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# Carnosinase inhibition enhances reactive species scavenging in high fat diet

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## ABSTRACT

**Aims:** Life expectancy is typically reduced by 2–4 years in people with a body mass index (BMI) of 30–35 kg/m<sup>2</sup> and by 8–10 years in people with a BMI of 40–50 kg/m<sup>2</sup>. Obesity is also associated with onset, or exacerbation of, multiple chronic diseases. Mechanistically, this, in part, involves formation of advanced glycation and lipidation end-products that directly bond with proteins, lipids, or DNA, thereby perturbing typical cellular function. Here we seek to prevent these damaging adduction events through inhibition of carnosinase enzymes that rapidly degrade the physiological reactive species scavenger, carnosine, in the body.

**Main methods:** Herein we performed *in silico* computational modelling of a compound library of ~53,000 molecules to identify carnosine-like molecules with intrinsic resistance to carnosinase turnover.

**Key findings:** We show that leading candidate molecules reduced reactive species in C2C12 myotubes, and that mice fed *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine alongside a high fat diet had significantly decreased amounts of damaging plasma 4-hydroxynonenal and 3-nitrotyrosine reactive species. Oral administration of *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine to high fat-fed mice also resulted in a modest ~10 % reduction in weight gain when compared to mice fed only high fat diet.

**Significance:** Our findings suggest that inhibition of carnosinase enzymes can increase the life-span, and thereby enhance the efficacy, of endogenous carnosine *in vivo*, thereby offering potential therapeutic benefits against obesity and other cardiometabolic diseases characterised by metabolic stress.

## 1. Introduction

Obesity represents an ongoing major global health challenge, with life expectancy reduced by about 2–4 years in people with a body mass index (BMI) of 30–35 kg/m<sup>2</sup>, and by about 8–10 years in people with a BMI of 40–50 kg/m<sup>2</sup> [1]. Moreover, between 1990 and 2017, the global deaths attributable to high body mass index (BMI) more than doubled

from 2.2 million to 4.7 million people [2]. Importantly, BMI is also associated with increased disability-adjusted life years, which increased from 65 million to 147.7 million people over the same period [2].

Obesity levels track closely with type 2 diabetes (T2D), where the figure has been projected to rise from an estimated 451 million adults worldwide in 2017, to 693 million by 2045 [3]. Importantly, over 80 % of patients with T2D are either obese or overweight, with elevated BMI

**Abbreviations:** 3-NT, 3-nitrotyrosine; 4-HNE, 4-hydroxynonenal; AGE, advanced glycation end-product; ALE, advanced lipidation end-product; BMI, body mass index; GLT, glucolipotoxic; GOLD, genetic optimisation for ligand docking; HOMA-IR, homeostatic model assessment of insulin resistance; HSkM, human skeletal muscle myoblast; NICE, National Institute for Health and Care Excellence; RCS, reactive carbonyl species; ROCS, rapid overlay of chemical structures; T2D, type 2 diabetes.

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found to be associated with 73.5 % of the increase in diabetes mortality rate since 1990 [4]. A successful response to the twin challenge of T2D and obesity therefore requires timely, effective interventions aimed at counteracting the disease burden generated by high BMI. This approach, in turn, would also help mitigate the risk from a range of associated diseases, including cardiovascular disease, kidney diseases, and cancer among others.

Obesity and T2D have been targeted by strategies aimed at raising physical activity. Whilst there is evidence showing that increased daily exercise can help reduce weight and manage T2D, thereby improving long-term patient health prospects [5,6], lifestyle changes of this nature can prove challenging to maintain over time. Furthermore, complications associated with diabetes and obesity may also make adherence to demanding exercise programmes difficult for certain individuals to sustain. This has led to a search for alternative strategies.

Glucose was first reported to be able to chemically bind to protein, lipid, or DNA through non-enzymatic pathways over a century ago [7], typically resulting in decreased functional capability of affected molecules. Individuals subject to chronically elevated levels of glucose are consequently often found to have increased levels of advanced glycation (AGE) and advanced lipidation (ALE) end-products. Therefore, a therapeutic strategy that has been adopted in several recent pilot studies is to attempt to reduce the level of oxidative stress in obese and diabetic individuals through dietary supplementation with carnosine [8–10], a naturally occurring physiological dipeptide comprised of  $\beta$ -alanine and L-histidine.

Carnosine is able to exert protective action against reactive carbonyl species (RCS) damage, as extensive carnosine glycation serves to reduce toxic RCS levels, thereby consequently reducing the extent of protein and lipid adduct formation that renders these molecules non-functional [11]. With specific regard to obesity, glycated forms of carnosine linked to RCS, such as carnosine-4-hydroxynonenal, an  $\alpha$ ,  $\beta$ -unsaturated hydroxyalkenal produced by lipid peroxidation in cells, have previously been reported in the urine of obese rats [12]. More recently, carnosine has also been shown to be an efficient RCS scavenger following exposure of cells to metabolic stress, removing, or preventing, 65–90 % of damaging 4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT) protein adduction, the latter being a relatively specific marker of oxidative damage mediated by peroxynitrite, which otherwise results in mitochondrial dysfunction and breakdown of stimulus-secretion coupling pathways [13]. A subsequent report also showed that  $\beta$ -alanine, the biosynthetic rate-limiting precursor for carnosine synthesis in the human body [14], similarly protects mitochondrial function in cells exposed to metabolic stress [15]. In addition, carnosine has also been shown to lead to a reduction of methylglyoxal-modified proteins in type-2 diabetic skeletal muscle cells [16].

