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RESEARCH PAPER

Synthetic (+)-antroquinonol exhibits dual actions against insulin resistance by triggering AMP kinase and inhibiting dipeptidyl peptidase IV activities

C Y Hsu[1,2]*, R S Sulake[3]*, P-K Huang[1], H-Y Shih[1], H-W Sie[1], Y-K Lai[2], C Chen[3] and C F Weng[1]

[1]*Institute of Biotechnology,* [3]*Department of Chemistry, National Dong-Hwa University, Hualien, Taiwan, and* [2]*Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan*

Correspondence
Ching Feng Weng, Institute of Biotechnology, National Dong-Hwa University, Hualien 974, Taiwan. E-mail: cfweng@mail.ndhu.edu.tw or Chinpaio Chen, Department of Chemistry, National Dong-Hwa University, Hualien 974, Taiwan. E-mail: chinpiao@mail.ndhu.edu.tw

*These authors equally contributed to this work.

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BACKGROUND AND PURPOSE

The fungal product (+)-antroquinonol activates AMP kinase (AMPK) activity in cancer cell lines. The present study was conducted to examine whether chemically synthesized (+)-antroquinonol exhibited beneficial metabolic effects in insulin-resistant states by activating AMPK and inhibiting dipeptidyl peptidase IV (DPP IV) activity.

EXPERIMENTAL APPROACH

Effects of (+)-antroquinonol on DPP IV activity were measured with a DPPIV Assay Kit and effects on GLP-1-induced PKA were measured in AR42J cells. Translocation of the glucose transporter 4, GLUT4, induced either by insulin-dependent PI3K/AKT signalling or by insulin-independent AMPK activation, was assayed in differentiated myotubes. Glucose uptake and GLUT4 translocation were assayed in L6 myocytes. Mice with diet-induced obesity were used to assess effects of acute and chronic treatment with (+)-antroquinonol on glycaemic control *in vivo*.

KEY RESULTS

The results showed that of (+)-antroquinonol (100 μM) inhibited the DPP IV activity as effectively as the clinically used inhibitor, sitagliptin. The phosphorylation of AMPK Thr^{172} in differentiated myotubes was significantly increased by (+)-antroquinonol. In cells simultaneously treated with S961 (insulin receptor antagonist), insulin and (+)-antroquinonol, the combination of (+)-antroquinonol plus insulin still increased both GLUT4 translocation and glucose uptake. Further, (+)-antroquinonol and sitagliptin reduced blood glucose, when given acutely or chronically to DIO mice.

CONCLUSIONS AND IMPLICATIONS

Chemically synthesized (+)-antroquinonol exhibits dual effects to ameliorate insulin resistance, by increasing AMPK activity and GLUT4 translocation, along with inhibiting DPP IV activity.

Abbreviations

AMPK, AMP-activated protein kinase; DIO, diet-induced obesity; DPP IV, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide 1; GLUT4, glucose transporter 4; KRH, Krebs-Ringers-HEPES; OGTT, oral glucose tolerance test; T2DM, type 2 diabetes mellitus; TEA, triethanolamine

Tables of Links

TARGETS
Enzymes[a]
AMPK, AMP kinase
DPP-IV, dipeptidyl peptidase 4
Transporters[b]
GLUT4, glucose transporter 4 (SLC2A4)

LIGANDS
GLP-1, glucagon like peptide-1
Insulin
Sitagliptin
Metformin

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b.).

Introduction

The increasing prevalence of diabetes in developing countries, such as China and India, presents a serious public health problem and financial burden for their governments. The recent long-term mega trials have shown that intensive glycaemic control can reduce the exacerbation of insulin resistance, type 2 diabetes mellitus (T2DM) and the risk of cardiovascular disease (Avogaro, 2012). Thus there is an urgent need for new and more effective agents for glycaemic control. Two compounds already widely used for glycaemic control in patients with T2DM are sitagliptin, a highly selective inhibitor of dipeptidyl peptidase-4 (DPP IV) (Goldstein *et al.*, 2007), and metformin, an activator of the AMP-activated protein kinase (AMPK) (Riddle, 1999; Zhou *et al.*, 2001; Fryer *et al.*, 2002; Leverve *et al.*, 2003). As these drugs lower blood glucose concentrations through different mechanisms, clinical trials have assessed the efficacy of initial combination therapy of sitagliptin and metformin in patients with T2DM and have demonstrated greater improvements than the corresponding monotherapies (Goldstein *et al.*, 2007; Williams-Herman *et al.*, 2012).

Antrodia cinnamomea is a parasitic fungus living on the inner cavity of *Cinnamomum kanehirai*, one of Lauraceae species exclusive to Taiwan (Wu and Ryvarden, 1997). The fruiting body of *A. cinnamomea* is expensive in Taiwan because *C. kanehirai* is rarely found and is thus protected by the Taiwan government. In recent years, good-quality basidiomes have sold for around $US15 000 per kg (Wu and Ryvarden, 1997). Therefore, it is becoming difficult to find *A. cinnamomea* in the wild. Extracts of this fungus have been used as a folk medicine to treat many conditions, including diabetes mellitus, cancer, diarrhea, abdominal pain, hypertension, viral infection, stomatitis, liver cirrhosis, hepatoma, influenza and car sickness (Ao *et al.*, 2009; Geethangili and Tzeng, 2011). However, the effects of *A. cinnamomea* are based on local experience (Ao *et al.*, 2009). (+)-Antroquinonol (Figure 1A) is only found in this fungus (Kuo and Lin, 2010) and is a major constituent of the fermentation of *A. cinnamomea* (unpublished results). This isomer (hereafter referred to as antroquinonol) has shown anti-cancer potential in human hepatocellular carcinoma cells (Chiang *et al.*, 2010), pancreatic carcinoma cells (Yu *et al.*, 2012a), non-small cell lung cancer (Kumar *et al.*, 2011), breast carcinoma and prostate carcinoma (Lee *et al.*, 2007). It is also the first non-toxic drug that is a highly effective cancer treatment agent under FDA phase II review (ClinicalTrials.gov Identifier: NCT01134016).

An earlier study found that antroquinonol displayed anticancer potential through the activation of AMPK (Chiang *et al.*, 2010). Because AMPK activation is also an effective strategy to treat diabetes through increasing glucose transporter 4 (GLUT4) translocations (Riddle, 1999; Zhou *et al.*, 2001; Fryer *et al.*, 2002; Leverve *et al.*, 2003), antroquinonol could also exert hypoglycaemic effects that might account for the antidiabetic properties of extracts of *A. cinnamomea*. In order to investigate this possibility, a reliable and plentiful source of antroquinonol was required. Traditionally, it could be extracted and purified from *A. cinnamomea* but such extraction and purification is expensive as the yield is low and the source fungus is scarce (see above). Thus, chemically synthesized antroquinonol was essential for a full investigation, and possible subsequent commercial exploitation, of the antidiabetic potential of this compound.

The present study was conducted to examine whether chemically synthesized (+)-antroquinonol exhibited beneficial metabolic effects in insulin-resistant states and models of diabetes through the activation of AMPK. Also, as we had found that antroquinonol could bind to DPP IV in molecular docking analysis (our unpublished results), we tested this compound as an inhibitor of DPP IV. Our present results have shown that antroquinonol did lower blood glucose in diet induced obese (DIO) mice and, *in vitro*, increased activation of AMPK and inhibited DPP IV activity. Thus, synthetic antroquinonol may exert antidiabetic effects through two separate pathways of glycaemic control.

