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Novel peptide calcitonin gene-related peptide antagonists for migraine therapy

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Abstract

Objectives. It has previously been shown that the peptide (^{34}Pro,^{35}Phe)CGRP$_{27–37}$ is a potent calcitonin gene-related peptide, CGRP receptor antagonist, and in this project we aimed to improve the antagonist potency through the structural modification of truncated C-terminal CGRP peptides.

Methods. Six peptide analogues were synthesized and the anti-CGRP activity confirmed using both *in vitro* and *in vivo* studies.

Key findings. A 10 amino acid-containing peptide VPTDVGPFAF-NH$_2$ (P006) was identified as a key candidate to take forward for *in vivo* evaluation, where it was shown to be an effective antagonist after intraperitoneal injection into mice. P006 was formulated as a preparation suitable for nasal administration by spray drying with chitosan to form mucoadhesive microcarriers (9.55 ± 0.91 mm diameter) and a loading of 0.2 mg peptide per 20 mg dose.

Conclusions. The project has demonstrated the potential of these novel small peptide CGRP antagonists, to undergo future preclinical evaluation as anti-migraine therapeutics.

Keywords: migraine; peptide; CGRP antagonist; chitosan microparticles; nasal delivery

Introduction

Migraine affects around 14% of the world population and can be a debilitating condition, with migraineurs experiencing intense headache pain often accompanied by sensitivity to light, sound, and touch, lasting up to 72 h [1]. Migraine symptoms are typically treated using the triptan family of serotonin/5HT-receptor agonists for example sumatriptan, which are available in a variety of dosage forms, including tablets, subcutaneous injection, and nasal spray, each with specific onset times and duration of action. However, these are not always effective in relieving symptoms [2].

Migraine is a complex disease and, as such, is difficult to understand and treat. The calcitonin gene-related peptide (CGRP) is a neuropeptide consisting of 37 amino acids, that plays key roles in cardiovascular homeostasis and nociception [3] and is important in the pathogenesis of migraine [4]. CGRP-targeted drugs have been shown to be effective with fewer side effects than that of earlier drugs such as the triptans [5].

Since 2018, the Food and Drug Administration (FDA) have approved eight drugs that either block CGRP or the CGRP receptor and Dubowchik *et al.* have provided a comprehensive review of this area [6]. The gepants, (small molecule CGRP receptor antagonists) ubrogepant, rimegepant, and atogepant are used to treat acute episodes and are delivered orally [7]. Rimegepant and Atogepant has also recently received FDA approval as a prophylactic treatment. Pfizer's nasally administered zavegepant recently (March 2023) received FDA approval for acute migraine. However, a phase 2/3 clinical trial of Zavegepant indicated it was only effective at reducing pain symptom at 2 h post dose in around 20% of participants [8].

Monoclonal antibody (mAb) CGRP antagonists act as preventative medicines and are delivered monthly *via* the subcutaneous route (erenumab, fremanezumab, and galcanezumab)

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or intravenously (eptinezumab) [9]. The mAbs offer higher specificity and a longer half-life than the gepants but rely on self-injection, which can reduce compliance and result in pain and inflammation at the injection site [10]. They are also expensive to manufacture. Three of the mAbs act by directly binding to and inactivating the CGRP peptide, whereas erenumab and the gepants are CGRP receptor antagonists, binding to and blocking the receptor-binding site [9, 10]. Many migraineurs do not respond fully to antibodies for example, only 48% patients, achieved ≥50% symptom reduction using Fremanezumab in a clinical trial NCT03308968 [11].

Long-term (5-year) studies have demonstrated that mAbs targeting the CGRP pathway are a safe and well-tolerated option [12]. However, there are still some concerns about the cardiovascular risks of continuous CGRP blockade provided by mAb injection given their long half-life [13].

The nasal delivery route is attractive as it avoids some of the metabolic liabilities associated with gastrointestinal degradation, permits rapid drug absorption with quick onset of action, and nasal medicines have a high degree of patient acceptability [14, 15]. These are all key requirements for acute migraine therapy and are particularly useful in patients for whom nausea and vomiting are problematic. A number of existing anti-migraine medicines are available, usually as liquid nasal preparations [15] for example zolmatriptan (Zomig®), sumatriptan (Imitrex®), and butorphanol tartrate (Stadol NS), demonstrate good patient acceptance, and the route has been exploited for large peptides such as insulin (Nasulin) [14, 15]. The pharmacokinetics can vary depending on the site of deposition and current migraine formulations migraine target the lower space so can be quickly cleared by dripping or swallowing reducing bioavailability compared to IV administration. The upper nasal cavity and olfactory region is more mucosal, vascular, and has a lower clearance rate [14]. For example, ONZETA sumatriptan nasal powder provided a higher C_{max} and reduced earlier exposure compared to the same dose of IMITREX sumatriptan liquid nasal spray [14]. With appropriate formulation, nasal administration can produce bioavailability close to that of parenteral delivery [15]. For dry powder nasal delivery, two important parameters to control are particle size, where the diameter should be above 10 μm to prevent inhalation to the lungs, and mucoadhesiveness. Absorption enhancers such as chitosan can improve adhesion to the nasal cavity, reduce mucocillary clearance and increase the very bioavailability of small molecules and peptides [14]. Mucoadhesion can be evaluated by measuring changes in charge that occur upon bonding due to electron transfer between the polymeric system and the mucus membrane epithelium [16].