While carnosine is clearly an effective reactive species scavenger at the molecular level, data from trials with humans or animals has been less impressive, with only moderate (human outcomes) or very low (rodent outcomes) reductions having been reported in fasting glucose, HbA<sub>1c</sub>, and HOMA-IR [17]. The likely reason for this is carnosine hydrolysis in the body by carnosinase enzymes. Carnosinase was originally identified in the kidney of a hog [18], with human serum carnosinase being subsequently identified as a separate and more highly expressed enzyme than the tissue carnosinase [19]. The combined action of the two carnosinase enzymes in the body, however, means that taking carnosine as a supplement is likely to require sustained administration of high doses to achieve even the modest beneficial effects reported. This would require a significant burden in terms of remembering to take all doses and would also incur high cost.

Alternative strategies to direct carnosine administration could involve the development of carnosine analogues that are resistant to hydrolytic degradation but capable of reactive species scavenging similar to carnosine, or instead simply inhibiting carnosinase enzymes to raise the level of available endogenous carnosine. Both approaches have been explored with the generation of scavengers [20] and inhibitors

[21] based on the carnosine skeleton which are resistant to carnosinase degradation. In the development of carnosinase inhibitors, the carnosine skeleton is an attractive starting point in terms of activity but, as a highly hydrophilic molecule, it is less attractive in terms of development of good pharmacokinetic properties. In this work we have sought to identify novel carnosinase inhibitors with more promising drug-like properties.

Herein, we screened a databank of ~53,000 candidate drug molecules for compounds with similar predicted 3D structure to carnosine, and hence capable of fitting into the catalytic cleft of the carnosinase enzymes and acting as carnosinase inhibitors. Potential candidates were then assessed for cellular toxicity, ability to reduce levels of reactive species, and finally body weight determined following 10-weeks of high fat-feeding in mice.

## 2. Materials and methods

### 2.1. Materials

Unless stated otherwise, all chemicals or reagents were purchased from Merck Life Science UK Ltd. (Gillingham, Dorset, UK) and all plasticware from VWR International Ltd. (Lutterworth, UK).

### 2.2. Lead compound identification

Maybridge HitDiscover 2015 dataset of ~53,000 compounds (ThermoFisher Scientific) was initially modelled using energy-minimised 3D structures to compare with a similarly prepared carnosine model using ROCS (rapid overlay of chemical structures, Cadence Molecular Sciences, Sante Fe, USA) to positively identify 500 carnosine-shaped structures. The top scoring 50 hits were then docked into the deposited structure of human carnosinase CN1 (3DLJ) [22] and mouse tissue carnosinase CN2 (2ZOG) [23] using GOLD (genetic optimisation for ligand docking) [24] and the twelve top scoring compounds in this docking exercise purchased for biological screening.

### 2.3. C2C12 and human skeletal muscle cell culture

Clonetics™ human skeletal muscle myoblasts (HSkM) were purchased from Lonza Bioscience, (Basel, Switzerland). HSkM cells were isolated from the upper arm or leg muscle tissue of a healthy donor (20-year-old, Caucasian male, BMI = 21, non-smoker). HSkM cells were incubated in Human Skeletal Muscle Growth Media with supplement pack (PromoCell, Heidelberg, Germany). Mouse C2C12 skeletal myoblasts were maintained in high glucose-DMEM supplemented with 10 % (v/v) foetal bovine serum, 10 % (v/v) heat inactivated newborn calf serum (Life Technologies, Paisley, UK), and 1 % (v/v) penicillin-streptomycin (Life Technologies) in a humidified atmosphere with 5 % CO<sub>2</sub> at 37 °C. C2C12 cells were switched to DMEM supplemented with 2 % (v/v) heat-inactivated horse serum (Life Technologies) for 7 days to facilitate myocytic differentiation, whereas HSkM cells were induced to differentiate in DMEM-F12 media. Cells were then incubated for a further 5 days in either control DMEM media (containing 11 mM glucose for C2C12 myotubes; 5 mM glucose for HSkM cells), or GLT media (DMEM with 28 mM glucose, 200  $\mu$ M oleic acid, 200  $\mu$ M palmitic acid for C2C12 cells; DMEM with 17 mM glucose, 200  $\mu$ M oleic acid, 200  $\mu$ M palmitic acid for HSkM cells), conditions representative of those seen in poorly controlled T2D, and carnosine analogue as indicated.

### 2.4. Cell viability

C2C12 cells were cultured in RPMI 1640 or GLT media for 5 days,  $\pm$  100  $\mu$ M of indicated compound, before media was aspirated and cells washed 3 times in Krebs-Ringer buffer (KRB). A final concentration of 5  $\mu$ M Calcein AM Cell Viability Dye (ThermoFisher Scientific) in KRB was loaded for 1 h before washing again with KRB. Cell viability was

measured via fluorescence, with excitation and emission at 490 nm and 520 nm.

