Modulation of adiponectin receptors AdipoR1 and AdipoR2 by phage display-derived peptides in \textit{in vitro} and \textit{in vivo} models

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Introduction

The global prevalence of diabetes mellitus is approximately 425 million affected individuals and is expected to reach 629 million by 2045 [1]. The prevalence has doubled over the past three decades as a result of obesity epidemic and poses a major health and socio-economic burden [2]. Obesity is nearly invariably associated with insulin resistance in muscle, liver and adipose tissue.

The long-term complications of diabetes (i.e. cardiovascular disease, visual disability, renal failure, and neuropathy with limb amputation) forecast diabetes as a major metabolic disease that already attained an epidemic extent. Considering that type 2 diabetes (T2D) is increasingly prevalent (about 85% of diabetic patients) and develops at younger age, patients’ life quality and expectancy are notably reduced consequent to severe diabetic complications. Absolute insulin deficiency is characteristic to type 1 diabetes (T1D), while T2D is caused by relative insulin deficiency coupled to reduced insulin action in the body’s tissues. Because T2D may be paucisymptomatic for many years, the diagnosis is often made late, when complications have already developed. Diabetes healthcare is very costly, especially when chronic complications are present, and could be largely reduced through prevention, early diagnosis and optimal treatment.

Metabolic syndrome assembles several risk factors that concur in the development of cardiovascular disease and T2D. The key risk factors for its development are central obesity and insulin resistance [3].

In addition to its participation to energy homeostasis, adipose tissue acts as an endocrine organ that secretes several types of adipokines, such as adiponectin, adipisin, leptin, plasminogen activator inhibitor-1, resistin, and TNF-α. They may link obesity to metabolic syndrome and associated morbidity through their implication in energy and vascular homeostasis but also in immune pathways. Pro-inflammatory adipokines are overproduced in obesity, whereas anti-inflammatory or insulin-sensitising adipokines, such as adiponectin, are decreased. The modulation of this altered adipokine production may thus have therapeutic potential in metabolic syndrome [4–6].

Adiponectin has been reported to improve insulin sensitivity and exert antidiabetic, anti-inflammatory and antiatherogenic effects [7]. Adiponectin binding to its main receptors, AdipoR1 and AdipoR2, triggers free fatty acid (FFA) oxidation and glucose uptake by skeletal muscle, while hepatic gluconeogenesis is prevented. AdipoR1 is principally expressed in skeletal muscle, where the signalling pathway of 5’ adenosine monophosphate-activated protein kinase (AMPK) is activated. AdipoR2 is mostly expressed in

ABSTRACT

Type 2 diabetes (T2D) is often linked to metabolic syndrome, which assembles various risk factors related to obesity. Plasma levels of adiponectin are decreased in T2D and obese subjects. Aiming to develop a peptide able to bind adiponectin receptors and modulate their signalling pathways, a 12-amino acid sequence homologous in AdipoR1/R2 has been targeted by phage display with a linear 12-mer peptide library. The selected peptide P17 recognises AdipoR1/R2 expressed by skeletal muscle, liver and pancreatic islets. In HepaRG and C2C12 cells, P17 induced the activation of AMPK (AMPKα2-pT172) and the expression of succinate dehydrogenase and glucokinase; no cytotoxic effects were observed on HepaRG cells. In db/db mice, P17 promoted body weight and glycaemia stabilisation, decreased plasma triglycerides to the range of healthy mice and increased adiponectin (in high fat-fed mice) and insulin (in chow-fed mice) levels. It restored to the range of healthy mice the tissue levels and subcellular distribution of AdipoR1/R2, AMPKα2-pT172 and PPARγ-pS12. In liver, P17 reduced steatosis and apoptosis. The docking of P17 to AdipoR is reminiscent of the binding mechanism of adiponectin. To conclude, we have developed an AdipoR1/AdipoR2-targeted peptide that modulates adiponectin signalling pathways and has therapeutic relevance for T2D and obesity associated pathologies.

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Artificial Intelligence

Developing an AI model to predict diabetes risk.

Keywords

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liver, where it results in peroxisome proliferator-activated receptor alpha (PPARα) activation [8]. The two receptors are also co-expressed in many other cell and tissue types [9]. Pancreatic β cells express both AdipoR1/R2 [10] that exert antiapoptotic effects of adiponectin by activating MEK-ERK1/2 and PI3K-Akt pathways [11]. Adiponectin receptors represent a highly interesting drug target for the management of T2D and obesity-related pathologies, as well as for cancer [12]. Several AdipoR-targeted molecules were recently developed, either derived from a chemical library [13] or from the amino acid sequence of adiponectin, that have shown therapeutic potential [12,14,15].

The two AdipoRs present 66.41% sequence identity and are composed of 7 transmembrane domains with a membrane topology that is reversed when compared to G-protein-coupled receptors (GPCRs), meaning that their N-terminus is intracellular, whereas the C-terminus is extracellular [16]. Within the AdipoR C-terminal domain, we identified a 12-amino acid sequence (AdiopoR-12C) that is homologous in AdipoR1 (Q96AS4) and AdipoR2 (Q86V24), both in humans and mice. In a search for AdipoR1/R2-targeted molecules as novel therapeutic agents, we targeted AdipoR-12C with a phage displayed linear 12-mer random peptide library. Here we identified the promising P17 peptide that presents AdipoR binding, activates downstream signalling and confers beneficial metabolic outcomes in cell, tissue and in vivo models.

Materials and methods

Targeting of AdipoR-12C by phage display and phage clone characterisation

Phage display panning and DNA sequencing of the selected phage clones

Cys was coupled to the N-terminal of AdipoR-12C via a polyethylene glycol (PEG) spacer, whereas its C-terminus was amidated. Cys was used to immobilise AdipoR-12C on the surface of magnetic beads (Dynabeads® M280 Tosylactivated, Life Technologies, Gent, Belgium) according to the manufacturer instructions. AdipoR-12C was synthesised by the PolyPeptide Laboratories (Strasbourg, France) and presented the following composition: Cys-8-amino-3,6-dioxaoctanoyl-His–Phe–Tyr–Gly–Val–Ser–Asn–Leu–Gln–Phe–Arg– CONH₂. Bovine serum albumin (BSA) immobilised on Dynabeads was used as control protein during the preselection steps of the 3 panning rounds to exclude non-specific phages. A combinatorial 12-residue linear peptide library fused to the minor pIII coat protein of M13 bacteriophage (PhD-12, New England BioLabs Inc., Bioké, Leiden, The Netherlands) [17] was screened against AdipoR-12C, using the ER2738 strain of Escherichia coli (Escherichia coli K12 ER2738, F⁺, tetracycline-resistant strain; New England BioLabs Inc., Bioké, Leiden, The Netherlands) to amplify phages and isolate selected clones. The DNA sequences of selected clones and encoded peptides were read with JamBW 1.1 software (http://bioinformatics.org/JamBW/) as previously described [17]. The alignment of peptide sequences with pertinent proteins was performed by BLAST (The Basic Local Alignment Search Tool). Additional information is available in Supplementary information – Materials and Methods.