Synthetic and truncated analogues of CGRP with the potential to act as CGRP antagonists offer the possibility of a new migraine therapy or potentially as a 'rescue' medicine for poor mAb responders.

Peptides are naturally occurring biologics, and peptides composed of all-natural proteinogenic amino acids would generally be expected to have better safety profiles than small molecules and the relatively short half-lives of peptide drugs are associated with lower accumulation in tissues and allows rapid reversal of adverse effects [17–19]. Compared with recombinant proteins and antibodies, short synthetic peptides are generally nonimmunogenic [17]. From a manufacturing point of view, small peptides are much easier and cheaper to produce and to quality control than mAbs [19].

The full-length 37 amino acid CGRP peptide acts as an agonist of the CGRP receptor, however, removal of CGRP residues 1–8 affords a peptide-based antagonist [20] that can be further truncated to just amino acids 27–37 [21]. Earlier structure-activity relationships studies by Rist *et al.* used truncated CGRP analogues to systematically study both the modification the terminal segments and Ala, Phe, and Pro scans. Analogues [D^{31}, P^{34}, F^{35}] CGRP(27–37) and [P^{34}, F^{35}] CGRP(27–37) increased receptor affinity by around 100-fold [22]. A decrease in GCRP-induced vascular conductance was also observed following administration of [P^{34}, F^{35}] CGRP(27–37) [23]. However, it was also observed that these high-potency analogues were much less soluble than the parent CGRP(27–37) peptide [24]. We also recently showed that the short peptide $Pro^{34}Phe^{35}$CGRP(27–37) is a potent CGRP receptor antagonist both *in vitro* and *in vivo*, and demonstrated that it can be formulated for nasal delivery without loss of potency [25].

This study takes an innovative approach to the development of a new treatment for migraine management through the use of novel short peptides specifically designed to target the CGRP receptor. We have shown, for the first time, that the peptide VPTDVGPFAF-NH_2 (P006) is a potent CGRP receptor antagonist both *in vitro* and *in vivo* and can be readily formulated for nasal delivery. This research demonstrates the potential of this peptide as a new, convenient, and effective treatment for migraine sufferers.

Materials and methods

Materials

Human α-CGRP (Bachem AG, Switzerland) was dissolved in pure water and stored as frozen (–18°C) aliquots until use. Fmoc-amino acids, Rink Amide ProTide resin and Oxyma Pure™ were purchased from CEM Microwave Technologies, UK. Diisopropylcarbodiimide (DIC) was obtained from Apollo Scientific. Peptide synthesis grade *N*,*N*'-dimethylformamide (DMF) was purchased from Fisher Scientific, UK. Diisopropylethylamine and piperidine were purchased from Merck Millipore. All reagents were used as received unless otherwise indicated. Earl´s salts, L-glutamine foetal bovine serum, sodium pyruvate, and nonessential amino acids were purchased from Life Technologies (Sweden). SK-N-MC cell membranes (endogenous CGRP receptor expressing) were purchased from Receptor Biology (USA) containing 23.8 mg protein per ml in a buffer containing 50 mM Tris, pH 7.4, 5 mM $MgCl_2$ and 10% sucrose. Membranes were frozen (–80°C) in aliquots. Trifluoroacetic acid (TFA), triisopropylsilane (TIPS), chitosan (CAS number 9012-76-4), and Porcine Mucin II were purchased from Sigma Aldrich. High-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific, UK.

Peptide synthesis

Peptides were synthesized using automated solid-phase peptide synthesis on a Liberty Blue microwave-assisted peptide synthesizer (CEM). Fmo-protected amino acids (0.2 M in DMF; 5 eq.) were sequentially coupled onto preswollen Rink amide ProTide resin (180 mg, 0.56 mmol/g loading; 0.1 mmol

scale) using DIC (1 M in DMF; 10 eq.) and Oxyma Pure (1 M, 5 eq). Generally, double coupling of each amino acid (2.5 min, 90°C) was performed. Between coupling steps, Fmoc-group removal was completed using piperidine (20%, v/v, in DMF; 587 eq., 4 ml). When synthesis was complete, the resin was washed with DMF and shrank in diethyl ether, and peptides were released from the resin as the C-terminal amide by acidolysis (5 ml; TFA, TIPS, and water (9: 0.5: 0.5, v/v) with shaking at room temperature for 4 h. Cleavage solutions were added dropwise to cold diethyl ether to precipitate the peptide, which was isolated following centrifugation. The pellet was washed twice by sequentially adding more cold diethyl ether and further centrifuging. The solids obtained were dissolved in H_2O/MeCN, flash frozen, lyophilized, and purified by semi-preparative HPLC.

High-performance liquid chromatography

Analytical HPLC and semi-preparative HPLC employed an Agilent 1100 Series and 1200 Series, respectively, comprising a diode-array detector (215 nm) and G1364C fraction collector (semi-prep only).

Analytical high-performance liquid chromatography

Post-cleavage peptide mixtures were analysed using analytical HPLC, employing a C18 analytical HPLC column (Phenomenex, 3.6 µm particle size, 4.6 × 150 mm) with a binary eluent system comprising MeOH/H_2O (18 min gradient: 5%–95% with 0.1% formic acid) as mobile phase.

