An Roinn Talmhaíochta,
Bia agus Mara
Department of Agriculture,
Food and the Marine

Food Institutional Research Measure

Final Report

'Translation of pharmaceutical drug delivery to nutraceutical delivery using in-vitro and in-vivo techniques. NUTRADEL

DAFM Project Reference No: 11 F 042

Start date: 01/02/2013

End Date: 31/1/17

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Collaborating Research Institutions and Researchers:
Technological University Dublin. Prof Hugh Byrne and Prof Jesus Frias
University College Dublin. Dr Sinead Ryan, Prof David Brayden

Please place *one* "x" below in the appropriate area on the research continuum where you feel this project fits

Basic/Fundamental → Applied → Pre Commercial

1	2	3	4	5 X	6	7

Please specify priority area(s) of research this project relates to from the National Prioritisation Research Exercise* (NRPE) report

Priority Area (s)	**H Food for Health** **I Sustainable Food Production and Processing**

Key words: food delivery systems, pre-hypertension, formulation, pre-clinical.

1. Rationale for Undertaking the Research

The present project aimed to develop products that can contribute to lowering blood pressure in mild hypertensive or pre-hypertensive patients as an additional treatment. The prevalence of pre-hypertension among adults in the USA is 31% (Hsia et al., 2007) and it has recently been categorised into the different degrees of severity of the disease (Collier et al., 2014)

Five major classes of drugs to counteract hypertension are currently available on the market; thiazide diuretics (indapamide, Napamide®), angiotensin converting enzyme (ACE) inhibitors (captopril, Capozide®), calcium channel blockers (nifedipine, Adalat®), angiotensin receptor type II blockers (losartan, Cozaar®) and beta-blockers (propanolol, Inderal®). ACE inhibitors have been proven to be successful therapeutic agents, especially in combination with other drugs. Unfortunately, patients with hypertension are required to take the drug on a long-term basis and drug-drug interactions have been found in antihypertensive drugs (for example with potassium supplements). Moreover, hypertension is generally under-diagnosed as it has no symptoms. This problem makes intervention through food origin supplementation a desirable, safe and long term measure for a large population.

The peptides of interest in the project, the tripeptides Isoleucine-Proline-Proline (IPP) and Leucine-Lysine-Proline (LKP) were originally derived from respective dairy and chicken muscle sources. This opens the possibility of further enhancing the marketing of the health benefits of two well established Irish food classes as the original sources.

Current *in vitro* studies provide no information on pharmacokinetics, stability, efficacy or clinical 'proof of concept' of these food origin derived peptides/nutraceuticals. To overcome these obstacles, the bioactives were formulated into a nanoparticle drug delivery system and this research project studied the formulation properties.

Studies involving the oral administration of foods rich in the ACE inhibitor milk peptide, IPP, reported an anti-hypertensive effect. However, the ability of bioactive peptides to exert a physiological effect *in vivo* over time is dependent on the bioavailability of the peptide at its target site beyond the small intestine. Isolated from chicken, the peptide LKP has been reported to elicit a similar anti-hypertensive effect, but due to low oral bioavailability, it was achieved only by intravenous administration at a high dose in a rat model.

Significant advancements have been made towards the design of commercial nanoparticle-based therapeutic formulations, improving the potency, efficacy and pharmacokinetics of orally delivered peptides by protecting and increasing their bioavailability at their target site. In this project IPP and LKP were loaded into nanoparticles composed of an excipient with a long safe history of oral use in humans, chitosan. Chitosan is mucoadhesive, promotes absorption and can modulate intestinal transepithelial permeability through the paracellular and/or transcellular route to increase oral bioavailability.

The oral route of delivery for peptides is problematic due to enzymatic degradation and acidic pH in the stomach and low epithelial permeability. To address this, IPP and LKP where formulated with the aim of enhancing stability, permeability and controlled release at the target site. Both nanoparticles and transport enhancers were tested at the pre-clinical level.

2. Research Approach

1. The main achieved project objectives achieved (in bullet point format).

- Development of a proof of concept for translating knowledge from the area of pharmacology oral macromolecule peptide delivery to nutraceutical delivery.
- Development of a suite of methods useful to the nutraceutical R&D sector, bringing the bioactive from the discovery stage to preclinical, specifically for bioactive peptides.
- Development and assessment of two model peptides formulated into a nanoparticle, one from dairy origin, IPP, and one from meat origin, LKP (chicken muscle). Both peptides were selected on the basis of a proven angiotensin inhibitory properties with lower IC_{50} compared to other peptides.
- Assessment of their mechanisms of transport using mass transport modelling.