Methods

Total synthesis of (+)-antroquinonol

The enantioselective synthesis has involved an iridium-catalysed olefin isomerization-Claisen rearrangement (ICR), a lactonization, and a Grubbs metathesis reaction for the establishment of three stereogenic centres. The requisite α,β-unsaturation was achieved using a selenylation/oxidation protocol. The synthesis of (+)-antroquinonol was obtained from Dr Chinpiao Chen (Department of Chemistry, National Dong-Hwa University, Hualien, Taiwan).

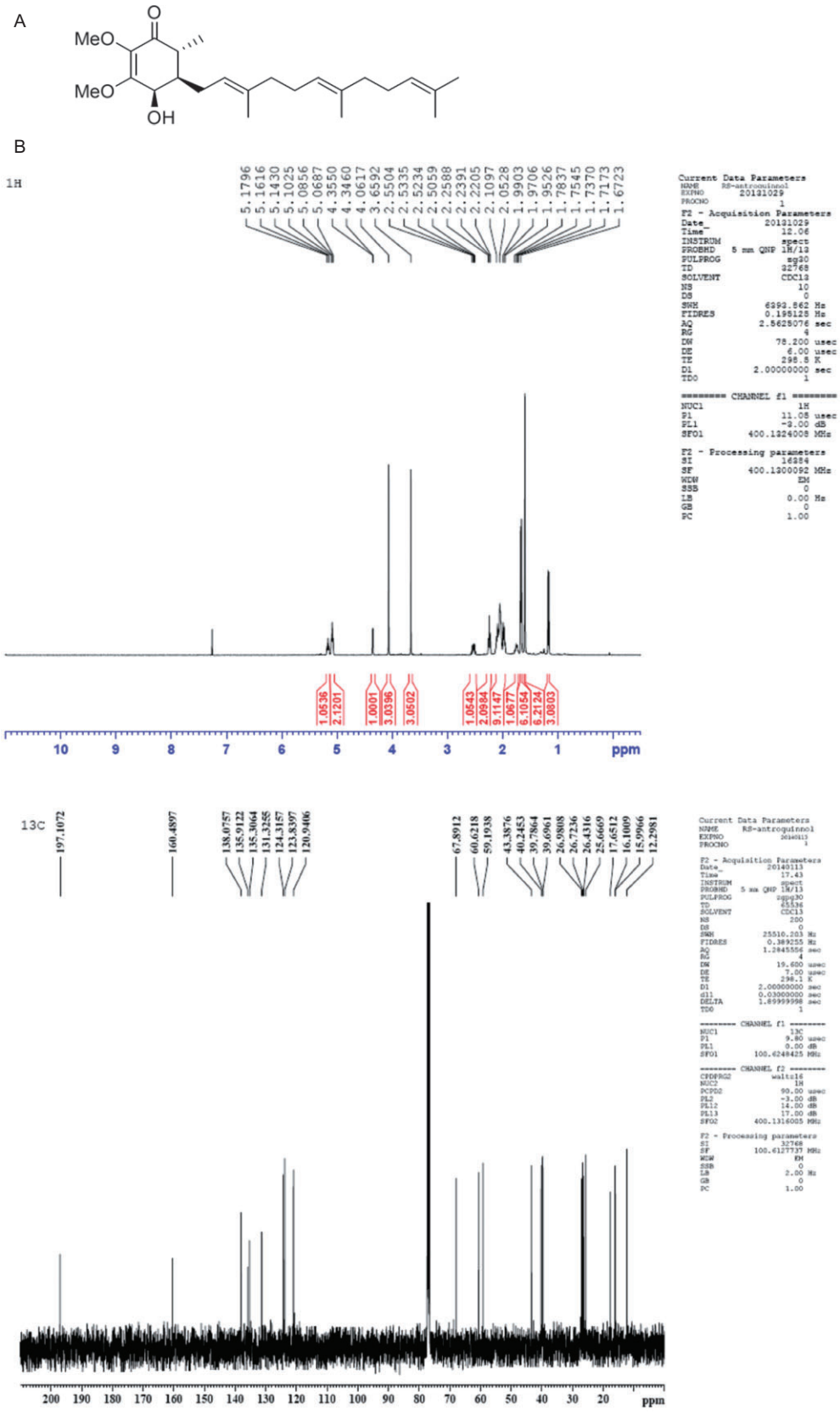

Figure 1

(A) Chemical structure of antroquinonol and (B) the H NMR and C NMR spectra of the final product, (+) antroquinonol.

Cell culture

Mouse muscle myoblast cell (C2C12) culture and maintenance. Mouse muscle myoblast cells (C2C12) were obtained from the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). The cells were grown and maintained in high-glucose DMEM (GIBCO, Carlsbad, CA, USA) containing 10% FBS (GIBCO) and 1% penicillin streptomycin (GIBCO) in an incubator with 5% CO_2 at 37°C. Prior to the experiment, cells were seeded and cultured at a density of 8×10^3 cells per well in 96-well plates or 2.5×10^5 cells per well in 6-well plates. After the myoblasts achieved 80% confluence, the cells were incubated for 4 days in high-glucose DMEM supplemented with 1% FBS and 1% horse serum to induce differentiation into the myotubes.

Mouse muscle myoblast cell (L6) culture and maintenance. Mouse muscle myoblast cells (L6) were a gift from Professor Hitoshi Ashida laboratory (Kobe University, Kobe, Japan). The cells were grown and maintained in α-minimal essential medium (α-MEM, 12000022; GIBCO) containing 10% FBS (GIBCO) and 1% penicillin streptomycin (GIBCO) in the cell culture incubator with 5% CO_2 at 37°C. Prior to the experiments, cells were seeded and cultured at a density of 8×10^3 cells per well in a 96-well plate or 2.5×10^5 cells per well in a 6-well plate. After the myoblasts achieved 80% confluence, the cells were incubated for 5 days in α-MEM supplemented with 2% FBS-induced differentiation to the myotubes.

Rat pancreas tumour cells (AR42J) culture and maintenance. Rats pancreas tumour cells (AR42J) were obtained from the FIRDI. The cells were grown and maintained in DMEM (GIBCO) containing 20% FBS (GIBCO) and 1% penicillin streptomycin (GIBCO), and 2 mM of L-glutamine in the cell culture incubator with 5% CO_2 at 37°C. Prior to the experiments, cells were seeded and cultured for 16–24 h.

Human colorectal adenocarcinoma cell (Caco-2) culture and maintenance. Human colorectal adenocarcinoma cells (Caco-2) were obtained from the FIRDI. The cells were grown and maintained in DMEM (GIBCO) containing 20% FBS (GIBCO) and 1% penicillin/streptomycin (GIBCO) in cell culture incubator with 5% CO_2 at 37°C. Prior to experiment, cells were seeded and cultured for 16–24 h.

