation of RXFP3 and RXFP4 using cell-based assays and for stability using serum assays.
Results: Grafting in conjunction with point mutations further stabilising structure using natural and non-natural amino acids generates high affinity and potency analogues for both RXFP3 and RXFP4. Modification of the Gly23-Gly24 linker in the C-terminal tail can control the conformation of the Arg26-Trp27 activation domain to favour either RXFP3 or RXFP4. All variants are highly stable in serum.
Conclusions: Grafting offers an attractive avenue for creating highly stable and potent analogues that are easy to synthesise and which can be tuned to favour RXFP3 or RXPF4 by modifications in the C-terminal linker region.

Minimally invasive, sustained-release relaxin-2 microparticles reverse arthrofibrosis

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Introduction: Substantial advances in biotherapeutics are distinctly lacking for musculoskeletal diseases. Musculoskeletal diseases are biomechanically complex and localized, highlighting the need for novel therapies capable of addressing these issues. All frontline treatment options for arthrofibrosis, a debilitating musculoskeletal disease, fail to treat the disease etiology – the accumulation of fibrotic tissue within the joint space. For millions of patients each year, the lack of modern and effective treatment options necessitates surgery in an attempt to regain joint range of motion (ROM) and escape prolonged pain. Human relaxin-2, an endogenous peptide hormone with antifibrotic and antifibrogenic activity, is a promising biotherapeutic candidate for musculoskeletal fibrosis. However, relaxin-2 has previously faltered through multiple clinical programs due to pharmacokinetic barriers. Here, we describe the design and in vitro characterization of a tailored drug delivery system for the sustained release of relaxin-2.

Objective: To evaluate the performance of a sustained-release relaxin-2 microparticle formulation for the treatment of arthrofibrosis.

Methods: Mechanism of action and cell studies were performed in *in vitro* 2D culture using primary human fibroblast-like synoviocytes. Human fibroblasts were treated with TGF-β1 and relaxin-2, and key players in the TGF-β1 and RLX-2 signaling pathways were investigated using western blot and RT-qPCR. Relaxin-2 was subsequently encapsulated in biocompatible microparticles composed of PLGA. We next performed a pharmacokinetic study in healthy Sprague Dawley rats using relaxin-2 radiolabeled with ^{125}I. Finally, we assessed the *in vivo* efficacy of relaxin-2-loaded microparticles utilizing a previously validated atraumatic rat model of shoulder arthrofibrosis.

Results: Relaxin-2-loaded, polymeric microparticles release relaxin-2 over a multi-week timeframe without altering peptide structure or bioactivity. *In vivo*, intraarticular administration of relaxin-2 loaded microparticles in rats results in prolonged, localized concentrations of relaxin-2 with reduced systemic drug exposure. Further, a single injection of relaxin-2-loaded microparticles restores joint ROM and architecture in an atraumatic rat model of arthrofibrosis with clinically derived endpoints. Finally, confirmation of relaxin-2 receptor expression, RXFP1, in multiple human tissues relevant to arthrofibrosis suggests the potential for rapid clinical translation of relaxin-2 when administered in a sustained and targeted manner.

Conclusion: Intraarticular injection of relaxin-2-loaded polymeric microparticles restores joint range of motion and reverses arthrofibrosis in rats.

Developing Single-B-Chain Specific Biologically Active Insulin-Like Peptide 5 (INSL5) analogues

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Constipation is an unappreciated but common gastrointestinal motility disorder that affects the general population, with a high prevalence in senior people and those with neurological disorders. Current therapies are inefficient and have adverse side effects. Therefore, there is an urgent need to develop new pharmacological drug leads for the treatment of constipation. Insulin-like peptide 5 (INSL5), the natural ligand for the relaxin family peptide receptor 4 (RXFP4), is a gut hormone that is produced by the endocrine cells of the colon[2]. Our laboratory has recently discovered that RXFP4 agonists have the potential to treat constipation. Our laboratory previously generated biologically active novel INSL5 analogues with simpler structures. While the simplified INSL5 analogous are easier to synthesize (yield ~14%) compared with native INSL5 (yield 0.8%), they still possess a complex two-chains and two-disulfide bond structure (Figure 1). Previous structure-activity data suggest that the B-chain of INSL5 contains all the key residues for RXFP4 binding and activity. Therefore, we hypothesize that it is possible to generate B-chain-specific bioactive INSL5 analogues. Here I present new designs and chemical approaches to produce B-chain-specific biologically active INSL5 analogues. A series of analogues were developed and one of these analogues was high yielding (41%) that exhibited high affinity and cAMP potency (~100 nM) at RXFP4 *in vitro.* Importantly, this single B-chain analogue was shown to reverse opioid-induced constipation in mice with similar efficacy to the currently available two-chain lead peptide, INSL5-A13. This new compound, therefore, is an important research tool and drug template for the potential treatment of constipation.

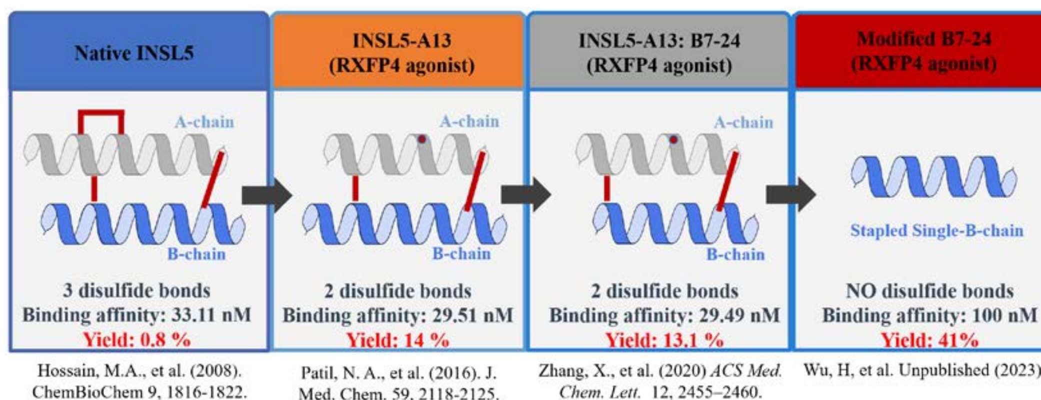

Figure 1: Development of simplified analogues of INSL5

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SESSION 3

Discovery Of AZD5462, The First Oral Small Molecule RXFP1 Agonist To Enter Clinical Trials.

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Introduction: An orally administered small molecule (SM) RXFP1 agonist could potentially overcome some of the shortcomings of infused relaxin-H2 such as the short plasma half-life. However, RXFP1 has been challenging to target by SM. At the start of our work there was but one notable exception, a series of anthranilic anilides as exemplified by ML290 in the pioneering work of Xiao et al. (2013).
Objective: To identify SM agonists of RXFP1 with balanced properties enabling oral administration and clinical evaluation for treatment of heart failure.
Methods: Several SM hit identification approaches were pursued including two corporate SM HTS-campaigns, relaxin-H2 beta-chain peptide stapling and virtual screening. We also set ML290 as a starting point for a traditional, iterative lead generation and optimization effort.
Results: SM optimization of a beta amino acid subseries led to identification of AZD5462 as a potent, selective RXFP1 agonist, which was well tolerated in toxicological studies in both rat and cynomolgus monkey. Like relaxin-H2, AZD5462 acutely increased blood pressure and heart rate in rat. Most importantly, significant improvements of cardiac ejection fraction were observed in aged, obese cynomolgus diagnosed with heart failure with reduced ejection fraction. AZD5462 mimicked relaxin-H2 in the signaling pathways downstream of RXFP1, was highly selective over RXFP2, and demonstrated species-dependent RXFP1 (cAMP) potency as similarly reported for ML290.
Conclusion: AZD5462 was identified as an oral, safe, potent and selective agonist of human RXFP1 and is currently in clinical development for treatment of heart failure.

Discovery of Selective Small Molecule Agonists of the Relaxin Family Receptor 2: An Emerging Therapeutic Target for the Treatment of Bone Loss.

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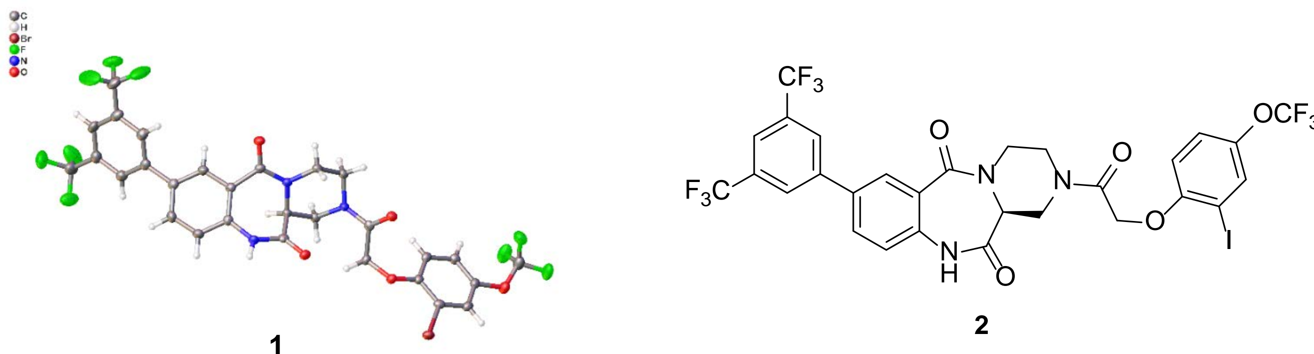
Introduction: The relaxin/insulin-like family peptide receptor 2 (RXFP2) belongs to the class A family of G-protein coupled receptors (GPCRs) and it is the only known target for the insulin-like factor 3 peptide hormone (INSL3). The importance of this ligand-receptor pair in the development of the gubernacular ligament during the transabdominal phase of testicular descent is well established. More recently, RXFP2 has been implicated in maintaining healthy bone formation.

Objective: To design an orally bioavailable specific small molecule agonist of RXFP2. The desired compound would display high specificity, oral bioavailability in target tissues, and show biological effects similar to that of native INSL3 hormone.

Methods: A quantitative high throughput screen (qHTS) of an NIH small molecular library was performed in HEK293 cells transfected with human RXFP2 receptor to identify compounds that modulate cAMP response. Counter-screens using parental cells and cells transfected with related receptors were performed. The initial hits were subjected to an extensive structure-activity campaign and functional studies *in vitro* and *in vivo*.

Results: The qHTS of > 120,000 compounds identified compound 1 with modest agonist activity and efficacy. An extensive structure-activity study was undertaken to optimize the potency, efficacy, and physical and metabolic properties of the series, resulting in the identification of compound 2 which has excellent *in vivo* PK properties with high levels of systemic tissue exposure. This series, exemplified by *S*-enantiomer 2, are first-in-class small-molecule selective agonists of RXFP2 that exhibit high specificity and preferred pharmacology. These compounds induced INSL3-like effects on gubernaculum differentiation in female embryos, increased human osteoblast mineralization *in vitro*, and demonstrated bone anabolic properties in female mice.

Conclusion: We have identified a first-in-class series of potent and specific agonists of RXFP2. The described RXFP2 agonists are orally bioavailable and display favorable pharmacokinetic properties, which allows for future evaluation of the therapeutic benefits of modulating RXFP2 activation in disease models.



Cryo-EM Structure Of RXFP4 And Development Of Small Molecule RXFP3 And RXFP4 Agonists.