Binding of phages to AdipoR-12C and AdipoR1

To evaluate phage binding to AdipoR-12C, this last one was immobilised in a Pierce® maleimide activated 96-well ELISA plate (Thermo Fisher Scientific, Erembodegem, Belgium) according to the manufacturer instructions. The binding to human AdipoR1 (Origene Technologies, Sanbio B.V., Uden, The Netherlands) was evaluated after protein immobilisation in a medium binding Microlon® ELISA plate (Greiner Bio-One, Wemmel, Belgium). Control wells were coated with BSA. Phage samples were incubated with AdipoR-12C, AdipoR1 or BSA coated wells for 1-2 h at RT. HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) was used to detect bound phages. 2,2’-Azino-bis(3-Ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich, Bornem, Belgium) (ABTS) solution completed with 0.05% H₂O₂ was employed to develop the staining reaction, followed by OD₄₅₀ measurement [17]. Additional information is available in Supplementary information – Materials and Methods.

In vitro evaluation of selected AdipoR-binding peptides

Based on the affinity tests, the peptides expressed by the most promising clones (5, 8, and 43) were synthesised (PolyPeptide Laboratories, Strasbourg, France) as biotinylated or not biotinylated 8-amino-3,6-dioxaoctanoyl derivatives and encoded as follows: P16 (clone 5: ADWYHWRSHSSS), P17 (clone 8: IPNYSMQSREYR) and P18 (clone 43: YDVPNKSWRTSW).

Binding of peptides to AdipoR1/R2 in mouse muscle and liver and human pancreas and colocalisation studies

The binding of peptides to healthy mouse (NMRI mice, mean body weight 23 g, Harlan Laboratories, Horst, The Netherlands) skeletal muscle and liver, and human pancreas (kindly provided by Prof Isabelle Salmon and Dr Sandrine Rorive, Pathology Department of the Erasme Hospital, ULB, Brussels, Belgium) was evaluated by immunofluorescence. Biotinylated peptides were incubated with tissue sections at a concentration of 20 µM. The bound peptides were revealed with anti-biotin antibody made in goat and with fluorescein anti-goat IgG made in rabbit (both from Vector Labconsult, Brussels, Belgium). Tissue sections were mounted with Vectashield Mounting Medium containing 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, Vector Labconsult, Brussels, Belgium) and observed with a Leica DM2000 microscope equipped with a DFC 425 C camera and light source EL 6000 (Leica Microsystems, Groot Bijgaarden, Belgium).

To confirm AdipoR1 (skeletal muscle and pancreas) and AdipoR2 (liver) binding, biotinylated peptides were co-incubated with goat anti-AdipoR1 or anti-AdipoR2 IgG (Santa Cruz Biotechnology, Heidelberg, Germany). Sections were then incubated with mouse anti-biotin antibody (Vector Labconsult, Brussels, Belgium), followed by a coincubation with horase anti-mouse IgG conjugated to Texas Red and rabbit anti-goat IgG conjugated to fluorescein (both from Vector Labconsult, Brussels, Belgium). The samples were mounted and observed as described above.

For the colocalisation studies, human pancreas sections were co-incubated with biotinylated peptides and mouse anti-human insulin IgG (Abcam, Cambridge, GB). NMRI mouse skeletal muscle sections were co-incubated with mouse anti-slow myosin IgG (Abcam, Cambridge, GB) and goat anti-AdipoR1 and anti-AdipoR2 IgG (both from Santa Cruz Biotechnology, Santa Cruz, CA) or biotinylated peptides. Then, sections were incubated with goat anti-biotin antibody (Vector Labconsult, Brussels, Belgium), followed by coincubation with secondary antibodies, mounting and observation as described above. Additional information is available in Supplementary information – Materials and Methods.
**Evaluation of peptides on cell models**

*Cell lines and induction:* The cellular effects of selected peptides were studied on HepaRG human hepatocytes (Life Technologies, Gent, Belgium) and C2C12 myoblasts (kindly provided by Dr Frédérique Coppée from the Molecular Biology Department of UMONS).

HepaRG and differentiated C2C12 cells were induced for 15, 60, and 135 min in culture medium supplemented with glucose (25 mM total glucose for HepaRG and 39 mM for C2C12), a solution of free fatty acids and cholesterol (FFAC, Chemically Defined Lipid Concentrate, Life Technologies, Gent, Belgium) or both. Except for control samples, the induction medium contained 40 μM of non-biotinylated peptides P16, P17 or P18 or 2 μM of the commercial AdipoR agonist (AgoAdipoR; compound 112254, Santa Cruz Biotechnology, Heidelberg, Germany).

For immunofluorescence studies, cells were seeded on coverslips placed in 6-well culture plates (CellStar™, Greiner Bio-One, Wemmel, Belgium). For the measurement of phosphorylated AMPKα [pT172] (Life Technologies), cells were seeded in 96-well culture plates (Greiner Bio-One, Wemmel, Belgium). Then, the cells were grown and induced as described above. Additional information is available in *Supplementary information – Materials and Methods*.

All experiments were done in triplicate or quadruplicate. For immunofluorescence studies, 6-10 microscopic fields per slide were photographed using a Leica DM2000 microscope. The fluorescent labelling was semi-quantitatively analysed using ImageJ software (National Institutes of Health, Bethesda, MD).

**Quantification of phosphorylated AMPKα [pT172]:** Cells were cultured and induced as described above, total protein was extracted with an extraction buffer (Life Technologies) according to manufacturer instructions, and cell lysates were stored at −80°C. AMPKα phosphorylated on Thr172 [pT172] (AMPKα-pT172) was quantified using a sandwich ELISA (Life Technologies). Results were normalised to protein content, measured with the Pierce™ BCA Protein Assay (Thermo Fisher Scientific, Erembodegen, Belgium). Additional information is available in *Supplementary information – Materials and Methods*.

**Immunofluorescent detection of glucokinase and succinate dehydrogenase:*** Glucokinase (GK) and succinate dehydrogenase (SDHA) were detected concomitantly by immunofluorescence on cell samples induced for 60 min (see above) and fixed with 100% methanol, followed by blocking/permeabilisation with 1% BSA and 0.3% Triton-X100. The antibodies were rabbit anti-GK and mouse anti-SDHA antibody (both from Abcam). Cells were then co-incubated with Texas red conjugated horse anti-mouse IgG and fluorescein conjugated goat anti-rabbit IgG (both from Vector Labconsult). Samples were mounted and examined as described above. Additional information is available in *Supplementary information – Materials and Methods*.

**Fluorescent colocalisation of AdipoR1 with lysosomes and caveolae on C2C12 cells:** AdipoR1 was colocalised with lysosomes and caveolae on differentiated C2C12 cells cultured and induced as described at point Cell lines and induction, excepting that induction time was limited to 60 min. The cells were fixed and blocked as explained at point Immunofluorescent detection of glucokinase and succinate dehydrogenase, and then were co-incubated (overnight, 4 °C) with 4 μg/mL of goat anti-AdipoR1 IgG and 4 μg/mL of either rabbit anti-LAMP-1 IgG or rabbit anti-caveolin-1 IgG (prepared in phosphate buffer pH 7.8, 0.5% BSA). Next day, the samples were co-incubated (1 h, RT) with 10 μg/mL of Dylight 594 conjugated horse anti-rabbit IgG (both from Vector Labconsult). The cell samples were finally mounted and observed at microscope as described above.

**MTT protocol for cytotoxic evaluation of P17:** To evaluate potential cytotoxic effects of P17, HepaRG cells were seeded in 96-well culture plates and incubated with 40 to 400 μM P17. The percentage of viable cells was determined using the *in vitro* MTT based cytotoxicity assay (Sigma-Aldrich) according to the supplier’s protocol. Additional information is available in *Supplementary information – Materials and Methods*.