Glucose uptake capacity

Measurement of translocation of GLUT4 to the cell surface. C2C12 cells were fully differentiated with DMEM supplemented with 1% FBS and 1% horse serum for 4 days. Cells were washed twice with PBS containing 0.1% BSA and subsequently incubated in PBS with or without insulin, metformin and antroquinonol treatment. Then cells were then placed on ice and immediately fixed with 1% glutaraldehyde in PBS at room temperature for 10 min. After quenching with 0.1 M glycine in PBS for 10 min, the cells were blocked with PBS containing 5% mouse serum for 30 min. To determine the levels of GLUT4 on the cell surface, the cells were then incubated with anti-GLUT4 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) diluted to 1 $\mu g \cdot mL^{-1}$ in PBS containing 3% mouse serum for 1 h. The cells were then treated with a secondary antibody-HRP-conjugated anti-goat IgG (Jackson ImmunoResearch, Suffolk, UK) diluted 1:300 in PBS containing 3% mouse serum for 1 h. Following a rinsing step with PBS, 3,3′,5,5′-tetramethylbenzidine substrate (BioLegend, San Diego, CA, USA) was added and incubated at room temperature for 30 min, and 2N H_2SO_4 added to stop the reaction. HRP activity was determined by measuring the absorbance at 450 nm with a spectrophotometer (EnSpire 2300 Multilabel Reader, Perkin Elmer, Waltham, MA, USA).

Glucose uptake assay. Glucose uptake was assayed as described by Yamamoto *et al.*, (2010), with minor modifications, and the techniques from Professor Hitoshi Ashida (Kobe University). Briefly, the differentiated L6 myotubes seeded in a 96-well microplate were incubated with 100 µL per well of α-MEM with 0.25% BSA in the presence of insulin, S961 (insulin receptor antagonist, a gift from Dr Lauge Schäffer; Novo-Nordisk, Bagsvaerd, Denmark), metformin (Glucophage; Bristol-Myers Squibb, Princeton, NJ, USA) or antroquinonol for 30 min. After incubation, the cells were washed twice with Krebs-Ringer HEPES (KRH) buffer (50 mM HEPES, 137 mM NaCl, 4.8 mM KCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$). Then the L6 myotubes were incubated with KRH buffer containing 1 mM of 2-deoxyglucose (2DG; Sigma-Aldrich, St. Louis, MO, USA) and 60 µL of 0.1% BSA in 5% CO_2 at 37°C for 20 min. After incubation, the cells were washed twice with KRH buffer and then 50 µL of 0.1 N NaOH was added. The microplate was dried by incubation at 85°C for 90 min. The components in the wells were then neutralized by the addition of 50 µL of 0.1 N HCl, and then 50 µL of 50 mM triethanolamine hydrochloride (TEA) buffer (200 mM KCl, 200 mM TEA, pH 8.1) was added. Uptake of 2DG into the cells was measured by an enzymic fluorescence assay. The fluorescence assay buffer was composed of 50 mM TEA buffer, 0.1% BSA, 2.5 mM β-NADP (Wako Pure Chemical, Osaka, Japan), 0.05 U diaphorase (Wako Pure Chemical), 150 U of *Leuconostoc mesenteroides* G6PDH (Sigma-Aldrich) and 0.5 mM resazurin sodium salt (Sigma-Aldrich). About 10 µL of 2DG sample with 100 µL of fluorescence assay buffer was reacted at 37°C for 30 min. At the end of the incubation, fluorescence at 570 nm with excitation at 540 nm was measured by spectrophotometry (EnSpire 2300 Multilabel Reader, Perkin Elmer).

Cell signaling and enzyme activity

Western blot. After treatments, cells were collected and washed twice in cold KRH buffer and then lysed in ice-cold RIPA buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM NaF, 1% NP40, 1 mM sodium orthovanadate, 0.5% sodium deoxycholate, 0.1% SDS, Roche protease inhibitors and phosphatase inhibitor (DE-68305, Mannheim, Germany)] incubation at 4°C for 60 min. After centrifugation at 12 000× *g* for 30 min at 4°C, the protein in the supernatant was quantified by Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins were separated using SDS-PAGE and subsequently transferred to PVDF (Perkin Elmer Life Sciences, Boston, MA, USA) membrane. The blots were blocked with 5% non-fat milk in TBS/T (20 mM Tris-Base, 137 mM NaCl at pH 7.4, and 0.05% Tween-20) at room temperature for 1 h and then incubated with the appropriate primary antibody at 4°C overnight. After washing, the blots were incubated with HRP-conjugated secondary antibody (General Electric, Little Chalfont, Bucks, UK) for 1 h. The signals were monitored using Western

Lightning™ *Plus*-ECL (Perkin Elmer Life Sciences) and the PVDF membrane was exposed to luminescent image analyser (LAS)-3000 (Fujifilm, Minato, Tokyo, Japan). Acquired data were analysed and the differences were compared among the treatments.

Antroquinonol induced phosphorylation of AMPK at Thr^{172}. C2C12 cells were fully differentiated with DMEM supplemented with 1% FBS and 1% horse serum for 4 days. Cells were washed twice with PBS containing 0.1% BSA and subsequently incubated in PBS containing 0.1% BSA with or without insulin, metformin or antroquinonol treatment for 30 min. The treated cells were collected and washed twice in KRH, then lysed in ice-cold RIPA buffer for 60 min. After centrifugation at 12 000× *g* at 4°C for 30 min, the supernatant was kept at –80°C until use. Protein levels including phospho-AMPK (Thr^{172}), AMPKα, phospho-Akt (Thr^{308}) and Akt (Cell Signaling, Boston, MA, USA) were detected and evaluated by Western blot with primary antibody.

Inhibition of DPP IV enzyme activity in the kit assay. DPP IV enzyme activity was measured by DPPIV/CD26 Assay Kit (Enzo, New York, NY, USA); antroquinonol and sitagliptin (Januvia, Merck & Co., Inc., Whitehouse Station, NJ, USA) were tested. This assay is based on the cleavage of p-nitroaniline (pNA) from the chromogenic substrate (H-Gly-Pro-pNA) to increase the absorbance at 405 nm. At the beginning, 50 µL of assay buffer (50 mM glycine, pH 8.7, 1 mM EDTA) was added to a 96-well clear microplate. Then 20 µL of DPP IV enzyme (13 µU·µL^{-1}), 20 µL of tested inhibitors (100 µM of antroquinonol or sitagliptin) and 10 µL of pNA substrate were sequentially added. The final concentration of tested inhibitors, antroquinonol or sitagliptin, was 20 µM. The reaction mixtures were incubated at room temperature for 30 min and the absorbance of samples was read at 405 nm by the ELISA plate reader (Thermo LabSystems, Opsys MR, Thermo Fisher Scientific, Waltham, MA, USA).

Inhibition of DPP IV enzyme activity in cultured cells. Caco-2 cells were treated with antroquinonol (4, 8 and 16 µM) or sitagliptin (10, 50 and 100 µM) for 12 h. Lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 0.04 U·mL^{-1} aprotinin, 0.5% Nonidet P40, pH 8.0) was then added and incubated at 4°C for 60 min. Cells were centrifuged at 13 000× *g* at 4°C for 30 min, and protein in the supernatant was quantified by the Bradford protein assay (Bio-Rad). About 30 µg of sample protein was taken and 70 µL of assay buffer (50 mM glycine, pH 8.7, 1 mM EDTA) was added into a 96-well microplate. Next, 10 µL of pNA substrate (H-Gly-Pro-pNA 5 mM; MW = 328.8) was added and the absorbance was read at 405 nm by the ELISA plate reader (Thermo LabSystems, Opsys MR, Thermo Fisher Scientific).

