Weight-reducing, lipid-lowering and antidiabetic activities of a novel arginine vasopressin analogue acting at the V1a and V1b receptors in high-fat-fed mice

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Abstract
Aim: To assess the beneficial metabolic effects of the nonapeptide hormone, arginine vasopressin (AVP), on metabolism.

Materials and Methods: We exchanged amino acids at position 3 and 8 of AVP, namely phenylalanine and arginine, with those of oxytocin, to generate novel analogues with altered receptor selectivity. Secondary modification by N-terminal acetylation was used to impart stability to circulating endopeptidases. Analogues were screened for degradation, bioactivity in rodent/human clonal beta cells and primary murine islets, together with evaluation of receptor activation profile.

Results: Analogue Ac3IV, which lacked effects at the V2 receptors responsible for modulation of fluid balance, was selected as the lead compound for assessment of antidiabetic efficacy in high-fat-fed mice. Twice-daily administration of Ac3IV, or the gold standard control exendin-4, for 22 days, reduced energy intake as well as body weight and fat content. Both interventions decreased circulating glucose levels, enhanced insulin sensitivity, and substantially improved glucose tolerance and related insulin secretion in response to an intraperitoneal or oral glucose challenge. The peptides decreased total- and increased HDL-cholesterol, but only Ac3IV decreased LDL-cholesterol, triglyceride and non-fasting glucagon concentrations. Elevations of islet and beta-cell areas were partially reversed, accompanied by suppressed islet cell proliferation, decreased beta-cell apoptosis and, in the case of exendin-4, also decreased alpha-cell apoptosis.

Conclusion: AVP-based therapies that exclusively target V1a and V1b receptors may have significant therapeutic potential for the treatment of obesity and related diabetes, and merit further clinical exploration.

KEYWORDS
enzymatic stability, obesity, receptor selectivity, type 2 diabetes, vasopressin

1 | INTRODUCTION

The last 15 years have witnessed a substantial increase in the treatment options available for type 2 diabetes with the timely introduction of glucagon-like peptide-1 (GLP-1) mimetics, as well as dipeptidyl peptidase-4 (DPP-4) and sodium-glucose co-transporter-2 (SGLT-2) inhibitors. In particular, the clinical success of GLP-1 mimetics has illustrated the benefit of simultaneous activation of...
multiple physiological pathways by a single peptide entity, conferring positive effects on insulin secretion and action, gut motility, appetite and body weight, as well as cardiovascular and neuronal function. Unimolecular dual- or even triple-acting peptides targeting GLP-1, gastric inhibitory polypeptide (GIP) and glucagon receptors are now being developed to modulate multiple receptor sites. Other peptides being explored include apelin, peptide YY (PYY), cholecystokinin (CCK-8), fibroblast growth factor-21 (FGF-21), oxyntomodulin, irisin, obestatin and xenin. Given present imperfections in attempts to normalize blood glucose and prevent the risk of diabetic complications, the search continues for other naturally occurring peptide hormones with beneficial action profiles that can be structurally modified to confer enzyme stability and long duration of action for potential exploitation in type 2 diabetes therapy.

Arginine vasopressin (AVP), also known as antidiuretic hormone, is a nonapeptide synthesized in the hypothalamus and secreted by the posterior pituitary gland. Although the classically recognized physiological action of AVP is in the regulation of fluid balance and cardiovascular function, an increasing body of evidence suggests that AVP plays an important role in glucose homeostasis and metabolic control. The biological effects of AVP are mediated through modulation of three separate G-protein coupled receptors (GPCRs), namely, Avpr1a (V1α), Avpr1b (V1b) and Avpr2 (V2). Whilst the V2 receptor is responsible for the effects of AVP on the kidney and water retention, V1α and V1b receptors are expressed in metabolically active tissues. Studies in AVP receptor knockout (KO) rodents confirm a role for AVP signalling in metabolism. Thus, plasma glucose levels are elevated in V1α receptor KO mice but are reduced in V1b KO mice. Mice with double V1α and V1b receptor KO present with unaltered circulating glucose levels, but this is accompanied by hyperinsulinaemia and glucose intolerance. Furthermore, an inherent genetic mutation leading to lack of AVP production in rats results in reduced plasma insulin concentrations. Genetic variation in AVP receptor expression in humans is also associated with obesity and increased prevalence of diabetes.