## 2.5. Glucose uptake

Following the indicated treatment, cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM  $\pm$ 100 nM insulin. Medium was replaced with PBS + 0.125 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min, and then terminated by addition of stop buffer (0.4 M HCl + 2 % dodecyl trimethylammonium bromide). 2DG6P detection reagent was applied and data were acquired using a CLARIOStar luminometer (BMG Labtech, Ortenberg, Germany).

## 2.6. High fat-fed mouse

For compound administration studies, eight-week-old C57Bl/6 mice (Envigo, UK) (6 per group) were fed high-fat diet (HFD; 60 % fat; D12492) or low-fat control (10 % fat; D12450B), (both Research Diets, USA) for 10 weeks. Mice were administered the putative carnosinase inhibitor *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine, referred to in figures as **M8** (45 mg compound/kg body weight/day), via drinking water throughout the 10-week feeding period. Body weight, water and food intake were monitored throughout. Mice were maintained on a 12 h light/12 h dark cycle. All animal experiments were conducted in accordance with UK Home Office Animals (Scientific Procedures) Act 1986, with local Ethical Committee approval (PP9071010).

## 2.7. Reactive species scavenging

### 2.7.1. DCFDA assay

C2C12 cells were cultured for 5 days in standard tissue culture media, or media supplemented with 28 mM glucose, 200  $\mu$ M oleic acid, and 200  $\mu$ M palmitic acid (GLT media)  $\pm$  100  $\mu$ M of indicated compound. Cells were washed 3 times in KRB, then 20  $\mu$ M 2',7'-dichlorofluorescein diacetate (DCFDA) loaded for 1 h. Radical species detection was measured via fluorescence, with excitation at 495 nm and emission at 530 nm. Radical species are expressed as percentage change relative to control.

### 2.7.2. 4-HNE/3-NT determination

Heparin was added to isolated plasma as an anticoagulant and samples were centrifuged for 5 min at 1000 g at 4 °C. Supernatant was collected and assayed immediately following manufacturer's 4-HNE or 3-NT ELISA kit protocol (Fine test, Wuhan Fine Biotech Co., Ltd.).

## 2.8. Carnosinase activity assay

10 ng/ $\mu$ L of recombinant human CN2 (rHCNDP2) was incubated  $\pm$ 100  $\mu$ M **M8** for 30 min at room temp. Reaction mix was then incubated with 2 mM carnosine for a further 1 h, protected from light. 1 % aqueous solution of trichloroacetic acid (TCA) was added to terminate the reaction. All sample solutions were centrifuged at 13000 rpm for 10 min, after which supernatant was transferred to a new tube with 5 mg/mL of ortho-phthalaldehyde (o-PA) in 2 M NaOH, vortexed and incubated at room temp for 30 min protected from light. Finally, reaction mix was loaded to a black 96-well plate and fluorescence readings taken at 360 nm (excitation) and 460 nm (emission), using an Infinite® 200 Pro multimode plate reader (Tecan Life Sciences, Switzerland).

## 2.9. Statistical analysis

Results are expressed as mean  $\pm$  standard error of the mean ( $n = 3$  or more independent experiments). Parameters were compared either using ANOVA and Tukey *post-hoc* test, or one-tailed student *t*-test assuming equal variance, with statistical significance determined using

an alpha value of 5 %. A *p* value  $<0.05$  was considered to be statistically significant.

### 2.9.1. Power calculation

With regard to animal numbers, based on previous data ( $n = 6$  animals), in which we typically observed a 2.5 fold change in resulting data generated, and with a standard deviation of 0.7, for an alpha of 5 %, we would have 93 % power to detect a difference. We therefore anticipated that groups of six mice per experimental condition would be adequate to determine statistical significance in reactive species.

## 3. Results

### 3.1. Computational modelling of carnosine-like molecules

The preliminary search for carnosinase inhibitors and potential lead compounds for screening was by design biased toward molecules with favourable solubility and stability profiles by the selection database of commercially available molecules chosen for drug-like properties. This database was then computationally screened for compounds with potential as carnosinase inhibitors. This initial choice effectively excluded molecules based on carnosine and other dipeptides from the pool of candidates and ensured potential issues with poor lipophilicity and hydrolytic instability inherent to the carnosine skeleton was mitigated from the outset. In the event, the Maybridge HitDiscover library [25] of commercially available, drug-like compounds was utilised to screen for potential lead carnosinase inhibitors. This was accomplished via a shape comparison screening approach, from which 500 potential candidates were selected, from which the top 50 were further assessed by structure-based docking into a known human CN1 carnosinase enzyme X-ray crystal structure [22] and mouse carnosinase CN2 structure [23] using GOLD [24]. Docking against each enzyme provided the same subset of carnosine-like molecules of which the top twelve commercially available, highest scoring, compounds (Fig. 1) were purchased and subjected to biological testing.

### 3.2. Effects on glucose uptake

Given that impaired insulin-stimulated muscle glucose uptake is common in people with obesity and is a major risk factor for type 2 diabetes [26,27], potential drug candidates were next investigated for biological action on skeletal muscle cell myotubes using glucose-uptake assays (Fig. 2A). Specifically, C2C12 myotubes were cultured in Dulbecco's Minimal Eagle's Medium (DMEM) media, or DMEM GLT media (DMEM supplemented to 28 mM glucose, 200  $\mu$ M oleic acid, 200  $\mu$ M palmitic acid) for 5 days  $\pm$ 100  $\mu$ M of indicated compound, the concentration determined to be optimum in preliminary optimisation assays (data not shown). Cells were then serum-starved overnight in DMEM containing 5 mM glucose and subsequently incubated for 1 h in glucose-free DMEM  $\pm$  100 nM insulin. Based upon their ability to prevent GLT-mediated inhibition of insulin-stimulated glucose uptake, five of the twelve molecules, namely **M4**, **M8**, **M14**, **M28**, and **M38**, significantly improved insulin-stimulated glucose uptake relative to GLT.