Semi-preparative high-performance liquid chromatography purification of peptides

Pure synthetic peptides were isolated from crude mixtures using semi-preparative HPLC using an XBridge® Peptide BEH C18 Prep 130 Å, 5 µm column (10 × 150 mm Waters) with a binary mobile phase comprising MeCN and H_2O (with 0.1%, v/v formic acid). Isolated pure peptides were concentrated under a vacuum to remove organic solvents and were then flash frozen (liquid N_2) and lyophilized.

Mass spectrometry

Peptide identify was confirmed using electrospray ionization mass spectrometry in positive ion mode (*m/z* range: 50–3200 Da), with a fragmentor voltage of 150 V, gas temperature of 325°C (flow 10 L/min) and sheath gas temperature of 400°C (flow 11 L/min). This used an Agilent 1260 Infinity II LC system with Agilent 6530 Accurate-Mass QToF spectrometer, and an Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HD analytical column (1.8 µm particle size, 2.1 × 50 mm) employing a binary mobile phase of MeOH/H_2O (12 min gradient: 1%–99% with 0.1% formic acid).

In vitro antagonist activity P001 to P006

Antagonist activity was evaluated by measuring the reduction of CGRP-induced cAMP accumulation in Chinese Hamster Overy (CHO) cells that over-express the human CGRP receptor complex [receptor activity-modifying protein-1 (RAMP1) and calcitonin receptor-like receptor (CLR)] using a commercial assay kit (DiscoveRx HitHunter) which is validated for this purpose. Increasing concentrations (0.01–30 nM) of αCGRP were added to the cells and the agonist EC_{50} values were measured in the presence of different concentrations of antagonist peptide (30, 100, 300 nM, or 1 µM). Antagonist affinity constants (K_B) were obtained from Schild analysis, by plotting Log_{10} (conc. ratio—1) versus $-log_{10}$ [antagonist conc.]. Log_{10} K_B was determined from the abscissa intercept of the straight line obtained by extrapolation of the line of best fit. In some experiments, K_B was estimated from shifts in CGRP concentration/response curves using a single antagonist concentration and applying the Schild equation: (agonist conc. ratio—1) = (antagonist conc.)/K_B. Note: concentration ratio = agonist Effective concentration, EC_{50} value in the presence of antagonist/EC_{50} value in the absence of antagonist.

Receptor binding was measured in membranes from SK-N-MC cells naturally expressing the human CGRP receptor incubated with ^{125}I-αCGRP and increasing concentrations of competing peptides.

Binding experiments were performed using 24 µg SK-N-MC membranes in a total assay volume of 200 µl. All components were diluted in a buffer containing 50 mM Tris, pH 7.4 at 20°C, 5 mM $MgCl_2$, 0.1% BSA and 100 µM Guanosine 5'-Triphosphate(GTP). GTP was added to assure that the receptor would show the same affinity for the agonist ^{125}I-αCGRP and an indication for more than one binding site would not be due to functional receptor heterogeneity. The nonlabelled and labelled substances were added to a 96-well plate (Nunc Brand Products, Nalge Nunc International) and mixed prior to addition of the membranes. After 1 h of incubation in room temperature the reaction was terminated by rapid filtration (Micro96 Harvester, Skatron Instruments, Norway) to separate bound ^{125}I-αCGRP from free. The filters used (Filtermat B, Wallac, Finland) were presoaked in 0.3% polyethylenimine for 15 min and then dried. The filtration buffer (50 mM Tris, pH 7.4 at 20°C, 5 mM $MgCl_2$) was kept cold. After filtration, the filters were dried and a scintillator sheet (MeltiLex B/HB, Wallac, Finland) was melted on the filter (microsealer 1495-021, Wallac, Finland). The amount of bound ^{125}I-αCGRP was measured by counting for β-radiation in a liquid scintillation counter (1450 Microbeta PLUS, Wallac, Finland). The results were presented as decompositions per minute.

In displacement binding experiments 20 pM ^{125}I-αCGRP was used. Concentrations from 20 pM to 50 µM of nonlabelled peptides were added. For each concentration of nonlabelled ligand, the amount of total bound ^{125}I-αCGRP was determined and specific binding was calculated.

In vivo activity

Animals

Experiments were performed on male Navel Medical Research Institute (NMRI) mice (28–40 g) purchased from Toxicoop Ltd. (Budapest, Hungary). Mice were kept in the Animal House of Szentágothai Research Centre of the University of Pécs (Pécs, Hungary) in individually ventilated cages at 24°C and a standard 12 h light-dark cycle with the provision of standard rodent chow and water *ad libitum*.

Measurement of skin perfusion

Perfusion of the hind paw skin was measured by a PeriCam PSI equipment (Perimed AB, Sweden) which applies the Laser Speckle Contrast Analysis technology. Perfusion measured by the equipment is expressed in arbitrary perfusion units which is a composite parameter derived from the number and

velocity of red blood cells moving through the investigated tissue. The response was quantified as a percentage change over an initial baseline measurement and area under the curve (AUC) over baseline.