Antroquinonol inhibits the potentiation by DPP IV of the induction of PKA by glucagon-like peptide-1 (GLP-1). In pancreatic beta cells, inhibition of DPP IV increases binding of incretin peptides to their GPCRs (GLP-1R and GIP-R) (Doupis and Veves, 2008). The downstream pathway is mainly regulated by cAMP as GLP-1 binds to GLP-1R and increases cAMP formation (Drucker *et al.*, 1987), which leads to the activation of PKA (Wang *et al.*, 2001; 2012) affecting the subsequent Ca^{2+}-stimulated insulin secretion (Doyle and Egan, 2007). AR42J cells (pancreatic tumour cell line) were treated with 10^{-9} M GLP-1 (ProSpecBio, East Brunswick, NJ, USA), 10^{-9} M exendin-4 (Ex-4; Byetta, Eli Lilly, Indianapolis, IN, USA), antroquinonol, antroquinonol plus GLP-1 or antroquinonol plus Ex-4 for 48 h. Levels of PKA protein were determined by Western blot.

Glycaemic control in vivo

Animals. All animal care and experimental procedures complied with the 'Guide for the Care and Use of Laboratory Animals' of National Dong-Hwa University and were approved by the National Dong-Hwa University Animal Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 20 animals were used in the experiments described here.

ICR mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and kept in controlled environmental conditions (room temperature. 22 ± 2°C: humidity, 50 ± 10%). A 12 h light (06:00–18:00 h) and 12 h dark cycle was maintained throughout the study. Mice had free access to food and water and were maintained on a standard laboratory diet (carbohydrates 60%; proteins 28%; lipids 12%; vitamins 3%).

Diet-induced obesity (DIO) in ICR mice. Six-week-old ICR male mice were induced with a high-fat diet and 60% fructose water for 10 weeks. The high-fat diet comprised 1 kg of conventional chow plus 150 g of conventional lard (23% total saturated fatty acids and 77% total unsaturated fatty acids) (Chinshan oil; Wei Li Foods Co., Changhua, Taiwan). The experimental mice were allocated to two groups: (i) group A was diet-induced glucose intolerance (n = 30) and group B was fed with a normal diet [control group (Con), n = 5]. After being fed with high-fat diet and 60% fructose water for 10 weeks, ICR male mice were given D-glucose (2 g·kg^{-1}, p.o.) after fasting for 12 h. At approximately 0, 30, 60, 90 and 120 min, blood was sampled by venepuncture from the tail vein. Blood glucose was immediately determined by the glucose oxidase method using a glucose analyser (Accu-Chek; Roche). Concentrations of blood sugar greater than 2 g·L^{-1} at 120 min after the oral glucose were defined as high blood sugar.

Oral glucose tolerance test (OGTT). Mice were used in this test after fasting for 12 h. Mice were treated with D-glucose by oral gavage (2 g·kg^{-1}). At approximately 0, 30, 60, 90 and 120 min, blood was sampled by venepuncture from the tail vein and blood glucose immediately determined by the glucose oxidase method using a glucose analyser (Accu-Chek; Roche). In order to understand the whole blood glucose change, the AUC was calculated from the different timed samples and the data were shown on a bar chart.

Short- and long-term effects of antroquinonol on glucose tolerance of DIO mice. For the short-term effects of antroquinonol, DIO mice were given antroquinonol (25 mg·kg^{-1}) or sitagliptin (20 mg·kg^{-1}), 15 min before oral gavage with D-glucose

(2 g·kg^{-1}) to assess glucose tolerance by the OGTT. For long-term effects of antroquinonol, antroquinonol (25 mg·kg^{-1}) and sitagliptin (10 mg·kg^{-1}) were given to DIO mice, once every other day for 4 weeks. At the end of long-term treatment, the OGTT was performed.

Data analysis. Data are expressed as means ± SEM. Statistical comparisons of the results were made using one-way ANOVA, with Tukey's *post hoc* test.

Results

Total synthesis of (+)-antroquinonol

(+)-Antroquinonol [(4*R*,5*R*,6*R*)-4-hydroxy-2,3-dimethoxy-6-methyl-5-((2*E*,6*E*)-3,7,11-trimethyldodeca-2,6,10-trienyl) cyclohex-2-enone] was prepared through enantioselective total syntheses. R_f (hexane/ethyl acetate 7:3) 0.45; $[\alpha]_D^{25}$ = +42.5 (c = 1.20 in $CHCl_3$); IR (film, cm^{-1}): 3435, 2926, 1659.3, 1622, 1451, 1358, 1240, 1141, 1017, 944, 832, 749; ^{1}H-NMR (400 MHz, $CDCl_3$, p.p.m.): δ 5.16 (m, 1H), 5.08 (m, 2H), 4.34 (d, *J* = 3.24 Hz, 1H), 4.06 (s, 3H), 3.66 (s, 3H), 2.52 (m, 1H), 2.23 (dd, *J* = 7.48, 7.44 Hz, 2H), 1.97–2.09 (m, 9H), 1.75 (m, 1H), 1.67 (s, 3H), 1.66 (s, 3H), 1.60 (s, 6H), 1.17 (d, *J* = 6.92 Hz, 3H); ^{13}C-NMR (100.6 MHz, $CDCl_3$, p.p.m.): δ 197.12, 160.49, 138.03, 135.92, 135.34, 131.31, 124.31, 123.85, 120.99, 67.91, 60.58, 59.19, 43.40, 40.27, 39.81, 39.71, 27.00, 26.74, 26.45, 25.69, 17.67, 16.12, 16.01, 12.31; HRMS-EI (m/z) $[M]^+$ calculated for $C_{26}H_{42}O_5$ 390.2770 found 390.2764. The results are shown in Figure 1B. Our values for the ^{1}H and ^{13}C NMR of synthetic (+)-antroquinonol (Supporting Information, Table S1) compare well with those from earlier studies (Lee *et al*., 2007; Liu *et al*., 2012). Note that the earlier ^{1}H NMR spectrum from Lee *et al*. (2007) contains many impurity peaks and in the patent publication (Liu *et al*., 2012) some peaks are missing.

Effects on glucose uptake

Antroquinonol enhanced GLUT4 translocation in C2C12 cells. One mechanism for controlling glucose uptake is through the recruitment of GLUT4 to the plasma membrane (GLUT4 translocation) where these transporters provide the major route of uptake. We therefore assessed the effects of antroquinonol on GLUT4 translocation , using metformin and insulin as positive controls. As shown in Figure 4A, of the increase in AMPK phosphorylation of AMPK at Thr^{172} was increased by antroquinonol, after 30 min exposure Based on these results, we measured GLUT4 translocation after 30 min treatment of the cells with antroquinonol at different concentrations (50, 100 150 μM) and compared the effects with those after similar treatment with insulin or metformin (Fig 2A) Antroquinonol enhanced GLUT4 translocation concentration-dependently, with the effects of the lowest concentration of antroquinonol (50 μM) being slightly greater than that of 16 mM of metformin (Figure 2A). When the cells were simultaneously treated with antroquinonol and insulin, the effects ere significantly greater than with either agent alone (Figure 2B). These experiments showed that antroquinonol alone was able to increase GLUT4 translocation *in vitro*, independent of the action of insulin.