2. Research approach.

- Formulations of IPP and LKP using nanoparticle drug delivery systems were developed. IPP and LKP nanoparticles were assessed as follows:
- Physico-chemical characterisation (particle morphology, Zeta-potential and particle size) was used to develop a formulation within the optimal range identified by literature for oral delivery of peptides.
- *In vitro* release of peptides from the nanoparticle using standard dissolution tests was used to observe the effect of formulations to control the release of the peptides.
- Stability analysis in gastrointestinal enzymes in simulated media for stomach and intestinal milieu and liver homogenate were used to establish the feasibility of the oral formulation.
- Toxicity studies against gastrointestinal and liver cells using high content analysis assessed the possible toxicity of the peptides and formulations.
- *In-vitro* transport studies using human epithelial monolayer models simulating the small intestine provided an initial indication of the transport capacity, mechanisms, possible interactions, active efflux transport and metabolic mechanisms.
- *Ex-vivo* transport studies across isolated rat intestinal mucosae in Ussing chambers provided further evidence as to the suitability of the formulations for the appropriate delivery of the bioactive cargo.
- *In-vivo* pharmacokinetic studies with a rodent model of hypertension provided 'proof-of-concept' of bioactive delivery.

3. Research Achievements/Results

- *Ex vivo* studies indicate a potential solution to enhance the low permeability of the IPP and LKP peptides using transport enhancers available in the pharmaceutical industry that are also used as ingredients in the food industry.
- A chitosan-based nanoparticle formulation of the LKP with an association efficiency of over 80% has been achieved using modified ionotropic gelation protocols and optimising the formulation parameters.
- Data suggested that IPP-loaded nanoparticles may present a higher variability in technological parameters than LKP-nanoparticles due to mismatches in conformation.
- The *in vitro* toxicity and stability assessment of the IPP and LKP formulations has been completed showing no incompatibility in the range studied for free or nanoencapsulated formulations.
- The IPP and LKP bioactivity assays are well developed
- The nanoparticle formulation has been tested for toxicity. stability and controlled release before in vivo testing indicating the potential of zein and caprylic acid to enhance the formulation.
- Toxicity in-vitro and in-vivo has been tested in the raw materials and the nanoparticles (on a preliminary basis).
- There is confirmation that intestinal permeation enhancers used in pharmaceutical formulation in current clinical trials may increase the transport of the tripeptides and present advantages in the oral formulation of these peptides.
- Dosage and efficacy of the peptides has been already tested and confirmed by injected and non-injected routes in-vivo.
- The formulation of LKP and IPP peptide oral delivery proposed has been tested in vivo in a spontaneously-hypertensive rat model The bioactivity of the formulations was on a par with the commercial therapeutic product, captopril, within a range of magnitude of concentration difference.
- Ex vivo and in vivo studies have contributed to the elucidation of the transport mechanism of peptides through the intestinal barrier and to the characterisation of the effect of transport inhibition and enhancers.
- The in vivo pharmacokinetic and pharmacodynamics of the two peptides and the peptide nanoformulation have been modelled and quantitative evidence gathered.

4. Impact of the Research

NUTRADEL has provided an evidence that food bioactive research can benefit from oral formulation approaches present in the large pharmacology research community. This first proof of concept demonstrated in NUTRADEL has been continued in another DAFM funded project, SELDEL. The Irish research grouping that has been developed in this collaboration is contributing with their expertise in other food and pharmaceutical research and industry initiatives.

The trained Ph.D researchers in the project are following two different research paths, Dr Khalid working in Research and Development at Devenish and Dr Gleeson at Carnegie Mellon University (USA).
The research impact of this project has allowed the establishment in Ireland an internationally recognised expertise in the area of food bioactive oral delivery systems, with emphasis on peptides and chitosan nanoformulations.

The outcomes and outputs of the project are outlined below.

4(a) Summary of Research Outcomes

(i) Collaborative links developed during this research

During this project the following industrial collaborative links have been developed: **AllTech** (Cathal Connolly), **Monaghan Mushrooms** (John Collier) **Anabio** (Dr Sinead Bleiel), and **Kerry** (Cal Flynn) have participated in the stakeholder group of the project. **Nutribio, Carbery** and **Nuritas** have developed collaborative links with the research groups.

(ii) Outcomes where new products, technologies and processes were developed and/or adopted

During this project an unexpected development was observed: experimental results showed nanoformulated IPP and LKP peptides with an in-vivo therapeutic action comparable with Captopril in terms of pharmacodynamics profile at the same magnitude of dose. The nanoformulation makes use of 1) chitosan nanoparticles as a mucoadhesive polymer, 2) a zein coating that protects the peptide from release in the stomach and delivers it in the intestine where receptors for the peptides are more expressed.