In addition, AVP receptors are present on pancreatic islets and their activation directly evokes insulin secretion and protects against beta-cell loss. AVP is also known to stimulate glucagon secretion, but AVP-induced insulin release only occurs in the presence of high glucose concentrations, whereas effects on glucagon secretion are only apparent when glucose levels are low. This represents a physiologically important action in the maintenance of glucose homeostasis, linked to prevention of both hyperglycaemia and hypoglycaemia. Such a biological characteristic is also evident with the incretin hormone GIP, which is now considered to possess bona fide therapeutic promise for diabetes. Taken together, these observations suggest that AVP may have untapped potential for exploitation in the treatment of diabetes and related metabolic disorders.

Interestingly, AVP has a strikingly similar structure to oxytocin, with the two nonapeptides only differing at positions 3 and 8, with phenylalanine and arginine in AVP being replaced by isoleucine and leucine in oxytocin. Although native oxytocin is best known for positive effects on mood and reproductive function, enzyme-resistant analogues have recently been developed and shown to exert notable benefits on body weight, glucose homeostasis, lipid metabolism and pancreatic architecture in high-fat-fed (HFF) mice. We hypothesized that the similarity in structure between the two hormones provides a good foundation for the generation of novel and therapeutically interesting peptides that could positively interact with both oxytocin and AVP receptor subtypes. In an attempt to generate such compounds, we designed six novel AVP peptides where amino acids at positions 3 and 8 in AVP were replaced by those of oxytocin (Table 1). We also examined the effects of removal of the characteristic disulphide bridge plus addition of an N-terminal acetyl group to impart enzyme resistance, as employed previously.

The AVP analogues were initially screened for enzymatic stability, and insulin secretory responses in vitro and ex vivo, together with receptor activation profile. The lead peptide emerging from these studies, namely Ac3IV, which lacked appreciable effects at V2 receptors, was progressed to antidiabetic efficacy testing in HFF mice in head-to-head comparison with the clinically approved GLP-1 mimic, exenatide. Our results suggest that such designer AVP analogues constitute a potentially exciting new drug class, meriting further exploration for the treatment of obesity and type 2 diabetes.

2 | MATERIALS AND METHODS

2.1 | Peptides

All peptides (Table 1) were obtained from Synpeptide (Shanghai, China) at 95% purity. Characterization of the peptides was carried out in-house by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS), as described previously. Abbreviated names are used for AVP analogues, denoting as appropriate, the position and type of amino acid substitutions in AVP (where AVP is abbreviated to V), namely, 3IV and 8LV, presence of N-acetyl group (abbreviated to Ac) and indication of reduced form without disulphide bridge by letter R.

2.2 | Plasma stability

To establish in vitro stability of the peptides, peptides (10 μg) were incubated with 5 μL of overnight (18 hours) fasted murine plasma at 37°C, with degradation profiles acquired using reversed phase (RP)-HPLC and MALDI-ToF, as described previously.

2.3 | In vitro and ex vivo insulin secretion

Rat BRIN BD11 and human 1.B4 cells were utilized to investigate the influence of test peptides on insulin release. The origin and secretory characteristics of these clonal beta cells have been detailed in full elsewhere. The insulin secretory activity (20 minutes) of test
peptides (10^{-12} to 10^{-6} M) at 5.6 and 16.7 mM glucose was determined as previously described. In a separate series, BRIN BD11 cells were incubated in 16.7 mM glucose with test peptides (10^{-6} M) alone, or in combination with 10^{-6} M of a selective oxytocin (L-351257; Tocris), V1a (SR-49059, Sigma-Aldrich), V1b (Nelivaptan; SR-149415, Axon Medchem) or V2 (Tolvaptan, Sigma-Aldrich) receptor antagonists, and percentage inhibition of insulin secretion recorded. Values are expressed as mean ± SEM. * * indicates presence of the disulphide bridge between the two cysteines at position 1 and 6. To assess enzymatic stability, peptides (n = 3) were incubated with plasma for 4 h and degradation profile followed by reversed phase high-performance liquid chromatography. For insulin secretory experiments, BRIN BD11 cell line have previously been reported. For isolated islet studies, receptor. Full details of the generation and characterization of the KO INS1 832/13 cells with CRISPR-Cas9-induced KO of the oxytocin receptor. Studies were conducted in 20-week-old HFF male NIH Swiss mice | 2.4 | Animals