Effect of P006 on calcitonin gene-related peptide-induced increase of skin perfusion

Mice were treated with P006 (20 mg/kg i.p.) 30 min before intraplantar (i.pl.) CGRP administration (0.1 mg/ml, 5 µl i.pl.). Approximately 18 min after pretreatment, mice were anaesthetized with a mixture of ketamine-xylazine (100–5 mg/kg i.p.). Anaesthetized mice were placed on a heating pad in prone position and their paws were fixed to the pad with thin strips of adhesive tape. CGRP or saline was administered i.pl. via PE-10 polyethylene cannula attached to 30G hypodermic needles which were separated from the hub. Needles were inserted subcutaneously into the hind paws at the level of metatarsophalangeal joints and carefully advanced 1–2 mm towards the heels. After 3–5 min of baseline perfusion measurement, saline was slowly injected into the left paw and CGRP into the right paw shortly after. The perfusion was measured for an additional 10 min. After the end of the measurement mice were euthanized with pentobarbital.

Ethical approval

All procedures were designed and performed according to the European legislation (Directive 2010/63/EU) and Hungarian Government regulation (40/2013., II. 14.) on the protection of animals used for scientific purposes. The project was approved by the National Ethics Committee on Animal Research of Hungary (license No.: BA02/2000-7/2018, issued on 26 February 2018 by the Government Office of Baranya County, Hungary).

Chitosan microparticle formulation

P006-containing microcarriers were obtained by spray drying a 0.5% acetic acid solution of low molecular weight (LWM) chitosan (2 %) and P006 (1%, w/w) using A Büchi B-290 spray dryer (Büchi Labortechnik AG, Postfach, Switzerland) equipped with a nozzle atomizer with a nozzle orifice diameter of 2.0 mm. The atomizing gas was nitrogen and a high-performance cyclone (Büchi Labortechnik AG) was used to separate the dry microparticles (MPs) from the airstream via centrifugal forces. Optimal operating parameters were previously determined [25] constant spray gas flow (60) and feed flow rate (15% of pump capacity, just under 5 ml/min), aspirator capacity 95%, and inlet temperature, 165°C. The experiment were carried out in triplicate for both blank and loaded MPs.

Microparticles size and morphology

Scanning electron microscopy (SEM) was used to observe the MP morphology and diameter. ImageJ 1.51 software was used to measure 100 particles for each sample in a single image and calculate the average diameter of the particles.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) by TGA Q5000 (TA Instruments, New Castle, DE, SAD) was used to establish the moisture content of blank chitosan microcarriers where water loss between 25 and 100°C was analysed. The spray-dried powder (10 mg) was loaded on a platinum TGA pan suspended from a microbalance and heated from 25°C to 250°C at 10°C per min.

Mucoadhesive test

A mucin particle method, developed by Takeuchi *et al.* [26] was employed to determine the charge and the mucoadhesive properties of blank chitosan MPs. Briefly, the mucin powder (Porcine Mucin type II) was suspended in phosphate buffer (pH 6.5) at a concentration of 1%, w/v. Then, 1 ml of this suspension was mixed with different volumes (1, 2, 3, and 4 ml) of a suspension of MPs 0.1% in phosphate buffer (pH 6.5) under mild magnetic stirring (200 rpm) and the Zeta potential of the mixtures was measured. All experiments were performed in triplicate.

Release from chitosan microparticles

Release of P006 from the chitosan MPs into phosphate saline buffer (pH 7.4, sodium chloride 137 mM, phosphate buffer 10 mM, potassium chloride 2.7 mM) was ascertained by suspending MPs (10 mg) in buffer (1 ml) in a 1.5-ml centrifuge tube and mixing at 20 rpm and 37°C. Unloaded MPs were used as blanks. For each replicate, three samples were prepared, and one sample was centrifuged (Eppendorf Centrifuge 5415 D) at 13 200 rpm for 30 min at each time point (5, 15, 30, and 60 min, 4, 8, and 24 h). RP-HPLC (Phenomenex Aeris 3.6 µm peptide XB-C18 LC-Column 100A 150 × 4.6 mm) solution using a linear gradient from 80% solution A (high-purity water 0.05% TFA) to 60% solution B (MeCN 0.05% TFA) over 10 min was utilized to analyse the supernatant. A flow rate of 1 ml/min was used, the absorbance detected at 215 nm and the analysis was performed at 65°C. A calibration curve of P006 (R^2 of 0.9999) was obtained by analysing peptide aqueous solutions (5 to 1000 µg/ml).

Data analysis and statistical calculations

Excel fit or GraphPad Prism were employed to analyse the results which were determined as means ± standard error. Students t-test or ANOVA were performed for statistical analysis where appropriate.

Results and discussion

We previously reported that Pro^{34}Phe^{35}CGRP(27–37) is a potent CGRP receptor antagonist both *in vitro* (pK_B = 8.1 ± 0.14 nM) and *in vivo* using a mouse extravasation model, in which it significantly reversed the effects of CGRP [25]. Subsequently, we have now investigated whether the design of additional CGRP peptide analogues could further improve antagonist activity.

Peptide synthesis

Six 8–11 amino acid peptides (Table 1) were synthesized by solid phase peptide synthesis, purified using preparative HPLC (>95% homogeneity) and characterized by analytical HPLC and Liquid Chromatography-Mass Specrometry (LC–MS). Modifications were designed to probe the effect of (i) mutation of the Pro-34 to Ala and (ii) sequential truncation of the N-terminal Val-Pro motif on antagonist activity. Proline (Pro) is unique in its ability to undergo conformational isomerism at the prolyl bond, unlike alanine (Ala), and replacement of Pro, therefore, can significantly impact the peptide conformation

Table 1. Sequences and biological evaluation of synthesized peptides.