Guangyao Lin[1], Yan Chen[2], Qingtong Zhou[2], Jiang Wang[3], Chenghao Li[3], Yang Feng[1], Xiaoqing Cai[1], Caihong Zhou[1], Lin Lin[1], Xiao-qing Cai[1], An-tao Dai[1], Yue Zhu[1], Jie Li[1], Lijun Shao[1], Yan Chen[1], Linhai Chen[1], Qing Liu[1], Qingtong Zhou[1], Ross A.D. Bathgate[4,5], H. Eric Xu[6], Hong Liu[3], Dehua Yang[1], Ming-Wei Wang[1,2,5]. *[1]The National Center for Drug Screening, [3]State Key Laboratory of Drug Research, [6]The CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China; [2]Department of Pharmacology, School of Basic Medical Sciences, Fudan University., Shanghai, China; [3]The Florey, and [4]Department of Biochemistry and Pharmacology, University of Melbourne, Parkville, Victoria 3010, Australia.*

Studies on the relaxin-3 and INSL5 receptors, RXFP3 and RXPF4, have been limited by the lack of specific small molecule agonists. Furthermore, development of such agonists would be aided by access to high resolution structures of the ligand-receptor complexes. High throughput screening of RXFP3 and RXFP4 for small molecule agonists was conducted at the National Center for Drug Screening in Shanghai, China. For both receptors screening was conducted in CHO cells stably expressing the receptors with the readout being inhibition of forskolin-induced cAMP signalling. RXFP3 screening was against 32,021 synthetic and natural product derived compounds and for RXFP4 52,000 compounds were screened. Only one RXFP3 hit compound was identified, WNN0109-C011, which was a full agonist of RXFP3 with micromolar potency while demonstrating 10-fold lower agonist activity at RXFP4. Initial screening of RXFP4 discovered 3 hits all with micromolar potency and minimal activity on RXFP3. To progress development of high affinity agonists of RXFP4 the cryo-EM structures of RXFP4–Gi protein complexes in the presence of INSL5 and small molecule agonists were solved. The small molecule agonist DC591053 was developed from the initial RXFP4 hit compound and is RXFP4 specific. The dual RXFP3/RXFP4 agonist compound 4 was developed by Novartis. The structures highlighted the predominant role of the INSL5 B-chain in binding to RXFP4 with the extended α-helix of the B-chain penetrating the orthosteric pocket to allow key interactions of the C-terminal Trp-24 to mediate receptor activation. Small molecule structures demonstrated both common and unique features of the two small molecule agonists. Both small molecules structurally mimicked Trp-24 binding in a manner similar to INSL5 and the varying extents to which they contact RXFP4-specific residues form the foundation that governs receptor subtype selectivity. The further structure-guided optimization of DC591053 towards nanomolar potency should be feasible with the support of the near-atomic level structural information.

SESSION 4

Characterizing RXFP3-Expressing Neurons Using Transgenic RXFP3-Cre/Fluorophore Mice, And The Effects Of Blood-Brain Barrier Penetrating RXFP3-Acting Drugs On Rodent Brain And Behaviour.

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Introduction: Two strains of transgenic mice have recently been developed to express fluorophores (tdTomato or YFP) within RXFP3-expressing cells. Although powerful, these strains have only been used to investigate RXFP3-expressing neurons within a small number of brain regions. In Aim 1 of this study, we expand this analysis and examine RXFP3 within other key regions of the brain. Furthermore, rodent studies targeting RXFP3 with peptide relaxin-3 mimetics have required direct brain injection due to low blood-brain barrier (BBB), permeability. In Aim 2, we assess the effects of recently developed BBB-penetrating RXFP3-acting drugs on mouse brain and behaviour.

Objectives: Aim 1: To validate RXFP3-Cre/fluorophore mice and use them to help determine the neurochemical identity of specific populations of RXFP3-expressing neurons within the brain. Aim 2: To characterise the behavioural effects of newly developed BBB-penetrating RXFP3-acting drugs, and gain insights into their neuronal mechanisms of action.

Results: Aim 1: Both RXFP3-Cre/tdTomato and RXFP3-Cre/YFP mice displayed neuroanatomical distributions of fluorophore that were similar to a previously published mapping of RXFP3 mRNA, however some discrepancies (e.g. within the cerebral cortex) were present. Within the hypothalamic arcuate nucleus, a small proportion of RXFP3-expressing cells co-expressed the dopamine marker, tyrosine hydroxylase. Aim 2: Intraperitoneal injection of two different RXFP3 antagonist peptides (0.5 – 2.0 µmol/kg), modified to cross the BBB, reduced palatable food consumption during the hour after injection in wildtype mice, but not in RXFP3 knockout mice. Transcriptome sequencing of the hypothalamus revealed changes in several gene sets and pathways that are associated with feeding, such as GLP-1 signalling, in wildtype mice injected with RXFP3 antagonist versus saline controls.

Conclusions: Aim 1: The validation of RXFP3-Cre/fluorophore mice will facilitate further studies of RXFP3-expressing cells. Our findings using these mice suggest that RXFP3 may regulate prolactin release, by modulating dopaminergic neurons within the arcuate nucleus. Aim 2: Peripheral injection of BBB-penetrating RXFP3-acting drugs within rodents represents one of the most powerful and clinically relevant methods for exploring the function and therapeutic potential of RXFP3. Our study highlights the ability of RXFP3 antagonists to reduce feeding by influencing hypothalamic satiety pathways and paves the way for future studies which investigate the influence of RXFP3-acting drugs on stress, arousal, and other relevant parameters in both naïve and disease model rodents.

Biased RXFP3 Ligands And Receptor Dynamics: Tools To Understand The Neurobiology Of RXFP3.

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Introduction: In this study, we aim to understand the mechanisms of biased signaling at RXFP3. Such biased agonists would be instrumental in elucidating molecular mechanisms behind the diverse neurobiological functions of the relaxin-3 mediated RXFP3 activation.
Objective: To determine the mechanisms of possible G-protein or beta-arrestin biases in signaling at RXFP3.
Methods: Concentration-response curves for the activation of $G\alpha_{i/o}$ and β-arrestin ½ were generated for H3 relaxin and known RXFP3 agonists using quantitative signaling assays and HEK-RXFP3 cells. Using H3 relaxin as a reference ligand, bias factors were calculated for each ligand. THE GRK subtype bias was determined using GRK2/3/5/6 KO HEK cells, upon transfecting the cells with each GRK subtype. Moreover, using homology modelling approaches, RXFP3-ligand complexes were modeled. These complexes were subjected to molecular dynamics simulations and the simulation trajectories were used in determining receptor-ligand interactions and dynamics.
Results: In this study, we observed that the stapling position of the relaxin-3 B chain stapled peptides may result in $G\alpha_{i/o}$ bias at RXFP3. The biased peptides did not recruit β-arrestin1/2 at the presence of GRK2/3/5/6. However, the endogenous ligand relaxin-3 did not show bias towards either pathway. Our in-silico experiments revealed that N-terminal residues of the B-chain of relaxin-3 interacted with ECL3, of RXFP3, resulting in pushing ECL3 away from the binding pocket. As a result, TM6 and TM7 of RXFP3 adopted open confirmations at the intracellular side, allowing GRK2/3 mediated recruitment of β-arrestins. However, the staple linker of biased stapled peptides did not interact with RXFP3 to induce the above conformational changes.
Conclusions: The relaxin-3 A-chain plays a role in inducing conformational changes in RXFP3, which leads to GRK2/3 mediated β-arrestin recruitment. In contrast, biased ligands studied did not induce such changes in the receptor. This knowledge will be useful in designing biased ligands to selectively activate certain signaling pathways leading to specific neurobiological functions of RXFP3.

Exploring the Role of the Relaxin-3/RXFP3 System in Neuropsychiatric Disorders: A Genetic Risk Variant Study Using the UK Biobank

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Introduction: The relaxin-3/RXFP3 system, a prominent neuropeptidergic network implicated in modulating behavioural alterations associated with clinical depression and anxiety, has been identified as a prospective target for therapeutic intervention. However, a systematic review assessing its role in these disorders yielded inconclusive results, highlighting a deficit of comprehensive clinical studies, particularly in human subjects.
Objective: The current investigation aims to address this research gap by conducting a retrospective analysis of genetic risk variants associated with the relaxin-3/RXFP3 system in the UK Biobank.
Methods: Implementing a candidate gene approach, we examined potential associations between single nucleotide polymorphisms (SNPs) integral to relaxin-3 signalling and several outcomes such as depression, atypical depression, anxiety, and metabolic syndrome. In silico tools were employed to identify a set of candidate SNPs associated with relaxin-3 signalling, and rigorously defined phenotypes were established for each outcome. The analysis incorporated subsample sizes spanning from 85,881 to 386,769 participants.
Results: The study found no associations between any candidate SNP and outcome phenotypes, even after adjusting for multiple testing burdens. Additionally, regression models integrating several SNPs per candidate gene as exploratory variables failed to predict any outcome.
Conclusion: These findings underscore the restricted utility of candidate gene approaches in genetic research for neuropsychiatric disorders, even when grounded in compelling biological hypotheses. This study serves to reaffirm the necessity for additional, high-quality human research to gain a more in-depth understanding of the role of the relaxin-3/RXFP3 system in neuropsychiatric disorders.

Impact of Relaxin-3/RXFP3 Signalling on Retrosplenial Cortex Processing of Contextual Fear Conditioning Acquisition and Extinction in Rats

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Introduction: The retrosplenial cortex (RSC) is a target of relaxin-3 (RLN3)-positive fibers arising from the pontine, nucleus incertus. Therefore, this RLN3 projection may participate in, or modulate, the functions associated with the RSC via the RLN3 receptor, RXFP3. The RSC is a pivotally positioned between sensory cortical areas and the hippocampus and is involved in the generation of spatial cognitive maps via head direction cells. RSC also participates in contextual-related processes, including context fear conditioning, which is affected by nucleus incertus manipulations. Thus, our hypothesis is that RLN3/RXFP3 signalling can regulate contextual processing in the RSC.

Objective: We examined the effect of acute or sustained RXFP3 activation with RXFP3 agonists (RXFP3-A2 and AAV1/2-FIB-R3/I5) before acquisition or extinction to study their effects on RSC-related context fear conditioning. We also examined the expression of RXFP3 mRNA in excitatory (vGLUT1 mRNA) and inhibitory (vGAT mRNA) neurons in different layers of the RSC.

Results: Using the AAV1/2-FIB-R3/I5 vector that constitutively produced and secreted the R3/I5 agonist into the RSC, we assessed any impairment in the context fear conditioning processes of acquisition, retrieval and extinction. AAV1/2-FIB-R3/I5 injected rats acquired and retrieved fear memory normally, but extinguished significantly slower than AAV1/2 control vector injected rats. We then deciphered if the impaired extinction was due to an effect on the acquisition or an effect on extinction itself by injecting the selective RXFP3 agonist, RXFP3-A2 (A2) into the RSC either before acquisition or before each extinction trial; and observed that rats injected with A2 before acquisition extinguished significantly slower than aCSF injected rats, although their acquisition was normal. In contrast, rats injected with A2 before each extinction trial displayed a normal extinction not significantly different to aCSF-injected rats. In our neurochemical studies, we observed that inhibitory (vGAT mRNA-positive) neurons accounted of a higher proportion of RXFP3 mRNA-positive cells than excitatory (vGLUT1 mRNA-positive) neurons. RXFP3 mRNA expressing neurons were concentrated in deeper layers IV-VI of the RSC, which contain a high density of RLN3 fibers in both the granular (area 29) and dysgranular (area 30) regions of the RSC. Thus, it is predicted that the main effect of RXFP3 activation will be a reduction in the inhibition by RSC inhibitory neurons on the populations of excitatory neurons that are concentrated in cortical layers II/III.

Conclusion: These results identify the ability of pharmacological RXFP3 activation to modify the acquisition process and make it more resistant to extinction, an effect which is likely mediated by a reduction of the inhibition by interneurons of the deep layer RSC neurons. Thus, endogenous RLN3/RXFP3 signalling may drive such plasticity in contextual related processes under specific physiological or pathological conditions.

SESSION 5

C1q Tumor Necrosis Factor-Related Protein 8 (CTRP8) is a novel marker of myeloid-derived innate immune cells.