### 3.3. Cellular toxicity screening

To investigate potential effects of the selected drug candidates on cell toxicity, fluorescence-based cell viability testing was conducted using calcein-AM dye. This was performed using both C2C12 mouse muscle myotubes (Fig. 2B) and a human skeletal muscle cell-line (Fig. 2C). None of the five selected molecules caused any significant toxicity to either cell population, so all five were consequently retained for further analysis.

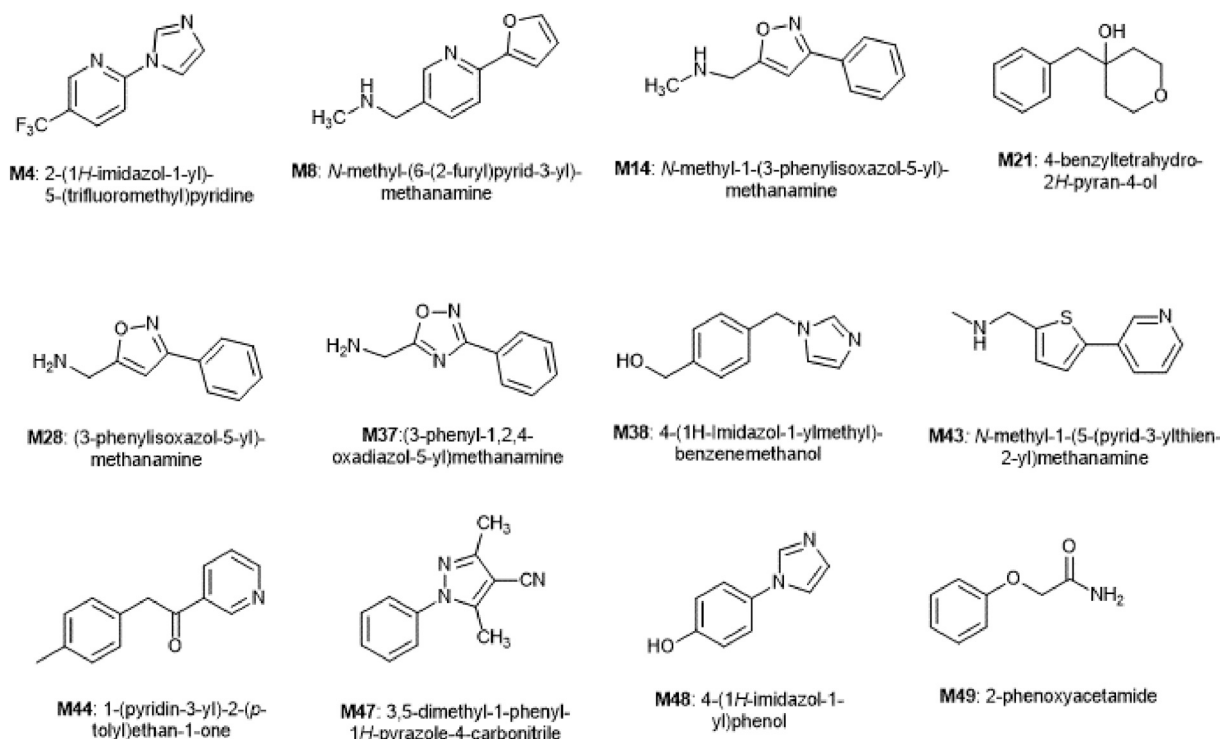


Fig. 1. Formal names and chemical structures of candidate compounds.

### 3.4. Effect on reactive species

As carnosine has previously been shown to protect cells from the damaging effects of metabolic stress through its ability to effectively scavenge both reactive oxygen and reactive carbonyl species [13,28], we investigated the action of the selected drug candidates on C2C12 myotubes (Fig. 3A). Whilst incubation in GLT media for 5 days resulted in a significant increase in reactive species ( $215.5 \pm 9.5\%$ ) compared to control, supplementation of GLT media with  $100 \mu\text{M}$  of either of the five drug candidates each resulted in a significant reduction of intracellular reactive species when compared to the GLT condition.

While all five drug candidates scavenged significant levels of GLT-associated reactive species, tissue culture experiments are not necessarily reflective of *in vivo* physiological responses. We therefore then went on to conduct a 10-week animal study to investigate the effects of potential drug candidates on reactive carbonyl scavenging in high fat-fed mice (with King's College London Ethics Committee approval, PP9071010). All compounds showed promise from the *in vitro* studies undertaken to this point, but, due to our desire to reduce animal welfare concerns, only two were taken forward for *in vivo* consideration. Based upon their ability to enhance insulin-stimulated glucose uptake, whilst at the same time showing no enhanced basal glucose uptake, the compounds selected were **M8** and **M28**. Unfortunately, once the study commenced, **M28** was not well tolerated by mice, so was instantly discontinued. No such adverse effects were, however, shown for **M8**.