**In vivo evaluation of peptide P17**

Animal experiments were approved by the Ethics Committee of the University of Mons [project codes MU/14/01 (June 2012–June 2016) and MU/14/02 (July 2016 – July 2020)] in compliance with Directive 2010/63/EU.

1. db/db mice (BKS(D)-Leprdb/JOnRj, Janvier Labs, St. Berthevin, France; 4 mice per group) received the following treatment and *ad libitum* diet: (a) 6-week-old mice injected with peptide P17 and fed Western high-fat diet (HFD) containing 60 kcal % fat (D12492 Rodent Diet, Research Diets Inc., New Brunswick, NJ); (b) 6-week-old mice injected with PBS and fed 60 kcal % fat HFD; (c) 7-week-old mice injected with P17 and fed on a standard chow diet (SCD) containing 10 kcal % fat (D12450 Rodent Diet, no sucrose, Research Diets Inc.); (d) 7-week-old mice injected with PBS and fed on a SCD. P17 was injected i.p. every evening at a dose of 40 μmol/kg body weight (60 μL/30 g body weight) for 4 days. An additional control group of 4 healthy NMRI mice (Janvier Labs) received SCD and no treatment.

Mouse body weight and glycaemia were evaluated daily during the experimental period. Blood glucose was measured in a drop of blood from the caudal vein, using the OneTouch® Verio Blood Glucose Metre (Johnson & Johnson Company, New Brunswick, NJ). The mice were killed (injection of 500 mg/kg body weight of pentobarbital and of 0.05 mg/kg body weight of buprenorphine) the fifth day after the start of treatment, and blood, liver and skeletal muscle were collected.

Blood was collected on heparin and plasma separated by centrifugation (30 min, 7000 rpm). Plasma triglycerides were quantified using the triglyceride assay from BioAssay Systems (Gentaur BVBA, KAMPENHOUT, BELGIUM). Mouse adiponectin was measured using an Invitrogen ELISA (Life Technologies), and mouse insulin was evaluated with a Eurobio ELISA (Courtaboeuf, France).

**Biomarkers of adiponectin signalling were analysed on histologic tissue samples (fixed in 4% paraformaldehyde and paraffin embedded).** AdipoR1 and AdipoR2 were detected with goat anti-AdipoR1 or anti-AdipoR2 IgG (Santa Cruz Biotechnology, Heidelberg, Germany) followed by horse anti-goat IgG conjugated to fluorescein (Vector Labconsult). To stain AMPKα-pT172, tissue sections were incubated with rabbit anti-AMPKα-pT172 antibody (Santa Cruz Biotechnology, Heidelberg, Germany), followed by Dylight 488 conjugated anti-rabbit IgG developed in horse. PPARα-pS12 was observed by incubating tissue sections with rabbit anti-PPARα-pS12 (Thermo Fisher Scientific), followed by horse anti-rabbit IgG coupled to fluorescein (Vector Labconsult).

Activated caspase-3 was stained on liver sections using rabbit anti-activated caspase-3 antibody (Thermo Fisher Scientific). Apoptotic livers from Balb/c mice injected with anti-Fas antibody (clone Jo2, isotype κ2, BD Biosciences Pharmingen, Erembodegen, Belgium) were used as positive controls. Sections were incubated with Dylight 488 conjugated horse anti-rabbit IgG.

Tissue sections were mounted with Vectashield Mounting Medium with DAPI before observing at microscope.
Liver morphology and integrity were evaluated by Masson’s Trichrome stain (Accustain® kit, Sigma-Aldrich). Briefly, nuclei were stained in black with Weigert’s iron haematoxylin, cytoplasm in red with Beibrich scarlet-acid fuchsin, and collagen in blue with aniline blue after treating sections with phosphotungstic and phosphomolybdic acid. Sections were then rinsed in acetic acid and distilled water and mounted in a permanent medium after dehydration.

Additional information is available in Supplementary information – Materials and Methods.

Prediction of P17 binding to AdipoR by a docking study

The binding of P17 to AdipoRs was explored using the HPEPDOCK web server (http://huanglab.phys.hust.edu.cn/hpepdock/) [18]. This docking algorithm considers linear peptides as flexible molecules able to adopt a wide range of spatial conformations and generates 10 top binding prediction models. The crystallographic structures of AdipoRs are provided by the server after introducing the PDB ID of the protein, i.e. 3WXV chain A for AdipoR1 and 3WXW chain A for AdipoR2. The root-mean-square deviation (RMSD) is calculated to evaluate the quality of docking by considering the atoms of the residues in peptide and protein situated within 10 Å of distance. A RMSD of ≤2.0 Å is considered as a successful docking prediction.

Statistical analysis

Results are expressed as means ± standard deviation (SD). Statistical differences between experimental groups were calculated by one-way ANOVA using SigmaPlot 11.0 software. Holm-Sidak and Bonferroni corrections were applied for groups with uneven variance. A p-value < .05 was considered significant.

Results and discussion

Targeting of adiponectin receptors by phage display

The homologous amino acid sequence (AdipoR-12C) identified within the C-terminal domain of AdipoRs corresponds to 351-HFYGVSNLQEFR361 in AdipoR1 and 362-HFHGVSNLQEFR373 in AdipoR2, respectively, the only difference between them being at the third amino acid. According to earlier publications [19], the C-terminal extracellular region of AdipoR1 starts at L358, whereas that in AdipoR2 has L294 as the starting residue. Tanabe et al. [16] published the crystal structures of AdipoRs, where AdipoR-12C was included at the end of the 7th transmembrane domain (TMD7) (Figure 1(A–D)). The UniProt protein database assigns the four last residues (QEFR) of AdipoR-12C. This may be involved in the recognition of adiponectin by AdipoR1.

As revealed in silico by BLAST, the three other extracellular loops of AdipoRs present either a lower degree of homology or a sequence length shorter than 12 amino acids. Regarding the homology, we preferred to simultaneously target AdipoR1 and AdipoR2 with the goal to concomitantly modulate both glucose (mainly regulated by AdipoR1) and lipid (mainly regulated by AdipoR2) metabolism, simulating physiological adiponectin action. Concerning the length of the targeted protein fragment, we observed in previous studies [17] that fragments shorter than 12 amino acid residues lead to selection of 7-mer peptide candidates exposed in duplicate (i.e. 14-mer peptides) on the phage capsid and are probably meant to equilibrate the molecular interaction between target and peptide ligands.

With these concepts and goals in mind, AdipoR-12C (HFYGVSNLQEFR) was screened by phage display using a linear 12-mer random peptide library. During three rounds of panning, the affinity for AdipoR-12C of the phage pools increased from 1.79 to 2.53 times over that for BSA (Figure 1(E)), demonstrating increased specificity. The specific binding to AdipoR1 was even better, the ratio over BSA increasing from 0.95 to 7.08 (2nd round) and 6.58 (3rd round) (Figure 1(F)). Fifty clones from the 3rd round of panning were assessed for binding to AdipoR-12C (Figure 1(G)). Twenty clones had an AdipoR-12C/BSA ratio above the mean (>1.6) and were selected for supplemental characterisation.

Amino acid sequence and in vitro characterisation of the selected peptide clones

The 20 lead phage clones expressed 12 different peptides (Table 1). The peptides were generally expressed by a single clone, except for peptides 3, 9, and 10 that were associated to 3 or 4 clones. Several amino acids (G, A, P, K, R, H, S, T, W) were more frequent (Supplementary Figure S1(A)); some of them form consensus motifs (i.e. SWR, GS, RTS) repeated in different clones (Table 1). These amino acids are either basic (K, R, H), uncharged polar (S, T) or hydrophobic (G, A, P, W), which is quite similar to the amino acid composition of AdipoR-12C (16.7% basic, 41.7% hydrophobic, 33.3% uncharged polar) (Table 2), meaning that their interaction could occur via hydrogen bonds, hydrophobic attraction and saline bridges. Hydrophobic amino acids could also favour proximity to the cell membrane.