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Introduction: Relaxin is a known modulator of innate immune cell functions. Human RXFP1 (Relaxin Family Peptide Receptor 1) is expressed in cells of the myelo-monocytic lineage as well as in breast and prostate cancer cells. Tumor-associated macrophages in the tumor microenvironment have important supportive roles in angiogenesis and cancer cell invasiveness. Recently, we have identified CTRP8 as a novel marker of a tryptase+ mast cell subpopulation in prostate cancer tissues. Activation of RXFP1 by CTRP8 led to increased proliferation and attenuated degranulation of the human mast cell line ROSA$^{kit\ WT}$. Currently, the role of CTRP8 on innate immune cell functions in the breast tumor microenvironment is unknown.
Objective: Our study had two major objectives: (1) determine the abundance of CTRP8-positive innate immune cells in human breast cancer tissues; (2) investigate the impact of RXFP1 ligands on M1 and M2 differentiation in the RXFP1+ monocytic cell model THP1.
Methods: Human breast and prostate cancer tissue microarrays were employed to identify CTRP8 expression in Tryptase+ mast cells and CD68+ macrophages by immunohistochemistry. The human monocytic cell line THP1 was differentiated into M1 and M2 polarized macrophages and exposed to CTRP8 or the small molecule RXFP1 agonist ML290. Cytokine gene expression changes and alterations in the secretion of cytokines were quantified. Recombinant human CTRP8 was produced in E. coli. The RXFP1 expressing human mast cell line ROSA KIT WT was exposed to CTRP8, RLN-2 and ML290 to assess changes in mast cell degranulation. CTRP8 expression human TNBC and prostate cancer cell lines was determined by immunocytochemistry.
Results: Tryptase+ mast cells in breast and prostate cancer tissues express CTRP8. In addition, CTRP8 colocalizes with CD68+ macrophages in breast cancer tissues. CTRP8+ cells were significantly more abundant in triple-negative breast cancer tissues (TNBC) than in breast cancer tissues expressing the hormone receptors ER, PR, HER2. TNBC tissues had significantly higher number of CTRP8+/tryptase+ mast cells compared to ER/PR/HER2+ BC tissues. TNBC tissues contained a higher number of CTRP8+/CD68+ macrophages than CTRP8+/tryptase+ mast cells. M1 polarized THP1 cells showed enhanced RXFP1 gene expression that was not observed for M2 polarization. Exposure of M1 polarized THP1 cells to CTRP8 and to RLN2 revealed common and CTRP8-specific changes in secreted cytokines. CTRP8 enhanced the secretion of proinflammatory cytokines while RLN2 increased secretion of pro-migratory and anti-inflammatory factors in M1 polarized myeloid cells. In mast cells, both RXFP1 ligands concordantly reduced degranulation.
Conclusions: Human RXFP1+ mast cells and M1 polarized macrophages responded to the RXFP1 ligands RLN-2 and CTRP8 as well as the small molecule agonist ML290 with attenuated degranulation and altered cytokine secretion, respectively. Myeloid-derived innate immune cell populations in human breast and prostate cancer tissues can be a source and target of CTRP8 and identify this RXFP1 agonist as a novel player in the tumor microenvironment.

Inhibition of relaxin autocrine signaling confers therapeutic vulnerability in ovarian cancer.

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Ovarian cancer (OC) is the most deadly gynecological malignancy, with unmet clinical need for new therapeutic approaches. The relaxin peptide is a pleiotropic hormone with reproductive functions in the ovary. Relaxin induces cell growth in several types of cancer, but the role of relaxin in OC is poorly understood. Here, using cell lines and xenograft models, we demonstrate that relaxin and its associated GPCR RXFP1 form an autocrine signaling loop essential for OC in vivo tumorigenesis, cell proliferation, and viability. We determined that relaxin signaling activates expression of prooncogenic pathways, including RHO, MAPK, Wnt, and Notch. We found that relaxin is detectable in patient-derived OC tumors, ascites, and serum. Further, inflammatory cytokines IL-6 and TNF-α activated transcription of relaxin via recruitment of STAT3 and NF-κB to the proximal promoter, initiating an autocrine feedback loop that potentiated expression. Inhibition of RXFP1 or relaxin increased cisplatin sensitivity of OC cell lines and abrogated in vivo tumor formation. Finally, we demonstrate that a relaxin-neutralizing antibody reduced OC cell viability and sensitized cells to cisplatin. Collectively, these data identify the relaxin/RXFP1 autocrine loop as a therapeutic vulnerability in OC.

RLN2 Is A Novel Biomarker For Differentiated Thyroid Carcinoma In Humans

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Introduction: Differentiated thyroid cancer (DTC) is the most common endocrine malignancy with 30% of patients experiencing progression where the prognosis is quite poor. Relaxin (RLN2) can regulate macrophage polarization and reduce fibrous extracellular matrix, creating a tumor microenvironment favorable for cancer cells. There has been data to suggest that RLN2 is produced by thyroid cells. Hence, we hypothesized that RLN2 and macrophages might be biomarkers for thyroid carcinogenesis in humans.

Objectives: To identify the role of RLN2 and its receptor RXFP1 in thyroid carcinogenesis via investigation of human thyroid tissue and cell lines.

Methods: We performed fluorescent immunohistochemistry (FIHC) for RLN2, CD68 (total macrophages), CD163 (M2 macrophages), and DAPI (nucleus) on thyroid tissue microarrays (TMA) with two cores per specimen. Fluorescence imaging and thresholding was performed, then the integrated density (total pixels) of staining for each channel was calculated. Control slides (using isotype matched immunoglobulin) were used to calculate background staining. Mean pixels/area in each channel was compared between tissue from n=181 non-distant metastatic DTC tumors (cases) and n=185 benign thyroid tissue (controls) via t-test assuming unequal variances. $P<0.05$ was used for statistical significance. We also used qPCR to determine the expression of RLN2, RLN1 and RXFP1 in human thyroid cancer cell lines including papillary (BCPAP, KTC-1, TPC-1), follicular (FTC133) and anaplastic (C643, Hth7) or a benign thyroid cell line (N-Thy).

Results: Amongst the cases, 91.2% were papillary; 65.2% were localized to thyroid; and only 11% were considered high-risk for recurrence. The cohort was followed for a mean of 5 years from thyroidectomy, during which 87.8% did not demonstrate any evidence of disease. Cases demonstrated higher expression compared to controls for RLN2 (difference of means 36.9; 95% CI 30.2 - 43.7; $p<0.0001$), CD68 (differences of means 9.7; 95% CI of 5.5 - 13.8; $p<0.0001$) and CD163 (differences of means 23.9; 95% CI 14.4 - 33.3; $p<0.0001$). Amongst the cases, these markers were not significantly different in relation to sex, age, AJCC stage, lymph node status or risk of recurrence categories. In the cell lines, RLN2 was elevated in FTC133, KTC-1 and N-Thy cells, reduced in C643, undetectable in TPC-1 cells. Only the C643 cells had increased RLN1 expression. Expression of RXFP1 was elevated in BCPAP cells, reduced in Hth7 and N-Thy cells, and undetectable in TPC-1.

Conclusions: In this overall fair-prognosis cohort of DTC, higher expression of RLN2 correlating with macrophage infiltration, specifically pro-tumoral M2 subtype, demonstrates its role as a biomarker for thyroid carcinogenesis. The undetectable expression of RLN2 and RXFP1 in TPC-1 cells which are also negative for differentiation marker TTF-1 provides an avenue to explore biomarkers for de-differentiation of DTC. The findings advance our understanding of thyroid carcinogenesis and will guide further investigations for therapeutic avenues against advanced thyroid cancer.

C1qTNF related protein 8 (CTRP8) and RXFP1 promote distinct brain tumor pathologies in mice

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Introduction: The C1Q-tumor necrosis factor related protein (CTRP) family has functions in metabolism, immunity, and cancer. Among the CTRP family, CTRP8 remains the least characterized in terms of functions, receptors, and binding partners. We showed in fatal high-grade glioma (HGG) brain tumor cells that CTRP8 impacts migration and treatment resistance through its receptor RXFP1. However, the in-vivo effects of CTRP8-RXFP1 signaling on brain tumor progression and the underlying molecular mechanisms are currently unknown.
Here, we have employed orthotopic xenografting of CTRP8-RXFP1 glioma transfectants into mouse brain to determine humane endpoints and used histological and proteomic analysis to characterize glioma tumors and their microenvironment in immunocompromised mice.
Objective: Characterize the role of the human CTRP8 and RXFP1 in glioma progression xenografts in mice.
Methodology: Human brain tumor cells (U251) stably transfected with single CTRP8 or RXFP1 or dual CTRP8/ RXFP1 were generated and orthotopically xenografted into the right brain hemisphere of immunocompromised mice. At humane endpoint, brains were collected for immunodetection of cellular markers. Right (tumor) and left (non-tumor) brain hemispheres were processed for global and phospho-proteomic analysis. Upon bioinformatic analysis, selected proteins identified in the proteomic data sets were validated.
Results: Mice orthotopically xenografted with U251-CTRP8/ RXFP1 transfectants generated fast-growing, highly aggressive glioma of mesenchymal phenotype and showed significantly shorter survival times than U251-CTRP8, U251-RXFP1, or U251 parental cells. Immunohistochemical analysis of these glioma tumor models revealed distinct differences in cellular and matrix composition of their tumor microenvironment. Comparative proteomic analysis of tumor-and non-tumor-bearing brain hemispheres identified novel putative proteins and pathways for future studies.
Conclusions: Our mouse orthotopic brain tumor studies identified the presence of CTRP8/RXFP1 signaling axis to cause aggressive glioma progression and short survival times in mice. Distinctly different glioma histopathology, including markedly different survival times, tissue phenotypes, and brain tumor proteomes, were obtained in mice xenografted with glioma cells expressing RXFP1 exclusively. Collectively, these xenograft studies provide novel molecular insights into new clinically relevant biology of the CTRP8 and RXFP1 in brain tumors.

SESSION 6

Role Of INSL5 And RXFP4 In The Control Of Colorectal Function: Implications For Constipation And Diarrhea Treatment

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Introduction: Insulin-like peptide 5 (INSL5), the natural ligand for the relaxin family peptide receptor 4 (RXFP4), is a gut hormone that is produced by endocrine cells of the colon.
Objective: The aims of these studies were to localise RXFP4 in the colon and to develop agonists and antagonists of the receptor to interrogate the roles of the INSL5-RXFP4 system in the colon..
Methods: We rationally designed and synthesised simplified INSL5 peptide analogs with agonist or antagonist activity and tested their binding affinity and activity in CHO cells stably expressing RXFP4. Lead agonists and antagonists were then investigated for their effects in WT and RXFP4 KO mice. RXFP4 reporter mice were used to locate sites of *Rxfp4* expression.
Results: We discovered the first simplified (2-chain and single chain) potent agonists and the first potent and specific antagonist of RXFP4. Cell-based assays demonstrated high affinity for RXFP4 and no activity on RXFP3 or any off-target effects in cells for the lead compounds. Testing in conscious mice showed that agonists stimulated propulsive reflexes in the colon and reversed opioid-induced constipation. Conversely, our antagonist inhibited propulsion, included accelerated propulsion caused by bacterially-produced short-chain fatty acids (SCFA). Localisation studies showed that the colonic endocrine cells that produce INSL5 (L cells) were commonly closely apposed to 5-HT containing endocrine cells, and that the majority of 5-HT cells expressed *Rxfp4*. Consistent with this relationship, propulsion induced by SCFAs was inhibited by our RXFP4 antagonist.
Conclusions: The INSL5-RXFP4 system has a physiological role in the control of colorectal propulsion. RXFP4 agonists have the potential to treat constipation, and antagonists to treat diarrhea.

Synthetic mRNA-induced Expression of H2 Relaxin by Bovine Vaginal and Cervical Tissues

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Introduction: Deficiencies in physiologic activity of relaxin (RLN) during parturition have demonstrated negative effects in some species (i.e., pigs and rodents) including prolonged delivery, increased rate of stillbirths, in part, due to incomplete softening of connective tissues essential for normal delivery. Conversely, cattle are incapable of synthesizing endogenous relaxin but have managed to maintain reproductive success except for high incidence of dystocia and its sequelae in domestic breeds, which contributes to significant economic loss to the cattle industry. Previous attempts using purified porcine or recombinant human RLN to reduce the incidence of dystocia in heifers present variable results. However, Human 2 (H2) RLN has a high affinity to the bovine RLN receptor (RXFP1) and given advancements in mRNA therapeutics, H2 RLN mRNA therapy may prove to be a more efficacious treatment for dystocia.

Objective: To deliver synthetic mRNA encoding human relaxin (H2 relaxin) to bovine vaginal epithelial cells and cervical mucosa, and to determine H2 relaxin synthesis of treated cells and tissues compared to controls.