At the conclusion of the study blood was taken and plasma isolated. This was then analysed for the presence of 4-HNE and 3-NT, reactive carbonyl species the concentrations of which we have previously shown to be elevated relative to healthy control individuals in human serum from patients with obesity, type 2 diabetes, or gestational diabetes [13]. Consistent with the data from humans, our analysis showed that high fat-fed animals had significantly elevated plasma levels of both 4-HNE (Fig. 3B) and 3-NT (Fig. 3C). Importantly, in high fat-fed mice given drinking water supplemented with **M8**, 4-HNE and 3-NT levels were significantly lower.

### 3.5. Carnosinase inhibitory action

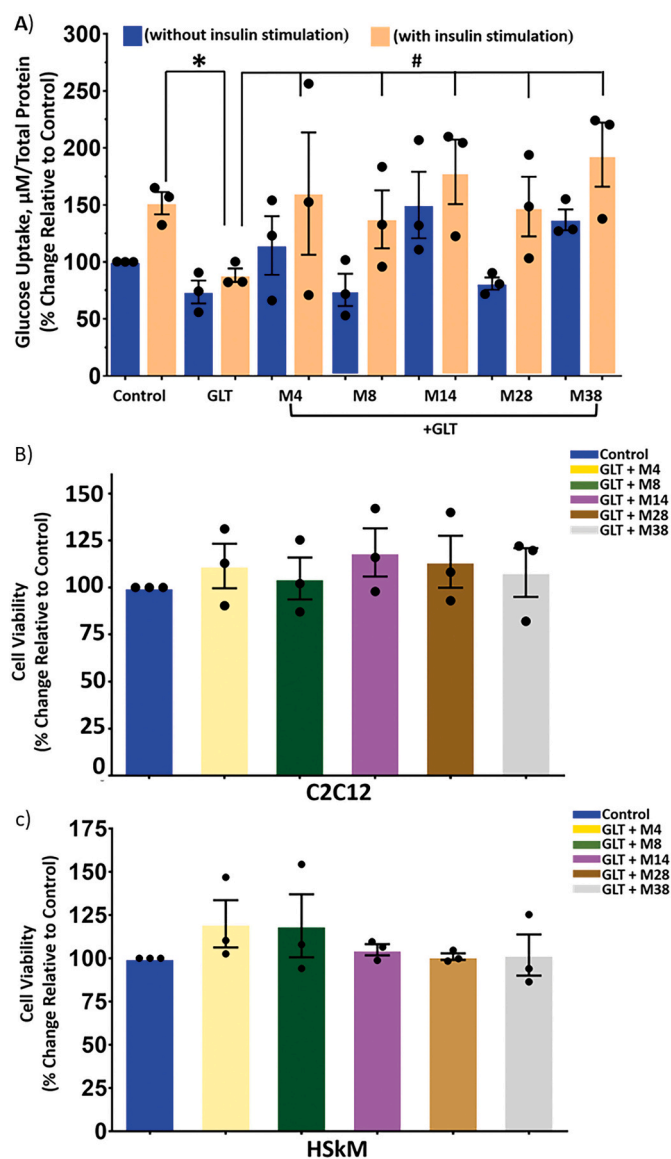
Given that the initial *in silico* screening strategy excluded dipeptides with carnosine-like scavenging properties from our pool of candidate drug-like molecules, the carnosinase inhibitory action of **M8** was next quantified directly using commercially available recombinant human CNDP2 (rhCNDP2) in conjunction with a fluorescence-based assay using carnosine as substrate. The fluorescence intensity produced was then used to assess the inhibitory action of **M8** based upon the complex formed between the histidine component from the cleaved carnosine substrate and the derivatisation reagent ortho-phthalaldehyde (o-PA) used in the assay. As shown in Fig. 4A, **M8** ( $p < 0.005$ ) significantly inhibited the carnosinase enzyme and protected carnosine from cleavage when compared to control (rhCNDP2 + carnosine only), thereby formally confirming **M8** action as a carnosinase inhibitor. The docking pose for **M8** in the active site of human carnosinase CN1 (3DLJ) is depicted in Fig. 4B.

### 3.6. *N*-Methyl-[6-(2-furyl)pyrid-3-yl]methylamine reduces weight gain in high fat-fed mice

Given the ability of **M8** (*N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine) to inhibit carnosinase, and thereby counter the increase in reactive carbonyl species that is associated with high fat diet and obesity, we sought to determine whether this would be of physiological benefit. Mice were either given a high fat diet, or high fat diet with drinking water supplemented with **M8**, and water intake and animal weight measured weekly. No significant changes were observed in physical activity or water intake ( $3.79 \pm 0.06 \text{ ml/mouse HFD}$ ;  $3.74 \pm 0.15 \text{ ml/mouse HFD} + \text{M8}$ ). However, high fat-fed mice receiving **M8** showed  $\sim 10\%$  less body weight gain relative to high fat-fed littermates that did not receive **M8** (Fig. 4C).