The affinity of the peptide clones for AdipoR1 was evaluated and compared to that for BSA, the latter being negligible. Based on Kd values, three clones were selected for additional characterisation, namely 5, 8, and 43 (Supplementary Figure S1(B)). They were encoded as P16 (ADWYHWRSHSSS), P17 (IPNYSMQSREYR), and P18 (YDVPNKSWRTSW). The three-dimensional structure and spatial conformation of these peptides are compared to AdipoR1-12C and AdipoR2-12C in Figure 2.

The exposure to the solvent of hydrophobic residues like Trp, Tyr, Phe at peptide extremities is remarkable both in the three hits P16, P17 and P18 and in AdipoR-12C. This may be advantageous for the interaction of the peptides with AdipoR in close proximity to the cell membrane. The proportion of residues with different chemical functions is almost identical in P17, P18 and AdipoR1-12C and similar to AdipoR2-12C (Table 2). All of them are rather hydrophilic, with a slightly higher tendency to hydrophobicity for AdipoR1, as suggested by their LogP and LogD values. At the same time, the aliphatic index and proportion of nonpolar residues suggest that P17 and P18 present a more hydrophobic character than P16, being closer to AdipoR-12C and predicting their ability to interact with both the target and cell membrane. Among the analysed peptides, P17 has the best theoretical half-life, namely 20h, which suggests it is a promising pharmacological candidate for in vivo applications.

The alignment of the three candidate peptides with relevant proteins was analysed using BLAST on the NCBI proteomics server, showing interesting homologies with proteins involved in signal transduction of various growth factors and hormones regulating the metabolism, cell proliferation and differentiation, and DNA repair (Tables 3–5).
**In vitro characterisation of hit peptides**

**Validation of peptide binding to AdipoR1 and AdipoR2 in muscle, liver, and pancreas**

AdipoR1 is mainly expressed in skeletal muscle, where it activates AMPK signalling, while AdipoR2 is predominantly expressed in liver, where it activates PPARγ [8]. AMPK phosphorylation (activation) increases FFA β-oxidation and glucose uptake via membrane translocation of glucose transporter GLUT4 (in muscle), and inhibits gluconeogenesis (in liver) [21,22]. PPARγ regulates lipid and glucose metabolism and is anti-inflammatory [23]. In pancreatic β cells, the level of AdipoR1 and AdipoR2 expression is comparable...
to that in liver and greater than in muscle [10], but AdipoR1 is predominantly located at the plasma membrane but also in the intracellular compartment. In mouse skeletal muscle, AdipoR1 is mainly expressed on type II fibres, whereas AdipoR2 is expressed by both fibre types, with a more homogeneous distribution in type I fibres. Peptides P17 and P18 bind both AdipoR1 and AdipoR2 in type I and type II muscle fibres, while P16 is more specific to AdipoR1 in type II fibres. P16 and P17 bind with high efficacy to AdipoR2 in liver and human pancreatic islets, where they probably recognise both AdipoR1 and AdipoR2.

### Cellular activity of peptides

**Modulation of AMPK activity:** When adiponectin binds to the extracellular region of AdipoRs, their intracellular region interacts with APPL1 adaptor protein, which activates liver kinase B1 (LKB1) and Ca2+/calmodulin-dependent protein kinase 2 (CaMKK2). AMPK is activated by AMP binding to its γ subunit, which induces a conformational modification of the α subunit harbouning the AMPK catalytic domain. This exposes its Thr172 residue that is phosphorylated by LKB1, resulting in full AMPK activation. The same residue is also phosphorylated by CaMKK2, but only when intracellular Ca2+ concentration is elevated following the APPL1-induced opening of Ca2+ channels during AdipoR1 activation by adiponectin. The two pathways of AMPK activation can operate simultaneously when both AMP and Ca2+ concentrations are increased intracellularly. AMPK promotes fatty acid oxidation and glucose uptake by peripheral tissues [26-28].

The ability of the three candidate peptides to bind AdipoRs and activate AMPK phosphorylation (AMPKα-pT172) was evaluated in HepaRG and differentiated C2C12 cells. P16 had no or weak effects on AMPK activation in HepaRG cells supplemented with glucose or FFAC (Figure 4(A-C)), and in C2C12 cells induced with FFAC, P16 even inhibited AMPK phosphorylation (Figure 4(H)).

Peptide P17 significantly increased AMPKα-pT172 in almost all experimental conditions and particularly in HepaRG cells challenged with high glucose and FFAC (Figure 4(C)), where a 2-3-fold increase in AMPK phosphorylation was observed. C2C12 cells responded to P17 in basal or high glucose medium (Figure 4(E,F)), but not in that supplemented with FFAC (Figure 4(G,H)). These results suggest on the one hand that P17 stimulates both AdipoR1 and AdipoR2, and on the other that glucose and FFAC provide an additional stimulus for AMPK phosphorylation in HepaRG cells. In C2C12 cells, only glucose promotes AMPK phosphorylation.
Peptide P18 induced AMPK activation in HepaRG cells in culture media containing glucose and/or FFAC (Figure 4(A–D)), demonstrating its activity on AdipoR2 expressing cells. In C2C12 cells, P18 had minor (Figure 4(G)) or no effects.

We used AgoAdipoR as a positive control to assess the efficacy of our hit peptides. AgoAdipoR increased AMPKα–pT172 in HepaRG cells, when culture medium was supplemented with FFAC alone or in combination with high glucose (Figure 4(C,D)) to levels that were comparable to P17 and P18. In C2C12 cells, AgoAdipoR activated AMPK only when culture medium was supplemented with high glucose (Figure 4(E–G)).

Taken together, these results demonstrate that P17 is the most potent activator of AMPK phosphorylation, likely by binding to both AdipoR1 and AdipoR2. FFAC promotes AMPK activation in

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Figure 2. Three-dimensional structure (A) and spatial conformation (B) of peptides P16, P17 and P18, compared to AdipoR1-12C and AdipoR2-12C. The three-dimensional structures of peptides were drawn with ACD/ChemSketch 2.0 software. The three-dimensional structure of AdipoR1-12C and AdipoR2-12C and the spatial conformations of all molecules were obtained with MarvinSketch 5.11.5 software (2013, http://www.chemaxon.com).
HepaRG cells, whereas glucose promotes it in C2C12 cells. Our results corroborate other studies that stipulate an allosteric modulation of AMPK by FFA, which renders AMPK susceptible to phosphorylation by LKB1 [29]. Palmitic acid stimulates glucose uptake through activation of PI3K/AMPK/ERK1-2 pathways, leading to GLUT4 translocation to the cell membrane [30].

**Modulation of succinate dehydrogenase and of glucokinase in HepaRG and C2C12 cells:** Activated AMPK regulates lipid metabolism by different mechanisms, such as activation of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1α, that stimulates mitochondrial biogenesis), carnitine palmitoyltransferase-1 (CPT-1, that enables FFA β-oxidation) and PPARγ (that expresses genes involved in FFA β-oxidation), and inhibition of 5-hydroxymethylglutarate-coenzyme A reductase (HMGCoA reductase, involved in cholesterol synthesis) [23,31]. Glucose metabolism is also regulated by AMPK at different levels, such as activation of GLUT via membrane translocation of GLUT4 and GLUT1 [21], inhibition of glucose efflux via GLUT2 [32], activation of 6-phosphofructo-2-kinase (PFK2) to stimulate glycolysis [33], and inhibition of glucogenes via downregulation of specific genes [28].