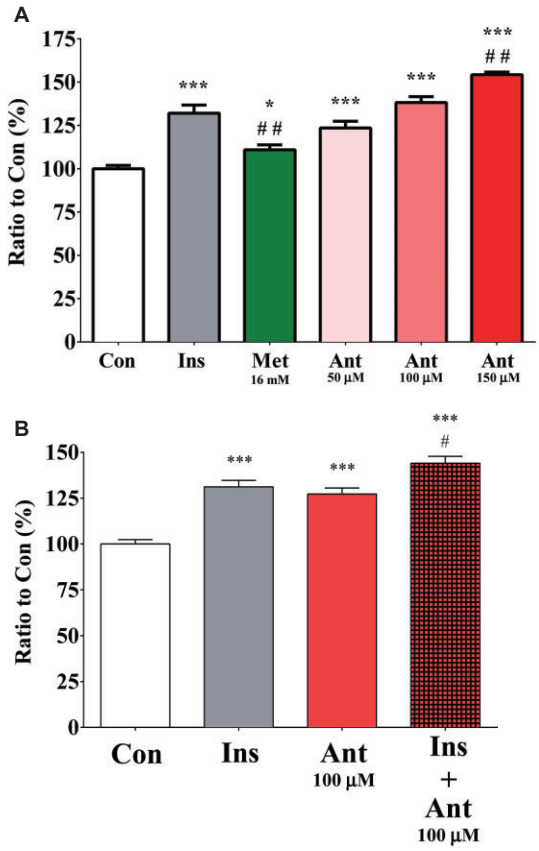

Figure 2

Antroquinonol enhanced the translocation of GLUT4 in C2C12 cells. Differentiated C2C12 cells after administration of (A) 185 μM insulin (Ins), 16 mM metformin (Met), and 50, 100 and 150 μM of antroquinonol (Ant) for 55 min. (B) Insulin, 100 μM antroquinonol, and insulin plus antroquinonol for 40 min (mean ± SEM; *n* = 5 in each group). The data are expressed as mean ± SEM (n = 5 in each group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the control (Con); $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 compared with Insulin only.

Antroquinonol enhanced cell glucose uptake in L6 cells. We then measured glucose uptake directly in L6 cells because in these cells only 1 μM insulin was needed to increase glucose uptake (Figure 3B). To investigate effective and significant In these cells, antroquinonol concentration-dependently (1, 10 and 100 nM) increased glucose uptake. The optimal concentration of antroquinonol was 10 nM which resulted in uptake threefold that of the control group (Figure 3A). Therefore, 10 nM antroquinonol used as a standard concentration in further uptake assays. This Figure also shows that antroquinonol or metformin enhanced glucose uptake by these cells, with or without insulin. When cells were treated simultaneously with S961 and antroquinonol, the uptake of glucose was still increased (Figure 3B).

Antroquinonol induced AMPK Thr^{172} phosphorylation; no response to insulin signalling. Translocation of GLUT4 requires activation of either the insulin-dependent, PI3K/Akt signalling or of the insulin-independent AMPK pathway, in skeletal muscles. Here we tested the effects of antroquinonol on these

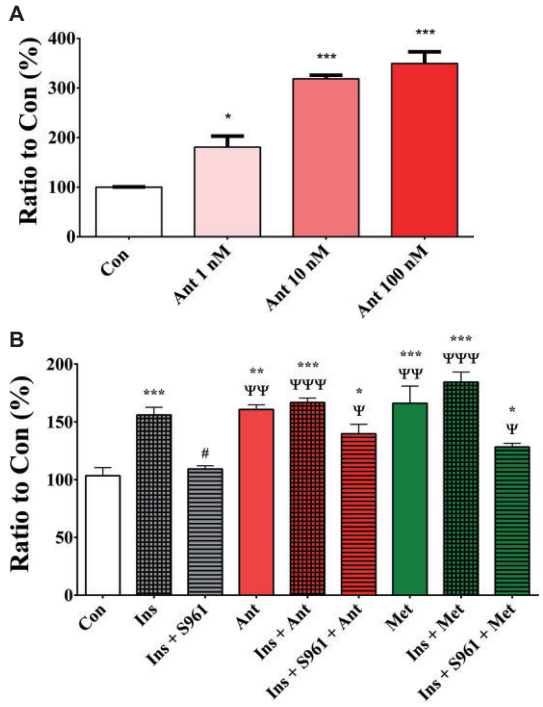

Figure 3

Antroquinonol increased the uptake of glucose in L6 myocytes. Differentiated L6 cells after administration of (A) different concentrations of antroquinonol (1, 10 and 100 nM) and (B) 1 μM insulin, 2 mM metformin (Met), 10 nM antroquinonol (Ant) and 400 nM S961 for 30 min (mean ± SEM, n = 5 in each group). The data are expressed as means ± SEM (n = 5 in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control (Con); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with Ins group; $^{\Psi}P < 0.05$, $^{\Psi\Psi}P < 0.01$, $^{\Psi\Psi\Psi}P < 0.001$, compared with Ins plus S961 group.

two pathways, using metformin to activate the AMPK pathway and insulin to activate the Akt pathway. The time course of AMPK Thr^{172} phosphorylation (Fig 4A) showed increased phosphorylation after 30 min incubation with antroquinonol and insulin, compared with the phosphorylation after insulin alone. In a comparative study (Fig 4B), insulin alone induced no such phosphorylation and 50 μM antroquinonol was more effective that the positive control metformin, at 16 mM.

For the Western blots shown in Figs 4C and D, we used 25 μM of antroquinonol and a 30 min incubation. Here, the levels of phospho-Akt were elevated by treatment with insulin, compared with the control group. However when the cells were treated with metformin or antroquinonol, levels of phospho-Akt were not changed (Figure 4C). Note that at this concentration (25 μM) antroquinonol, but not metformin (16 mM), increased phosphorylation of AMPK at Thr^{172} (Figure 4D) in mouse myoblast C2C12 cells.

Antroquinonol inhibited DPP IV enzyme activity. Our initial experiments using the DPPIV/CD26 Assay Kit, showed that antroquinonol inhibited DPP IV activity and that 20 μM of antroquinonol was as effective as 20 μM of sitagliptin, a known inhibitor of DPP IV (Figure 5A). We then used Caco-2 cells and a range of concentrations of antroquinonol (2, 8 and 16 μM) or sitagliptin (10, 50 and 100 μM) to confirm inhibition of DPP IV activity. As shown in Figure 5B, a 12 h incubation with antroquinonol inhibited cellular DPPP IV activity concentration-dependently, with 20 μM antroquinonol being equivalent to 10 μM sitagliptin.

Antroquinonol inhibited DPP IV potentiated GLP-1 to induce PKA protein level. In pancreatic beta cells, inhibition of DPP IV preserves incretin peptides and potentiates their effects at the corresponding GPCRs (GLP-1R and GIP-R) (Doupis and Veves, 2008), which leads to the activation of PKA (Wang *et al.*, 2001; 2012) and subsequent Ca^{2+} stimulated insulin secretion (Doyle and Egan, 2007). The results of the experiments in AR42J cells showed that, as expected, GLP-1 or exendin-4 increased the levels of PKA protein in these cells, compared with control cells (Figure 6) A comparable increase in PKA was also observed after treatment of the cells with either concentration (5 or 25 μM) of antroquinonol alone.