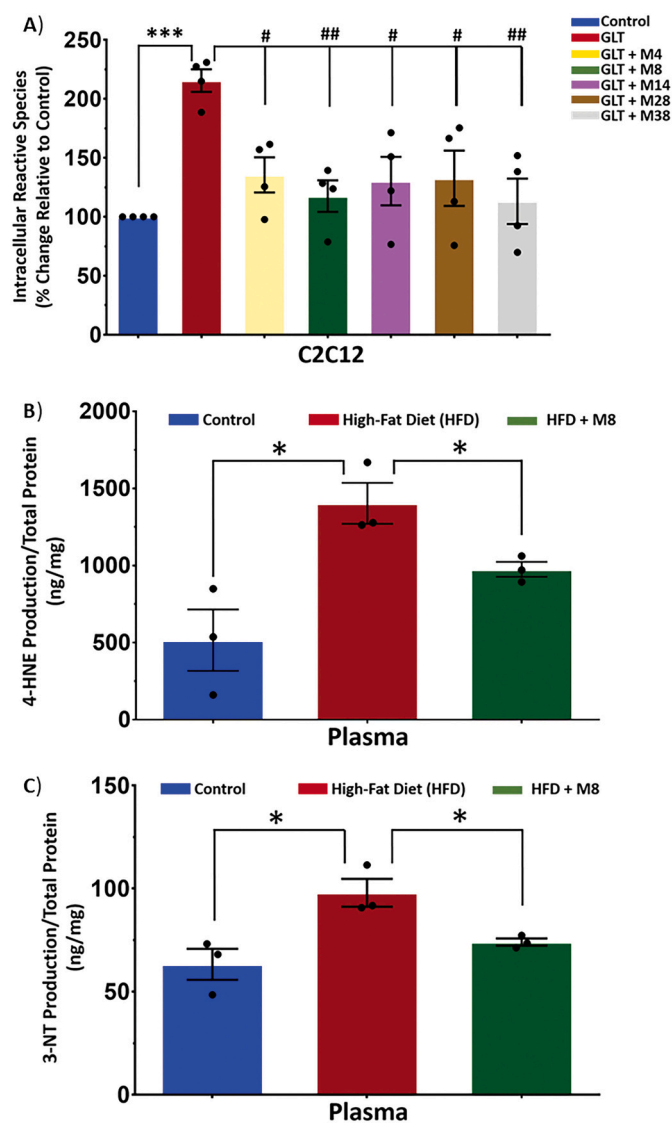
## 4. Discussion

Carnosine is a physiological histidine-containing dipeptide that has



**Fig. 2.** Determination of candidate molecule effect on glucose uptake and cell viability. A). C2C12 myotubes were cultured in DMEM media, or DMEM GLT media for 5 days  $\pm 100 \mu\text{M}$  of indicated compound. Cells were serum-starved overnight in DMEM supplemented with 5 mM glucose, then incubated for 1 h in glucose-free DMEM  $\pm 100 \text{ nM}$  insulin. Medium was replaced with PBS + 0.125 mM 2-deoxy glucose (2-DG). Glucose uptake reactions were conducted for 30 min. 2DG6P was detected using a luminometer. Data are expressed as means  $\pm$  SEM of 3 independent experiments. (\* $p < 0.05$  vs Insulin-stimulated Control, # $p < 0.05$  vs Insulin-stimulated GLT; Tukey's test). B and C). Cells indicated were cultured in control or GLT media + compound for 5 days. After 1 h incubation with 5  $\mu\text{M}$  solution of calcein AM, fluorescence intensity was measured using excitation and emission of 490 nm and 520 nm. Results shown are expressed as percentage change relative to control from 3 or more independent experiments  $\pm$  SEM. ( $p > 0.05$ ; ANOVA for all treatments). A. C2C12 myotubes. B) HSkM cells.

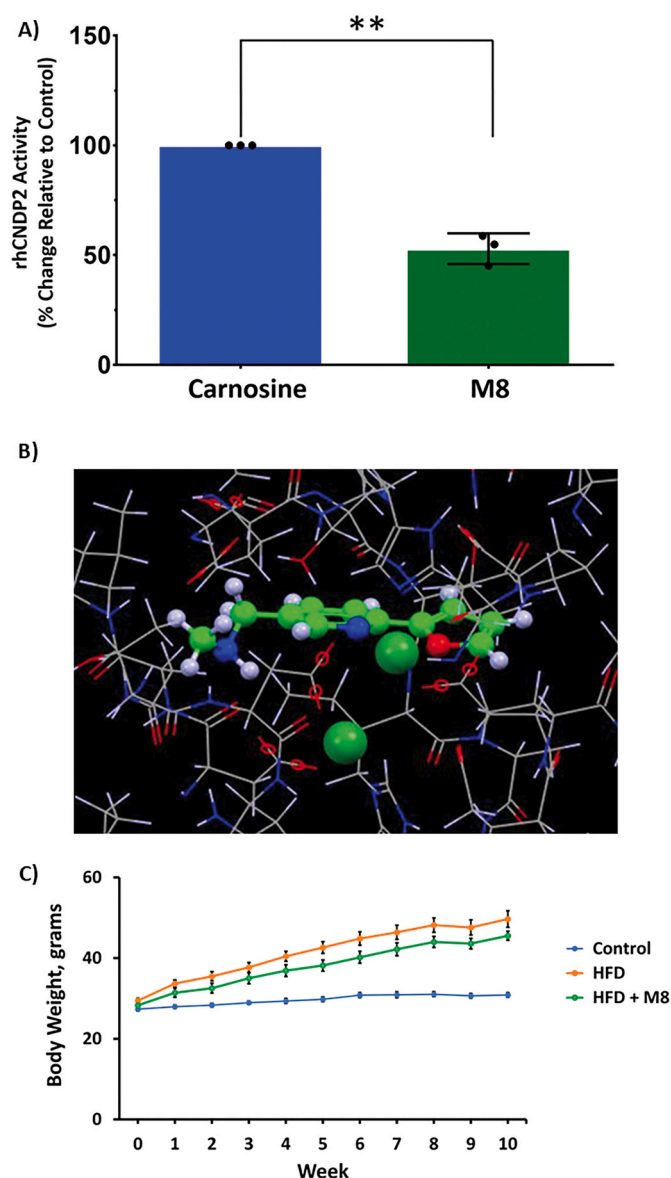
previously been proposed as an aid to healthy ageing [29,30]. Research has more recently also begun to focus on reactive carbonyl scavenging as a strategy to combat the effects of metabolic stress in cardiometabolic diseases. In this context, carnosine has been reported to effectively scavenge RCS in a mouse model of diabetes [31]. At the cellular level, similar carnosine scavenging actions have been shown to increase both insulin secretion and glucose uptake [28], and to protect against damaging carbonyl adduction that is associated with metabolic stress in cells and tissues associated with the control of glucose homeostasis [13].



