Output Report: Bowel Project, Animal proof of principle

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Submitted to:
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Executive Summary

This report concerns the animal proof of principle experiments that have been conducted since the grant period was commenced with the signing of the funding agreement by all parties (8 July 2014) and the presentation of the first invoice (1 July 2014). Also included is a summary of the analysis of the first clinical trial; this analysis was completed and published in this period.

The purpose of the animal proof of principle experiments is to evaluate the suitability of compounds that act at ghrelin receptors to stimulate defecation as treatments for the constipation that occurs in spinal cord injury. We have further investigated the first generation drug, capromorelin, for its effectiveness in an animal model of constipation. This had not been done before. Capromorelin was found to be similarly effective in stimulating defecation in constipated and normal rats. Thus capromorelin remains a good candidate for human studies.

We investigated a suspected off-target effect of a second generation drug, ulimorelin. We found (for the first time) an off-target effect that we identified to be at the alpha receptor for catecholamines. This off target effect has the potential to lower blood pressure. We therefore conclude that ulimorelin is not a good option for the treatment of people with constipation that results from spinal cord injury.

Background

A major problem identified in spinal cord injury (SCI) is an inability to empty the bowel when defecation is convenient and a leakage of bowel contents that occurs at inappropriate times; bowel problems are amongst the commonest secondary effects of spinal cord injury (Snoek et al. 2004; Krogh et al. 2009; Liu et al. 2010). In many patients this is the most distressing aspect of SCI, due to the social embarrassment associated with leakage and the difficulties of assisted bowel emptying (Coggrave and Norton 2010). Disturbances of bowel function occur in over 80% of people with SCI (Widerström-Noga et al. 1999). Failure of normal neural control of the bowel can have significant side effects, including impaction, hemorrhoids, rectal bleeding, prolapse, formation of anal fissures and chronic constipation leading to megacolon requiring operative diversion (Lynch et al. 2001). The most common approaches to bowel management are manual emptying and use of laxatives.

Another possible approach would be activation of the defecation centres in the lower spinal cord. Most SCIs are at cervical and thoracic levels, whereas the defecation centres are located at lumbo-sacral levels. We have discovered that centrally-penetrant compounds that are agonists of the ghrelin receptor stimulate defecation by an action within the spinal cord, at the lumbo-sacral defecation centres (Shimizu et al. 2006). Defecation can be initiated by either intravenous or oral application of ghrelin receptor agonists that cross into the central nervous system (Shimizu et al. 2006; Shafton et al. 2009). The effects of peripheral administration are mimicked by direct application to the spinal cord of ghrelin itself or of ghrelin receptor agonists (Shimizu et al. 2006; Hirayama et al. 2010; Pustovit et al. 2014).

This research program is aimed at rigourously testing the applicability of ghrelin receptor agonists for treatment of bowel disorders consequent on spinal cord injury in animal models in order to provide data necessary to translate the knowledge to human, and conducting adequately powered
and controlled human trials. We also aim to update and promote guidelines for bowel care in those living with spinal cord injury.

References:

The Research Question(s)
For the part of the program covered by this report, the questions were

- Did the initial clinical trial show that the lead compound, capromorelin, was safe to use in Spinal Cord Injury?
- Is capromorelin less effective, similarly effective or more effective in an animal model of constipation?
- Are there off-target effects of the newer compound, ulimorelin?

Discussion/Recommendations/ Impact for WorkSafe/TAC
The results indicate that capromorelin is safe to use in people with spinal cord injury, it is effective in an animal model of constipation and it should be progressed to an adequately powered double-blind study in people with spinal cord injury.
The later compound, ulimorelin, is more effective than capromorelin but it has a side effect that might possibly preclude its use in people with spinal cord injury.

Results
A major aim of the first six months has been to work towards the next clinical trial, which includes continuation of animal proof of principle studies. For the development of the next clinical trial, we have concentrated on the detailed analysis of the previous trial, on the widening of our animal studies to a model in which constipation can be clearly demonstrated, on investigating further second generation drugs, and in getting agreements in place for the trial.

We have completed the analysis and writing up of the first trial, which was for safety and pharmacokinetics of the first generation drug, capromorelin. Capromorelin, given orally, was well tolerated in people with spinal cord injury and in normal volunteers, there were no adverse side effects. The metabolism of the drug was slightly slower in people with spinal cord injury, but reached a similar peak plasma concentration, at about 60-90 minutes. Defecation occurred at an average of 90 minutes after oral administration. This work has now been published (Ellis et al. 2015). The data on time to peak plasma concentration in spinal cord injured people, and the time to defecation, are being used in design of the up-coming trial, a double-blind, placebo controlled, single ascending dose study, with subjects acting as their own controls.

