Original research

A novel peptide protects against diet-induced obesity by suppressing appetite and modulating the gut microbiota

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ABSTRACT
Objective  The obesity epidemic and its metabolic complications continue to be a major global public health threat with limited effective treatments, especially drugs that can be taken orally. Peptides are a promising class of molecules that have gained increased interest for their applications in medicine and biotechnology. In this study, we focused on looking for peptides that can be administrated orally to treat obesity and exploring its mechanisms.

Design  Here, a 9-amino-acid peptide named D3 was designed and administered orally to germ-free (GF) mice and wild-type (WT) mice, rats and macaques. The effects of D3 on body weight and other basal metabolic parameters were evaluated. The effects of D3 on gut microbiota were evaluated using 16S rRNA amplicon sequencing. To identify and confirm the mechanisms of D3, transcriptome analysis of ileum and molecular approaches on three animal models were performed.

Results  A significant body weight reduction was observed both in WT (12%) and GF (9%) mice treated with D3. D3 ameliorated leptin resistance and upregulated the expression of uroguanylin (UGN), which suppresses appetite via the UGN-GUCY2C endocrine axis. Similar effects were also found in diet-induced obese rat and macaque models. Furthermore, the abundance of intestinal Akkermansia muciniphila increased about 100 times through the IFN-Irgm1 axis after D3 treatment, which may further inhibit fat absorption by downregulating Cd36.

Conclusion  Our results indicated that D3 is a novel drug candidate for counteracting diet-induced obesity as a non-toxic and bioactive peptide. Targeting the UGN-GUCY2C endocrine axis may represent a therapeutic strategy for the treatment of obesity.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Uroguanylin (UGN) is an anorexic hormone that can target the GUCY2C receptor of the hypothalamus and activate anorexigenic pathways.
⇒ Regulation of the gut microbiota can improve host metabolism and reduce obesity.
⇒ Polypeptides such as glucagon-like peptide-1 (GLP-1) analogue used to prevent obesity represents a new direction for drug design, but with poor adherence of patients to injectable therapies.

WHAT THIS STUDY ADDS
⇒ Oral administration of D3, a 9-amino-acid peptide but not a GLP-1 analogue, can counteract diet-induced obesity in mice, rat and rhesus maca.
⇒ D3 can effectively inhibit appetite through the UGN-GUCY2C endocrine axis.
⇒ D3 restores the gut microbiota disorder caused by obesity and specifically increases the abundance of Akkermansia muciniphila in the gut via the IFN-Irgm1 axis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ D3 is a promising candidate of weight reducing drug that can be taken orally to manage excess weight and obesity.
⇒ The UGN-GUCY2G axis represents a potential therapeutic target for the development of anti-obesity drugs.

INTRODUCTION
According to the WHO, 650 million adults had obesity in 2016, and 39 million children under the age of 5 were overweight or obese in 2020.1 Obesity is becoming a worldwide epidemic and poses major therapeutic challenges due to its associated high risk for developing non-communicable diseases, including type 2 diabetes, cardiovascular disease, stroke, cancer and depression.2 Obesity is also linked with decreased life expectancy and increased morbidity and mortality and economic burden on healthcare systems.3 Various strategies have been developed to combat obesity and overweight conditions, especially through reducing energy intake or enhancing metabolic expenditure rate. However, recent studies found that excessive dietary interventions may lead to physical and mental health disorders,4 which further increase the demand for effective, safe and acceptable therapeutic options.

Extensive studies have shown that obese humans and mice display an altered gut microbiota with reduced diversity and increased capacity to absorb energy.5 The intestinal microbiota is essential in regulating host energy homeostasis, fat accumulation and mucosal barrier integrity.6 Disordered microbiota are closely associated with metabolic...
disorders such as obesity, insulin resistance and glucose intolerance.\(^7\) Intervention strategies that modulate the gut microbiota have been proposed to prevent and treat obesity. For example, temgol can decrease the weight gain of mice by preferentially reducing the genus *Lactobacillus*.\(^8\) Cranberry extract\(^9\) and metformin\(^10\) can protect mice from diet-induced obesity by increasing *Akkermansia muciniphila* in the gut microbiota. Several recent studies have shown that daily administration of *A. muciniphila* can counteract the development of high-fat-diet (HFD)-induced obesity.\(^11\) Additionally, oral gavage with either *Bacteroides thetaiotaomicron*\(^12\) or *Lactobacillus rhamnosus* GG\(^13\) can also alleviate diet-induced body weight gain and adiposity in mice.