Studies were conducted in 20-week-old HFF male NIH Swiss mice (Envigo, UK) previously maintained on a high-fat diet for 12 weeks (45% fat, 20% protein, 35% carbohydrate; percentage of total energy 26.15 kJ/g; Dietex International, Witham, UK; catalogue number 824018) to evoke dietary-induced obesity-diabetes. All mice were housed individually and kept in a temperature-controlled environment (22 ± 2 ºC), with a 12-hour light/12-hour dark cycle. Experiments were carried out in accordance with the UK Animal Scientific Procedures Act 1986. All animal studies were approved by the University of Ulster Animal Welfare and Ethical Review Body. No adverse effects were observed during the in vivo procedures.

2.5 | In vivo experimental design

HFF mice were allocated to comparable groups (n = 8) based on blood glucose and body weight and were administered saline vehicle (0.9% w/v NaCl, intraperitoneal [i.p.]), Ac3IV or exendin-4 (both at 25 nmol/kg, body weight [bw], i.p.) twice-daily for 22 days. This peptide dose was chosen based on our previous studies assessing the metabolic effects of AVP in mice. An additional control group of HFF mice receiving either native AVP or oxytocin was not employed because of the short biological half-lives of these peptides. Cumulative food and fluid intake, body weight, non-fasting glucose and insulin concentrations were monitored at regular intervals. At the end of the treatment period, plasma was collected for assessment of circulating glucagon and lipids. In addition, i.p. and oral glucose (18 mmol/kg) tolerance tests were performed in mice fasted for 18 hours, with peripheral insulin sensitivity (15 U/kg bw, i.p.) tests in non-fasted mice, at the end of the study. At termination, body composition was assessed by dual-energy X-ray absorptiometry (DEXA) scanning using a PIXIImus densitometer (GE Medical Systems, USA) prior to subsequent analyses, as detailed below.

2.6 | Terminal analyses

Following completion of DEXA analysis, pancreatic tissues were excised and processed for either histological analysis or hormone

| Peptide/analogues | Sequence | % degradation at 4 h | % reduction in insulin secretion compared with the control in the presence of receptor antagonists |
|-------------------|----------|---------------------|------------------------------------------------------------------------------------------|
| AVP               | C“YFQNC”PRG-NH₂ | 100 ± 0.1           | OTra: 42 ± 5*  V1aRa: 64 ± 3***  V1bRa: 57 ± 6***  V2Ra: 20 ± 10 |
| AVP               | CYFQNCPRG-NH₂ | 65 ± 0.4            | V1aRa: 59 ± 6**  V1bRa: 42 ± 5*  V2Ra: 39 ± 5 *  0 |
| Oxytocin          | C“YFQNC”PLG-NH₂ | 65 ± 0.9            | V1aRa: 42 ± 5*  V1bRa: 15 ± 7  V2Ra: 62 ± 4***  1 ± 6 |
| BLV               | C“YFQNC”PLG-NH₂ | 67 ± 0.1            | V1aRa: 42 ± 11*  V1bRa: 46 ± 5***  V2Ra: 76 ± 5***  92 ± 2*** |
| BLVR              | CFYQNCPLG-NH₂ | 88 ± 0.7            | V1aRa: 27 ± 20  V1bRa: 8 ± 15  V2Ra: 58 ± 5 **  0 |
| 3IV               | C“YFQNC”PRG-NH₂ | 68 ± 0.2            | V1aRa: 68 ± 3***  V1bRa: 82 ± 3***  V2Ra: 45 ± 9*  22 ± 10 |
| 3IVR              | CYIQNCPRG-NH₂ | 64 ± 0.4            | V1aRa: 13 ± 5  V1bRa: 34 ± 11*  V2Ra: 46 ± 13*  0 |
| Ac3IV             | Ac-C“YFQNC”PRG-NH₂ | 0                 | V1aRa: 17 ± 4  V1bRa: 24 ± 3***  V2Ra: 22 ± 5**  0 |
| Ac3IVR            | Ac-CYIQNCPRG-NH₂ | 0                 | V1aRa: 50 ± 4***  V1bRa: 12 ± 5  V2Ra: 15 ± 5  0 |
content. Briefly, pancreatic tissues were divided longitudinally with half snap frozen for hormonal content measurement as described previously, and the other half processed for immunohistochemical analysis using standard laboratory methods, as previously described from our laboratory.