Peptide name	Sequence	CGRP antagonist potency (pK_B)	CLR binding affinity (pK_i)
*hα*CGRP	SCNTATCVTH-RLAGLLSRSG-GVVKDNFVPTNVGSEAF-NH_2 (disulfide bridge: 2-7)	–	9.7
$Pro^{34}Phe^{35}$CGRP(27–37)	F V P T N V G P F A F—NH_2	8.1	8.0
P001	T D V G A F A F—NH_2	6.7	6.8
P002	P T D V G A F A F—NH_2	6.7	6.8
P003	V P T D V G A F A F—NH_2	6.9	7.3
P004	T D V G P F A F—NH_2	7.5	7.4
P005	P T D V G P F A F—NH_2	7.7	7.7
P006	V P T D V G P F A F—NH_2	7.9	7.8

The data represent an initial screen of the modified peptides (n = 2, with each conducted in triplicate).

[27]. It has previously been identified that the full-length *hα*CGRP peptide can be truncated to the C-terminal 27–37 residues without significant loss of receptor affinity [20–23], however, further structural minimisations would be advantageous from the point of view of synthetic tractability, reduced metabolic sensitivity and increased ligand efficiency, leading to more 'drug-like' compounds [18]. In all cases, a further modification of the $Pro^{34}Phe^{35}$CGRP (27–37) peptide saw the Asn-31 residue replaced with Asp, bearing a carboxylic acid side chain to enhance water-solubility properties.

In vitro potency

CGRP receptor-antagonist potency was measured using a commercial DiscoverRx HitHunter cAMP assay kit (n = 2, Table 1). Peptide P006 (pK_B = 7.9) was confirmed as the most potent new antagonist, bearing a proline at position 34 and retaining the N-terminal Val-Pro motif. In contrast, analogues P001-3 lacking the proline were around 10-fold lower potency. This suggested that the conformational arrangement of the peptide, perhaps through a turn motif centred at Pro-34 is important for avid receptor binding and has been previously reported for related analogues [21, 22, 28]. The alanine mutant would be expected to find adoption of this conformation more challenging, and the trend of lower antagonist affinity was consistently observed in compounds that lacked the Pro-34.

Truncation of P006 by sequential removal of Val-28 (P005) and Val-28-Pro-29 (P004) afforded a lower binding affinity (Fig. 1) and may be attributed to the loss of important ligand-receptor interactions or subtle effects on the conformation of the antagonist. Pro-29 is thought to help induce a β-turn in solution based on Nuclear Magnetic Resonance (NMR), Fourier Transform Infra-Red (FTIR), and molecular dynamics experiments and is important for target engagement [28]. Overall, replacement or deletion of prolines in the parent sequence was found to be detrimental to antagonist activity, as underlined by P001—the Pro34 to Ala mutant lacking the Val-Pro motif, which was the weakest antagonist studied. Further stabilization of the binding conformation could enhance antagonist activity; while other modifications to improve proteolytic stability, cellular permeability, and lipophilicity would also be therapeutically beneficial. It is known that CGRP can also bind to other receptors, such as AMY1, and future selectivity studies will investigate whether these peptides can also antagonize other CGRP-family receptors.

$Pro^{34}Phe^{35}$CGRP(27–37) and PYP006 models were generated through computational mutagenesis using the

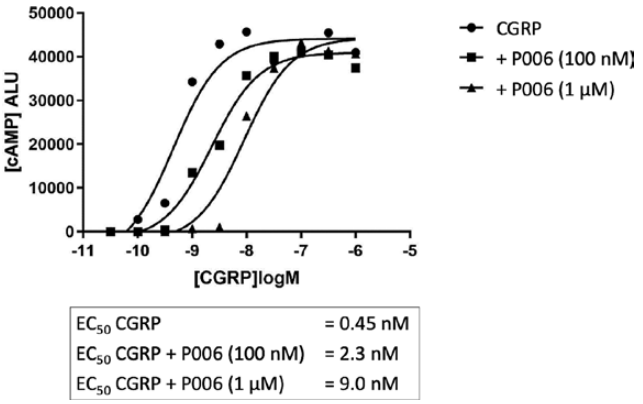

Figure 1. CGRP stimulated cAMP accumulation in the absence and presence of P006 (P6) in concentrations of 100 nM and 1 μM. Each data point indicates triplicate determinations in a single representative experiment.

natural CryoEM CGRP 27–37 structure (PDB accession code: 6E3Y) [29], then energy minimized (with respect to the receptor) using calculation method XED Accurate within the molecular modelling software FLARE V5.0 (cresset software, https://www.cresset-group.com/software/flare/). Modelling the interactions between $Pro^{34}Phe^{35}$CGRP(27–37) and the CGRP-receptor complex (Fig. 2) indicated that the removal of Phe27 may lose a possible π–π interactions between Phe27 of the ligand with His132 from the CLR protein. This offers a possible explanation for the subtle reduction of both potency and affinity of P006 (the most potent new antagonist) compared with $Pro^{34}Phe^{35}$CGRP(27–37), while Val28 does not seem to for any productive interactions with the receptor. Asn31 also did not appear to interact with the receptor but is likely important for maintaining the conformational stability of the ligand. Therefore, mutation of Asn31 to Asp31 (PYP006) provides a more negatively charged sidechain, but probably has little impact upon binding affinity. Overall, the combination of removal of Phe27 and introducing an ionizable side chain, ultimately afforded improved water solubility without any major impact of biological activity.