Consisting of 20 natural amino acids and a multitude of non-natural amino acids, peptides have attractive pharmacological profiles, excellent safety and tolerability, which represent an excellent starting point for the design of novel therapeutics.\(^14\) Currently, a number of peptides have been developed to prevent obesity, such as glucagon-like peptide-1 (GLP-1),\(^15\) atrial natriuretic peptide and brain natriuretic peptide.\(^16\) Many of these peptides, however, are usually longer than 20 amino acids and are therefore less likely to escape degradation by proteases in the gastrointestinal tract. On the other hand, recent studies on apelin\(^17\) and semaglutide\(^18\) indicate that oral drugs represent an excellent starting point for the design of novel therapeutics.\(^14\) When challenged with additional insulin and glucose, D3-treated mice showed similar kinetics for glucose clearance with mice in the NC group, indicating that D3 could reduce the insulin resistance caused by obesity (figure 1E,F). Significant decreases in the tissue weight of inguinal, epididymal and perirenal fat were detected after D3 treatment (p<0.05, Wilcoxon test), which may be attributed to the reduced volume of fat cells (figure 1H). Additionally, D3 treatment also protected the liver from steatosis (figure 1H).

Based on these findings, we further examined the impact of 4 weeks of oral administration of D3 in a new cohort of DIO mice (n=5) after 10 weeks of HFD feeding. A significant reduction in body weight was observed in DIO mice (p<0.01, Wilcoxon test) (figure 1I), whereas the trend tended to be modest in the lean mice (n=5) fed a chow diet for 10 weeks in parallel (p<0.05, Wilcoxon t-test) (figure 1J). Notably, similar effects were observed after exchanging their diets at the 14th week (figure 1I,J). Taken together, these findings indicated that D3 treatment can prevent the onset and worsening of DIO, particularly on a HFD (online supplemental figure 1e).

D3 maintains energy homeostasis by inhibiting appetite

Considering that obesity occurs when energy intake exceeds energy expenditure, we therefore evaluated whether oral administration of D3 could affect food intake and/or energy metabolism. Notably, both in the specific pathogen-free (SPF) mice and the GF mice, D3 treatment significantly reduced food intake over time, with a significant reduction in 24-hour food intake compared with vehicle-treated animals (p<0.05, Wilcoxon test) (online supplemental figure 1d and figure 2A,B). In addition, indirect calorimetry analysis was performed to address whether changes in energy expenditure contributed to the observed D3-mediated weight loss. As shown in figure 2C, there was almost no difference in energy expenditure between the two groups (p>0.05, Wilcoxon test), indicating that D3 treatment prevented weight gain in socio-economical scenarios.

**RESULTS**

**Oral administration of D3 counteracts obesity**

Increasing electric charge and hydrophobicity can improve the membrane permeability of peptides. Therefore, to enhance hydrophobicity, four peptides named D1–4 were modified from one of the fragments of HD5 degraded by proteases in human duodenal fluid, HD5(1–9), the first nine amino acids at the N-terminus of defensin HD5.\(^22\) All these peptides have a positive charge, among which D3 shows the strongest potential to penetrate cell membranes (online supplemental table 1 and figure 1A). We then evaluated the cytotoxicity of D1–4 in mammalian cells. D1–3 showed a slight inhibition of cell activity (2.71%–5.42%) at a very high concentration (2 μM), indicating their low cytotoxicity (online supplemental figure 1A). Additionally, D1–4 was assayed for potential toxic effects on mouse red blood cells in vitro. As shown in online supplemental figure 1b, the haemolytic rate of D1–4 at 2 μM was 1.06%–3.61%, respectively, indicating their low haemolytic effect. Therefore, the safe concentration range of these small peptides was set to 0–2 μM.

We next compared the effects of daily administration of D1–4 on HFD-fed mice. Strikingly, HFD-fed mice orally administered D3 rather than other peptides caused a 12.06%±2.33% decrease in body weight gain compared with the HFD-fed control mice over 8 weeks of HFD exposure (figure 1B-D). Recent findings indicate that obesity and its accompanying metabolic state are increasingly implicated in the composition of the gut microbiota.\(^1\) To examine whether the gut microbiota may contribute to this effect, the same experiment was repeated in gnotobiotic (antibiotic cocktail, ABX) and germ-free (GF) mice. Similarly, 9.65%±4.26% and 9.14%±2.93% weight loss was observed in ABX (online supplemental figure 1C) and GF mice (figure 1D), respectively, suggesting that the reduction in adiposity by D3 oral gavage may be partially independent of the gut microbiome.