2.7 | Biochemical analysis

Blood samples were collected from the cut tip of the tail vein of conscious mice. Blood glucose was measured immediately using a handheld Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, UK). For plasma insulin and glucagon, blood was collected in chilled fluoride/heparin-coated micro-centrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged using a Beckman micro-centrifuge (Beckman Instruments, Galway, Ireland) for 10 minutes at 12,000 rpm. Plasma was separated and stored at -20°C until determination of plasma insulin by radioimmunoassay and glucagon by a commercially available ELISA kit (EZGLU-30K, Merck Millipore). Plasma lipid profile was assessed by an ILab 650 Clinical Analyser (Instrumentation Laboratory, Warrington, UK).

2.8 | Statistical analysis

Analyses were performed using GraphPad PRISM software (version 5.0). Values are expressed as mean ± SEM (standard error of the mean). Comparative analyses between groups were carried out using a one-way ANOVA with Bonferroni’s post hoc test or Student’s unpaired t-test, as appropriate. The difference between groups was considered significant if \( P < 0.05 \).

3 | RESULTS

3.1 | Plasma degradation

AVP was fully degraded by a 4-hour incubation in plasma (Table 1). In addition, oxytocin and all non-N-terminally acetylated AVP analogues were also degraded by more than 60% during the 4-hour incubation (Table 1). N-acetylation of the cysteine residue in 3IV or 3IVR generated AVP analogues that were completely stable (Table 1).

3.2 | Effects of AVP analogues on in vitro insulin secretion

Representative dose-dependent insulin secretory responses of native AVP and oxytocin, as well as the two enzymatically stable analogues, namely Ac3IV and Ac3IVR, in both cell lines, are depicted in Figure 1A-D. As expected, AVP induced prominent insulinotropic effects, with both AVP analogues also displaying clear dose-dependent actions in each cell line (Figure 1A-D). Full concentration-response curves could not be obtained, thus precluding calculation of

![Figure 1](image-url) **Figure 1** Effects of arginine vasopressin (AVP), oxytocin and AVP analogues Ac3IV, Ac3IVR on insulin secretion and receptor selectivity. (A-D) Effects of AVP and related analogues \( 10^{-12} \text{ to } 10^{-6} \) M on insulin secretion (20 min) at (A and C) 5.6 and (B and D) 16.7 mM glucose from (A and B) rodent BRIN BD11 or (C and D) human 1.1B4 beta cells. (E) Isolated islets from male mice were incubated (60 min) at 16.7 mM glucose and the effects of AVP, oxytocin and Ac3IV \( 10^{-8} \) and \( 10^{-6} \) M on insulin secretion examined. (F) Effects of AVP and Ac3IV on insulin secretion (20 min) from oxytocin receptor KO INS1 832/13 beta cells at 16.7 mM glucose. Values are mean ± SEM. (A-D, F) \( n = 8 \) and (E) \( n = 3 \). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) compared with respective glucose alone control. Δ\( P < 0.05 \) compared with AVP at the same concentration.
Effects of twice-daily administration of Ac3IV or exendin-4 on (A) body weight, (B) total body fat, cumulative (C) energy and fluid intake, as well as (E) glucose and (F) insulin concentrations in high-fat-fed (HFF) mice. (A, C-F) Variables were measured at regular intervals prior to, and during, 22 days twice-daily treatment with test peptides (25 nmol/kg bw) in HFF mice. (B) Variables were measured on day 22. (E, F) Insets depict AUC data for glucose and insulin over the 22-day treatment period. All values are expressed as mean ± SEM for seven mice. *P < .05, **P < .01, ***P < .001 compared with HFF-saline control mice.
significant \( (P < .01) \) reductions compared with HFF controls on day 22 (Figure 2E). This was accompanied by reduced overall glucose exposure in exendin-4 and Ac3IV mice during the entire treatment period (Figure 2E inset). Corresponding daily plasma insulin concentrations were largely unaltered during the study, but there was a significant decrease \( (P < .05) \) in this variable in both treatment groups on day 16 (Figure 2F), as well as in overall insulin levels during the 22 days (Figure 2F inset). Plasma glucagon levels were not changed in exendin-4-treated HFF mice, but significantly reduced \( (P < .05) \) by Ac3IV treatment (Figure 3A).