Methods: Using a H2 RLN-NanoLuciferase mRNA construct with a secretion signal, we transfected bovine kidney (BK) and primary bovine vaginal epithelial cells (BVEC) with 0.5, 1 or 2 µg synthetic mRNA. At 3, 6, 12, 24 and 48 h post-transfection, cell lysates and supernatants were collected for detection of H2 RLN indirectly via Nano-Glo Assay® (Promega) or directly via H2 RLN ELISA (R&D Systems). Additionally, two approximately 4-year-old Holstein cows were treated with H2 mRNA with secretion signal by aerosol application at t=0 h and retreated at t=48 h. Prior to treatment, the cows' reproductive tracts were evaluated to confirm the absence of gross abnormalities and estrous cycles were synchronized. Vaginal secretions were collected at 0, 6, 12, 24, 48, 72, 96 and 120 h post-transfection using 75 cm double-guarded cotton swabs placed at the cervical-vaginal interface for 3 mins. Tissues were harvested after 5 days of data collection for detection of H2 RLN directly via Western Blot.

Results: Luminescence and ELISA demonstrated BK and BVEC expressed H2 RLN in cell lysates for all observed time points with a decline only observed at 48 h in the BVEC cells. Cell supernatants exhibited increasing levels of luminescence and concentration determined by ELISA, indicating secretion of H2 RLN. *In vivo* transfections of bovine reproductive mucosa with H2 RLN mRNA resulted in detectable levels of expression of H2 RLN in vaginal, cervical, and uterine tissues by western blot. Additionally, H2 RLN was detected in cervical-vaginal secretions except for 0 h when secretions were collected prior to administration of mRNA.

Conclusions: These data provide evidence in support of the potential use of H2 RLN mRNA therapy as a novel approach in reducing the incidence of dystocia in heifers.

Relaxin Receptor RXFP1 Is Targeted By Novel Interaction Partners CTRP1, CTRP6 And CTRP8 In Ocular Surface Wound Closure

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Introduction: Relaxin/insulin-like family peptide receptor 1 (RXFP1) signaling plays an important role in various physiologic as well as pathophysiologic processes, including ocular surface wound healing. In particular, binding of relaxin to RXFP1 promotes corneal wound healing through increased cell migration and modulation of extracellular matrix formation. C1q/tumor necrosis factor related proteins (CTRP) 8 has been identified as novel interaction partner of RXFP1 and CTRP1 and CTRP6 share high homology in the putative RXFP1 binding site. The role of CTRP1, CTRP6 and CTRP8 at the ocular surface remains unclear.
Objective: In this study, we investigated the effects of CTRP1, CTRP6, and CTRP8 on epithelial ocular surface wound closure and their dependence on the RXFP1 receptor pathway**.**
Methods: CTRP1, CTRP6 and CTRP8 expression was analyzed by RT-PCR and immunohistochemistry in human tissues and cell lines of the ocular surface and lacrimal apparatus. Effects of CTRP1, CTRP6 and CTRP8 on ocular surface epithelial wound closure were analyzed in an in vitro wound model (scratch assays) in human corneal and conjunctival epithelial cell lines. Dependence on RXFP1 signaling was determined by inhibition of ligand binding to RXFP1 by a specific anti-RXFP1 antibody.
Results: We detected CTRP1, CTRP6 and CTRP8 expression in human tissues and cell line of the ocular surface and lacrimal apparatus. Treatment with CTRP1, CTRP6 or CTRP8 resulted in a significantly increased surface defect closure rate in human corneal epithelial cells but not in conjunctival epithelial cells. Inhibition of RXFP1 fully attenuated the effect of CTRP8 on the closure rate of surface defects in human corneal epithelial cells, whereas CTRP1 and CTRP6 effects were not completely suppressed.
Conclusions: Our findings demonstrate a novel role for CTRP1, CTRP6 and CTRP8 in corneal wound closure in vitro by targeting the relaxin receptor RXFP1.

Anti-inflammatory And Neurogenic Actions Of Relaxin-3 In Zebrafish Following Traumatic Brain Injury.

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Introduction: Inflammation of nervous tissue following brain injury, or resulting from neurodegenerative diseases such as Alzheimer's Disease, increases tissue damage and cell death. In humans, the ability for new neurons to form and re-establish relevant synaptic connections is compromised by prolonged inflammation and scarring. Zebrafish and other non-mammalian vertebrates experience a brief bout of inflammation following brain injury which subsides and initiates neuroregenerative processes. Subsequently, neural pathways are re-established resulting in recovery of function. Members of the relaxin peptide family have anti-inflammatory and antifibrotic actions in a variety of tissues and organs and could enhance recovery following brain injury.

Objective: To determine if relaxin-3 suppresses inflammation and promotes neurogenesis in adult zebrafish following traumatic brain injury (TBI).

Methods: Adult male and female zebrafish were anesthetized by immersion in 0.02% buffered MS-222 for 30-45s. Anesthesia was maintained by placing each fish onto a MS-222 soaked sponge and a 33G insulin needle was inserted through the left nostril to create injury of the forebrain on that side. In each fish, the undamaged side served as a control. Following injury, fish were placed in fresh, aerated water in 1L beakers without any treatment (control) or containing 3×10^{-9}M H3 relaxin for 60 min during which time fish recovered from anesthesia. Fish were returned to their home aquarium for two days post-injury. Subsequently, fish were euthanized using an overdose of MS-222 and brains were removed and fixed in the skull for 24 hours in 4% paraformaldehyde at 4°C, then removed and fixed for an additional 3 hours at 4°C. Brains were impregnated and blocked in paraffin wax and serial coronal sections (10µm) of each brain at the level of the telencephalon were collected and mounted onto microscope slides for immunohistochemical detection of L-plastin (a marker of microglial activation), IL-1ß (a pro-inflammatory cytokine), and PCNA (a marker of radial glial activation).

Results: L-Plastin (but not IL-1ß) immunoreactivity was elevated in injured hemispheres compared with undamaged controls 2 days post-injury. In H3 relaxin treated fish, L-Plastin immunoreactivity in injured hemispheres was significantly reduced compared with saline-treated controls. Furthermore, PCNA immunoreactivity around the site of injury was increased in H3 relaxin-treated fish.

Conclusions: Due to its anti-infammatory, anti-fibrotic and angiogenic actions, relaxin 3 may promote healing of nervous tissue following injury.

SESSION 7

Engineering Bone Marrow-derived Stromal Cells To Deliver Anti-fibrotic Cargo As A Novel Treatment Option For Hypertensive Kidney Disease

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Introduction: Human bone marrow-derived mesenchymal stromal cells (BM-MSCs) have emerged as a promising treatment option for chronic kidney disease (CKD), based on their anti-hypertensive, immunomodulatory and tissue-reparative effects. However, the build- up of fibrosis (scar tissue) in CKD significantly impairs the viability and efficacy of transplanted BM-MSCs. To address this, we previously combined the separate administration of BM-MSCs with the anti-fibrotic drug, serelaxin (RLX), which enhanced the renoprotective effects of BM-MSCs in normotensive and hypertensive models of CKD. To advance the clinical application of this combination therapy, we utilized lentiviral transduction to engineer BM-MSCs to express RLX and GFP (BM-MSCs-eRLX+GFP) or GFP alone (BM-MSCs-eGFP), which enabled the delivery of the combined treatment via injection(s) or BM-MSCs-eRLX+GFP.

Objective: To investigate the renoprotective effects of BM-MSCs-eRLX+GFP in a high salt (HS)-induced model of hypertension.

Methods: BM-MSCs were transduced with a lentivirus containing human relaxin-2 cDNA and green fluorescent protein, under a eukaryotic translation elongation factor-1α promoter (BM-MSCs-eRLX+GFP). The ability of BM-MSCs-eRLX+GFP to differentiate, proliferate, migrate and produce RLX was evaluated in vitro, whilst BM-MSCs-eRLX+GFP homing to the injured kidney and renoprotective effects were evaluated in an 8-week HS (2% NaCl)-induced hypertensive murine model in vivo. Over the last two weeks of HS loading, subgroups of HS-fed mice were treated with i) BM-MSCs-eRLX+GFP (i.v, once-weekly); ii) BM-MSCs-GFP (i.v; once- weekly); iii) osmotic minipump-delivered RLX (s.c, 0.5mg/kg/day) and i.v-injected BM-MSCs (all 1x106 cells/mouse); or iv) the ACE inhibitor, perindopril (as a current standard of care treatment for hypertensive CKD).

Results: Flow cytometry revealed transduction efficiencies of 72.4% and 94.4% at a multiplicity of infection of 2 for BM-MSCs- eRLX+GFP and BM-MSCs-eGFP, respectively. When cultured for 3- and 7-days, 1x106 BM-MSCs-eRLX+GFP produced therapeutic levels of RLX (~30ng/ml) without compromising their proliferation and differentiation capacity compared to naïve BM-MSCs in-vitro and retained the therapeutic effects of RLX. The combined effects of RLX and BM-MSCs, administered separately or via BM-MSCs- eRLX+GFP (which homed to the injured kidney), equivalently abrogated the HS-induced tubular epithelial damage and interstitial fibrosis, and lowered the HS-induced hypertension, kidney inflammation, glomerulosclerosis, and proteinuria to a broader extent than BM-MSCs-eGFP or perindopril. Interestingly all cell-based treatments promoted gelatinase (MMP-9 and MMP-2) activity, which perindopril failed to affect.

Conclusions: BM-MSCs-eRLX+GFP produced equivalent renoprotection to that of RLX and BM-MSCs administered separately and may represent a novel treatment option for hypertensive CKD.

Enhancing the therapeutic applicability of relaxin and related peptides

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Introduction: Despite the pleiotropic organ-protective actions of H2 relaxin (RLX) and related peptide mimetics (B7-33) that have been well-documented at the pre-clinical level, the clinical application of these peptides has been limited by their short half-lives and poor oral absorption. Hence, improving the longer-term activity and oral applicability of these RXFP1-targeting peptide agonists would vastly improve their therapeutic application for a host of indications including cardiovascular and other fibrosis-related diseases.

Objective: To use biodegradable glycine-coated super-paramagnetic iron oxide nanoparticles (SPIONs) to act as a drug delivery vehicle that could facilitate the longer-term activity and oral applicability of RXFP1-targeting peptide agonists.

Methods: The N-terminus RLX or B7-33 were conjugated to glycine-coated SPIONs using carbodiimide chemistry and characterised for their hydrodynamic size, charge and stability. These SPIONs were designed to degrade within 72 hours, to release the peptides they were conjugated to. In a short-term (14-day) isoprenaline (ISO)-induced murine model of cardiomyopathy, i.p (systemic)-administered SPION-RLX was compared to daily drinking water (p.o)-administered SPION RLX, osmotic minipump (Pump)-delivered RLX or Empty-SPIONs alone, from days 7-14 post-injury. In a longer-term (42-day) ISO-induced murine model of cardiomyopathy, the effects of oral gavage (p.o)-administered SPION-RLX or SPION-B7-33 were compared to unconjugated RLX or B7-33 or Empty-SPIONs alone, from day 14-42 post-injury. In each case, measures of left ventricular (LV) inflammation, remodelling, fibrosis and function were assessed at the respective time-points investigated.

Results: ISO-injured mice underwent a significant increase in LV inflammation (at day-14) as well as cardiomyocyte hypertrophy, interstitial fibrosis, vascular rarefaction and systolic dysfunction at each time-point studied. In the short-term model established, i.p or p.o-administered SPION-RLX was taken up by infiltrating dendritic cells and detected in the circulation of treated mice, which resulted in similar circulating RLX levels being detected after 7-days of treatment to that produced by Pump-RLX. Furthermore, daily p.o- administered SPION-RLX (via drinking water over 7-days) significantly attenuated each of the above-outlined measures to an equivalent extent as Pump-RLX or i.p-delivered SPION-RLX. In the longer-term model established, oral gavage-administered SPION-RLX or SPION-B7-33 abrogated several measures evaluated after a 4-week treatment period. These effects, however, were not induced by the p.o-administration of unconjugated peptides alone or Empty-SPIONs alone.

Conclusion: These findings collectively demonstrated that the conjugation of short-acting and poorly absorbed peptides to glycine- coated SPIONs provided a means to improve their longer-term therapeutic activity and oral applicability.