(iii) Outcomes with economic potential

The potential of using 1) and 2) with 3) transport enhancers in formulations that have proven to improve peptide intestinal transport present the potential to develop a pre-hypertensive formulation with economical potential.

(iv) Outcomes with national/ policy/social/environmental potential

4 (b) Summary of Research Outputs

(i) Peer-reviewed publications, International Journal/Book chapters.

Brayden, D. J. Gleeson, J. & Walsh, E. (2014). A head-to-head multi-parametric high content analysis of a series of medium chain fatty acid intestinal permeation enhancers in Caco-2 cells. *Eur. J. Pharm. Biopharm.* **88** (3) 830-839.

Ryan, S.M. and Brayden, D. J. (2014). Progress in the delivery of nanoparticle constructs: towards clinical translation. Curr Opin Pharmacol. 2014 Oct;18:120-8. doi: 10.1016/j.coph.2014.09.019. Epub 2014 Oct 15.

Gleeson, JP, Heade, J, Ryan, S. and Brayden, DJ. (2015). Stability, toxicity and intestinal permeation enhancement of two food-derived antihypertensive tripeptides, Ile-Pro-Pro and Leu-Lys-Pro. *Peptides*. 71, 1-7.

Gleeson, J.P., Ryan, S.M. and Brayden, D.J., 2016. Oral delivery strategies for nutraceuticals: Delivery vehicles and absorption enhancers. Trends in Food Science & Technology, 53, pp.90-101.

Vozza, G., Khalid, M., Byrne, H.J., Ryan, S. and Frias, J., 2016. NUTRITION—NUTRIENT DELIVERY. Nutrient Delivery, p.1.

Gleeson, J.P., Brayden, D.J. and Ryan, S.M., 2017. Evaluation of PepT1 transport of food-derived antihypertensive peptides, Ile-Pro-Pro and Leu-Lys-Pro using in vitro, ex vivo and in vivo transport models. European Journal of Pharmaceutics and Biopharmaceutics, 115, pp.276-284.

Danish, M. K., Vozza, G., Byrne, H.J., Frias, J.M. and Ryan, S.M., 2017. Comparative study of the structural and physicochemical properties of two food derived antihypertensive tri-peptides, Isoleucine-Proline-Proline and Leucine-Lysine-Proline encapsulated into a chitosan based nanoparticle system. Innovative Food Science & Emerging Technologies. 44, pp.139-148.

Danish, M. K., Vozza, G., Byrne, H.J., Frias, J.M. and Ryan, S.M., 2017. Formulation, characterisation and stability assessment of a food derived tripeptide, Leucine-Lysine-Proline loaded chitosan nanoparticles. Journal of Food Sciences. 82(9), pp.2094-2104.

(ii) Popular non-scientific publications and abstracts including those presented at conferences

Gleeson, J. P., Ryan, S. M. and Brayden, D. J. (2014). Medium chain fatty acids increase the permeability of food derived antihypertensive bioactive tri-peptides across isolated intestinal tissue. The 43rd Annual Food Research Conference (IFSTI), Dublin, Ireland. December, 2014. Best Oral Presentation Award – Nutrition

Khalid, M. Byrne, H.J., Ryan, S.M. and Frias, J.M. (2014) Application of a mixture amount design for the formulation and characterization of LKP-loaded nanoparticles into an oral drug delivery system. The 43rd Annual Food Research Conference (IFSTI), Dublin, Ireland. December, 2014. Oral Presentation – Food Engineering and Processing.

Gleeson, J. P., Heade, J., Ryan, S. M. and Brayden, D. J. (2014). Investigation of the permeability, stability and toxicity of a food-derived antihypertensive peptide. The Controlled Release Society (CRS), 41st Annual Meeting and Exposition, Chicago, Illinois. July, 2014. Poster No. 241.

Khalid, M, Byrne, J. H, Ryan, S. M. and Frías, M. J. (2014) Development and Optimisation of IPP-Loaded Nanoparticles into an Oral Drug Delivery System using the Design of Experiment Approach. United Kingdom and Ireland Controlled Release Society (UKICRS), 2014 Workshop and Symposium, Cork, Ireland. April, 2014. Poster.

Gleeson, J. P., Ryan, S. M. and Brayden, D. J. (2014). Investigation of the application of intestinal permeation enhancers to increase absorption of food-derived antihypertensive peptides. United Kingdom and Ireland Controlled Release Society (UKICRS), 2014 Workshop and Symposium, Cork, Ireland. April, 2014. Poster

Gleeson JP, Ryan SM, and Brayden DJ. Intestinal permeation enhancers improve flux of food-derived tripeptides across rat intestinal mucosae in vitro. *Controlled Release Society, 42nd Annual Meeting*, Edinburgh, Scotland. July 2015.