In vivo potency

CGRP is known to induce peripheral blood vessel dilation, so vascular changes upon administration of CGRP antagonists (compared to nonadministration as a control) can be used to

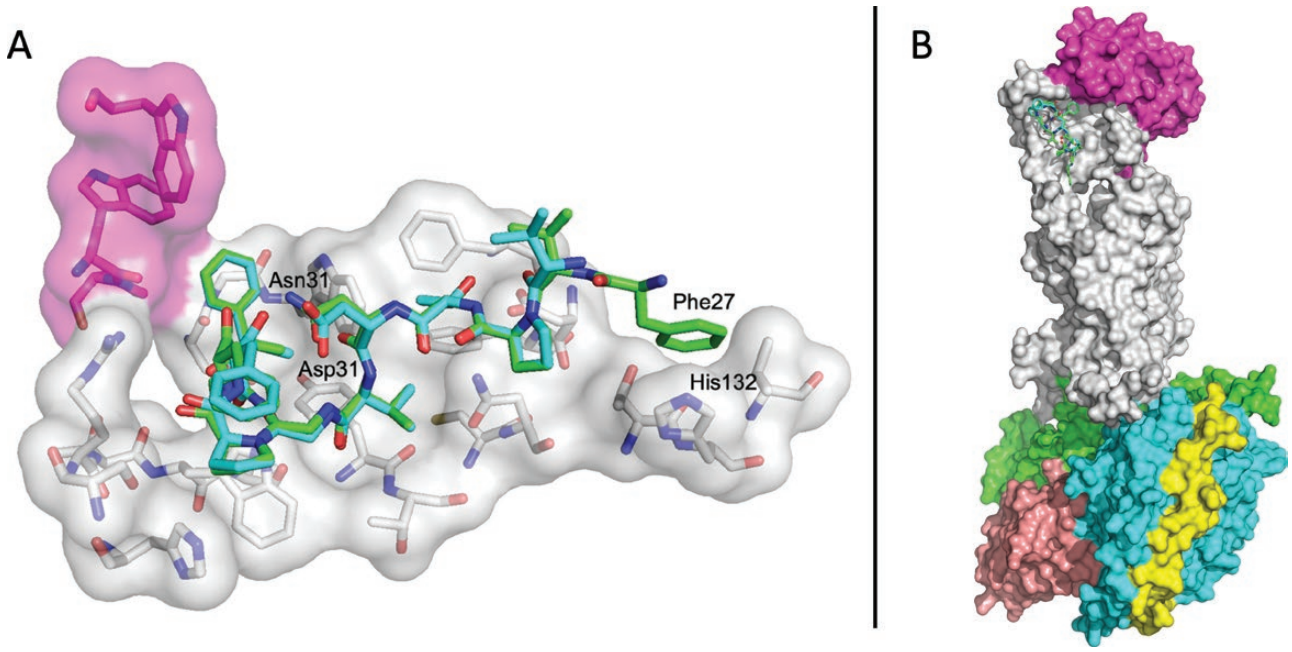

Figure 2. A) Molecular modelling (FLARE V5.0, cresset software) shows Pro[34]Phe[35]CGRP(27–37) (green sticks) overlayed with PYP006 (blue sticks) at the extracellular agonist-binding site of the CryoEM CGRP receptor complex (grey sticks show CLR protein, pink sticks show RAMP1 protein, PDB accession code 6E3Y). B) Pro[34]Phe[35]CGRP(27–37) (green sticks) and PYP006 (blue sticks) docked at agonist-binding site on whole CryoEM CGRP receptor (surface representation, produced using PDB accession code 6E3Y).