As a next step of peptide validation, we characterised their ability to modulate two metabolic enzymes, namely GK and SDHA. Being the most potent inducer of AMPK phosphorylation, peptide P17 was selected as lead compound from the three candidate peptides. GK and SDHA were simultaneously detected by immunofluorescence (Supplementary Figure S4), and the relative ratio of fluorescent labelling (RRFL) was semi-quantitatively evaluated using ImageJ (Figure 5).

HepaRG and differentiated C2C12 cells cultured in media supplemented with glucose and/or FFAC and induced with peptide P17 presented significantly enhanced labelling of GK and SDHA (compared to control samples), suggesting these enzymes are induced by P17 (Figure 5). The only condition where GK was not modified was in C2C12 cells cultured at high glucose, which could possibly downregulate GK in these cells. FFAC induced a striking activation of GK in both HepaRG and C2C12 cells stimulated with P17, probably because of the permissive effect of FFA on glucose uptake. It has been shown that palmitate stimulates glucose uptake via the activation of PI3K/AMPK/Akt and PI3K/ERK1-2 pathways [30]. In differentiated C2C12 cells, P17 has also induced a marked activation of SDHA independently of FFAC.

These results corroborate the findings on AMPK activation. Peptide P17 induces GK and SDHA expression in parallel to inducing AMPK phosphorylation (Figure 4) in HepaRG and C2C12 cells, likely due to AdipoR1 and AdipoR2 binding.

**Colocalisation of AdipoR1 with lysosomes and caveolae in C2C12 cells:** Under basal conditions, AdipoR1 and AdipoR2 can self-associate to form homomers or heteromers at the plasma membrane. In response to full-length adiponectin (FLAdipoQ), AdipoRs redistribute to early endosomes within the first 5 min after stimulation and disappear 30 min later. The globular fragment of adiponectin (GAdipoQ) induces AdipoRs endocytosis after 30 min of stimulation. After endocytosis, AdipoRs can return to the membrane or be degraded within lysosomes to down-regulate adiponectin signalling [9]. It has also been shown that AdipoR1 and caveolin-1 associate at the plasma membrane of endothelial cells, this association being critical for adiponectin transmembrane signalling. Under high glucose/high lipids concentration, AdipoR1 interaction with caveolin-1 is downregulated primarily because of the reduced caveolin-1 expression [34].

We hypothesised that peptide P17 modulates AdipoR1 endocytosis via lysosomes (detected with anti-LAMP-1 antibody) or caveolae (detected with anti-caveolin-1 antibody) in differentiated C2C12 cells incubated for 60 min in culture medium supplemented with glucose or glucose and FFAC. AdipoR1, LAMP-1 and caveolin-1 were observed by immunofluorescence, and the level of immunostaining was semi-quantitatively measured with ImageJ. The colocalisation of AdipoR1 with LAMP-1 or caveolin-1 was analysed using the MCC M1 coefficient.

Glucose supplement decreased AdipoR1 expression (p < 0.1 versus basal medium) in control cells. Caveolin-1 immunodetection was increased (p < 0.1 versus basal medium) by glucose, but not that of LAMP-1 (Figure 6 and Supplementary Figure S5), to assist the glucose uptake as described in literature [35]. M1 coefficient reveals an excellent colocalisation of AdipoR1 with caveolin-1 (M1 = 0.945, p < 0.01 versus basal medium) and LAMP-1 (M1 = 0.911, p < 0.01 versus basal medium), suggesting that caveolin-1 could assist the signalling cascade of this receptor, while lysosomes could contribute to its downregulation.

In the same culture conditions, P17 induced the increase of AdipoR1 expression (p < 0.1 versus control; p < 0.05 versus basal medium) (Figure 6 and Supplementary Figure S5), which was associated with increased lysosome (p < 0.05 versus control) and decreased caveolae immunodetection (p < 0.01 versus control). However, both lysosomes and caveolae remained at the level of control cells incubated in basal medium. The colocalisation with LAMP-1 was lower than in control cells: challenged with glucose (M1 = 0.449, p < 0.01), while that with caveolin-1 was similar (M1 = 0.893, implying that AdipoR1 downregulation via lysosome pathway could be restrained by P17.

Glucose and FFAC in culture medium (Figure 6 and Supplementary Figure S6) induced a prominent and significant increase of AdipoR1 expression (p < 0.01 versus control; p < 0.05 versus basal medium), which was associated with an increased content (p < 0.05 versus basal medium) of both lysosomes and caveolae. The relatively good colocalisation of AdipoR1 with LAMP-1 (M1 = 0.612) and better with caveolin-1 (M1 = 0.870) suggests that AdipoR1 signalling cascade could be regulated by the two endocytic pathways to assist both nutrient metabolism and receptor downregulation. In cells stimulated with P17, lysosome content was decreased (p < 0.01 versus control cells), whereas caveolae were comparable to both controls. AdipoR1 colocalisation with LAMP-1 was relatively weak (M1 = 0.563), while that with caveolin-1 was very good (M1 = 0.774, p < 0.05 versus control), suggesting that P17 diminishes the lysosome degradation of AdipoR1. In the same culture conditions, P17 induced a significant activation of AMPK (Figure 4(G)) and of GK and SDHA expression (Figure 5(B)).
mice. No significant effects of P17 were seen on body weight of SCD-fed db/db mice compared to PBS-treated SCD-fed mice. 4-day treatment (Supplementary Figure S8). P17 stabilised the body weight of the 5 groups of mice was followed over the 5 weeks of studies in db/db mice, which developed findings above, we selected peptide P17 for in vivo studies in db/db mice, which develop weight loss without inhibition of food intake, as a result of increased lipid oxidation and thermogenesis [37]; this was associated with reduced serum glucose and lipid levels. Accordingly, P17 significantly improved glycaemia in the SCD- and HFD-fed mice after 66–90 h of treatment (Figure 7(A)).

P17 treatment markedly improved plasma triglyceride levels (Figure 7(A)), reducing them by half in HFD- and SCD-fed db/db mice, to levels of healthy NMRI mice. This may be due to increased lipid oxidation through AdipoR binding, and activation of AMPK and PPARγ pathways. Adiponectin also reduces plasma triglycerides by increasing expression and activity of lipoprotein lipase (LPL) in skeletal muscle, as well as the expression of VLDL receptor (VLDLr), thereby enhancing VLDL-triglyceride catabolism [38].

P17 administration induced a slight (non-significant) increase in plasma adiponectin levels (Figure 7(B)), probably as a consequence of body weight stabilisation and lipid oxidation [7]. Adiponectin levels were significantly higher in P17-treated HFD-fed db/db mice compared to healthy NMRI mice. Plasma insulin was significantly higher in db/db mice compared to NMRI mice (Figure 7(B)). Its concentration significantly increased with P17 treatment in SCD-fed db/db mice, in keeping with the improvements in glycaemia in these mice.

Taken together, P17 stabilises body weight, improves glycaemia, strikingly reduces plasma triglycerides, and increases insulin levels, the latter in chow-fed mice.