Arthrofibrosis: Prevalence and Clinical Shortcomings

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Introduction: Arthrofibrosis (AF) is a common pathologic condition characterized by excessive periarticular scar-tissue formation, resulting in joint stiffness and periarticular soft-tissue contractures. AF may occur in any joint secondary to even modest injury, post-surgical inflammation, trauma, hemarthrosis, infection, or it may even occur idiopathically. In the shoulder it is characterized by an early acute inflammatory phase followed by a longer “frozen” phase with significant fibrosis development. In addition to long standing loss of motion, patients commonly experience chronic pain and swelling. All present treatment options for AF fail to treat the disease etiology—the accumulation of fibrotic tissue within the joint space. For millions of patients each year, the lack of modern and effective treatment options necessitates prolonged physical therapy or surgical intervention in an attempt to regain functional joint range of motion (ROM), often with uncertain and inconsistent outcomes.

Objective: To characterize the prevalence and clinical shortcomings in treating AF, and introduce the potential use of relaxin as a therapeutic option

Methods: We review the primary orthopedic etiologies and clinical deficiencies in the management of AF and propose the development of intra-articular relaxin as a possible therapeutic modality.

Results: Anterior cruciate ligament reconstruction, total knee arthroplasty, periarticular knee fractures, proximal humerus fractures, periarticular elbow fractures, and idiopathic adhesive capsulitis, are the most common non-congenital etiologies of AF treated by orthopedic surgeons. Physical therapy can extend for many months with incomplete resolution of AF, often requiring further manipulation under anesthesia, arthroscopic open lysis of adhesion, or open surgical releases as secondary surgical interventions. For shoulder contractures, less common surgical alternatives include distension arthrography, rotator interval debridement, or subscapularis tendon lengthening procedures.

Conclusion: There are no pharmacological options in present clinical practice except for pain management and anti-inflammatory modalities as complements to Physical Therapy. Collagenase based intraarticular treatments can be highly damaging to healthy articular cartilage and are not an option for joint AF. Based on anecdotal clinical observation of long-standing improvements in ROM in patients with end stage AF who were able to restore motion during pregnancy, we propose the use of intraarticular administration of relaxin as a possible treatment option to be investigated.

Histone modification and transcriptional regulation of RXFP1 gene expression in lung fibroblasts

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Introduction: Relaxin/relaxin insulin-like family peptide receptor 1 (RXFP1) signaling has anti-fibrotic effects, but clinical trials of relaxin-based therapies in systemic sclerosis (SSc) failed to demonstrate benefit. Downregulation of RXFP1 expression in lung fibroblasts of patients with SSc and idiopathic pulmonary fibrosis (IPF) resulted in unresponsiveness of these cells to relaxin. The molecular mechanisms associated with RXFP1 down-regulation in fibrotic lung fibroblasts are not well known.

Objective: Characterize the molecular mechanisms of RXFP1 down-regulation in fibrotic lung fibroblasts.

Methods: Human primary lung fibroblasts were cultured from lungs of organ donors (controls) and from IPF or SSc patients undergoing lung transplant. Transcriptional regulation was characterized using bioinformatics, reporter system, EMSA and ChIP techniques. Lung gene expressions of AP1 transcription factors and correlation with lung function were analyzed using the Lung Genomics Research Consortium (LGRC) data. A ChIP-seq experiment was performed using Cut &Tag with an antibody specific for H3K27ac (Active Motif, Carlsbad, CA) and next-generation sequencing. Differential histone acetylation modifications of the normal and fibrotic lung fibroblasts were analyzed using Galaxy platform (https://usegalaxy.org/).

Results: We identified a distal enhancer 200kb upstream of the putative RXFP1 transcription start site. The transcription factor AP1 transactivates the RXFP1 gene expression through this distal enhancer. Co-transfection of expression plasmids for AP-1 components, JUN and FOS, resulted in a 12-fold increases in enhancer activity compared to pcDNA3 expression vector while a dominant negative JUN abolished the activation. The expression of JUN and FOS were reduced in IPF lungs and their expression levels were positively correlated RXFP1 expression. The expression levels of all four FOS members were negatively correlated with force vital capacity (FVC) in IPF patients. Consistent with our finding, within the RXFP1 locus, most of the histone acetylation peaks were localized to the distal enhancer region although there was no global difference between control and fibrotic lung fibroblasts.

Conclusion: A distal enhancer contributes to the differential regulation of the RXFP1 gene in control and fibrotic lung fibroblasts. Transcriptional regulation and histone modification are important in RXFP1 regulation. Our study provides insight into the molecular mechanisms associated with the downregulation of RXFP1 gene expression in lung fibrosis and the possibility of restoring the effectiveness of relaxin-based therapy in SSc and IPF patients.

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Investigation Of Relaxin-2 As A Treatment For Hypertrophic And Keloid Scars.

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Introduction: Hypertrophic and keloid scars result from aberrant wound healing in response to trauma to the reticular dermis of the skin. These scars are common cosmetic ailments, with hypertrophic scars developing in over 50% of surgical patients and after 90% of burn injuries. While hypertrophic scars remain within the boundaries of the wound, keloids grow incessantly into surrounding normal skin tissue and can develop idiopathically with high rates of recurrence. Current treatment options are limited and involve toxic or painful procedures including radiotherapy, cryotherapy and surgical removal. Thus, there is a need for a therapeutic that targets the underlying pathology of scar development. Here, we explore the effects of the natural antifibrotic peptide hormone, relaxin-2 (RLX-2), on hypertrophic and keloid scars.

Objective: To characterize the RLX-2 antifibrotic mechanism of action in human skin tissue at the molecular and mechanical level and to create a sustained-release hydrogel that delivers targeted RLX-2 to treat hypertrophic and keloid scars.

Methods: Mechanism of action and efficacy studies were performed *in vitro* using primary human dermal fibroblasts, in *ex vivo* culture of human normal skin and scar tissue biopsy punches, and using an *in vivo* murine chemical burn wound model. Human dermal fibroblasts or normal human skin tissues were treated with TGF-β1 and RLX-2 for 24 hours and 14 days, respectively, and keloid tissue was treated with RLX-2 alone. Protein and gene expression of key players in the TGF-β1 and RLX-2 signaling pathways (collagen-I/III, α-SMA, psmad2, MMP-1/9, TIMP-1) were investigated using western blot and RT-qPCR. Skin tissues were further assessed using histological staining, pathological evaluation and collagen fiber analyses. The proposed hydrogel is generated via a recombinant expression construct to attach a cysteine-polyethylene glycol (PEG)-DBCO linker to the RLX-2 C-terminus using intein chemistry. This linker conjugates RLX-2 into a DBCO-N_3-linked PEG hydrogel and subsequently hydrolyzes to release unmodified RLX-2.

Results: RLX-2 inhibits the profibrotic effects of TGF-β1 in both 2D dermal fibroblasts and human skin tissue by reducing protein and gene expression of collagen-I/III, α-SMA, fibronectin, psmad2, CTGF, TIMP-1, as well as upregulating MMP-1/9. Further, RLX-2 reduces collagen fiber alignment and density in keloid scar tissues. RLX-2 also improves wound healing and reduces scar size in an *in vivo* murine chemical burn wound model when applied over 10 days. The sustained-release hydrogel formulation studies are ongoing.

Conclusion: RLX-2 inhibits profibrotic signaling by TGF-β1 in human dermal fibroblasts and skin tissues, as well as promotes reversal of the keloid scar phenotype. It also demonstrates the ability to promote proper wound healing and reduce excessive scarring in deep dermal wounds *in vivo*. Together, these findings support the use of relaxin-2 as a novel treatment for hypertrophic and keloid scars.

SESSION 8

Metabolic Effects Of Relaxin-2 At Cardiac Level And Its Potential As A Biomarker For Atrial Fibrillation

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Introduction: Intense translational research has highlighted the potential role of the pleiotropic hormone relaxin-2 in the physiology and physiopathology of the cardiovascular system, where it induces numerous beneficial effects including vasodilatation, the prevention of cardiac damage induced by ischemia-reperfusion, the stimulation of angiogenesis, the inhibition of cardiomyocyte apoptosis, the decrease of oxidative stress and inflammatory mechanisms, the reversion of cardiac fibrosis and hypertrophy, or the suppression of atrial fibrillation (AF), as well as inotropic and chronotropic effects. Recent preclinical and clinical outcomes have encouraged the use of relaxin-2 or serelaxin as a potential therapeutic strategy in patients suffering from different cardiovascular disarrangements, especially heart failure. However, it is at present unknown the possible regulatory role of relaxin-2 on cardiac metabolism as well as its use as a biomarker for AF.

Objective: We aim to study the role of relaxin-2 on cardiac metabolism regulation and determine the potential association between endogenous relaxin-2 and AF physiopathology.

Methods: Primary cultures of neonatal rat cardiomyocytes and normal human atrial cardiac fibroblasts (NHCF-A) were treated with 1-10 ng/mL of serelaxin, and Sprague-Dawley rats were treated with 0.4 mg/kg/day serelaxin for 2 weeks using osmotic minipumps. In vitro, we determined metabolic activity, glucose and fatty acid uptake, and activation of intracellular signalling pathways through western blot, and performed wound-healing assays. Rats' body composition, respiratory quotient, locomotor activity and energy expenditure were measured with a nuclear magnetic resonance imaging system and a calorimetric system. Metabolic profiling of rat atria was carried out using ultra-high-performance liquid chromatography (UHPLC)-Time of Flight-MS based platforms. Cardiac and NHCF-A gene expression levels of key enzymes of polyunsaturated fatty acids (PUFAs) synthesis or pro-fibrotic markers were assessed by Real Time-PCR. A commercial ELISA kit was employed for the determination of relaxin-2 plasma levels in the peripheral vein and left atrium (LA) plasma samples from AF patients subjected to pulmonary vein radiofrequency catheter ablation in the Cardiovascular Area of the University Clinical Hospital of Santiago de Compostela.

Results: Relaxin-2 is able to regulate cardiomyocyte metabolism activating AMPK, ERK1/2, AKT and AS160, and increasing glucose uptake. In rat atria, relaxin-2 modifies the lipidome inducing changes in the levels of lipids of the membrane bilayer: glycerophospholipids (phosphatidylcholines and phosphatidylethanolamines) and sphingolipids (ceramides and sphingomyelins). Relaxin-2 also stimulates the endogenous synthesis of n-3 and n-6 long-chain PUFAs increasing mRNA levels of elongase 5 (Elovl5), desaturase Δ5 (Fads1) and desaturase Δ6 (Fads2) in rat atria. In NHCF-A, relaxin-2 inhibited its migration and decreased mRNA and protein levels of the pro-fibrotic molecule transforming growth factor-β1 (TGF-β1). Peripheral vein relaxin-2 plasma levels were higher than LA relaxin-2 plasma levels in men while, in women, peripheral vein relaxin-2 levels were increased compared to men. AF patients with higher levels of relaxin-2 exhibited a reduction in H_2O_2 plasma levels and in mRNA levels of alpha-defensin 3 (DEFA3) and IL-6 in leucocytes from LA plasma.

Conclusion: Relaxin-2 can regulate cardiac energetic metabolism and modifies cardiac lipidomic profile; while its plasma levels are associated with molecules involved in fibrosis, inflammation and oxidative stress in AF patients. Our outcomes will expand the knowledge of the functions performed by relaxin-2 in cardiac physiology and pathology, which could influence the therapeutic strategies with this hormone and could strengthen the relevance of relaxin-2 in AF physiopathology, diagnosis and treatment.

Relaxin reduces inflammation, reverses fibrosis, and suppresses atrial fibrillation in aged hearts

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Introduction: Healthy aging results in cardiac structural and electrical remodeling that increases susceptibility to cardiovascular diseases. Relaxin has shown broad cardioprotective effects including anti-fibrotic, anti-arrhythmic and anti-inflammatory outcomes in multiple models of heart disease. We report here the cardioprotective effects of Relaxin in a rat model of aging.