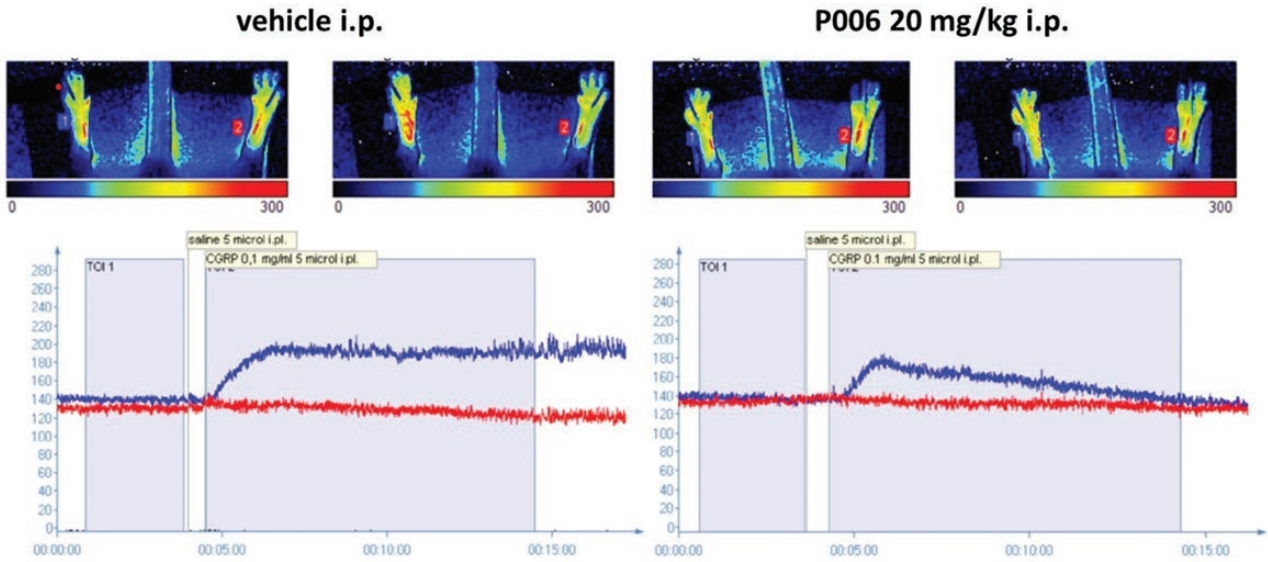

Figure 3. Representative perfusion measurement images of the mouse paw as determined with Laser Speckle Contrast Imaging. The *y* axis shows arbitrary units of perfusion determined by the Pericam PSI equipment and the *x* axis is time in minutes. After baseline measurement, 5 µl saline was administered into the left paw (red) and 5 µl CGRP 0.1 mg/ml into the right paw (blue) by slow infusion. The left panel shows a representative recording of a vehicle-treated animal, while the right panel shows a representative recording of an animal pretreated with P006 (20 mg/kg i.p.).

investigate the functional role of CGRP analogues *in vivo*. *In vivo* CGRP-induced cutaneous vasodilation in a mouse model was assessed by blood perfusion measurement using Laser Speckle Contrast Imaging (Fig. 3). Intraplantar injection of 5 µl CGRP 0.1 mg/ml in anaesthetized mice produced a highly significant enhancement of blood flow compared with saline control (mean increase above baseline: 23.24 ± 0.2%) (Fig. 4). The mean perfusion increase was significantly inhibited by PYP006 pretreatment (20 mg/kg i.p. 30 min before), compared with the vehicle-treated group ($P < 0.05$, Student's t-test for unpaired samples). The AUC above baseline was also reduced, but the difference was not statistically significant.

Microparticle formulation

Chitosan was selected for the MPs preparation due to its high mucoadhesive properties that would enable the particles to

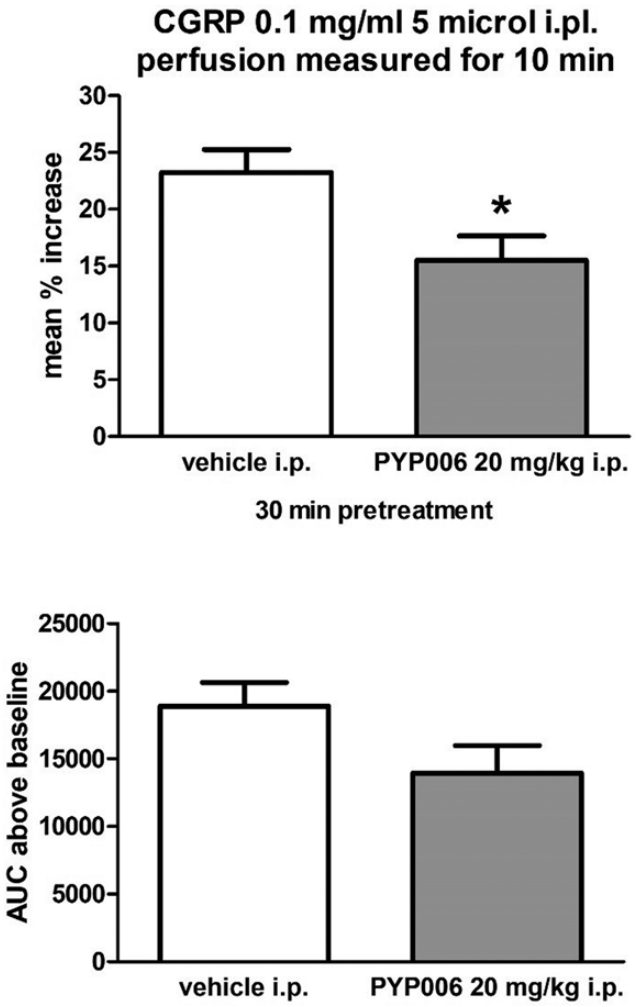

Figure 4. Mean percentage increase of perfusion induced by CGRP (upper graph) and AUC above baseline of the CGRP-induced response (lower graph). Asterisk marks statistically significant difference between vehicle (n = 11) and drug-treated animals (n = 12; $^{**}P < 0.05$, Student t-test for unpaired samples).

deposit and stick to the nasal mucosa and deliver the peptide through the olfactory nerve [30]. Parameters previously optimized by our group [25] to obtain MPs of 10 µm diameter were employed. Chitosan MPs containing 5 mg of P006 (1%, w/w) in 0.5 g of LMW Chitosan were prepared with a yield of 75% for the blank formulation and 68% for the loaded formulation, much higher than the typical resulting powder obtained when spray-drying polymers with a great tendency to stick to the walls of the cyclone [14] even 27–37 MPs yielded 45%.