When challenged with additional insulin and glucose, D3-treated mice showed similar kinetics for glucose clearance as mice in the NC group, indicating that D3 could reduce the insulin resistance caused by obesity (figure 1E,F). Significant decreases in the tissue weight of inguinal, epididymal and perirenal fat were detected after D3 treatment (p<0.05, Wilcoxon test), which may be attributed to the reduced volume of fat cells (figure 1H). Additionally, D3 treatment also protected the liver from steatosis (figure 1H).
Gut microbiota may affect energy intake rather than energy expenditure. Pair-feeding was performed to explore the effects of lower food overload. As a result, mice orally administered D3 caused a 10.04%±2.19% (p<0.01, Wilcoxon test) decrease in body weight gain compared with the HFD control mice over 8 weeks of HFD exposure (figure 2D). Additionally, mice in the pair-fed group caused a 6.02%±2.03% decrease in body weight gain. These results suggest that the physiological effects of D3 are largely based on the inhibitory on appetite in mice. To further explore the mechanism of D3, FITC-labelled D3 (FITC-D3) was used to visualise its systemic distribution in mice. Three hours after oral administration of FITC-D3 or FITC, intense fluorescence was visible in the intestinal tract of FITC-D3-treated mice, especially in the distal small intestine, but not...
Figure 2. D3 treatment decreases food intake by upregulating uroguanylin (UGN) expression in the ileum. (A, B) Food intake was measured once a week. (C) Effect of D3 treatment on acute food intake, which was measured every other day for 1 week. For A and B, (A) (specific pathogen-free (SPF) mice, n=10–12), (B) (germ-free (GF) mice, n=6–8). (C) Energy expenditure of SPF mice measured for 20 hours (n=6 mice/group). (D) Grams of weight gain measured over time in mice of pair-feeding, starting at 4 weeks of age (n=6 mice per group). (E) Localisation of FITC-labelled D3 visualised by confocal microscopy. The cell nucleus was stained with DAPI (blue). Swiss rolls (above, scale bars: 500 µm) and intestinal cross sections (below, scale bars: 200 µm) of SPF mice were obtained after 3 hours of oral administration. The last column shows the localisation of FITC-D3 after 1 hour of coincubation with HCT cells. The magenta colour presented by FITC is a pseudocolour that was replaced later to distinguish it from the immunofluorescence staining of UGN. Images are representative of three independent experiments with similar results. (F) RNA sequencing and KEGG pathways of genes in the ileum. (G) Volcano plot showing relative gene expression in the D3-treated group versus the HFD control group. All genes were plotted, and each circle represents one gene. The diameter of the circle represents the value of FPKM, p<0.05 (green) and p<0.01 (red). The X-axis shows the log2-fold change, and the Y-axis shows the −log10 of the p values. (H) Relative Guca2b mRNA levels in the ileum of mice gavaged with D3 or phosphate buffered saline (PBS). (I) The concentration of UGN (Guca2b protein) (ng/mL) in the serum of mice was determined by ELISA. (J) Double immunofluorescence in ileal sections for UGN (green dots located at the base of intestinal epithelial cells) and nuclei (blue). The diffuse green thin layer refers to background noise. Scale bars: 10 µm. For A, B, H and I, p values were determined by a two-tailed Wilcoxon test, and data are presented as the means±SEM; *p<0.05; **p<0.01; ***p<0.001.
Intact leptin signalling contributes to the anti-obesity effect of D3

We sought to identify whether D3 treatment could protect against obesity in genetically obese (ob/ob) mice. As shown in figure 3A, no significant difference was observed in the body weight gain of the ob/ob mice between the D3 group and the HFD group after 6 weeks of oral administration. To validate that the anti-obesity effect of D3 was leptin associated, we next retrospectively examined the changes in leptin expression in SPF mice treated in previous experiments. Indeed, consistent with previous reports, feeding an HFD significantly upregulated the expression of leptin in both SPF and GF mice, which could be restored by oral administration of D3 (p<0.05, Wilcoxon test) (figure 3B). To further demonstrate that D3 can ameliorate the leptin resistance, we first investigated the response of body weight to leptin administration by using an acute method, in which WT mice were treated with leptin intraperitoneally for 3 days, and then their body weight change was determined. As shown in figure 3C, marked body weight reduction was observed in the mice that received D3 after exogenous leptin treatment (p<0.05, Wilcoxon test) but not in the mice of the HFD group. Second, we detected the leptin-stimulated cytokines, including signal transducer and activator of transcription 3 (STAT3) and phosphatidylinositol 3-kinase (PI3K) pathways. We found that within the negative regulators of leptin signalling, there was no significant difference in the mRNA expression level of PTB1B (online supplemental figure 4b) between the HFD and D3 groups, but SOCS-3 was significantly downregulated in D3-treated mice. The overexpression of SOCS-3 was found to inhibit leptin-induced STAT3 in vivo and consequently caused leptin resistance. In contrast, the expression level of PI3K, whose level is positively correlated with the total expression level and can reflect the leptin sensitivity, was significantly upregulated, which may represent an enhanced response to leptin (online supplemental figure 4b). Taken together, these results suggest that D3 treatment can restore leptin-induced signalling pathways in DIO mice and the leptin sensitivity.