The drug is intended to empty the bowel in periods of constipation of people living with spinal cord injury. However, it is difficult to quantify constipation in animals with spinal cord injury. We have thus developed a diet induced model of constipation, in which constipation is induced by a low fibre diet. In this model we were able to quantify changed colorectal responsiveness in constipation. We found that capromorelin was in fact more effective in causing defecation in the constipated animals. This work has also now been published (Pustovit et al. 2015).

We had done work on a second generation compound, ulimorelin, leading up to this research program. We showed that ulimorelin is effective in causing defecation, and has a similar mechanism of action to capromorelin (Pustovit et al. 2014). However, we were concerned to observe a cardiovascular effect of ulimorelin, a drop in blood pressure soon after it was given. We have now investigated the reason for this hypotensive effect. We found that ulimorelin causes vasorelaxation through a competitive antagonist action at α1-adrenoceptors, which are receptors on blood vessels and in other organs in humans (Broad et al. 2015). We therefore conclude that ulimorelin is unlikely to be a useful therapeutic compound.

Professor Furness has undertaken trips to the USA in July and to Europe in September to meet with representatives of companies making new compounds that act on ghrelin receptors (the target receptor for capromorelin). In the US, he discussed a possible trial using relamorelin with Rhythm Pharmaceuticals (Boston). A confidentiality agreement has been signed and discussions are ongoing. In France discussions were held with Alizé Pharma about their drug, AZP531. This drug does not seem to have the specificity required. In Switzerland discussions were held with Helsinn Healthcare. Their agonist of ghrelin receptors is being developed for the constipation of Parkinson’s Disease. A materials transfer agreement has been signed, the compound has been delivered to Professor Furness’s laboratory and animal experiments have commenced. Initial studies show that the compound is potent in stimulating defecation.

References:

Methods
The methods of the clinical trial are described in Ellis et al. In brief:

The study was approved by the Austin Health Human Research Ethics Committee and performed at the Austin Centre for Clinical studies. All applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during the course of this research.

Participant eligibility:

All participants underwent initial screening and were fully informed and signed protocol-specific informed consent was obtained. Spinal cord injured candidates from the Victorian Spinal Cord Service at Austin Health with spinal injuries between T6 – T12 and not otherwise excluded as per exclusion criteria applied to able bodied candidates were invited to participate.

Safety/Tolerability:

Physical examinations were performed at pre-study screening and on the day of each dose plus follow-up at + 1 week and + 4 weeks following the final dose and included body measurements, vital signs, electrocardiogram, blood and urine tests plus urine drug screening and pregnancy test. Adverse events and any concomitant medications were monitored throughout the study. Defecation was recorded and was not an expected target measure in this safety study.

Procedure:

Participants received the lowest dose of capromorelin (20 mg) initially. Subsequent higher doses (50 mg and 100 mg) were received after a minimum 1 week wash-out period between doses and only if safety and tolerability assessments were acceptable. Doses of capromorelin tartrate were prepared as capsule formulation and administered orally with 240 mL water following a 12 hour fasting period.

Pharmacokinetics:

Blood sample (5 mL) collections for capromorelin analysis were taken from an indwelling catheter in the cubital vein at -30 mins (pre-dose) and at times up to +12 hr. Red cells were separated and plasma aliquoted prior to freezing at -20°C and transfer within 24 hours to storage at -70°C until analysis. Capromorelin levels were measured at Austin Health Melbourne Australia using a liquid chromatography tandem mass spectrometry (LCMSMS) validated assay. Capromorelin concentration was interpolated from multipoint standard curve ranging from 20 pg/mL to 100 µg/mL. Validation of the method (accuracy, precision, recovery, stability, and interference) followed laboratory standard operating procedures.
Noncompartmental pharmacokinetic analyses were performed using PK Solutions Version 2.0 (Summit Research Services, Montrose Colorado, USA) and Excel 2007 (Microsoft, Sydney Australia).

Capromorelin tartrate was supplied by RaQualia Incorporated, Nagoya, Japan, who also supplied confidential details of in-house safety and drug metabolism data.