Glycaemic control by antroquinonol in vivo

Acute effects of antroquinonol on glucose tolerance in DIO mice. The OGTT measures the changes in blood glucose for 2 h after an oral glucose load and provides an indication of the glycaemic control processes in each animal. We calculated the AUC under the time-glucose concentration curve in order to assess the overall blood glucose change. Following the oral glucose load, the AUC of the DIO group was twice that of normal mice, as expected (Figure 7A). However, after pretreatment of the DIO mice with sitagliptin (20 $mg{\cdot}kg^{-1}$) or with antroquinonol (25 $mg{\cdot}kg^{-1}$), the response to the oral glucose load was the same as observed in normal mice. These results showed that a single treatment with either antroquinonol or sitagliptin corrected the dysfunctional glycaemic control processes in DIO mice.

Effects of chronic treatment with antroquinonol on glucose tolerance in DIO mice. DIO mice were treated with antroquinonol (25 $mg{\cdot}kg^{-1}$) or sitagliptin (10 $mg{\cdot}kg^{-1}$) every other day for 4 weeks. At the end of this treatment, mice were given an OGTT and the AUC of the response was calculated. As shown in Figure 7B, the chronic treatment with either antroquinonol or sitagliptin reduced the responses to the OGTT in DIO mice. Both treatments restored the OGTT responses to the levels observed in control mice.

Discussion and conclusions

The quality of most naturally occurring *A. cinnamomea* is highly variable and the fungus and their extracts are easily contaminated by heavy metals. Such contaminations or adulterations with heavy metals such as lead, mercury, cadmium, arsenic or thallium are of concern because there is poor quality control of these extracts which creates a health hazard. Total synthesis of (+)-antroquinonol should provide a safe and reliable source of the compound. Crude extracts of *A. cinnamomea* ferments, in which antroquinonol is a major constituent, have been used as a dietary supplement for liver health in Taiwan and possibly in other Asian countries (Ao

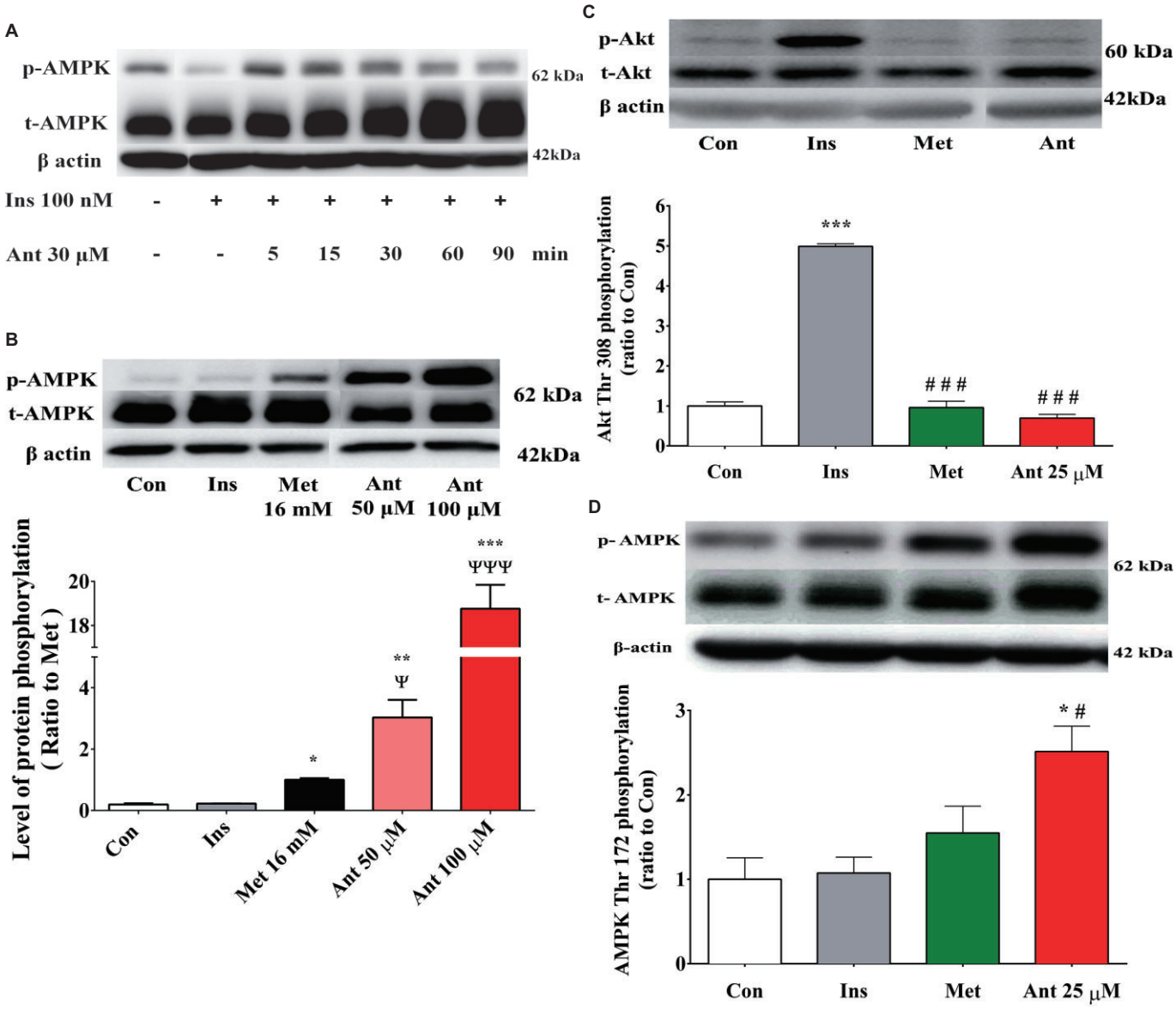

Figure 4

Effects of antroquinonol on activation of AMPK and Akt. (A) Time-dependent phosphorylation of AMPK Thr^{172}, (B) concentration-dependent phosphorylation of AMPK Thr^{172}, (C) phosphorylation of Akt Thr^{308}. In (D), AMPK Thr^{172} phosphorylation was significantly increased by a low concentration of antroquinonol (25 μM). Differentiated C2C12 cells were incubated with 100 nM insulin, 16 mM metformin (Met), and 25, 30, 50 or 100 μM antroquinonol (Ant) at 37°C for 30 min. Then the cell lysates were separated by SDS-PAGE and analysed by immunoblotting. Data are expressed as means ± SEM (n = 4 in each group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control (Con); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared with Ins group; $^{\Psi}P < 0.05$, $^{\Psi\Psi}P < 0.01$, $^{\Psi\Psi\Psi}P < 0.001$, compared with Met group.

et al., 2009). Its most common application is as an antitumour agent (Chiang *et al.*, 2010; Kumar *et al.*, 2011; Yu *et al.*, 2012b) and there have been no reports of its potential use for the treatment of human diabetes. Our present data suggested that antroquinonol could reduce blood sugar by inhibiting DPP IV activity and increasing AMPK activation.