Effects on body weight and biomarkers
The body weight of the 5 groups of mice was followed over the 4-day treatment (Supplementary Figure S8). P17 stabilised the weight of SCD-fed db/db mice compared to PBS-treated SCD-fed mice. No significant effects of P17 were seen on body weight of HFD-fed db/db mice (Supplementary Figure S8). Our results corroborate the effects of intracerebroventricular or systemic adiponectin administration in Lepob/db mice, which exhibited weight loss without inhibition of food intake, as a result of increased lipid oxidation and thermogenesis [37]; this was associated with reduced serum glucose and lipid levels. Accordingly, P17 significantly improved glycaemia in the SCD- and HFD-fed mice after 66–90 h of treatment (Figure 7(A)).

To conclude, these results reveal on the one hand that P17 limits the lysosomal degradation of AdipoR1, and thus its downregulation, and on the other hand that it improves the AdipoR1/caveolin-1 interaction to assist the signalosome formation. This last event agrees with the other cellular events upregulated by P17, namely the AMPK phosphorylation and GK/SDHA expression.

Cytotoxicity of P17 in HepaRG cells: The eventual cytotoxic effects of P17 were evaluated by an MTT assay on HepaRG cells incubated with 40 μM and 400 μM of the peptide for 2 h or 24 h (Supplementary Figure S7). No cytotoxic effects were observed, clearing the way for the in vivo evaluation of this pharmacological compound.

In vivo evaluation of peptide P17
Adiponectin protects from metabolic syndrome, T2D and cardiovascular disease by regulating food intake and body weight, lipid and glucose metabolism, and improving insulin sensitivity. It has antiatherogenic activity and is inversely correlated with cardiovascular risk factors such as blood pressure, low-density lipoproteins (LDL) and triglycerides. Adiponectin expression and secretion are decreased in subjects with obesity, hypertension, and T2D. Its expression and secretion are regulated by TNFα, probably via increased IL-6 production [36].

Based on the promising in vitro findings above, we selected peptide P17 for in vivo studies in db/db mice, which develop obesity, insulin resistance and hyperglycaemia by 4–5 weeks of age. Mice were fed HFD (60 kcal %) or SCD (10 kcal %). Healthy SCD-fed NMRI mice were used as control.

Effects on adiponectin signalling pathway
FLAdipoQ and GAdipoQ interact with AdipoR1/R2 and stimulate AMPK and PPARγ activation. PPARγ is a nuclear receptor that is indirectly activated by AMPK via the activation of p38 mitogen-activated protein kinase (p38 MAPK), which in turn phosphorylates

### Table 4. Sequence alignment of peptide P17 with relevant human protein sequences.

<table>
<thead>
<tr>
<th>Protein</th>
<th>SwissProt ID</th>
<th>Peptide alignment</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syntaxin-4 Q12846</td>
<td></td>
<td>143MQS-EYR149</td>
<td>Involved in the docking of intracellular vesicles to the plasma membrane, including that of intracellular GLUT4-containing vesicles in adipocytes</td>
</tr>
<tr>
<td>Insulin-like growth factor-binding protein 3 P17936</td>
<td></td>
<td>284YSMQS290</td>
<td>Role in the regulation of IGFs effects on cell proliferation</td>
</tr>
<tr>
<td>Cytoplasmic tyrosine-protein kinase BMX P51813</td>
<td></td>
<td>228PN-MQ---RE238</td>
<td>Involved in signal transduction of growth factor receptors, cytokine receptors or G-protein coupled receptors and in cell differentiation induced by IL-6, against extracellular stress</td>
</tr>
<tr>
<td>Coenzyme Q-binding protein COQ10 homolog A, mitochondrial Q96MF6</td>
<td></td>
<td>95YSMQ------S--REYR111</td>
<td>Involved in the function of coenzyme Q in the mitochondrial respiratory chain</td>
</tr>
<tr>
<td>Torsin-1A O14656</td>
<td></td>
<td>285MQVR290</td>
<td>Role in the control of protein folding</td>
</tr>
</tbody>
</table>

Alignment was done with BLAST on the NCBI proteomics server using the UniProtKB/Swiss-Prot database.

### Table 5. Sequence alignment of peptide P18 with relevant human protein sequences.

<table>
<thead>
<tr>
<th>Protein</th>
<th>SwissProt ID</th>
<th>Peptide alignment</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine/threonine-protein kinase tousled-like-1 Q9UK8</td>
<td></td>
<td>496NKSWR94</td>
<td>Role in cell cycle, the repair of DNA double-stranded breaks, intracellular trafficking of proteins and signal transduction</td>
</tr>
<tr>
<td>Lanosterol 14-alpha demethylase Q16850</td>
<td></td>
<td>145YDPN149</td>
<td>Role in steroid biosynthesis</td>
</tr>
<tr>
<td>Glucocorticoid receptor P04150</td>
<td></td>
<td>540YD-VP+--WR558</td>
<td>Role in cell response to glucocorticoids, transcription factor, cell proliferation and differentiation, inflammatory response, may play a negative role in adipogenesis</td>
</tr>
<tr>
<td>Serine/threonine-protein phosphatase CPPED1 Q98BF8</td>
<td></td>
<td>96P-KWR102</td>
<td>Role in AKT protein dephosphorylation, may play an inhibitory role in glucose uptake by adipocytes</td>
</tr>
<tr>
<td>Glycogen debranching enzyme P35573</td>
<td></td>
<td>92YDP+PN---W28</td>
<td>Involved in glycogen degradation</td>
</tr>
<tr>
<td>Acyl-coenzyme A thiosterase THEM4 QST1C6</td>
<td></td>
<td>48VPN-SW53</td>
<td>Role in fatty acid metabolism, involved in insulin receptor signalling pathway</td>
</tr>
</tbody>
</table>

Alignment was done with BLAST on the NCBI proteomics server using the UniProtKB/Swiss-Prot database.
several serine residues (S6, S12, and S21) of PPARα. Tissues that obtain most of their energy from FFA oxidation, such as liver, heart, kidney and skeletal muscle, have high levels of PPARα expression [39].

AdipoR1/R2: db/db mice showed significantly higher expression of AdipoR1 in muscle (Figures 8(A) and 9(A)) and AdipoR2 in liver (Figures 8(B) and 9(B)) compared to NMRI mice (Figures 8(F) and 9(A,B)). This high AdipoR1/R2 expression was independent of the

Figure 3. Colocalisation of peptides P16, P17, and P18 with mouse AdipoR1 in muscle (A) and AdipoR2 in liver (B) by immunofluorescence. AdipoR1 and AdipoR2 are detected with fluorescein (channel 2), peptides with Texas Red (channel 1), and nuclei with DAPI. The Manders' colocalisation coefficients M1 (overlap of channel 1 over channel 2) and M2 (overlap of channel 2 over channel 1) were evaluated using the JACoP plugin of ImageJ software.
Figure 4. The effect of peptides and AdipoR agonist (AgoAdipoR) on AMPK T172 phosphorylation (AMPKα-pT172) in HepaRG (A, B, C, D) and C2C12 cells (E, F, G, H). The cells were incubated for 15, 60, and 135 min in culture medium containing glucose supplement (A, C, E, G) or basal glucose concentration (B, D, F, H). Medium was supplemented (C, D, G, H) or not (A, B, E, F) with a mixture of free fatty acids and cholesterol (FFAC). Results are expressed as means ± SD. Each test group is compared to control, incubated in the same culture medium without peptides or AgoAdipoR. NS stands for non-significant. The peptides that present a significant effect are framed with the same colour as their graphical representation.
diet (i.e. HFD or SCD), but it was relatively weaker and more variable in liver of SCD-fed mice. The elevated AdipoR1/R2 expression in the skeletal muscle and liver of db/db mice could be related to their known insulin resistance and non-alcoholic steatohepatitis (NASH), as in T2D and obese patients [40]. In these subjects, AdipoR1 but not AdipoR2 promoter activity seems to be enhanced by insulin via the PI3K/Foxo1 pathway [40].