Experimental Design and Methods: Young (9-month-old) and aged (24-month-old) male and female F344/Brown Norway F1 rats were treated with vehicle or Relaxin (400μg/kg/day) for 14 days via subcutaneous osmotic minipumps. Rat hearts were excised, perfused on a Langendorff apparatus, and stained with voltage and Ca^{2+} indicator dyes. Optical mapping was used to test arrhythmia vulnerability and electrophysiological changes. After the collection of optical mapping data, atrial and ventricular myocytes and fibroblasts of control animals were isolated and cultured for further analysis using immunocytochemistry methods.

Results: Sustained atrial or ventricular fibrillation was readily induced in the hearts of aged but not young control animals. Relaxin treatment suppressed arrhythmias. Relaxin treatment increased conduction velocity, decreased fibrosis and promoted substantial remodeling of the cardiac fibers. Substantial anomalies in the subcellular localization of β-catenin and connexin 43 was observed in the ventricles of aged animals; these changes were reversed by Relaxin treatment. Relaxin treatment resulted in a significant increase in the levels of: Nav1.5, Cx43, β-catenin and Wnt1 in the hearts of treated aged animals. In isolated cardiomyocytes, treatment with Relaxin for 24-48 hours increased the localization of β-catenin in the nucleus and increased Nav1.5 expression. These effects were mimicked by the GSK3 inhibitor CHIR 99021 but blocked by the canonical Wnt inhibitor Dickkopf1. Relaxin prevented TGFβ-dependent differentiation of cardiac fibroblasts into myofibroblasts while increasing the expression of Wnt1; the effects of Relaxin of cardiac fibroblast differentiation were blocked by Dickkopf1.

Conclusions: Relaxin reduces arrhythmogenicity in the hearts of aged rats by a mechanism that involves a reduction of fibrosis and an increase of conduction velocity. These changes are accompanied by a substantial remodeling of the cardiac fibers and appear to be mediated by an increase in canonical Wnt signaling. The mechanisms by which Relaxin increases the expression of Wnt ligands and promotes Wnt signaling remain to be determined.

Long-acting relaxin (LY3540378) demonstrated improved renal hemodynamics response in preclinical and clinical studies

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Introduction: LY3540378 is a novel long-acting relaxin analog designed to capture the relaxin physiology and pharmacology as a chronic treatment for heart failure (HF) and chronic kidney disease (CKD).
Objective: LY3540378 is a single chain fusion protein of human relaxin-2 with an albumin-binding single domain antibody.
Methods: LY3540378 is a potent and selective agonist of relaxin family peptide receptor 1 (RXFP1) in multiple species. This novel relaxin analog demonstrated desirable pharmacokinetic (PK) properties suitable for chronic administration in preclinical and clinical studies.
Results: In normal rats, LY3540378 given intravenously (IV) and subcutaneously (SC) increased renal blood flow (RBF) by up to 73% and 34%, respectively. In a randomized, double-blind, and placebo-controlled trial in healthy human subjects, LY3540378 increased eRPF after acute (1.50 [1.33, 1.69]) and chronic (1.44 [1.28, 1.62]) administration (placebo-adjusted difference in estimated ratio to baseline [90% CI]) while maintaining mGFR (p=NS). Following repeated dosing, the corresponding mean percentage of reductions from baseline in filtration fraction are approximately 5% in placebo cohort and up to 28% in the LY3540378 treated cohorts.
Conclusions: These data demonstrated translatability of renal effects of relaxin from a preclinical model species to healthy human subjects. In this Phase 1 trial, LY3540378 was well-tolerated with an acceptable safety profile. This profile in healthy humans supports the need for future clinical investigation of LY3540378 in patients with chronic HF and/or CKD.

SESSION 9

RXFP1 Gene Therapy for Treatment of Pressure Overload Induced Chronic Heart Failure

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Introduction: Human Relaxin-H2 (H2) has been appreciated as a potential therapeutic for acute heart failure. However, H2 failed to meet the endpoint criteria in the RELAX-AHF2 trial. This may in part be attributable to the atrial restricted cardiac expression pattern of the cognate receptor relaxin family peptide receptor 1 (RXFP1).
Objective: To assess potential therapeutic effects of ventricular RXPF1 signaling in heart failure.
Methods: An adeno-associated viral vector harboring RXFP1 (AAV.RXFP1) was used for *in vivo* and *in vitro* RXFP1 overexpression. An AAV carrying a luciferase transgene (AAV.Luc) served as control. To evaluate potential therapeutic effects *in vivo* pressure overload was induced in wildtype mice by transverse aortic constriction (TAC). At day 7 following TAC animals were randomized to AAV.RXFP1 or AAV.Luc transduction via tail vein AAV injection. At day 28, when full transgene expression could be expected, mice were implanted with mini osmotic pumps delivering recombinant H2 or saline for the duration of 4 weeks until day 56 post TAC. Cardiac function was monitored by repeated echocardiography. At study end, organs were harvested and snap frozen for subsequent molecular analysis. The underlying molecular RXFP1 signaling was assessed in ventricular neonatal rat cardiomyocytes (NRVCM).
Results: Echocardiography revealed attenuated HF progression in AAV.RXFP1 treated mice compared to AAV.Luc mice. Interestingly, additional exogenous RLN supplementation significantly enhanced this effect and could almost restore cardiac function. RXFP1/H2 treatment mitigated activation of the fetal gene program. While cardiac hypertrophy was similar in AAV.RXFP1 and AAV.Luc control mice, ventricular dilation was restricted to AAV.Luc control mice. Gene expression patterns of typical remodeling genes were restricted to AAV.Luc control animals. To enlighten the underlying mechanism, cAMP response in NRVCM upon H2 Stimulation was assessed. We found not only an immediate dose dependent rise in cAMP levels, but also a significant rise in phospholamban (PLN) phosphorylation. Although RXFP1 shares critical signaling mediators with classical beta-adrenergic receptors, we found significant differences in temporal activation, amplitude, and specificity of downstream targets. Classical beta-adrenergic inotropes like isoprenaline (ISO) induce an immediate surge in PLN phosphorylation at both the protein dependent kinase (PKA) and Calcium/Calmodulin dependent Kinase II (CamKII) phospho-sites. In contrast, RXFP1 stimulation led to a less pronounced but specific PKA activation, resulting in predominant phosphorylation of PLN(S16) potentially explaining its protective properties.
Results: Ventricular RXFP1 overexpression with extrinsic H2 stimulation could rescue this pressure overload induced heart failure model. The unique PKA-specific activation profile of RXFP1/H2 beneficially influenced cardiac inotropy and remodeling.
Conclusion: Harnessing RXFP1 dependent signaling represents a novel and adjustable approach for heart failure therapy.

Therapeutic effects of relaxin receptor agonist ML290 vascular calcification in combination with lifestyle intervention in a model of atherosclerosis

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Introduction: Vascular calcification contributes to the rupture of atherosclerotic plaques—the leading cause of heart attacks. No therapeutics exist to treat vascular calcification. We hypothesized that relaxin, a vasoprotective and anti-fibrotic small peptide hormone of the insulin/relaxin family, may provide a therapeutic option for vascular calcification. However, recombinant relaxin has short stability *in vivo*, poor bioavailability, and is expensive to synthesize, limiting its clinical utility for chronic conditions such as vascular calcification.
Objective: ML290 is a biased allosteric agonist of the human relaxin receptor (hRXFP1) previously shown to attenuate vascular calcification in *Apoe*$^{-/-}$ mice. This study aimed to determine if ML290 may reverse arterial remodeling with a clinically relevant intervention - lifestyle changes.
Methods: A high-fat diet-induced atherosclerosis and vascular calcification in humanized (hRXFP1/hRXFP1) *Apoe*$^{-/-}$ mice for 15 weeks. To simulate lifestyle changes, mice were returned to the chow diet for the last 10 weeks of the experiment. Mice were divided into one of four groups: vehicle (60% Phosal and 40% PEG300) with atherogenic diet (N=16), ML290 with atherogenic diet (N=16), vehicle with chow diet (N=16), and ML290 with chow diet (N=16). In addition, we are developing an *in vitro* assay to compare multiple relaxin agonists on alkaline phosphatase activity, a key enzyme in the development of vascular calcification, in human aortic vascular smooth muscle cells (haVSMC).
Results: Preliminary data suggest that relaxin agonism reduces alkaline phosphatase activity dose-dependently in haVSMCs.
Conclusion: This study will demonstrate the potential for ML290 to prevent vascular calcification formation *in vivo* in combination with lifestyle interventions for atherosclerosis and determine the most suitable small molecule relaxin agonists for the treatment of vascular calcification.

Phase 1 Safety, Pharmacokinetics, and Pharmacodynamics of the RXPF1 Agonists AZD3427 and AZD5462 in Healthy Volunteers and Heart Failure Patients.

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Introduction: Despite recent advances in pharmacological treatments, morbidity and mortality due to heart failure (HF) remain alarmingly high and novel medicines are needed to treat these patients and slow progression. Activation of relaxin receptor RXPF1 is hypothesised to induce changes in systemic and renal hemodynamics and function which may be beneficial for these patients. AZD5462 is the first and only small molecule RXPF1 agonist, while AZD3427 is a relaxin fusion peptide engineered for extended half-life. Both compounds are in development as complementary new RXPF1 therapies for heart failure to ensure the best treatment is available for the individual needs of each patient.
Objective: To assess safety and tolerability of AZD5462 in healthy volunteers (HV) and AZD3427 in both HV and HF patients.
Methods: AZD5462 and AZD3427 were assessed in separate Phase 1, randomized, double-blind, placebo-controlled studies. AZD5462 is an oral solution that was evaluated in HV as a single dose at five dose levels, and as a twice daily dose given for 10 days at four dose levels. In contrast, AZD3427 is designed as both a subcutaneous (SC) injection and an intravenous (IV) infusion. It was evaluated as a single SC dose at five dose levels in HV, a single IV dose at one dose level in HV, and as a weekly SC injection given for 5 weeks at three dose levels in HF patients with ejection fraction ≥ 41% and ejection fraction ≤ 40%. Each study evaluated safety, pharmacokinetics, and exploratory pharmacodynamics and biomarkers.
Results: AZD5462 was evaluated in 98 HV (56 single dose, 42 repeat dose) and AZD3427 was evaluated in 56 HV (single dose) and 48 HF patients (repeat dose). Both compounds were safe and well-tolerated.
AZD5462 was rapidly absorbed in HV with a tmax between 0.5 and 1.8 hours and an estimated terminal half-life between 3 and 6 hours. The most common AEs in these HV were GI disorders (nausea, abdominal pain, vomiting) and headache, but without clear relationship to dose. Trends in systolic and diastolic blood pressure decrease and heart rate increase were observed over the 10 days of repeat dosing. No AEs of hypotension were reported. There was also a dose-dependent increase in plasma renin.
Complimentarily, AZD3427 had a slower absorption and a terminal half-life of 13–14 days. The most common AE in HF patients was hypotension, reported in 5 participants with EF ≥ 41% and 1 participant with EF ≤ 40%. All AEs of hypotension were mild and did not lead to discontinuation of the compound. Overall, no clinically relevant trends were observed in vital signs during this study. In HF patients, AZD3427 tended to increase stroke volume and plasma renin, and reduce systemic vascular resistance and plasma creatinine.
Conclusions: AZD5462 and AZD3427 were both generally well tolerated, and have the potential to be the first small molecule RXFP1 agonist and long-lasting large molecule RXFP1 agonist co-positioned for comprehensive treatment of heart failure patients.

Relaxin reverses diastolic dysfunction in heart failure with preserved ejection fraction

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Background and Significance: Heart Failure (HF) is the leading cause of cardiovascular deaths and ~50% of HF patients have HF with preserved ejection Fraction (HFpEF). Patients with chronic HFpEF have a near normal ejection fraction, reduced left ventricular (LV) diastolic compliance, increase in LV pressure, fibrosis, and ion channel remodeling leading to supraventricular arrhythmia which increases morbidity and mortality. HFpEF patients typically have co-morbidities such as atrial fibrillation (AF), diabetes, lung edema and hypertension. The latter is a predictor of mortality and is targeted to prolong survival given the lack of a direct therapy for HFpEF. To address this unmet public health problem, we investigate the actions of exogenous human Relaxin (RLX, RLN2) in a rat model of HFpEF that recapitulates most aspects of clinical HFpEF.