When carbohydrates are ingested, the major mechanism for lowering blood glucose is through insulin and increased glucose transport into skeletal muscles, following activation of AMPK. Skeletal muscles store glucose as glycogen and oxidize glucose to produce energy. A recent report has shown that activation of AMPK played a major role in the antitumour properties of antroquinonol (Chiang *et al.*, 2010). In the present study, we have extended the earlier investigation to provide more evidence that antroquinonol can increase glucose uptake by two effects, enhanced translocation of GLUT4 and action with insulin. The present data also indicated that AMPK could be a major mediator of the increased uptake of glucose into cells induced by antroquinonol. The principal glucose transporter protein, GLUT4, mediates glucose uptake and thus plays a key role in regulating whole body glucose homeostasis (Huang and Czech, 2007; Niu

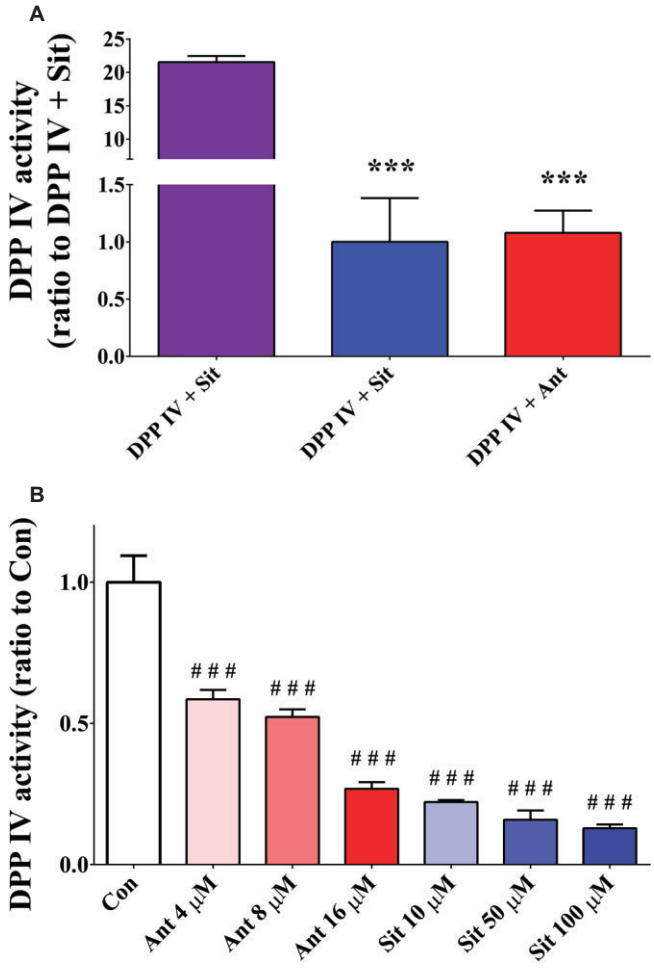

Figure 5

Inhibition of DPP IV activity by antroquinonol. (A) Uisng the DPP IV assay kit, DPP IV activity was inhibited by 20 μM antroquinonol (Ant) or sitagliptin (Sit). In (B), the DPP IV activity in Caco-2 cells was inhibited by incubation with antroquinonol (4, 8 and 16 μM) or sitagliptin (10, 50 and 100 μM), for 12 h. The data are expressed as means ± SEM (n=3 in each group). *P < 0.05, **P < 0.01, ***P < 0.001, compared with the DPP IV group; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, compared with control group (Con).

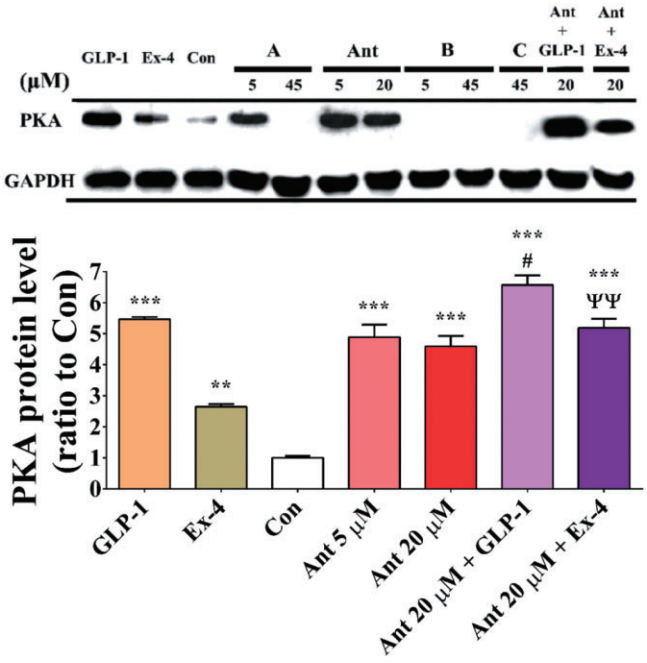

Figure 6

Antroquinonol potentiated GLP-1-induced PKA protein level in AR42J cells. AR42J cells were treated with 1 nM GLP-1, 1 nM exendin-4 (Ex-4), two concentrations (5 or 20 μM) of antroquinonol alone (Ant), Ant plus GLP-1 and Ant plus Ex-4 for 48 h. Protein levels were determined by Western blot. GAPDH was the internal control. A, B and C are not relevant to this data set. A typical Western blot is shown in the upper part of the Figure. The lower graph shows the summary data (means ± SEM) from 3 experiments. *P < 0.05, **P < 0.01, ***P < 0.001, compared with control (Con). $^{\#}P$ < 0.05, compared with GLP-1 alone; $^{\Psi\Psi}P$ < 0.01, compared with exendin-4 alone; one –way ANOVA with Tukey's test.

et al., 2010). The most important aspect of this work has demonstrated that antroquinonol activated AMPK activity in myocytes, along with increased GLUT4 translocation and glucose uptake.

An association between diabetes and cancer has already been published in the early part of the 20th century (Maynard, 1910). More credible direct evidence between diabetes and cancer is that, following the introduction of insulin therapy and the consequently increased lifespan of patients with diabetes, there was increased cancer prevalence (Marble, 1934). Furthermore, the association between cancer and diabetes has been extensively investigated by many meta-analyses that have confirmed diabetes (predominantly T2DM) has a higher incidence in many cancers (Czyzyk and Szczepanik, 2000; Vigneri *et al.*, 2009; Noto *et al.*, 2010; Osorio, 2013). Although the cause of this association is not clear, several possible mechanisms have been proposed such as hyperinsulinaemia, hyperglycaemia and inflammation (Vigneri *et al.*, 2009; Shikata *et al.*, 2013). Postmenopausal women with breast cancer are more likely to develop diabetes mellitus than women without breast cancer. This might be related to chemotherapy or glucocorticoid therapy, which is often used to treat breast cancer (Osorio, 2013). Antroquinonol has displayed anticancer potential (Lee *et al.*, 2007; Chiang *et al.*, 2010; Kumar *et al.*, 2011; Yu *et al.*, 2012a) and thus the same drug may be able to prevent diabetes mellitus in patients with cancer.