We observed that leptin treatment caused a marked tendency towards a reduction in body weight in ob/ob mice, and leptin+D3 induced a more significant reduction than D3 alone (figure 3D), implying that supplementation with exogenous leptin can restore the function of D3 in ob/ob mice. Then, we quantified and compared the expression level of Guca2b in the ileum of ob/ob mice with that of WT mice. A 2.22-fold increase in the expression of Guca2b was observed after D3 treatment in ob/ob mice (OB-D3) compared with untreated ob/ob mice (OB-NC), whereas the WT mice of the SPF-D3 group achieved a 3.67-fold increase (figure 3E). Furthermore, ob/ob mice treated with leptin alone (OB-Lep) showed a 2.12-fold increase, which was lower than those treated with leptin+D3 (OB-Lep+D3, 3.46-fold increase) (figure 3E), indicating the synergistic effect of leptin and D3 on regulating the expression of UGN. The expression of UGN in the ileum of ob/ob mice treated with D3, leptin and D3+leptin was further visualised and verified by immunofluorescence (figure 3F). Taken together, these findings indicate that leptin may play a significant role in mediating the anorexigenic and anti-obesity effects of D3.

Some neurons in the hypothalamus associated with appetite are activated on fasting, which is accompanied by dramatic induction of c-Fos expression in these neurons. To observe the effect of D3 treatment on the appetite of mice, 8-week-old SPF mice were gavaged with D3 or vehicle (saline) at 10:00 a.m. Transcardial and c-Fos staining were subsequently performed every 1 hour for 3 hours. The expression of c-Fos increased significantly at 1 hour after D3 administration, and then gradually returned to the normal level (online supplemental figure 4c), which was confirmed by subsequent immunofluorescence staining (figure 3HJ). We further measured the expression of AgRP and POMC during this process to identify the population of hypothalamic neurons implicated in the observed effects. The expression of POMC was significantly improved at 2 and 3 hours after D3 administration (p<0.05, Wilcoxon test), but with no significant changes in AgRP (p>0.05, Wilcoxon test) (figure 3GK). Moreover, the colocalisation of c-Fos and POMC expressing neurons was observed in arcuate nucleus (figure 3HJ). These results suggest that D3 administration may indirectly affect POMC neurons in arcuate nucleus, which
Gut microbiota signals satiety and reduces food intake, to achieve the effect of appetite suppression.

D3 treatment increases the abundance of *A. muciniphila* in the gut microbiota

The gut microbiota is an important contributor to metabolic function and has been linked with the development of human diseases, such as infantile autism and obesity. We next examined whether D3 protection against obesity was correlated with gut microbiota alteration. There was an obvious difference in the faecal microbiota composition between D3-treated and vehicle-treated mice (figure 4A-B). The linear discriminant analysis showed that *Oscillospira*, *Lawsonia* and *Desulfovibrio* were significantly enriched in the vehicle-treated mice, whereas the genera *Bacteroides*, *Bilophila* and *Akkermansia* were significantly enriched in the D3-treated group (figure 4C).

Faecal microbiota transplantation (FMT), which entails the transfer of gut microbial communities from healthy donors to patients, has emerged as a promising treatment option for
Gut microbiota a range of chronic disorders. Considering that compositional shifts in the gut microbiota may play a role during D3 treatment, we adopted FMT to determine whether the gut microbiota contributed to the rescue of obesity. The faecal microbiota from mice treated with D3 for 10 weeks were collected and orally given to the DIO mice. After 4 weeks, FMT promoted a 6.50%±2.91% weight reduction in the recipient mice compared with the HFD control mice, whereas the heat-inactivated faecal sample showed no effect (figure 4D,E). We wondered whether any particular faecal bacterial species exerted such protective effects. Therefore, we used two significantly enriched taxa (B. vulgatus and A. muciniphila) in the D3-treated DIO mice for 4 weeks. The colonisation of A. muciniphila led to a significant body weight reduction (p<0.05, Wilcoxon test), while the
colonicisation of *B. vulgatus* did not have such an effect (p>0.05, Wilcoxon test) (figure 4F). This finding suggests that individual members (eg, *A. muciniphila*) of the gut microbiota transferred to recipient mice may deter obesity development in DIO mice, which is in agreement with previous studies. We then returned to the initial experiment on the SPF mice and found that the abundance of *A. muciniphila* in the faeces of mice treated with D3 was approximately 100-fold higher than that in the HFD mice (figure 4G).

Recently, *A. muciniphila* was identified as a mucin-degrading bacterium that resides in the mucus layer. The specialisation of *A. muciniphila* in mucin degradation makes it a key player at the mucosal interface between the lumen and host cells. By staining the mucus of the colonic epithelium, we found that the thickness of the mucus layer was significantly decreased in the HFD-fed mice (SPF). Both D3 and *A. muciniphila* treatment counteracted this decrease (p<0.05, Wilcoxon test). Interestingly, this effect disappeared when using D3 to treat GF mice (figure 4H). Therefore, we speculate that the thickening of the mucus layer is due to the colonisation of *A. muciniphila* rather than the direct action of D3.