UCD Seed Funding Travel Bursary to attend CRS Annual Meeting in Edinburgh Awarded
Rooney R, Srivastava A, Gleeson JP, McCartney F, Pandit A, and Brayden DJ. Synthesis of hyaluronic acid-based nanoparticles for oral insulin delivery. *Controlled Release Society, 42nd Annual Meeting*, Edinburgh, Scotland. July 2015. UCD Seed Funding Travel Bursary to attend CRS Annual Meeting in Edinburgh Awarded
Gleeson, JP - Attendee, Semi-Finals Chemistry Champions, American Chemical Society, Washington DC. July 2015. All expenses paid by ACS.

Gleeson JP, Ryan SM and Brayden DJ. Assessment of the paracellular- and PepT1-mediated transport of the antihypertensive peptide IPP across Caco-2 monolayers. *44th Annual Food Research Conference*, Cork, Ireland. Dec 2015.

Gleeson JP. When Food met Pharma… Delivery Strategies for Nutraceuticals. *Royal Society of Chemistry – Public Lecture Series*, London, UK. Invited Lecture – Jan 2016

Khalid, M, Byrne, Frías, M. J and J. H, Ryan, S. M. (2015) Formulation and Characterisation of LKP-loaded nanoparticles into an oral drug delivery system using a Design Of Experiment (DOE) approach. 12th International Congress on Engineering and Food (ICEF12), June, Quebec, Canada. Oral Presentation

Khalid, M, Byrne, J. H, Ryan, S. M. and Frías, M. J. (2015). DEVELOPMENT AND OPTIMISATION OF IPP-LOADED NANOPARTICLES INTO AN ORAL DRUG

DELIVERY SYSTEM USING THE DESIGN OF EXPERIMENT APPROACH. The Delivery of Functionality in Complex Food Systems: Physically inspired approaches from the nanoscale to the microscale (DOF). 6th International Symposium, July, Paris, France. Poster Presentation.

Khalid, M., Vozza, G. Byrne, H.J. Ryan, S. and Frias, J.M. (2016) Comparative study of the characteristic properties of two food derived antihypertensive peptides, Isoleucine-Proline-Proline and Leucine-Lycine-Proline in a nanoparticle oral delivery system. Oral presentation at the IUFoST 18th World Congress of Food Science and Technology, Dublin, Ireland

Khalid. M., Vozza. G., Byrne. H. J., Frías, J. M and Ryan, S. M (2016). Development of isoleucine-proline-proline chitosan nanoparticles coated with zein to help increase the loading efficiency for an oral drug delivery system. Poster presentation United Kingdom and Ireland Controlled Release Society (UKICRS), 2016 Workshop and Symposium, Cardiff, UK.

Gleeson, J. P., Ryan, S. M. and Brayden D. J. Overcoming low intestinal transport of a food-derived bioactive peptide, Ile-Pro-Pro: Sodium caprate as a transport enhancer. 18th World Congress of IUFoST, Dublin, Ireland.

(iii) National Report

(iv) Workshops/seminars at which results were presented

Attendee, Postgraduate and Postdoctoral Summer Institute, American Chemistry Society, Publications Division, Washington DC. August, 2014. Awarded - Certificate in Innovation and Stipend (All expenses paid by ACS)

(v) Intellectual Property applications/licences/patents

(vi) Other

Gleeson, J. P., Heade, J., Ryan, S. M. and Brayden, D. J. (2014). Investigation of the Permeability, Stability and Toxicity of a Food-Derived Antihypertensive Peptide. *CRS Newsletter*. **31** (6) 11-13.

Gleeson, J. P., Postgraduate and Postdoctoral Summer Institute, American Chemistry Society, Publications Division, Washington DC. August, 2014. Awarded - Certificate in Innovation and Stipend (All expenses paid by ACS).

5. Scientists trained by Project

Total Number of PhD theses: 2

Minna Khalid, TU Dublin, Formulation and characterisation of food derived peptides into a nanoparticle oral drug delivery system, January 2017.
John Gleeson, UCD, Oral delivery of the food-derived antihypertensive tripeptides, Ile-Pro-Pro and Leu-Lys-Pro: in vitro and in vivo, January 2017.