3.5 | Effects of twice-daily administration of Ac3IV on pancreatic hormone content, lipid profile and bone mineral density in HFF mice

Pancreatic insulin content was significantly reduced \( (P < .05 \text{ to } P < .01) \) in both treatment groups compared with HFF control mice (Figure 3B), whereas pancreatic glucagon content was not altered (Figure 3C). In terms of lipid profile, Ac3IV and exendin-4 therapy were both associated with reduced total cholesterol (Figure 3D) and increased HDL-cholesterol (Figure 3E) levels, but only Ac3IV significantly \( (P < .05) \) decreased LDL-cholesterol and triglyceride concentrations (Figure 3F,G). Bone mineral density \( (P < .01) \) and content \( (P < .05) \) were increased on day 22 in both the HFF treatment groups of mice (Figure 3H,I).

3.6 | Effects of twice-daily administration of Ac3IV on glucose tolerance and insulin sensitivity in HFF mice

Administration of exendin-4 twice-daily for 22 days significantly \( (P < .05 \text{ to } P < .01) \) decreased individual and overall blood glucose levels following an i.p. glucose challenge (Figure 4A,B). Ac3IV treatment was also associated with significantly \( (P < .05) \) decreased glucose 0-120-minute AUC values compared with HFF controls (Figure 4B). In addition, individual glucose-induced insulin concentrations were only increased \( (P < .05 \text{ to } P < .01) \) by Ac3IV (Figure 4C), with both treatment regimens increasing overall AUC insulin values.

**FIGURE 3** Effects of twice-daily administration of Ac3IV or exendin-4 on pancreatic circulating glucagon, hormone content, lipid profile and body composition in high-fat-fed (HFF) mice. Variables were measured after 22 days of treatment with a twice-daily injection of test peptides (25 nmol/kg bw) in HFF mice. (A) Plasma glucagon was determined using a commercially available ELISA kit. (B and C) Pancreatic tissue was isolated at the end of the study and (B) insulin or (C) glucagon content was determined following acid-ethanol extraction. (D-G) Terminal non-fasted plasma (D) total cholesterol, (E) HDL-cholesterol, (F) LDL-cholesterol and (G) triglycerides were measured by an ILab 650 clinical analyser. (H) Bone mineral density and (I) bone mineral content were measured by DXA. Values are mean ± SEM for seven mice. \* \( P < .05 \), \* \( P < .01 \) compared with HFF-saline control mice.
In response to an oral glucose challenge, the treatment interventions significantly decreased individual (P < .05 to P < .001) and overall (P < .01) blood glucose levels compared with saline controls (Figure 4E,F). There were no changes in plasma insulin concentrations at individual time points (Figure 4G), but exendin-4 and Ac3IV both increased (P < .01 to P < .001) 0-120-minute overall plasma insulin concentrations in response to an oral glucose load (Figure 4H). Furthermore, the glucose-lowering activity of exogenous insulin was significantly increased (P < .01 to P < .001) by both treatments on day 22 (Figure 4L,J), which was supported by a significant decrease (P < .01 to P < .001) in homeostatic model assessment for insulin resistance (HOMA-IR) values in these groups of mice compared with saline-treated HFF controls (Figure 4K).