To further explore the mechanism for the increase in the abundance of *A. muciniphila* that occurs with D3 treatment, we first measured the growth curve of *A. muciniphila* associated with exposure to different concentrations of D3 in vitro. As shown in figure 4I, there was no significant change under different D3 concentrations, indicating that D3 cannot promote the growth of *A. muciniphila* in vitro. Previous studies found that *A. muciniphila* was significantly increased in IFNγ-deficient mice, and the restoration of IFNγ levels could reduce *A. muciniphila* abundance. IFNγ can induce the secretion of antimicrobial proteins from Paneth cells through Irgm1, a downstream molecule in its signalling cascade. Previous studies suggest that impaired production of antimicrobial peptides can lead to the overgrowth of *A. muciniphila* when the level of IFN or Irgm1 is reduced. In this study, we observed that D3 treatment significantly suppressed the expression of both IFNγ and Irgm1 in the ileum of mice relative to the HFD group (figure 4K,L). Therefore, we speculate that the increased abundance of *A. muciniphila* by D3 treatment may be attributed to the negative regulation of the IFNγ-Irgm1 axis.

To investigate whether *A. muciniphila* contributed to improving the phenotype of obese, we transplanted *A. muciniphila* into DIO mice for 8 weeks. Notably, *A. muciniphila* gavage with 1×10^9 CFU/mouse every 2 days led to a significant reduction (p<0.01, Wilcoxon test) in the body weight of mice, similar to the D3 treatment (figure 4M). We further compared the effects of *A. muciniphila* and D3 on lipid metabolism in DIO mice. We observed an increase in the expression of the Fasn and CD36 mRNAs, genes that regulate the synthesis and absorption of lipids, a decrease in the mRNA level of Adipoq that promotes lipid oxidation in epididymal fat, after the administration of D3 or *A. muciniphila* in the SPF mice but not in GF mice (online supplemental figure 5I), suggesting additional positive effects of the increased *A. muciniphila*. Interestingly, a dramatic upregulation of Acox2, which can increase fatty acid oxidation and further inhibit lipid accumulation in the liver, was observed only in D3-treated SPF mice (figure 4N and online supplemental figure 5J). These results indicate that the regulation of lipid metabolism should be a combined effect of D3 and *A. muciniphila*.

Recent studies showed that *A. muciniphila* could repair the thinning of the intestinal mucus caused by high-fat (HF) diet. Therefore, we further investigated how the administration of D3 or *A. muciniphila* affects the mucus in the colon. We found that the expression levels of MUC2 and IL4 were significantly increased after the administration of D3 or *A. muciniphila* in SPF mice (p<0.05, Wilcoxon test), but not in other markers of goblet cell development, such as Klf4 (p>0.05, Wilcoxon test) (online supplemental figure 5d-e). However, GF mice treated by D3 did not show the same results, suggesting that the thickening of the mucus layer may be attributed to the increased abundance of *A. muciniphila* rather than the direct action of D3.

**The anti-obesity effect of D3 in other animal models**

To investigate the anti-obesity effect of D3 in other animals, we further treated two additional DIO models, rats and macaques, with D3 (figure 5A,H). Notably, D3 treatment resulted in an 8.96%±3.11% decrease in body weight gain in rats compared with untreated controls over the 10 weeks of HFD exposure (p<0.05, Wilcoxon test) (figure 5B,C,I). As expected, we observed that D3 treatment significantly suppressed food intake in DIO rats compared with HFD control rats (p<0.05, Wilcoxon test) (figure 5D). The expression of Guca2b in the ileum was significantly increased (p<0.05, Wilcoxon test) and leptin in epididymal fat was significantly decreased (p<0.05, Wilcoxon test) (figure 5E,F). Similarly, the abundance of *A. muciniphila* was increased (p<0.05, Wilcoxon test) (figure 5G). For DIO macaques, the average increase in body weight gain in the D3 group was 7.71%±2.97% lower than that in the control group at the 6th week (figure 5i). (figure 5I). Moreover, we observed that the food intake of macaques in the D3 group was significantly decreased (p<0.05, Wilcoxon test) (figure 5J), with an increased abundance of *A. muciniphila* (p<0.05, Wilcoxon test) (figure 5K).