**Fig. 3.** Reactive species scavenging. A) C2C12 myotubes were cultured in control or GLT media for 5 days  $\pm 100 \mu\text{M}$  of indicated compound. 20  $\mu\text{M}$  DCFDA reagent in KREBS buffer was loaded for 1 h and reactive species detected via fluorescence with excitation and emission of 495 nm and 530 nm. Reactive species are expressed as percentage change relative to control from 4 independent experiments  $\pm$  SEM. \*\*\* $p < 0.0001$  vs control, # $p < 0.05$  vs GLT, ## $p < 0.005$  vs GLT; Tukey's test. B and C). Eight-week-old C57Bl/6 mice were fed high-fat diet (HFD; 60 % fat) or low-fat control (10 % fat) for 10 weeks. Mice were administered N-methyl-[6-(2-furyl)pyrid-3-yl]methylamine, referred to in figures as M8 (45 mg compound/kg body weight/day), via drinking water throughout the 10-week feeding period. Mice were culled and plasma isolated. Samples were then assayed for content of either, B) 4-HNE, or C) 3-NT. Data shown are from 3 independent experiments. \* $p < 0.05$  relative to high fat diet; Tukey's test.

Furthermore, preliminary data has shown a modest reduction in plasma glucose levels in obese and diabetic individuals who received regular dietary supplementation with carnosine [8–10]. Carnosine is, however, turned over relatively quickly within the human body by the enzyme carnosinase 1 (CN1) in blood, and within tissues by the enzyme carnosinase 2 (CN2), which may explain why the beneficial effects reported were only modest. Therefore, we sought to determine whether an alternative strategy, through the investigation of carnosine-like, carnosinase inhibitors, might pave the way for future more effective alternative options.

To this end, the Maybridge HitDiscover (ThermoFisher Scientific)



**Fig. 4.** *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine inhibition of carnosinase activity and weight gain. A) Carnosine was allowed to react at room temperature with rhCNDP2 for 1 h (following a prior 30 min reaction period in the presence of **M8**). After the reaction was stopped by addition of trichloroacetic acid, *o*-phthalaldehyde detection reagent was added and incubated for 30 min. Fluorescent complex was then measured at 360 nm (excitation) and 460 nm (emission). Data reported as average values  $\pm$  SEM of  $n = 3$ . (\*\* $p < 0.001$  vs carnosine Control; Dunnett's test). B) Docking pose for **M8** in active site of human carnosinase CN1 (3DLJ). C green, H white, N blue, O red and  $Zn^{2+}$  ions dark green. C). Eight-week-old C57Bl/6 mice (6 per treatment group shown) were fed high-fat diet (HFD; 60 % fat) or low-fat control (10 % fat) for 10 weeks. Mice were administered *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine, referred to in figure as **M8** (45 mg compound/kg body weight/day), via drinking water throughout the 10-week feeding period. Mouse body weight was measured weekly.

dataset of  $\sim 53,000$  structurally diverse, drug-like compounds was computationally screened; first by Rapid Overlay Comparison of the minimised chemical structures onto similarly prepared carnosine, to identify 500 compounds of similar pharmacological shape. The top 50 candidates were then carried forward and screened for docking into the active site of the X-ray crystal structure of human CN1 and mouse CN2 enzyme; both docking screens identified the same high scoring molecules. From the candidate ranking, based upon docking performance,

the top twelve commercially available compounds were purchased for assessment. It was noteworthy that in the docking process, using a number of scoring functions in GOLD, the top docking hits all lacked an amide bond, and therefore are unable to be hydrolysed by carnosinase.