Methods: ZSF1 diabetic rats (9-weeks old) were placed on a high fat diet (HFD) for 11-weeks and serial echocardiograms were used to track the development of diastolic dysfunction. At week 20, osmotic mini-pumps were implanted to continuously release vehicle (Control, Na-acetate) or RLX (400µg/kg/day for 2-weeks). After 2-weeks, hearts were perfused with a voltage-sensitive dye (RH237) and a Ca^{2+} indicator (Rhod-2/AM) in a Langendorff apparatus to optically map action potentials (APs) and Ca^{2+} transients (CaTs) to analyze the arrhythmia phenotype. Alternatively, left ventricular (LV) tissue sections were used for immune-fluorescence (IF) imaging to examine changes in fibrosis (collagen 1), connexin 43, Wnt1 and β-catenin in LV myocytes. IF data were confirmed by Western blot analysis. Blood samples were taken before the HFD, before and after RLX-treatment to measure changes in serum NT-pro-ANP, ET-1 and RLX. In preliminary studies, alternative approaches were tested to deliver RLX in rats with diastolic dysfunction.

Results: ZSF1 rats on a HFD developed HFpEF with E/e' (an echo marker of diastolic dysfunction) decreasing to -24.4 from -17.9 MV (n=12) and was reversed to -18.6 MV by RLX (n=6, p<0.0001). In HFpEF rats that received the vehicle (n=6), a premature stimulus (S1-S2= 30 ms) elicited: a) no arrhythmia b) non-sustained AF or c) sustained arrhythmia, with (1/3 of rats in each group). RLX treatment blocked sustained supraventricular (n=0/12) and n=4/12 had non-sustained AF. RLX improved the conduction velocity (CV), particularly at short cycle lengths (150 ms) from 0.74 to 0.9 m/s (n=4/group). IF indicated that RLX increased Cx43 expression (26.8 ± 0.03%, p<0.0001, n=6), and β-catenin (52.8± 0.05 %, p<0.0001) at intercalated disks. As expected, RLX reduced collagen deposition in HFpEF rats (25 ± 0.04%, p<0.04) or back to normal levels. RLX caused a marked increase of cytosolic Wnt1 (47.3±0.06%, p<0.0001) compared to HFpEF rats treated with the vehicle. In IF data, values are given as mean ± SEM.

Conclusions: The ZSF1 diabetic rat on a high-fat diet recapitulates most of the phenotypes associated with human HFpEF, including supraventricular arrhythmias, fibrosis, and lung edema. RLX treatment post-development of HFpEF reversed the pro-arrhythmic phenotype, increased conduction velocity particularly at fast heart rates, reversed fibrosis, reduced NT-pro-ANP and ET-1 in male rats. Most intriguing, RLX treatment activated Wnt1 and β-catenin and genomic remodeling of the heart. Possible mechanisms of action will be discussed.

Neuroanatomical Basis For The Anti-Nociceptive Role Of The Relaxin-3/RXFP3 Peptidergic System.

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Introduction: Affecting around 10% of world population, chronic pain and its related psychiatric comorbidities (e.g., depression and anxiety) are major health issues. Pathways and modulation of pain are well documented in the spinal cord, but the implication of neuropeptides remains poorly described in the brain. The relaxin-3 neuropeptide displays antidepressant and anxiolytic effects, and our preliminary results indicate an analgesic role in rat and mouse. Relaxin-3 is expressed by nucleus incertus (NI) neurons that project to different cortical (e.g., anterior cingulate cortex (ACC)) and subcortical (e.g., amygdala) areas of the pain matrix.
Objective: Because of the prominent expression of the relaxin-3 G protein-coupled receptor (RXFP3) in those areas, we aim at studying the pain modulatory effects of relaxin-3 by using pharmacological, behavioral and anatomical approaches in a mouse model of persistent inflammatory pain.
Methods: Persistent inflammation has been induced by injection of Complete Freud's Adjuvant (CFA) in the hind paw of the animal. To study the effect of the acute activation of RXFP3 on pain sensitization, RXFP3 agonists (A2 or πH3B) were injected in the ACC or amygdala. In addition, RXFP3 chronic activation has been studied at 4 weeks after stereotaxic injections of AAV-FIB-R3/I5 in ACC and amygdala. In both acute and chronic conditions, mechanical and thermal sensitivity have been assessed with von Frey (VF) and plantar tests, in pain (CFA) and no pain condition (NaCl). Anterograde tracing experiments have been performed using AAV-DIO-eGFP injection in the NI of relaxin-3 Cre mice. A whole-brain mapping of eGFP-labelled profiles was performed to estimate the density of projections from NI in physiological and pain conditions. Multiplex fluorescent in-situ hybridization (RNAscope) was performed to describe the expression of RXFP3 mRNA in the ACC and in the amygdala. A protocol for 3D quantification of immunolabelling has been developed to identify the contacts made by relaxin-3 containing fibers and determine the organization of relaxin-3/RXFP3 microcircuits in specific regions.
Results: Intra-amygdalar injection of RXFP3 agonists alleviated both mechanical and thermal pain, while intra-ACC injection had an effect only on mechanical sensitization. The effect of AAV-mediated chronic release of another RXFP3 agonist (R3/I5) confirmed these effects in the ACC and amygdala. Tracing experiments (using AAV-DIO-eGFP) of NI relaxin-3 neurons showed a dense but heterogenous network. In the amygdala, RLN3 projections were mostly found in central (CeA), medial (MeA) and basolateral (BLA) nuclei of the amygdala. In the ACC, this expression was seen ventrally and in deep layers of the ACC.
In situ hybridization experiments demosntrated RXFP3 mRNA expression in somatostatin interneurons both in the ACC and amygdala, with an increase of RXFP3 expression in the ACC in pain condition. 3D quantification in the ACC indicated an increase in the number RLN3 profiles, but a decrease in their volume under inflammatory conditions.
Conclusion: Our data highlight the plasticity of the RLN3/RXFP3 system and a novel antinociceptive role for this peptide family, suggesting its therapeutic potential in persistent pain conditions

Examining the Role of Relaxin in Fibrotic Endometriosis

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Introduction: Endometriosis is an estrogen dependent disorder, phenotyped by the growth of endometrium-like lesions outside of the uterus. Relaxin's role within the disease is not well established, yet endometriosis exhibits similar fibrotic dysregulation to other diseases, which respond to exogenous relaxin delivery. Here we show that relaxin can potentially mitigate the fibrotic nature of endometriosis in endometriotic epithelial cells and endometrial stromal cells.

Objective: To determine if relaxin has an antifibrotic effect in endometriosis pathology.

Methods: Endometriotic epithelial cells (12Z) and endometrial stromal cells (HESC) were cultured in complete media and split every 48 hours. After adherence to a 6-well plate, both cell types were pretreated with transforming growth factor beta (TGF-β) in a serum diluted medium to induce a fibrotic phenotype. After TGF-β treatment, 12Zs and HESCs were treated with β-estradiol, progesterone, and relaxin in serum diluted media at concentrations mimicking the highest serum concentration of each respective hormone during the menstrual cycle. Lysis or RNA extraction was performed within 48 hours of treatment for downstream analysis, including western blotting and quantitative polymerase chain reaction (qPCR) to identify changes in protein and RNA expression, respectively. A dose-dependent relaxin study was also conducted on both cell types in the same serum diluted media conditions to determine if a concentration less than the highest serum concentration during the menstrual cycle is sufficient to induce anti-fibrotic effects. All treatments were compared to just TGF-β treated or no treatment controls.

Results: Preliminary western blot data suggests relaxin prevents the deposition of extracellular matrix (ECM) proteins collagen-I and fibronectin following TGF-β induced fibrosis in both 12Zs and HESCs, suggesting that relaxin modulates fibrosis in both diseased and healthy cells of the endometrium. Studies of changes to ECM protein expression at the mRNA level are ongoing. Further studies are being conducted to investigate how relaxin either circumvents or potentiates other hormonal effects involved in the menstrual cycle and endometriosis progression, including that of β-estradiol and progesterone, to potentially identify relaxin as a treatment for endometriosis. Additionally, preliminary results reveal that relaxin downregulates estrogen receptor alpha following TGF-β induced fibrosis in 12Zs, indicating a negative feedback loop between relaxin and β-estradiol.

Conclusions: Relaxin's canonical inhibition of fibrosis likely occurs in endometriosis and relaxin may serve as a potential anti-fibrotic therapy for later stages of the disease.

RXFP1-CTRP8/ RLN2-CDC42 Axis Alters Golgi-Apparatus And Vesicular System In Patient Glioblastoma (GB) Cells.

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Introduction: Glioblastoma (GB) is the most aggressive type of brain cancer with very short survival times. Available treatments for GB are very limited and ineffective long term. There is an urgent clinical need to identify new targets for the development of novel and effective GB therapies. We have shown that CTRP8/ RLN2 upregulates small GTPase CDC42 with significant impact on the actin cytoskeletal system in GB cells, leading to filopodia formation and enhanced migration. Here, we have identified the Golgi-apparatus and vesicular system as a novel target of a functional RXFP1-CTRP8/ RLN2-Cdc42 axis in patient GB cells.
Objective: To determine the importance of the CTRP8 (RLN2)-RXFP1 ligand receptor system for the regulation of the vesicular system and its impact on malignancy of RXFP1 + patient GB cells.
Methods: Western blot (WB), siRXFP1, siCDC42, immunofluorescence (IF), real time migration assays, cell culture
Results: We have shown that CTRP8/ RLN2-RXFP1 receptor activation upregulates CDC42 protein levels and increases CDC42 activity. This coincided with enhanced lysosomal cathepsin B release and invasiveness of patient GB cells. Here we show that CTRP8 or RLN2 treatment caused increased intensity of IF signal for trans Golgi marker Golgin 97 and enlargement of the trans-Golgi system in GB cells. Silencing of RXFP1 or CDC42 abolished these changes in the trans-Golgi system and CTRP8 or RLN2 were unable to rescue this phenotype. It is known that alterations in the trans-Golgi system can affect lysosomal functions, including the production of lysosomal hydrolases, like cathepsin B (cathB). CTRP8/ RLN2 induced changes in Golgin 97 coincided with increased numbers of LAMP1+ vesicles. Golgi-derived cytoplasmic vesicles travel along microtubules to different cytosolic compartments or secretory pathways. CTRP8/ RLN2 induced microtubular structural assembly in GB. We observed that siCDC42 dramatically altered the microtubular phenotype, caused aggregation of LAMP1+ vesicles, and decreased secretion of cathB.
Conclusions: Our data have identified the trans-Golgi and lysosomes as novel targets of CTRP8/ RLN2-RXFP1-CDC42 signaling in patient GB.

The Pursuit Of High Throughput Cell-Based Assays To Characterize Functional Activity Of Small Molecule RXFP1 Agonists

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Introduction: The human relaxin receptor 1 (RXFP1) is a therapeutic target for a variety of diseases including fibrosis, heart failure, and pulmonary hypertension. Small molecule RXFP1 agonists provide advantages to peptide ligands for clinical use. Following our discovery of ML290 as a small molecule RXFP1 agonist, we have pursued improved analogs with favorable PK/PD properties. Through these studies, we have learned that in vitro assays using cAMP readouts are not sufficient to make decisions, as they are not predictive of activity in more complex models.

Objective: To identify RXFP1-dependent cell-based assays to guide SAR studies and the selection of compounds for advancing to *in vivo* studies.

Methods: Various cell-based models were investigated using small molecule and peptide agonists of RXFP1. Cell migration was studied using pulmonary arterial smooth muscle cells (WT and RXFP1-KO) using a Platypus migration assay. Migration was also examined in HEK293 cells (WT and RXFP1 overexpressing) using an Incucyte Scratch Assay. A calcification model using human aortic vascular smooth muscle cells was examined with an alkaline phosphatase readout. VEGF production was examined in THP-1 cells by measuring the endogenous protein (LANCE assay) and a HiBiT tagged VEGF luminescent reporter produced by CRISPR/Cas9 editing. Activity in these assays was compared to cAMP data from HEK-RXFP1 cells.