**DISCUSSION**

Although obesity management has improved significantly over the years, therapies that target its underlying processes are still lacking. There is a growing interest in drug therapies that can support weight loss. Currently, some endogenous peptides in humans have been used to treat type 2 diabetes and obesity, such as a K-casein-derived glycogenous peptide and casein. Moreover, some intestinal peptides (eg, α-defensin 5) were used to reverse dyslipidaemia and improve glucoregulatory capacity in DIO mice. We found that oral administration of D3 led to a 12.06%±2.35% decrease in body weight gain and ameliorated gut microbiota dysbiosis in DIO mice. We further demonstrated that D3 can upregulate the expression of UGN and leptin resistance, both of which can inhibit the appetite of mice. Our study provides a novel perspective and evidence that endogenous polypeptides derived from degraded fragments of protein precursors may play an important role in regulating obesity and other metabolic diseases (figure 5).

To date, five medications (orlistat, phentermine plus topiramate, naltrexone plus buproprion, liraglutide and semaglutide), all of which have an anorexigenic effect, have been approved by the Food and Drug Administration (FDA) for long-term weight management in adults. In this study, we found that after D3 treatment, the food intake of both SPF mice and GF mice was significantly reduced, accompanied by an increased expression of UGN in the small intestine. UGN, a 16-amino-acid anorexigen hormone secreted by enterochromaffin cells in the small intestine, has been shown to target the GUCY2C receptor of the hypothalamus and activate anorexigenic pathways through blood circulation. Intravenous injection of prouroguanylin could induce satiety in mice. Kim et al also found that transgenic UGN under a control of brain-specific promoter could produce...
physiological levels to induce durable anorexic responses opposing weight gain for at least 24 weeks without altering the metabolic rate in DIO mice. We found that D3 treatment could resist the onset and worsening of diet-induced obesity in mice by upregulating the expression of UGN and increasing its content in serum. Such a UGN (gut)-GUCY2C (hypothalamus) endocrine axis may have the potential to serve as a novel therapeutic target for appetite control and metabolic syndrome. Additionally, we found that D3 restored the compensatory leptin elevation caused by a HF diet and increased leptin sensitivity. Leptin can decrease appetite by inhibiting the activity of neurons in the ARC, the centre of appetite regulation in the hypothalamus. A dramatic induction of c-Fos expression occurs when neurons in the ARC are activated by starvation, which can be reversed...
Gut microbiota

by the administration of appetite inhibitors (eg, estradiol\textsuperscript{50} and leptin\textsuperscript{51}) or feeding.\textsuperscript{52} Notably, the upregulated c-Fos expression and its colocalisation with POMC expressing neurons in mice after D3 administration, probably due to the combined effect of D3 promoting UGN expression and acting as a leptin sensitiser.

Currently, there is accumulating evidence of the role of gut microbiota in various metabolic disorders, and the manipulation of the gut microbiota might be a novel therapeutic option.\textsuperscript{2,53} In our study, we found that D3 treatment could reduce the abundance of \textit{Desulfovibrio} and \textit{Ruminococcus}. The administration of \textit{Desulfovibrio} to GF mice led to an increase in body fat percentage and the expression of CD36,\textsuperscript{41} a critical regulator of lipid absorption and homeostasis within mammals.\textsuperscript{54} In addition, we observed a higher abundance of \textit{Bacteroides} in D3-treated mice. Many observational and interventional studies have correlated leanness or weight loss to increased levels of \textit{Bacteroidetes}, which can produce short-chain fatty acids.\textsuperscript{55} However, this relationship is controversial because observational studies have also found increased abundances of \textit{Bacteroides} to be associated with Western diets, which are related to obesity.\textsuperscript{56} Here, we found that significantly enriched colonisation with the taxon \textit{B. vulgatus} for 4 weeks did not lead to a significant change in the body weight of DIO mice. In contrast, the obesity phenotype was reversed by transplanting \textit{A. muciniphila} in obese mice, which is consistent with previous studies.\textsuperscript{38,57} Notably, the difference between the percentage of body weight lost in GF mice (9.14±2.93%) and SPF mice (12.06±2.35%) treated with D3 further demonstrates the role of the gut microbiota in treating obesity. In fact, many drugs and chemicals that have been found to reduce body weight can affect the gut microbiota. For example, liraglutide changed the overall composition as well as the relative abundance of weight-relevant phylotypes, such as a reduction in \textit{Proteobacteria} and an increase in \textit{A. muciniphila} in treated DIO mice.\textsuperscript{58} Interestingly, D3 treatment increased the abundance of \textit{A. muciniphila} by 100 times, and this effect was much stronger than that of the previous two drugs (three to five times).