Having completed initial computational modelling, we next sought to test the putative carnosinase inhibitors for biological activity. Obesity is known to contribute to insulin resistance through decreased insulin sensitivity in skeletal muscle, which, in turn, plays a key role in the pathogenesis of obesity-associated cardiometabolic complications [32]. Therefore, we initially chose to investigate the effect of the selected molecules on insulin-stimulated glucose uptake into C2C12 myoblasts incubated under conditions of glucolipotoxic metabolic stress. Despite being incubated in GLT media, several of the selected compounds were able to restore glucose uptake to a similar profile seen under control conditions, where C2C12 myotubes were incubated without metabolic stress. From the initial screen, we selected **M4**, **M8**, **M14**, **M28**, and **M38** for a more comprehensive analysis of glucose uptake. All five significantly enhanced insulin-stimulated glucose uptake in C2C12 myotubes incubated in GLT media (Fig. 2A). Of these, **M8** and **M28** generated the largest insulin-stimulated response relative to non-stimulated glucose uptake in their presence.

As none of the five selected molecules had toxic effects on either C2C12 myoblasts or a human skeletal muscle cell-line (Fig. 2B and Fig. 2C), we went on to investigate their ability to reduce metabolic stress-induced reactive species in C2C12 cells. All five again produced promising data, with each preventing the generation of  $>75\%$  of metabolic stress-induced reactive species (Fig. 3A). Given the similarity between the five compounds in terms of their ability to reduce reactive species, the superior ability of **M8** and **M28** to increase insulin-stimulated glucose uptake was used as a basis for selection of these molecules to take forward to a 10-week animal study.

Unfortunately, **M28** was not well tolerated by mice, so was discontinued from the study. However, there were no such issues with **M8**. After 10 weeks of feeding on a high fat diet, analysis of plasma indicated a significant increase in 4-HNE and 3-NT levels in these animals. Importantly, high fat-fed animals administered **M8** showed significantly lower 4-HNE and 3-NT reactive species levels in plasma compared to high fat diet fed mice not given **M8** (Fig. 3B and C). It should be noted that, based upon the structural differences between carnosine and **M8**, it is unlikely that **M8** acts directly as a functional carnosine surrogate, scavenging HNE for example.

Testing of carnosinase activity to cleave carnosine in the presence of **M8** (Fig. 4A), showed **M8** to be an effective carnosinase inhibitor, as hypothesised in the earlier computational modelling, with **M8** inhibiting  $52.97 \pm 4.11\%$  of the activity of CNDP2. In further support of a carnosinase inhibition mode of action, close examination of the original docking data, and further redocking experiments, showed **M8** occupying the active site of CNDP1 with contact distances consistent with potential interactions between the furan O atom and the pyridyl N and one of the zinc ions in the active site. This suggests, that in modelling in GOLD at least, the metal binding is an important factor (Fig. 4B). We therefore hypothesise that **M8** inhibition of carnosinase activity would increase the life-span, and thereby enhance the efficacy, of endogenous carnosine in vivo, although future studies will be required to formally confirm this hypothesis.

Future studies will focus on the specific impact of *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine on dyslipidaemia. Carnosine has, however, been reported to inhibit glycation of low-density lipoproteins that promote foam cell formation in vitro [33]. Given that glycation of low-density lipoprotein is an important contributor to atheroma development in T2D, there are, therefore, potential key benefits of enhancing levels of endogenous carnosine for the prevention of atherosclerosis and downstream clinical outcomes, such as myocardial infarction, pulmonary embolism and stroke. It remains to be determined whether *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine has similar properties in humans to those reported in this study. Should this prove to be the case,

however, then there would clearly be therapeutic potential of *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine beyond simply prevention of weight gain (Fig. 4C).

## 5. Conclusions

In silico computational modelling of a compound library of ~53,000 molecules enabled us to identify and characterise carnosine-like molecules with intrinsic resistance to carnosinase turnover. Importantly, mice fed *N*-methyl-[6-(2-furyl)pyrid-3-yl]methylamine alongside a high fat diet showed significantly decreased amounts of damaging plasma 4-hydroxynonenal and 3-nitrotyrosine reactive species, and reduced weight gain when compared to mice fed high fat diet only.

## CRedit authorship contribution statement

**Charlie Jr. Lavilla:** Writing – original draft, Investigation, Formal analysis, Data curation. **Merell P. Billacura:** Writing – original draft, Investigation, Formal analysis, Data curation. **Suniya. Khatun:** Writing – original draft, Investigation, Formal analysis. **Daniel P. Cotton:** Writing – original draft, Investigation, Formal analysis. **Vivian.K. Lee:** Writing – original draft, Investigation, Formal analysis. **Sreya. Bhattacharya:** Writing – original draft, Investigation, Formal analysis. **Paul W. Caton:** Writing – original draft, Supervision, Formal analysis, Data curation. **Craig Sale:** Writing – original draft, Supervision, Conceptualization. **John D. Wallis:** Writing – original draft, Supervision, Formal analysis, Conceptualization. **A. Christopher Garner:** Writing – original draft, Supervision, Formal analysis, Conceptualization. **Mark D. Turner:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

## Declaration of competing interest

CS received funding to support a PhD studentship relating to the effects of carnosine on cardiac function from Natural Alternatives International; a company formulating and manufacturing customised nutritional supplements, including CarnoSyn beta-alanine. The same company has also provided CS with supplements for other studies free of charge and has contributed to the payment of open access publication charges for some manuscripts on  $\beta$ -alanine supplementation. MDT and ACG have registered a patent (GB2110230.6) for the use of molecules detailed in this study for the treatment of metabolic disorders. All other authors declare that they have no conflicts of interest with the contents of this article.

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## Data availability

Data will be made available on request.

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