Total Number of Masters theses: N/A

6. Permanent Researchers

Institution Name	Number of Permanent staff contributing to project	Total Time contribution (person years)
UCD	2	0.7
TU Dublin	2	0.6
Total	**4**	**1.3**

7. Researchers Funded by DAFM

Type of Researcher	Number	Total Time contribution (person years)
Post Doctorates/Contract Researchers		
PhD students	2	4 years x2= 8 person years
Masters students		
Temporary researchers		
Other		
Total	**2**	**8**

8. Involvement in Agri Food Graduate Development Programme

Name of Postgraduate / contract researcher	Names and Dates of modules attended
Minna Khalid and John Gleeson	Science Writing for Agri-Food Researchers (Period 2) Teaching in Higher Education (Period 2) Innovation in the Bioeconomy (Period 2)
Minna Khalid	Industrial Scale R&D (Period 3) Next Generation Food Formulation module (Period 3)

9. Project Expenditure

Total expenditure of the project:	€ 382,001.02
Total Award by DAFM:	€ 477,815.00
Other sources of funding including benefit in kind and/or cash contribution(specify):	€ 0.00

Breakdown of Total Expenditure

Category	TU Dublin	UCD	Total
Contract staff			
Temporary staff			
Post doctorates			
Post graduates	80,666.51	76,337.49	157,004.00
Consumables	18,746.22	105,784.13	124,530.35
Travel and subsistence	6,169.51	6,143.08	12,312.59
Sub total	105,582.24	188,264.70	293,846.94
Durable equipment			
Other			
Overheads	31,674.67	56,479.41	88,154.08
Total	**137,256.91**	**244,744.11**	**382,001.02**

10. Leveraging

Enterprise Ireland innovation voucher (2015) entitled "Nutribio innovation partnership feasibility application". DIT participates with an objective to build an innovation partnership (led by Dan O'Sullivan from Nutribio).

Public private research partnership NURITAS-Beacon (2019). The BEACON Bioeconomy SFI Research Centre and Nuritas have joined forces to investigate the question of oral bioavailability in naturally occurring molecules, called peptides, from food or food by-products. The public-private research partnership is led by Professor David Brayden, UCD School of Veterinary Medicine, a senior scientist at BEACON, and Dr Nora Khaldi, CSO and Founder of Nuritas. Professor Brayden, will be using established and innovative techniques to create oral dosage forms of natural peptides discovered by Nuritas.
http://www.ucd.ie/innovation/news-and-events/latest-news-items/nuritasjoinforcestoinvestigateoralbioavailability/name,437233,en.html

11. Future Strategies

Research results with future impact for development
The recently completed NUTRADEL project (11 F 042) presented a number of novel findings which helped to better understand the requirements for oral delivery of anti-hypertensive angiotensin converting enzyme (ACE) inhibitor tripeptides, IPP and LKP: i) the oral delivery of these peptides is mediated by a specific peptide transporter (PepT1) with the potential for broad and significant competition from other dietary peptides ii) the blood-pressure lowering effect of the peptide was not completely elucidated and may comprise a combination of systemic ACE inhibition along with possible local effects in the GIT circulatory system iii) the use of intestinal permeation enhancers with a history of use in pharmaceutical clinical trials may contribute significantly to increasing the bioactive effect of a tripeptide formulation and iv) the spontaneously hypertensive rat model demonstrated equivalent blood-pressure lowering for food-derived tripeptides with the commercial ACE inhibitor (captopril)

How-to further develop the results of this research
The future development of this research will aim to bring these results closer to industrial application by i) developing experiments in which industrial grade tripeptides will be further optimised in nanoparticles made with chitosan and zein via the method of ionotropic gelation to develop oral peptide formulation ii) undertaking in vitro experiments to elucidate local effects using study of tripeptide effects on dilation of the smooth muscle of rat vascular rings iii) the development of chitosan/zein particle formulations of the peptides with intestinal permeation enhancers (C10) and inhibitors of PepT1 to further understand the relative contribution of the PepT1 and epithelial tight junction routes and iv) benchmarking the optimal formulations against the commercial oral small molecule ACE inhibitor Captopril in further in-vivo experiments.

Impact of this development
The project will progress the present state of the art to further identify the feasibility of using hydrolysated purified peptide ingredients that will enable further scale up processes. It will also further assess the transport and the therapeutic effect of the identified peptides, with a clear commercial benchmark. As a result of this project, the peptide formulations will be brought to a level of readiness to initiate human trials.

12. Consent to Publish Final Report on the DAFM Website and/or Through Other Dissemination channels

I consent to this report being made available to the public, through the Department's website and other dissemination channels.

Yes X No □

13. Declaration

I declare that the information contained in this final report is complete and true to the best of my knowledge and belief.

Signed: Jesus Maria Frias Celayeta Project Coordinator

Date: 3/9/19