A number of polypeptides have been used to act as signalling molecules in the regulation of the neuroendocrine system to prevent obesity, including ligandirulide and semaglutide.\textsuperscript{44} glucagon-like peptide-1 (GLP-1) agonists and adrenomedullin 2.\textsuperscript{14} However, several reasons may explain the slow adoption of injectable GLP-1R agonists, including poor adherence of this patient population to injectable therapies and the general patient preference for oral medicines compared with injectable therapies.\textsuperscript{59} Interestingly, it is worth noting that in our study, oral administration of D3 significantly reduced the body weight of mice, indicating the potential of D3 as an effective and preferred treatment option for obesity. The use of medications for obesity has also found increased abundances of \textit{Bacteroides} to be associated with Western diets, which are related to obesity.\textsuperscript{56} Here, we found that significantly enriched colonisation with the taxon \textit{B. vulgatus} for 4 weeks did not lead to a significant change in the body weight of DIO mice. In contrast, the obesity phenotype was reversed by transplanting \textit{A. muciniphila} in obese mice, which is consistent with previous studies.\textsuperscript{38,57} Notably, the difference between the percentage of body weight lost in GF mice (9.14±2.93%) and SPF mice (12.06±2.35%) treated with D3 further demonstrates the role of the gut microbiota in treating obesity. In fact, many drugs and chemicals that have been found to reduce body weight can affect the gut microbiota. For example, liraglutide changed the overall composition as well as the relative abundance of weight-relevant phylotypes, such as a reduction in \textit{Proteobacteria} and an increase in \textit{A. muciniphila} in treated DIO mice.\textsuperscript{58} Interestingly, D3 treatment increased the abundance of \textit{A. muciniphila} by 100 times, and this effect was much stronger than that of the previous two drugs (three to five times).

METHODS

Rational optimisation of hydrophobic peptides from human defensin HD5

A hidden Markov model as implemented in TMHMM Server V2.0 was used to evaluate the peptides’ abilities to penetrate cell membranes. The peptide secondary structure was modelled using SWISS-MODEL\textsuperscript{62} with human HD5 (1zmp) as the template. The amphipathic surface and the three-dimensional structures of D1–4 were modelled using PyMol V2.3\textunderscore 2.\textsuperscript{63} D1–4 were synthesised using solid phase synthesis by Mimotopes (Wuxi, China).

Bacterial strains and administration and faecal microbial transplantation

\textit{Bacteroides vulgatus} ATCC 8248 and \textit{Akkermansia muciniphila} (ATCC BAA-835) were grown anaerobically at 37°C in Gifu anaerobic medium (HB8518-1, Hopepiol, Qingdao). Mice were treated with \textit{B. vulgatus} and \textit{A. muciniphila} by oral gavage at a dose of 1×10\textsuperscript{9} CFU/0.2 mL suspended in sterile anaerobic phosphate buffered saline (PBS). A similar concentration of glycerol (2.5% (vol/vol)) was used as control. Treatments were continued for 4 weeks.

For the FMT experiment, 2–3 fresh faecal pellets were resuspended by vortexing in anaerobic PBS, and then the supernatant was harvested and mixed. Bacteria were harvested and resuspended in sterile anaerobic PBS before FMT, and 100 µL of the suspension was administered to the mice by oral gavage two times per day for 4 weeks. Additionally, inactivated bacteria treated with high temperature were also administered to the mice by oral gavage two times per day for 4 weeks, which served as a control.

Animals and experiments

Operation of SPF mice and rats

All C57/B16J mice and Sprague Dawley (SD) rats (SPF Biotechnology, Beijing, China) and ob/ob mice (Hua Fukang Biotechnology, Beijing, China) were housed in groups of 4–6 animals per cage in a pathogen-free facility with standard bedding and enrichment in a temperature-controlled and humidity-controlled room on a 12-hour light/dark cycle with free access to food and water. All animals were fed a standard laboratory chow diet (growth and reproduction diet, SPFSLFZ003, SPF Biotechnology, Beijing, China) unless otherwise stated. HFD mice or rats were fed a HF diet (Rodent diet with 60 kcal\% fat, D12492; Research diets, New Brunswick, New Jersey, USA). For rats, only D3 was used. After 1 week of recovery, animals were randomly assigned to treatment groups, and treatment continued for 10 weeks. Food intake and body weight were monitored weekly. Fresh excreta from animals were harvested and stored at −80°C for further analysis. All of the above experiments in mice and rats were started at 4 weeks of age, unless otherwise noted.

Operation of GF mice

GF C57Bl/6 mice obtained from the Department of Laboratory Animal Science of the Army Medical University (Chongqing, China) were used. Mice and rats were sacrificed by cervical dislocation. Relevant organs were collected and immediately immersed in liquid nitrogen and stored at −80°C for further protection.
analysis. Subcutaneous and visceral adipose deposits were precisely dissected and weighed.

Pair-feeding
The pair-feeding experiment was performed according to previously reported procedures. Pair feeding was accomplished by measuring the food intake of the ad libitum-fed D3-treated mice every 24 hours and presenting this amount of food to the pair-fed PBS-treated mice in two times (half within 12 hours). Blood samples were obtained, and tissues were harvested as described above.