P17 treatment significantly decreased AdipoR1/R2 expression in muscle and liver. AdipoR1 was homogenously distributed in skeletal muscle fibres in all experimental groups, including NMRI mice. AdipoR2 was homogenously distributed in hepatocyte cytoplasm and membrane of HFD-fed db/db mice, while in SCD-fed mice it presented a predominant plasma membrane localisation. P17 treatment induced a more restrictive localisation of AdipoR2 at the plasma membrane, similar to that of NMRI mice. The cytoplasmic distribution of AdipoR1/R2 could be related to their recycling and/or degradation in lysosomes after endocytosis, which is one of the mechanisms involved in the regulation of adiponectin signalling [9]. The membrane localisation of AdipoR2 in the liver of db/db mice treated with P17 could be associated with caveolin binding and signalosome formation, a phenomenon that could sustain the decrease in plasma triglycerides (Figure 7(A)) as a consequence of FFA oxidation in liver [34].

AMPK-pT172: The cellular localisation of AMPK-pT172 in skeletal muscle and liver presented a similar distribution as that of AdipoR1/R2, i.e. predominantly cytoplasmic in skeletal muscle and concentrated at the cell membrane in liver, including in NMRI mice (Figure 8(C,D,F)). The membrane recruitment of AMPK could be related to its activation by LKB1, whereas its cytoplasmic location would be advantageous for activation via the CaMKK2 pathway [26]. After activation, AMPK phosphorylates several downstream targets, the global effect being to inhibit the ATP consuming pathways (i.e. protein and fatty acid synthesis), whereas the ATP producing pathways (i.e. glycolysis and FFA

Figure 5. Semiquantitative evaluation of SDHA and GK stained by immunofluorescence in HepaRG (A) and differentiated C2C12 (B) cells induced with peptide P17 and incubated for 60 min in culture medium enriched with glucose (Glc, 25 mM for HepaRG; 39 mM for C2C12) and/or free fatty acids and cholesterol (FFAC). The relative ratio of fluorescent labelling (RRFL, normalised to nuclei number and background) was evaluated with ImageJ and represented graphically. The results are expressed as means ± SD.
oxidation) are upregulated [27]. The predominant cytoplasmic localisation of AMPK-pT172 in the liver of HFD-fed db/db mice was similar to that of AdipoR2 in the same tissue and could be related to downregulation mechanisms.

In our study, AMPK was significantly activated in the skeletal muscle and liver of db/db mice independently of diet, although it was higher in SCD-fed mice (Figure 9(C,D)). Concomitantly, this activation was more variable in the liver of these mice. The lower level of AMPK activation in HFD-fed mice could be associated to insulin resistance, as explained above for AdipoR1/R2. Moreover, adiponectin resistance could also contribute to this effect. Obese subjects present a reduced AMPK activation that is not related to reduced expression of AdipoR, which tends to be higher [41].

P17 significantly decreased AMPK activation in skeletal muscle and liver of HFD-fed db/db mice. AMPK-pT172 in liver was restricted to the cell membrane in P17-treated db/db mice, which could be related to its activation by LKB1.

PPARα-pS12: PPARα is a transcription factor that regulates expression of genes involved in FFA oxidation, ketogenesis, lipid transport and gluconeogenesis. Its dysregulation has been involved in the aetiology of diabetes, obesity, hyperlipidaemia, atherosclerosis, cancer and inflammation [42]. PPARα is dynamically shuttled between nucleus and cytoplasm, although it is present constitutively and predominantly in the nucleus. PPARα recruitment in the cytoplasm plays a role in its turnover by ubiquitination and proteolysis [42].

Figure 6. Semiquantitative evaluation of AdipoR1 colocalised with lysosomes (LAMP-1) and caveolae (Caveolin-1) stained by immunofluorescence in differentiated C2C12 cells induced with peptide P17 and incubated for 60 min in culture medium enriched with glucose (Glc, 25 mM for HepaRG; 39 mM for C2C12) and/or free fatty acids and cholesterol (FFAC). Plain control cells were incubated in basal culture medium. The relative ratio of fluorescent labelling (RRFL, normalised to nuclei number and background) was evaluated with ImageJ and represented graphically (A). The Manders’ colocalisation coefficients M1 (overlap of channel 1 over channel 2) were evaluated using the JACoP plugin of ImageJ software (B). The results are expressed as means ± SD.
db/db mice had higher PPARα phosphorylation (PPARα-pS12), especially those fed SCD (Figures 8(E,F) and 9(E)). PPARα-pS12 was distributed both in hepatocyte nuclei and cytoplasm, which could correspond to a mechanism of PPARα downregulation by proteolysis.

P17 treatment significantly decreased PPARα activation (and probably expression) in HFD-fed db/db mice to a level identical to that of healthy NMRI mice (Figure 9(E)). In SCD-fed db/db mice, only a trend for decreased PPARα-pS12 was seen. P17 treatment promoted localisation of PPARα-pS12 to the hepatocyte nuclei, which was comparable to its subcellular localisation in NMRI mouse liver. This nuclear localisation of PPARα-pS12 suggests that its transcriptional activity in lipid metabolism was restored, in keeping with the normalisation of plasma triglycerides (Figure 7(B)).

Figure 7. The effect of peptide P17 on glycaemia and triglyceridemia (A) and on plasma adiponectin and insulin (B) of db/db mice fed on an HFD (60 kcal %) or on a SCD (10 kcal %). The results of glycaemia are shown in box-and-whisker plots. Plasma triglycerides, adiponectin and insulin are expressed as means ± SD.
Figure 8. The effect of peptide P17 on the expression of AdipoR1 in skeletal muscle (A) and of AdipoR2 in liver (B) of db/db mice fed on a Western high-fat diet (HFD, 60 kcal %) or on a standard chow diet (SCD, 10 kcal %). Phosphorylated AMPK (AMPK-p) in skeletal muscle (C) and liver (D) and phosphorylated PPARα (PPARα-p) in liver (E) were also analysed on tissue samples of the same mice. The results obtained for healthy NMRI mice are shown in F. The different biomarkers are observed by immunofluorescence with fluorescein (AdipoR1, AdipoR2, and PPARα) or with Dylight 488 (AMPK-p); nuclei are stained with DAPI. All microphotographs were acquired with identical acquisition parameters to allow the quantification of fluorescent labelling in different experimental groups as reported in Figure 9.
The effects on cell survival and fatty liver disease  
Apoptosis is an important feature of T2D and metabolic syndrome in pancreatic β cells and hepatocytes. β cell death in T2D is caused by chronic exposure to high glucose and FFA concentrations [43]. Non-alcoholic fatty liver disease (NAFLD) is a common complication of obesity and T2D [44]. NAFLD can occur in two clinical presentations, non-alcoholic fatty liver (NAFL), characterised by steatosis, and NASH, which is characterised by steatosis, inflammation and hepatocyte apoptosis, although the two states can be intertwined [45]. Adiponectin has anti-apoptotic properties in various tissues, such as heart, liver and pancreas [24,46]. Adiponectin is furthermore involved in the stimulation of insulin gene expression and secretion by pancreatic β cells [24], while in liver it contributes to an enhanced lipid oxidation, reduced lipogenesis and prevention of hepatic steatosis [47].

db/db mouse livers showed increased staining for activated caspase-3 compared to NMRI mice (Figure 10). The staining level was in the range of the positive control, namely Balb/c mice injected with anti-Fas antibody.