From SEM analysis of blank and loaded Low Molecular Weight chitosan MPs, the diameter and morphology were obtained. No significant difference was observed between the diameter of blank MPs (9.00 ± 0.74 mm) and loaded MPs (9.55 ± 0.91 mm). Variation in particles morphology was observed by microscopic analysis, with most particles having a rounded shaped with some presenting a 'cheesy rough wrinkled surface'.

Blank chitosan microcarrier powder was analysed using TGA and a weight loss of around 7.1 ± 1.28% was detected, meaning that the powder had a moisture content of around 7%, probably due to the hydrophilic nature of chitosan.

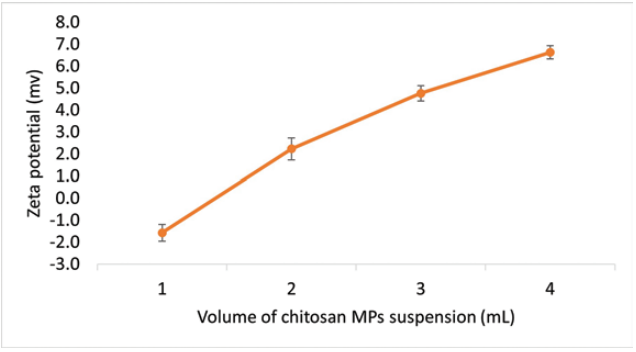

Figure 5. Volume of Chitosan MPs versus Zeta Potential Chart, showing positive correlation between mucine and chitosan MPs.

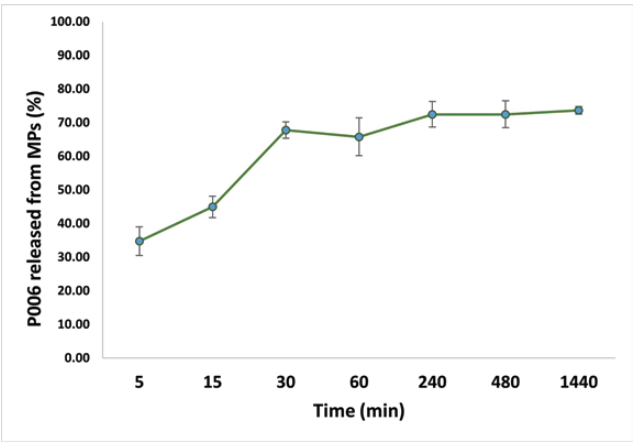

Figure 6. Release of P006 from chitosan microcarriers in PBS buffer solution over 24 h.

The mucoadhesion test showed that the charge of the MPs was proportional to the chitosan concentration in the mixtures which correlates to the bond strength between the mucin solution and the MPs. Figure 5 illustrates the change from negative to positive with the addition of higher quantities of the MP suspension. This result confirmed that chitosan has a high affinity to mucin particles and as expected, surface properties of the mucin particles are altered by the adhesion of a mucoadhesive polymer such as chitosan.

Release from chitosan microparticles

PYP006 was spray dried from chitosan solutions at 1%, w/w, peptide (loading of µg peptide/mg MPs). A standard 20 mg dose loaded into an Aptar nasal spray device, would provide a peptide dose of 200 µg. Approximately 68% of peptide was released from the MPs in PBS buffer at 37°C within 30 min (Fig. 6). After 24 h, 74% of the peptide was released from the chitosan microcarrier.

The peptide integrity was maintained during the spray-drying process as confirmed by observation of the expected parent ion and retention time in LC–MS analysis. MPs based on chitosan are considered a viable option for moving the project towards preclinical (Good laboratory practice (GLP) toxicity) and Phase I clinical trials. Chitosan has been shown to be effective for the nasal delivery of therapeutic drugs including peptides and is 'generally regarded as safe' for

dietary use. The preclinical safety of intranasally administered chitosan has been comprehensively demonstrated and in clinical trials, more than 3000 intranasal doses of chitosan have been administered to more than 1000 human subjects [15]. The migraine drug Alniditan has been given as a nasal chitosan solution in Phase I and II trials [16]. and a morphine-chitosan solution (Rylomine) has undergone Phase III trials in the USA [31].

Conclusion

A number of modified linear peptide antagonists ranging from 8 to 11 amino acids showed nanomolar antagonist potency and, of these, a 10 amino acid peptide, PYP006, was selected as the most promising for further study. Peptides cannot normally be administered orally and nasal delivery is regarded as an attractive alternative. Encapsuslation into mucoadhesive MPs is one strategy for improving the efficiency of nasal drug delivery.

PYP006 was spray dried with chitosan at 0.5%, w/w, peptide-producing MPs of 9.55 mm diameter with a loading efficiency of 67% (8.8 µg peptide/mg MPs). Release of 68% over 30 min.

Author contributions

Participated in research design: Capel, Coxon, Helyes, Hutcheon, Kendall, Killoran, and von Mentzer. Conducted experiments: Capel, D'Aloisio, Aczél, Bölcskei, and Killoran. Performed data analysis: Capel, Coxon, Helyes, Aczél, Bölcskei, D'Aloisio, Hutcheon, Kendall, and Killoran. Wrote or contributed to the writing of the manuscript: Coxon, Helyes, Aczél, Bölcskei, D'Aloisio, Hutcheon, Kendall, and Killoran.

Conflict of interest

DAK and BvM are Directors of Innovipharm Ltd. ZH is a Director of PharmInVivo Ltd.

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Data availability

The data underlying this article are available in the article and in its online supplementary material.

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