### 3.7 Effects of twice-daily administration of Ac3IV on pancreatic islet morphology in HFF mice

Representative histological images of pancreatic islets stained for insulin and glucagon are shown in Figure 5A. High-fat feeding was associated with insulin resistance and islet expansion, but both exendin-4 and Ac3IV significantly decreased islet and beta-cell areas compared with HFF saline controls (Figure 5B,C), with effects being more prominent in Ac3IV-treated mice. There was no significant change in alpha-cell area between the groups of HFF mice (Figure 5D). Proliferation rates of alpha and beta cells were decreased by both exendin-4 and Ac3IV treatment (Figure 5E,F). Apoptotic rates were also examined by co-staining glucagon or insulin, respectively, with TUNEL dye (Figure 5G,H). Both exendin-4 and Ac3IV reduced (P < .01 to P < .001) beta-cell apoptosis rates compared with saline control mice (Figure 5G), with exendin-4 treatment also increasing (P < .05) the rate of alpha-cell apoptosis (Figure 5H).

## 4 DISCUSSION

The presence of AVP receptors on pancreatic beta cells, as well as related positive effects of AVP on insulin secretion shown many years ago,\(^{37}\) have since been confirmed by several laboratories.\(^{18,38,39}\) AVP also has established satiety actions,\(^{40}\) as well as an ability to avert the onset of hypoglycaemia.\(^{37}\) Indeed, hypothalamic AVP neurons regulating glucagon secretion are considered to function as metabolic glucose sensors,\(^{41}\) supporting an important role for AVP in glucose homeostasis. Such actions are believed to be largely mediated through

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**FIGURE 4** Effects of twice-daily administration of Ac3IV or exendin-4 on glucose tolerance and insulin sensitivity in high-fat-fed (HFF) mice. Variables were measured after 22 days of treatment with twice-daily injections of test peptides (25 nmol/kg bw) in HFF mice. (A and E) Blood glucose and (C and G) plasma insulin were measured prior to and after (A and C) intraperitoneal (i.p.) or (E and G) oral administration of glucose alone (18 mmol/kg) at t = 0 min in 18-h fasted mice. (B, D, F and H) Respective AUC data for 0-120- min postglucose injection are shown. (I and J) Blood glucose was measured after i.p. administration of insulin (15 U/kg bw) at t = 0 min in non-fasted mice. (K) HOMA-IR was calculated using fasting insulin (mU/L) multiplied by fasting glucose (mmol/L) divided by 22.5 on day 22. All values are expressed as mean ± SEM for seven mice. *P < .05, **P < .01, ***P < .001 compared with HFF-saline control mice.
activation and modulation of V1a and V1b receptor signalling. Despite this knowledge, the potential therapeutic utility of AVP has been overlooked to date. The reason for this is probably 2-fold; first, AVP has an extremely short half-life in the circulation and, second, AVP also activates V2 receptors that are linked to aquaporin-mediated fluid reabsorption and concomitant elevation of blood pressure. The current study was undertaken to directly overcome these drawbacks, through generation of enzymatically stable AVP peptides that possess a receptor activation profile that is more compelling for long-term benefits in obesity-diabetes.

Given the similarity in the amino acid sequence of AVP and oxytocin, plus the established benefits of oxytocin receptor activation in diabetes, introduction of key oxytocin residues into the sequence of AVP analogues might be envisaged to generate peptides with additional beneficial oxytocin receptor activation properties. However, it should be noted that oxytocin has a much less prolonged and diminished effect on insulin secretion than AVP, as observed in this study and elsewhere, highlighting the major importance of V1a and V1b receptor activity for beta-cell actions. Interestingly, AVP is also believed to play a role in somatostatin secretion, indicating a complex mode of action on islet cells. Although it has been suggested that the stimulatory effects of AVP on insulin secretion are indirectly mediated by glucagon via paracrine islet interactions, we and others clearly show involvement of direct beta-cell stimulatory effects.