Operation of macaques
The rhesus experiment was undertaken by Beijing Prima Biotech. Nine rhesus monkeys feeding on a standard chow diet (#2150230401, Prima Biotech, Beijing, China) were randomly assigned to three treatment groups: D3, NC and HFD.

Short-term food intake measurement
Food weights were recorded every other day over a period of 1 week, and the average intake per day for each 2-day period was determined and averaged over the week measurement period for each individual.

Antibiotic treatment
SPF WT mice were treated with combined antibiotics (ABX) containing 100 g/L neomycin (Sigma), 100 g/L penicillin (Sigma), 50 g/L vancomycin (Sigma) and 100 g/L metronidazole (Sigma) for 8 weeks.

Morphology and histology
Liver and adipose tissue samples were quickly collected and fixed in 4% paraformaldehyde (PFA) solution for 24 hours and embedded in paraffin. Sections of 10 µm were stained with H&E. ABPAS staining was performed using a previous method. A minimum of 10 different measurements were made perpendicular to the inner mucus layer per field and were manually delineated and analysed by ImageJ.

Localisation of D3 after oral administration
FITC-D3 is used to trace drugs in target organs and their action pathways. The Swiss rolls were made according to previously published methods, with minor modifications. Briefly, after oral administration of 0.8 mg/kg FITC-D3 for 3 hours, the mice were sacrificed, and the lumen of small intestine was cut open from one side of the intestine using spring scissors. Cotton swabs were used to roll up the intestines that had been unfolded. The intestines were gently rolled around the self-closing forceps evenly, ensuring that each successive roll nearly laid over the previous roll with edges flush. The roll was gently slid off the cotton swabs and placed into an appropriately labelled pot containing 10 times the tissue volume of formalin. Whole-tissue slide scans at ×40 magnification were performed under a microscope slide scanner (Pannoramic MIDI, 3DHISTECH). Images were analysed with ZEN Imaging Software by ZEISS. Pictures were obtained by using an LSM 880 (Zeiss) or a spinning disc confocal microscope (Olympus). For the localisation of D3 uptake in cells, HCT116 cells were inoculated into confocal dishes (1×10^5 cells/well) and incubated for 18 hours at 37°C. Free FITC (10 µM) or FITC-labelled D3 (10 µM) was added to cultures for 1 hour. Following staining, the cells were washed twice with PBS and later observed under a spinning disc confocal microscope (Olympus) after the addition of fresh medium.

Immunofluorescence of the ileum and hypothalamus
Tissue segments were similarly removed, washed with PBS, mounted in embedding medium (Tissue-Tec, O.C.T. Compound) and stored (~8°C) until use. Immunofluorescence was performed using an anti-UGN (1:200; MBS7605526; MyBioSource) antibody. Detection and labelling were performed with secondary antibodies conjugated to Alexa Fluor-488 donkey anti-rabbit IgG (H+L) (1/500, A21206, Invitrogen).

Neuronal identification and c-Fos staining
Twenty-four mice (male, 8 weeks old) feeding on a standard chow diet were equally divided into eight groups (NC-0h, NC-1h, NC-2h, NC-3h; D3-0h, D3-1h, D3-2h, D3-3h). For gene expression assays, the hypothalamus was harvested 0–3 hours following D3 administration (NC: saline), and the expression levels of c-Fos, AgRP and POMC were quantified by quantitative RT-PCR.

The same experiment was carried out for the immunofluorescence. c-Fos staining was performed as described in a previous study. Briefly, mice were anaesthetised with a lethal dose of pentobarbital and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed, placed in 4% paraformaldehyde overnight and dehydrated in 30% sucrose for 1 week. Brains were cut into 25 mm sections. The sections were treated as described above and incubated overnight at room temperature in mouse anti-FOS (1:500; ab208942; Abcam) or rabbit anti-POMC (1:100; ab254257; Abcam). Detection and labelling were performed using secondary antibodies conjugated to Alexa Fluor-594 anti-mouse IgG (H+L) (1/500, ab150108, Abcam) or Alexa Fluor-488 donkey anti-rabbit IgG (H+L) (1/500, ab150073, Abcam), and imaging was performed as described above. Images were pseudocoloured using Photoshop software (Adobe) or ImageJ (NIH).

Contributors FZ is responsible for the overall content as the guarantor. FZ conceived and supervised the study. FZ and ZL designed the study, interpreted the results and wrote the manuscript. WH’s team fed the GF mice and recorded the experimental data. Real transcriptome and faecal flora diversity analyses were performed by BZ, ZL, HW, ZZ and NW performed all the other experiments and the data analysis and prepared the figures and tables.

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Competing interests None declared.

Patient consent for publication Not applicable.

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