P17-treated db/db mice showed significantly lower activated caspase-3 (Figure 10). The inhibition of liver apoptosis by P17 corroborates with its ability to restore AMPK and PPARα activation and cellular localisation in the range of healthy controls (Figures 8 and 9). The reduced NAFLD in P17-treated db/db mice was confirmed by immunohistochemistry (Figure 11). PBS-treated db/db mice showed clear steatosis with hepatocellular ballooning. With P17 treatment, liver morphology recovered the aspect of healthy tissue especially in HFD-fed db/db mice. No inflammation or fibrosis was observed in this animal model.
Docking of P17 binding to AdipoR

To analyse the interaction of P17 with AdipoRs at the molecular level, we performed a docking study using the HPEPDOCK programme [18]. P17 docking was examined either against the complete AdipoR proteins or against a molecule comprising AdipoR-12C and CTD (AdipoR-12C-CTD). Among the top 10 binding models provided by HPEPDOCK programme, we selected those that seemed optimal in the amphipathic cell membrane environment. Figure 12(A) shows P17 docked to AdipoR1, whereas the docking to AdipoR2 is presented in Figure 12(B). The docking to entire AdipoR proteins revealed that P17 spans the three extracellular loops (ECL) of each protein, with its intermediary residues (SMQSR) placed within the large internal cavity surrounded by the ECL, just below CTD; this disposition of P17 is mainly observed for AdipoR2. Adiponectin itself has been proposed to bind AdipoR via the three ECL and the C-terminal turns of TMD7 [16]. When P17 is docked to AdipoR-12C-CTD, it surrounds AdipoR-12C, projecting its hydrophilic C-terminus (EYR-COOH) likely towards the polar heads of membrane phospholipids, while its hydrophilic N-terminus (H2N-IPN) may be projected towards the aliphatic chains of fatty acids in membrane phospholipids. The C-terminal turns of TMD7 and residues downstream to Glu366 of CTD are required for adiponectin stimulation of AMPK phosphorylation [16]. Whatever the mechanism of P17 binding to AdipoR, AMPK phosphorylation and downstream pathways of AdipoR activation seem to be corroborated by the above described studies.

Conclusions

Adipokines are important molecular actors in the pathophysiology of obesity-linked disorders by their ability to regulate inflammatory and metabolic processes. Adiponectin replenishment has an
Figure 11. Masson’s trichrome staining of liver tissue from db/db mice fed on an HFD (60 kcal %) or on a SCD (10 kcal %). Apoptotic livers from Balb/c mice treated with anti-Fas antibody were used as positive controls.

Figure 12. Docking of P17 interaction with AdipoR1 (A) and AdipoR2 (B) predicted with HPEPDOCK programme (http://huanglab.phys.hust.edu.cn/hpepdock/) [18]. The predicted docking models were selected among the top 10 binding models. The first and the second rows show the binding models docked against the entire AdipoR1/R2 proteins; the binding regions in AdipoR1/R2 are zoomed in the second row to better observe the docking model. The model of P17 interaction with AdipoR-12C-CTD is displayed in the third row.
anti-diabetic effect by improving insulin sensitivity and cell survival, including that of $\beta$ cells [8,19], and produces pleiotropic beneficial effects in obesity [7], metabolic syndrome [8], fatty liver disease, and liver fibrosis [47].

Here, we developed an AdipoR1/AdipoR2-targeted peptide that modulates adiponectin signalling pathways and regulates glucose and lipid metabolism (Figure 13). The peptide P17 binds to a 12-mer sequence comprised in the C-terminal region of AdipoR1/AdipoR2, which is homologous for both receptors, in humans and mice. The biochemical properties of amino acid residues in P17 are identical to AdipoR1-12C and similar to AdipoR2-12C; it has the ability to bind AdipoR in the close proximity of cell membrane due to the equilibrated proportion of hydrophilic (58.4%) and hydrophobic (41.7%) residues. The theoretical half-life of 20 h and its homology with proteins involved in insulin sensitivity, cell proliferation, defence against cell stress and control of protein folding are favourable to its pharmacological use.

We confirmed P17 binding to AdipoR1/R2 by immunofluorescent colocalisation in skeletal muscle, liver and pancreatic islets, with no interaction with exocrine pancreas. P17 was a potent activator of AMPK phosphorylation and GK and SDHA expression in HepaRG and C2C12 cells, while reducing lysosomal degradation of AdipoR1 in conjunction with caveolin-1 colocalisation in C2C12 cells. After excluding cytotoxic effects, P17 was studied in vivo in the db/db mouse model. Considering that Mus musculus is a nocturnal species, we treated the mice with P17 during the evening time, just before the beginning of feeding and locomotion. P17 treatment for four consecutive days promoted body weight stabilisation, lowered glycaemia, decreased triglyceridemia, and slightly increased adiponectin secretion. The effect on glycaemia and plasma adiponectin was more evident in HFD-fed db/db mice, perhaps due to the one-week age difference between the two groups (i.e. 6 weeks for HFD vs. 7 weeks for SCD) or by the effect of FFA on AMPK activation and glucose uptake [29,30]. In SCD-fed db/db mice, P17 increased insulin secretion, in keeping with findings that adiponectin may stimulate insulin secretion [48]. Globular adiponectin has been shown to modulate insulin secretion via a pathway independent of AMPK and dependent on fatty acid oxidation [49].

P17 treatment reduced AdipoR1/R2 expression as well as AMPK-pT172 and PPAR$\gamma$-pS12 levels and modified their cellular distribution. Liver steatosis and hepatocyte apoptosis was reduced in db/db mice treated with P17. Finally, the docking of P17 binding to AdipoR and AdipoR-12C reveals an interaction mechanism that is similar to that of adiponectin.

Future studies should evaluate the effects of P17 administration for longer periods of time and at different ages. For longer treatment periods, smaller doses of P17 could be tested. Oral administration of P17 should become feasible by peptide encapsulation in a nanocarrier formulation [50]. Another therapeutic strategy could be conceived by cloning the oligonucleotide coding for P17 within an expression vector that would render cells able to endogenously express the peptide after transfection, in a similar manner as aptamers exploited as intramers [51].

Moreover, our present work paves the way for future explorations to better understand the cellular networks that are regulated by AdipoR and the selected peptide. For instance, other signalling pathways (i.e. IRS/Pi3K/mTOR, MAPK/ERK, TNF-$\alpha$/NF$\kappa$B, TGF-$\beta$/SMAD, etc.) related to AdipoR1/R2 would be interesting to be investigated both in healthy and pathological models (i.e.
diabetes, obesity, NAFLD, cardiovascular diseases, cancer, Alzheimer’s disease). Although caspase-3 activation is a prominent feature of experimental and human NAFLD and is correlated with disease severity, JNK (cJun N-terminal kinases), proapoptotic Bcl-2 proteins (Bax, BH3, and PUMA) are also activated in these pathological conditions and would be interesting to be investigated. In addition, cytokeratin-18 fragments generated by caspase-3 are predicting NASH in patients with suspected NAFLD [52]. Interestingly, adiponectin plays a role in the clearance of apoptotic cells by interacting with calreticulin, a chaperone of the endoplasmic reticulum that is exposed on the surface of apoptotic cells [53].

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Disclosure statement

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