In agreement, we observed only moderate insulinotropic actions of the AVP analogues in clonal beta cells, with much more prominent effects in isolated murine islets, confirming the importance of paracrine islet cell signalling in this regard.

In the current study, native AVP and some related analogues modulated both oxytocin and AVP receptor signalling pathways in BRIN BD11 cells, but preservation of V2 receptor activity and associated effects on the kidney and fluid retention were not considered favourable. Interestingly, reduced AVP exhibited bioactivity at oxytocin, V1a and V1b, with no interaction at V2 receptors, but this peptide was comparatively unstable. It should also be noted that removing the disulphide bridge appeared to lead to slightly different effects on the insulin secretory activity of native AVP and Ac3IV. This probably reflects distinct changes in the three-dimensional structure of the peptides and their interaction with receptors on target cells. However, further studies and detailed structure/function analysis are required to clarify this aspect. In addition, investigation of specific downstream signalling pathways would also have been useful to help further confirm receptor selectivity of the AVP analogues. However, upon closer inspection, our in vitro characterization data for 3IV, and the daughter

**FIGURE 5** Effects of twice-daily administration of Ac3IV or exendin-4 on pancreatic islet architecture as well as alpha- and beta-cell proliferative and apoptotic rates in high-fat-fed (HFF) mice. Variables were measured after 22 days of treatment with twice-daily injections of test peptides (25 nmol/kg bw) in HFF mice. (A) Representative images of islets showing insulin (red) and glucagon (green) immunoreactivity from each treatment group. (B-D) Islet architectural analysis included assessment of (B) total islet, (C) beta- and (D) alpha-cell areas. (E and G) Beta- and (F and H) alpha-cell proliferation or apoptotic rates were measured by co-staining with insulin or glucagon, respectively, and (E and F) Ki-67 antibody or (G and H) TUNEL reagent, as appropriate. Values are mean ± SEM for seven mice. *P < .05, **P < .01, ***P < .001 compared with HFF-saline control mice.
analogue Ac3IV, led to selection of the N-terminally acetylated analogue to evaluate the potential benefits of combined V1a and V1b receptor signalling in diabetes. This included careful consideration of enzymatic stability, receptor activation profile and in vitro bioactivity in both rodent and human beta cells. Key in this decision was the lack of effect of Ac3IV on V2 receptors and resistance to enzymatic degradation. In this regard, AVP is known to be rapidly degraded by vasopressinase enzymes in the circulation, as well as being subject to renal elimination.9 Whilst our in vitro system suggests excellent enzymatic stability of Ac3IV, it did not assess the influence of kidney filtration on peptide bioactivity. To assist with more pragmatic quantification of the ability of Ac3IV to reverse metabolic dysregulation present in HFF mice, beneficial effects were compared directly with the approved antidiabetic drug, exendin-4. Importantly, there was no obvious change in mouse behaviour during, and gross tissue anatomy was unaltered at the end of the Ac3IV treatment regimen, suggesting lack of toxicity of this compound. Further longer term studies are required to fully assess safety issues, but it is noteworthy that Ac3IV is structurally identical to native AVP, barring addition of an N-terminal acetyl group and substitution of the third amino acid residue, phenylalanine for isoleucine, which already exists in the closely related peptide hormone oxytocin.