Results: A poor correlation between cAMP and other cell-based readouts was observed. The migration assays demonstrated RXFP1-dependent effects but were low-throughput and variable. Calcification assays showed reproducible responses to RXFP1 agonists but were limited by throughput. VEGF assays showed favorable reproducibility and throughput. The VEGF-HiBiT assay was used to test 595 ML290 analogs in dose-response.

Conclusions: Our studies indicate that cAMP assays are insufficient, in isolation, to guide the development of small molecule RXFP1 agonists. We have validated several cell-based phenotypes that are dependent on the presence of RXFP1. Our current interpretation is that the VEGF production assay shows better agreement with in vivo results, for the small number of molecules where *in vivo* data is available, however additional data from relevant animal models is needed to substantiate these findings.

Involvement Of RXFP3 N-Terminus In Relaxin Family Peptide Binding And Activation.

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Introduction: Among the relaxin family of peptides, both relaxin-3 and relaxin-2 are known for their ability to bind and activate relaxin family peptide receptor 3 (RXFP3). While relaxin-3, the endogenous ligand, acts as a full agonist at RXFP3, relaxin-2 is known for its biased activation of RXFP3. Even though the binding modes and key binding residues involved in relaxin-3 binding to RXFP3 have been studied, the involvement of RXFP3 N-terminus for its binding with relaxin-3 is not yet understood. Moreover, the binding modes and key binding residues for RXFP3 binding with relaxin-2 are yet to be established.

Objective: To investigate the involvement of RXFP3 N-terminus for its binding with both relaxin-3 and relaxin-2, and to identify the major structural movements associated with biased activation of the receptor by relaxin-2.

Methods: The structure of the C-terminus-omitted RXFP3 was constructed using multiple template homology modelling and deep-learning-based predictions. Multiple template homology modelling was used to construct the transmembrane helices and the deep-learning-based structure prediction algorithm, RoseTTAFold, was used to predict the structure of the N-terminus. The N-terminal-stitched RXFP3 model (^{NT}RXFP3) was embedded in a lipid bilayer composed of 400 POPC/POPE/cholesterol lipids at a 2:2:1 ratio. The system was energy minimized, equilibrated and subjected to 400 ns long molecular dynamics (MD) simulation via GROMACS 2023. Next, the ^{NT}RXFP3-relaxin-3 and ^{NT}RXFP3-relaxin-2 complexes were constructed using mutational data and distant restraints using MODELLER 10.3. Both complexes were subjected to short MD simulations using GROMACS.

Results: Analysis of the trajectory, RMSD and RMSF plots revealed that the ^{NT}RXFP3 model remains stable. This N-terminus consists of 3 helical regions and 4 loop regions. The MD simulation of the constructed model shows that in the sub-microsecond time scale, the N-terminus of RXFP3 caps the binding pocket. The ligand-receptor complexes of ^{NT}RXFP3, formed with both relaxin-3 and relaxin-2, were stable during short MD simulations and revealed that relaxin-2 occupies a region closer to the rim of the binding pocket in comparison to relaxin-3. In addition, the N-terminus was found to be constantly contacting both the ligands and remain positioned over them in a partially enclosed conformation.

Conclusions: MD simulations of the N-terminal-stitched RXFP3 (^{NT}RXFP3) model, ^{NT}RXFP3-relaxin-3 and ^{NT}RXFP3-relaxin-2 complexes revealed the direct involvement of the RXFP3 N-terminus in modulating the ligand binding.

C1qTNF related protein 8 (CTRP8) receptor choice determines outcome in brain tumors

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Introduction: Members of the C1Q-tumor necrosis factor related protein (CTRP) family have functions in metabolism, immunity, and cancer. Among the CTRP family, CTRP8 remains the least characterized in terms of functions, receptors, and binding partners. We showed in fatal high-grade glioma (HGG) that CTRP8 impacts migration and treatment resistance through its receptor RXFP1. However, we also noticed CTRP8 signaling in glioma devoid of RXFP1, suggesting the presence of an additional receptor for CTRP8 in human glioma. Here, we present a new CTRP8 receptor and provide in-vivo evidence for strikingly different brain tumor formation and humane endpoints in xenografted mice, depending on the receptor choice of CTRP8.

Objective: Identify new CTRP8 binding partners, determine downstream signaling, and in-vivo outcome in human HGG.

Methodology: We used yeast-two hybrid (Y2H) screens to identify new CTRP8 interaction partner and co-immunoprecipitation (co-IP) to characterize the interactions. Human brain tumor cells (U251) stably transfected with CTRP8/ RXFP1 and CTRP8/ new receptor were used to assess stemness in tumor sphere formation assays, glioma stem cell (GSC) marker expression. We determined proteomic profiles of both mouse orthotopic xenografts to identify potential mechanisms mediated by the two CTRP8 receptors in human HGG.

Results: Our Y2H analysis identified the membrane-anchored Delta-like noncanonical NOTCH ligand 1 (DLK1) as a new CTRP8 interaction partner. We showed by co-IP that CTRP8 interacts with full-size DLK1 and a soluble, truncated form of DLK1. While DLK1 enhanced sphere formation and GSC marker expression in U251, CTRP8 mitigated this pro-stemness effect of DLK1 to initiate differentiation. In U251 cells expressing DLK1, treatment with CTRP8 increased DLK1 intracellular domain (ICD) release and nuclear translocation of DLK1 ICD which is known to act as a transcriptional co-factor to impact gene regulation. This revealed a new signaling function of CTRP8 in glioma. Upon orthotopic xenografting into mouse brain, U251-CTRP8/ RXFP1 transfectants were highly aggressive and resulted in short survival times, whereas U251-CTRP8/ DLK1 tumors showed long survival and strikingly different cell differentiation. This was reflected in proteomic analysis of brain tumor tissues with activation of distinct pathways depending on the CTRP8 receptor choice.

Conclusions: This is the first study identifying stem cell factor and noncanonical NOTCH ligand DLK1 as a new CTRP8 receptor. DLK1 was identified as a promoter of glioma stemness and CTRP8 altered this differentiation. CTRP8 activation of RXFP1 and DLK1 in mouse orthotopic xenografts elicited markedly different in-vivo tumor outcome which included distinctly different glioma survival times, tissue phenotypes, and brain tumor differentiation pathways. Collectively, CTRP8 receptor choice triggers distinctly different regulatory receptor signaling systems with impact on the glioma stem cell niche and potential clinical relevance.

Dexamethasone Regulation of *RXFP1* Gene Expression in Human Primary Fibroblast-Like Synoviocytes.

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Introduction: As an antifibrotic treatment, relaxin signalling occurs via RXFP1 receptor. However, recent studies document the downregulation of *RXFP1* expression in the affected tissues and this downregulation contributes to reduced relaxin efficacy. We report that dexamethasone (DEX) upregulates *RXFP1* expression *in vitro* in primary human fibroblast-like synoviocytes (hFLS). In this study, we investigate the molecular mechanisms underlying the crosstalk between the glucocorticoid and relaxin signalling pathways that govern this switch.
Objective: To elucidate the molecular mechanisms responsible for the upregulation of *RXFP1* expression by DEX in primary hFLS cells.
Methods: We used qPCR and RNAseq as primary methods to analyze changes in gene expression upon DEX treatment. Primary hFLS and HEK293T cells were cultured under standard conditions until reaching full confluence, and then treated with varying concentrations of DEX. Total RNA was isolated at different time points post-treatment for longitudinal evaluation of gene expression changes. HEK293T cells were used as a control, as *RXFP1* expression in these cells was unresponsive to treatment. Untreated cells also served as a negative control. Gene expression analysis was performed using qPCR with TaqMan probes, and paired-end sequencing was conducted using Illumina NextSeq 2000. All experiments were performed in triplicate.
Results We determined the optimal time and concentration of DEX treatment for upregulating *RXFP1* expression *in vitro* in primary hFLS cells. *RXFP1* expression maximally increased by 1.88 upon treatment with DEX at 50 nM concentration compared to control. This effect was evident as early as 4 hours after addition of the compound. HEK293T cells, where *RXFP1* gene is downregulated, were non-responsive to DEX treatment. Preliminary results also indicated that DEX upregulates the expression of RXFP1 isoforms both from proximal and distal promoters of the gene, narrowing down potential targets for glucocorticoid receptor signaling. Finally, we analyzed the overall changes in gene expression landscape induced by DEX in hFLS and control HEK293T cells.
Conclusions: *RXFP1* may be a new target for glucocorticoid signalling pathway in primary hFLS cells. These results provide a new perspective for the development of therapeutic strategies targeting fibrotic conditions.

Exploring Novel Interaction Partners of RXFP1 through MiniTurbo Biotin Proximity Labeling

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Introduction: RXFP1 (Relaxin Family Peptide Receptor 1) receptor plays a pivotal role in various physiological processes, including reproduction system, cardiovascular function, tissue remodeling and cancer development and progression. Its functions are mediated through interactions with different protein partners. Recently, Biotin Proximity Labeling (BPL) techniques have emerged as powerful tools to study protein-protein interactions and protein complexes in living cells. These techniques involve the application of engineered proteins for the biotinylation of protein interaction partners in close proximity. Biotinylated interaction partners can then be easily detected and isolated using streptavidin affinity purification methods. We have generated an RXFP1-miniTurbo (mT) fusion protein for highly efficient BPL of RXFP1 interaction partners within 10 minutes in the presence of non-toxic cell permeable biotin.
Objective: To explore new interaction partners of RXFP1 in HEK RXFP1-mT-V5 tag stable clones.
Methods: RXFP1-mT-V5-tagged fusion construct was confirmed by sequencing. Stably transfected HEK 293 clones expressing RXFP1-mT or mT alone, used as negative control, were selected using V5 immunofluorescence (IF). qPCR, Western blot (WB) and immunoprecipitation (IP) were used to confirmed RXFP1 expression clones. Biotin incorporation by BPL was confirmed by IF with streptavidin-AF594 and WB with streptavidin-HRP. Streptavidin beads were used to isolate biotinylated proteins followed by gel separation and mass spectrometry (MS) analysis.
Results: We isolated stable HEK transfectants with membrane localization of RXFP1-mT as confirmed by detection of V5-tagged RXFP1 membrane localization by IF. RXFP1-mT mRNA transcription level was confirmed by qPCR. WB and IP were used to detect full-size RXFP1-mT protein expression. Streptavidin affinity purification identified distinct differences in biotinylated protein bands by WB. Currently, gel-separated proteins are isolated for MS analysis of putative biotinylated RXFP1-mT interacting partners. We plan to validate novel RXFP1 interaction partners by co-IF, IP followed by WB analysis. Newly, identified RXFP1 protein partners are subjected to bioinformatics analysis to identify functional signaling pathways and cellular processes.
Conclusions: BPL identification of protein partners interacting with the C-terminal end of human RXFP1 in living cells represents a valuable tool to unravel interactomes and signaling cascades associated with RXFP1. The RXFP1-mT cell system also has great potential as a new screening tool for the discovery of drugs and small molecules that can interact with RXFP1. We hope that our study will expand our understanding of the in-vivo pathophysiology and molecular mechanisms underlying the complex RXFP1 signaling networks. Newly identified RXFP1 interaction partners may unveil new therapeutic strategies for RXFP1 targeted signaling.

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NOTES

Relaxin

Antibodies against ...

- **human Relaxin (hRLX)**
  - pro-hRLX-1
  - pro-hRLX-2
  - pro-hRLX-1/2
  - hRLX-1
  - hRLX-2
  - hRLX-1/2
  - hRLX 1 A Chain (aa 1-8)
  - hRLX 1 B Chain (aa 1-9)
  - hRLX 2 A Chain (aa 3-10)
  - hRLX 2 A Chain (aa 14-24)
  - hRLX 2 B Chain (aa 4-10)
  - hRLX 2 B Chain (aa 14-27)
- **porcine Relaxin (pRLX)**
  - pRLX

Proteins/Peptides

- **human Relaxin (hRLX)**
  - hRLX-2
  - hRLX 2 A Chain (aa 3-10)
  - hRLX 2 A Chain (aa 14-24)
  - hRLX 2 B Chain (aa 4-10)
  - hRLX 2 B Chain (aa 14-27)

human Relaxin-2 (hRLX-2)
fully native sequence

Relaxin ELISA
for the determination of Relaxin in serum, plasma, urine, seminal plasma and tissue

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