The methods of testing defecation in constipation is described in Pustovit et al. In brief:

Sprague-Dawley rats of either sex that were 6 weeks old at the commencement of dietary interventions were used. Animals were fed either a control diet (AIN93G, containing 4.7% crude fibre; Specialty Feeds, Perth, Australia) or a low fibre diet (AIN93G with 0% crude fibre; Specialty Feeds) for 5 weeks. All procedures were conducted according to the National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Measurements of fecal output from conscious rats

A total of 24 Sprague-Dawley rats was used to investigate the effect of the ghrelin receptor agonist, capromorelin, on defecation in conscious rats. These animals were maintained in individual cages in a quiet room for the period of the experiment and were familiarised with two experimenters by regular handling and daily gavaging with a 5% sucrose solution for 10 days. Following familiarisation, rats from both groups were randomly assigned to experimental (capromorelin) and control (5% sucrose solution) groups. Rats received 50 g of chow at 1pm every day and the residual chow was weighed the day after. Animals were weighed daily between 1pm and 3pm, and fecal pellets were removed, counted, weighed and moisture content determined. At 3pm, capromorelin (10 mg/kg) or vehicle (5% sucrose solution) was administered orally by gavage. Faecal output was measured every 30 min for the 2 hours following drug or vehicle administration. The oral gavage experiment lasted for three weeks, during the first and the last week, all rats were given sucrose for the first 5 days, and in the second week rats received capromorelin for the first 5 days. Fecal outputs for the 5 day administration periods were averaged.

Recording colorectal propulsion in anesthetised rats

Rats were sedated with ketamine hydrochloride (50-60 mg/kg, i.m.) and anesthesia was induced with α-chloralose (55-60 mg/kg, into the lateral tail vein). The femoral artery and vein were cannulated and anesthesia was maintained by intra-arterial infusion of α-chloralose (12-20 mg/kg/hr) combined with ketamine hydrochloride (3-5 mg/kg/hr) in PBS. The cannulated femoral vein was used for delivery of drugs. The distal colon was cannulated at the colonic flexure and a second cannula was placed at the anus. The colon was left in situ, and the muscle and skin were closed around the proximal cannula. The oral cannula was connected to a Mariotte bottle filled with warm phosphate buffered saline, and the distal cannula was connected to a pressure transducer and a fluid outlet via a one-way valve. The baseline intraluminal pressure was maintained at 7-10 mmHg. Blood pressure and heart rate, intraluminal pressure and expelled fluid were recorded continuously.

Water avoidance

Rats were familiarised with handling over a period of 2 weeks. On the experimental day, the rat was placed on a platform, located in the middle of a large plastic tub that was filled with water up to
7 cm (1 cm below the top of the platform), for a period of 60 min. The numbers of fecal pellets produced by the rat in the first 10 minutes on the platform were counted.

**Histology**

Histological examination was made in tissues from rats that had not been subjected to recording of colorectal motility. These rats were subjected to a dietary regime identical to rats used for defecation studies. Sections were cut transversely at a thickness of 5 μm and processed for standard hematoxylin and eosin (H&E) staining. Preparations were examined on a Zeiss microscope fitted with an Axio-Cam imaging system.

**Statistical analysis**

Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., San Diego, USA). Data are presented as mean ± SEM. Differences between data sets were tested by one-way and two-way ANOVA followed by post-hoc analysis using the Tukey-Kramer method for multiple group comparisons. For comparisons between two groups, an unpaired two-tailed Student's t-test was used. Differences were considered statistically significant at P<0.05.

**The methods of assessing off-target effects of ulimorelin, described in Broad et al. In brief**

Male Sprague-Dawley rats, mice with knockout of the gene for the ghrelin receptor, and wild-type C57BL6 mice were used. All animal procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee. The procedures abided by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

The saphenous artery was dissected from its origin at the femoral artery to a point just above the knee joint. Second/third order mesenteric arteries supplying the distal 15 cm of ileum were isolated from the mesentery. The whole of the basilar artery was isolated. After dissection, all vessels were maintained in physiological saline.

The arteries were cut into 1.2 – 2 mm length sections, and mounted isometrically onto stainless steel wires. The vessels are mounted at close to the peak of their length-tension relationship.

Different tissues were used to assess the effects of each concentration of ghrelin receptor agonist on contractions induced by phenylephrine, and other agonists. When applied on their own, the concentration of ghrelin receptor agonists was increased cumulatively, with at least 8 min between each increase in concentration. For cumulative concentration curves to phenylephrine, at least 4 min was allowed to elapse between each addition of phenylephrine. When the effects of receptor antagonists, L-NAME or ion channel blockers were also investigated.

Contractions to ghrelin receptor agonists were measured as a % of the peak force of contraction to phenylephrine or 60 mM [K⁺]. Both cumulative and non-cumulative curves were fitted using three parameter models. Linear regression was performed using the EC₅₀ values obtained from the phenylephrine concentration response curves. 1- or 2- way ANOVAs, followed by Dunnett’s post-tests were used where indicated. P < 0.05 was considered significant.
References to publications resulting from the project:


Pustovit, RV, Furness, JB, Rivera, LR: A ghrelin receptor agonist is an effective colokinetic in rats with diet induced constipation. Neurogastroenterol Motil. (published on line, 2015, DOI: 10.1111/nmo.12517).