Both peptide interventions decreased circulating glucose, body weight and fat content in HFF mice, presumably in part linked to prominent satiety actions. This is in full agreement with the established metabolic benefits of GLP-1 receptor activation,2 and in harmony with the actions of V1a receptor activation to suppress appetite.52 Studies in mice pair-fed to mimic Ac3IV-induced reductions of food intake would be required to help establish if benefits on body weight and metabolism occurred independently of changes in energy intake. Consistent with a lack of V2 action that might otherwise cause fluid retention, Ac3IV had only marginal effects on water intake, with even a small cumulative increase being apparent towards the end of the study, but further studies are required to fully assess the effects of Ac3IV on renal function. Somewhat surprisingly, given the established glucagonostatic effects of GLP-1 receptor agonism2 and reported glucagonotrophic actions of AVP,20 circulating glucagon concentrations were reduced by Ac3IV, but not by exendin-4. This is in the face of increased pancreatic alpha-cell apoptotic rates in exendin-4-treated HFF mice, and a tendency for reduced pancreatic glucagon content. In relation to this, there was a reduction in islet- and beta-cell areas, as well as pancreatic insulin content, in both treatment groups of HFF mice, accompanied by decreased proliferation of both alpha and beta cells. Thus, changes in islet-cell proliferation rates, or in recently reported islet cell transitioning events,44 probably outweigh decreased beta-cell apoptosis observed with both peptides. Moreover, GLP-1 receptor signalling has recently been linked to positive effects on alpha- and beta-cell transdifferentiation.45 Taken as a whole, it could be assumed that reductions in islet- and beta-cell areas are directly linked to the improved diabetic state evoked by both treatment regimens, leading to reduced metabolic demand.

In keeping with this, circulating insulin concentrations were largely unaltered and peripheral insulin sensitivity significantly improved in peptide-treated HFF mice, in accordance with notable GLP-1 and V1b receptor mediated benefits on insulin action.14,33 Moreover, both Ac3IV and exendin-4 reduced total- and increased HDL-cholesterol levels. However, only Ac3IV significantly reduced circulating LDL-cholesterol and triglyceride levels, highlighting this as a distinct advantage over exendin-4, given that diabetes and obesity represent key risk factors for cardiovascular disease.56 In addition, obesity-driven forms of diabetes are also associated with increased occurrence of bone fragility fractures,47,48 and both Ac3IV and exendin-4 augment bone mineral content and density, implying another potentially important therapeutic benefit.

Despite this, it should also be noted that increased levels of copeptin, a peptide co-secreted with AVP and considered as an excellent surrogate marker for assessing circulating AVP concentrations,49 is associated with metabolic dysregulation and onset of diabetes.9 However, this effect could well be linked to AVP receptor desensitization and inherent adaptations to increase AVP secretion, and as such requires further detailed study. Indeed, a very similar phenomenon is reported for the incretin hormone GIP in type 2 diabetes50 that possesses significant parallels with the bioactive profile of AVP, and this issue now appears to be more than surmountable from a therapeutic viewpoint.23 Moreover, both oral and i.p. glucose tolerance were substantially improved by Ac3IV in HFF mice, and consistently linked to the glucose-dependent insulinotropic actions. Such positive effects are particularly notable given aforementioned reductions in beta-cell area and pancreatic insulin content. Interestingly, AVP has been shown to stimulate V1b receptor-dependent secretion of GLP-1 from mouse and human intestines,51 and this could be one potential reason for the observed similar benefits of Ac3IV and exendin-4 on glucose disposal. Furthermore, we and others have previously illustrated similarities in beta-cell signalling pathways for AVP and the other hormones, including incretin hormones.18 However, further studies would be required to confirm the potentially positive impact of Ac3IV on the secretion and action of GLP-1 and other gut hormones.

In conclusion, combined sustained activation of V1a and V1b receptor pathways by Ac3IV exerts notable benefits on body weight and energy regulation, glucose homeostasis, insulin action and lipid metabolism, as well as reversing the detrimental effects of high-fat feeding on pancreatic architecture. Metabolic benefits were at least equivalent, and at times superior, to those exerted by the clinically approved GLP-1 mimetic, exendin-4. Future investigations into the therapeutic potential of AVP for the treatment of obesity and related diabetes may also want to consider further peptide optimization and approaches capable of combining the specific metabolic benefits of GLP-1 mimetics alongside V1a and V1b receptor activation. In this respect, it is not altogether uninteresting that effective strategies exist for the oral administration of both AVP and GLP-1 analogues.52

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CONFLICT OF INTEREST
All authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
PRF and RCM conceived/designed the study. SM, NI and PRF drafted the manuscript. SM participated in the conduct/data collection and analysis and interpretation of data. All authors reviewed the manuscript critically for intellectual content and approved the final version of the manuscript.

PEER REVIEW
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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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