The lowering of blood glucose by antroquinonol appeared not to involve activation of Akt. In pancreatic beta cells, inhibition of DPP IV helps incretin peptides to bind to GPCRs (GLP-1R and GIP-R) (Doupis and Veves, 2008), which leads to the activation of PKA (Wang *et al.*, 2001; 2012) and stimulation of insulin secretion (Doyle and Egan, 2007). In this study, antroquinonol increased PKA activation. Furthermore, antroquinonol and sitagliptin showed comparable inhibition of the activity of DPP IV. Thus the lowering of blood glucose by antroquinonol may derive from the inhibition of endogenous DPP IV.

In our preliminary experiments (our unpublished results), mice were given the insulin receptor antagonist-S961 to

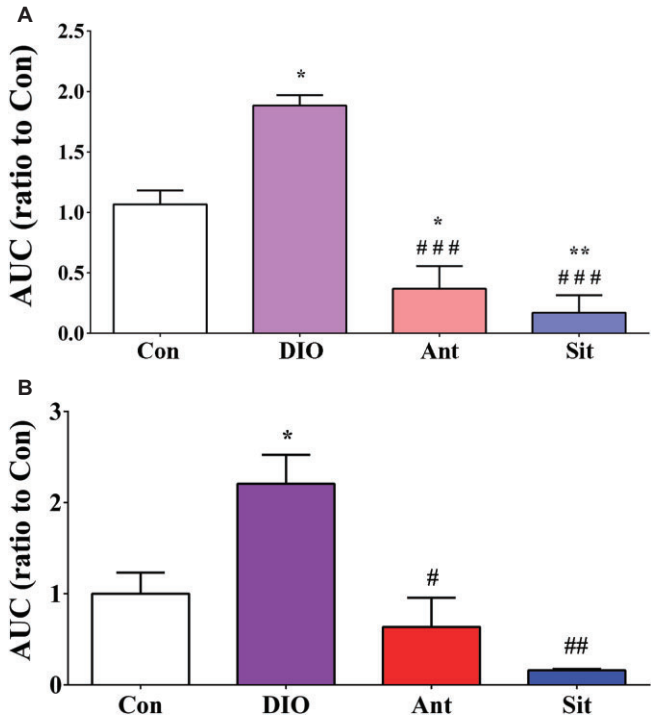

Figure 7

Effects of antroquinonol or sitagliptin on OGTT in DIO mice. (A) Effects of a single dose of antroquinonol (Ant, 25 mg·kg^{-1}) or sitagliptin (Sit, 20 mg·kg^{-1}) given to DIO mice, 15 min before the OGTT. Blood glucose was then followed for 2 h after the oral glucose load and the AUC for the glucose time curve calculated over this time. In (B), the same doses of antroquinonol (Ant) or sitagliptin (Sit) were given every other day to DIO mice for 4 weeks. At the end of this treatment, the OGTT was administered and AUC calculated as before. The data are expressed as means ± SEM (n = 5 in each group). *P < 0.05, **P < 0.01, ***P < 0.001, compared with the control (Con); $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, compared with untreated DIO group.

mimic insulin resistance. Then mice were given antroquinonol (25 mg·kg^{-1}), sitagliptin (10 mg·kg^{-1}) or metformin (100 mg·kg^{-1}), before performing the OGTT. Antroquinonol was as effective as the clinically used drugs, sitagliptin and metformin, in mice with S961-induced insulin-resistance. The DIO model in mice mimics some features of clinical T2DM patients and this model was also used to assess the effects of antroquinonol on blood glucose. Using acute or chronic treatments, both antroquinonol and sitagliptin lowered blood glucose similarly in DIO mice. Thus antroquinonol was an effective anti-hyperglycaemic agent in two models of insulin resistance *in vivo*.

Metformin, a biguanide in clinical use, is the least expensive among all oral anti-diabetic agents (DeFronzo and Goodman, 1995; Krentz and Bailey, 2005). However, the gastrointestinal side effects including abdominal discomfort and diarrhoea are the most common adverse events occurring in 20–30% of patients (DeFronzo, 1999; Yki-Jarvinen, 2002; Cheng and Fantus, 2005; Krentz and Bailey, 2005). The DPP IV inhibitors, such as sitagliptin, are based on the anti-diabetic action of the incretin hormone GLP-1 and provides a novel approach to lowering blood glucose in T2DM. This incretin-based therapy can reduce HbA1c without weight gain and some GLP-1 receptor agonists will actually reduce body weight. In clinical terms, this approach is relatively safe with very few adverse events apart from the gastrointestinal side effects frequently observed with GLP-1 receptor agonists (Russell, 2013). However, alternative sources of anti-diabetic drugs are still needed, particularly from natural products. Although antroquinonol can be isolated from *A. camphorate*, the natural source (the fungus) is rare, expensive and can only be found in Taiwan. Thus only a total chemical synthesis of antroquinonol would provide a safe and more readily available source to meet the demand of the pharmaceutical market. Furthermore, antroquinonol is the first non-toxic cancer treatment agent under FDA phase II review (ClinicalTrials.gov identifier: NCT01134016). These potential advantages of antroquinonol over metformin or sitagliptin were some of the reasons for choosing antroquinonol for further investigation.

The most recent ADA standards of medical care in diabetes on 'Pharmacological Therapy for Hyperglycaemia in T2DM Recommendations' show that metformin is the preferred pharmacological agent for T2DM, not contraindicated but tolerated. If non-insulin monotherapy at maximum tolerated doses, does not achieve or maintain the A1C target over 3 months, the recommendation (American Diabetes Association, 2014) is to add a second oral agent, GLP-1 receptor agonist, sulfonylurea or one of the thiazolidinediones (glitazones). However, a smaller percentage of patients may reach the desired therapeutic goal. Therefore, most patients with T2DM require combination therapy to achieve acceptable glycaemic control. Moreover, T2DM is a progressive disease. A patient-centred approach should be used to guide the choice of pharmacological agents. Considerations should include efficacy, cost, potential side effects, effects on weight, comorbidities, risk of treatment-induced hypoglycaemia and patient preferences (American Diabetes Association, 2014). In this context, antroquinonol exhibits some particular advantages in that the two paths to overcoming insulin resistance, by activating AMPK and inhibiting DPP IV activity, lead to an additive action allowing a reduction of the dose for a given effect.

In conclusion, synthesis of (+)-antroquinonol and its assessment for glycaemic control has revealed good efficacy and a pattern similar to that of metformin and sitagliptin, *in vitro* and *in vivo*.. This study provides the first evidence that antroquinonol may serve as a potential agent for glycaemic control via the inhibition of DPP IV activity and the activation of AMPK signalling pathways with the enhancement of GLUT4 translocation, to promote lowering of blood glucose.

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Author contributions

C. Y. H., R. S. S., P. K. H., Y. K. L., C. C. and C. F. W. wrote the manuscript. C. Y. H., P. K. H., Y. K. L., C. C. and C. F. W. designed the research. C. Y. H., P. K. H., H. Y. S. and H. W. S. performed the research. C. Y. H. and P. K. H. analysed the data. R. S. S. and C. C. contributed new reagents/analytical tools.

Conflict of interest

A provisional patent has been filed in relation to this work. The authors declare no additional competing financial interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12828

Table S1 The ^{1}H and ^{13}C NMR of synthetic (+)-antroquinonol (this article) compared with values obtained earlier with the natural product (Liu *et al.*, 2012; Lee *et al.*, 2007).