Novel mitochondrial complex I inhibitors restore glucose-handling abilities of high-fat fed mice

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Abstract

Metformin is the main drug of choice for treating type 2 diabetes, yet the therapeutic regimens and side effects of the compound are all undesirable and can lead to reduced compliance. The aim of this study was to elucidate the mechanism of action of two novel compounds which improved glucose handling and weight gain in mice on a high-fat diet. Wildtype C57Bl/6 male mice were fed on a high-fat diet and treated with novel, anti-diabetic compounds. Both compounds restored the glucose handling ability of these mice. At a cellular level, these compounds achieve this by inhibiting complex I activity in mitochondria, leading to AMP-activated protein kinase activation and subsequent increased glucose uptake by the cells, as measured in the mouse C2C12 muscle cell line. Based on the inhibition of NADH dehydrogenase (IC_{50} 27 μmol L^{-1}), one of these compounds is close to a thousand fold more potent than metformin. There are no indications of off target effects. The compounds have the potential to have a greater anti-diabetic effect at a lower dose than metformin and may represent a new anti-diabetic compound class. The mechanism of action appears not to be as an insulin sensitizer but rather as an insulin substitute.

Key Words
- type 2 diabetes
- metformin
- complex I
- NADH dehydrogenase
- AMPK
- insulin resistance
- ATP

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Introduction

Whether administered in the early stages of the development of type 2 diabetes or as a combination therapy with injectables, metformin is the main drug of choice for treating the condition. Yet the therapeutic regimens, the side effects of the compound, and the probable off-target actions are all undesirable and can lead to reduced compliance (Donnan *et al*. 2002, Florez *et al*. 2010, Kirpichnikov *et al*. 2002). The principal mode of action of metformin responsible for its anti-diabetic effects centers around its role in inhibiting liver gluconeogenesis (Bailey & Turner 1996, Madiraju *et al*. 2014), antagonising glucagon action (Miller *et al*. 2013), and activating AMP-activated protein kinase (AMPK) (Li *et al*. 2015, Owen *et al*. 2000). This is achieved via either a reduction in the activity of complex I of the mitochondrial respiratory chain (Bridges *et al*. 2014,

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Brunmair *et al*. 2004, El-Mir *et al*. 2000, Fontaine 2014, Owen *et al*. 2000) or the inhibition of mitochondrial glycerophosphate dehydrogenase (Madiraju *et al*. 2014). Other biguanides have similar and more potent effects on complex I (Bridges *et al*. 2014, Matsuzaki and Humphries 2015), with phenformin dramatically reducing the oxygen consumption rate in HepG2 cells (Bridges *et al*. 2014).

The effect of activating the intracellular metabolic sensor, AMPK, is to maintain ATP levels. This is achieved via further signaling resulting in an increase in glucose uptake by the cell (Pehmøller *et al*. 2009), an increase in fatty acid oxidation (Winder *et al*. 1997) and decrease in fatty acid synthesis (Munday *et al*. 1988), and a decrease in glycogen synthesis (Jørgensen *et al*. 2004) and an increase in glycolysis (Marsin *et al*. 2002). AMPK is activated during exercise as the cell seeks to replenish levels of ATP (Winder & Hardie 1996). Lowering high blood glucose levels in this way would appear to be as close to a natural physiological phenomenon as any other anti-diabetic mechanism of action.

This study deals with two related, novel compounds (PCT/EP2012/071286; see Fig. 1 and Supplementary Materials and methods (see section on supplementary data given at the end of this article)) with the aim of elucidating their mechanism of action. As can be seen, their principal mode of action is to inhibit complex I, thereby very effectively stimulating AMPK and increasing glucose uptake by muscle cells, a major site of insulin action within the body. These compounds represent a possible new anti-diabetic class and a move away from the well-known biguanides. In these respects, the compounds activate signaling pathways that are influenced in a similar way by exercise (Winder & Hardie 1996), and presumably also by metformin. Analyses also indicate that these compounds have no effects on other anti-diabetic targets and present no accepted toxic indicators.

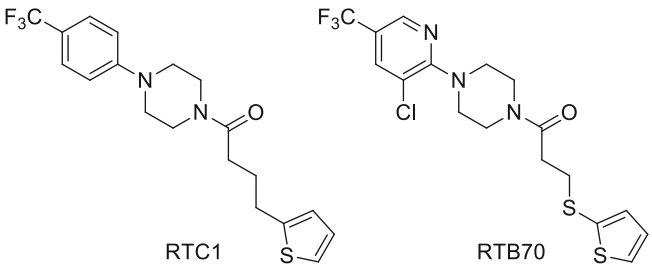

Figure 1
The chemical structures of the potential anti-diabetic compounds assessed in this study.

Materials and methods

Animal studies

All procedures were approved by the UK Home Office and local ethical committee of the University of Leeds, UK. In a prevention study, wildtype C57Bl/6 male mice (~20 g; Charles River Laboratories) were housed (4 mice per cage) in a conventional animal facility with a 12 h light:12 h darkness cycle, given access to water and an obesogenic diet *ad libitum* (Diet F3282; 5450 kcal kg^{-1}; Bio-serv, Flemington, NJ, USA). Each group comprised 8 mice, which were fed for 8 weeks with the high-fat diet (HFD) containing DMSO (vehicle control) or RTB-70 at 0.04% (w/w) relative to the diet (~1 mg/mouse/day). In a second series of experiments, mice were fed normal chow or the HFD for 16 weeks. The mice then underwent a glucose tolerance test which indicated that those on the high-fat diet indeed had impaired glucose handling abilities (mean AUC values – normal chow 17.64 ± 3.7, high-fat diet 36.08 ± 2.9). This latter HFD group was then sub-divided (each group had 8 mice) with one group maintained on the diet and the other on the diet plus the addition of RTC-1 at 0.04% (w/w) relative to diet (~1 mg/mouse/day), for a further 16 weeks. At the end of both feeding paradigms, glucose (GTT) and insulin tolerance tests (ITT) were carried out. Following a period of fasting (12 h and 4 h for GTT and ITT, respectively), repeated whole-blood sampling was carried out on conscious mice at 30-min intervals after an initial i.p. injection of either glucose (1 mg g^{-1} body weight; GTT) or insulin (1 U kg^{-1} body weight; ITT). Body weight was recorded throughout the protocol.

Cell culture

Mouse muscle cells (C2C12; passages 5–25; ECACC (STR authenticated)) were differentiated in DMEM supplemented with 2% (v/v) horse serum for 3 days. RTC-1 and RTB-70 were dissolved in 100% DMSO (to 50 mmol L^{-1}) and diluted to 1 mmol L^{-1} in Krebs Ringers Buffer (KRB; in mmol L^{-1}: 136 NaCl, 20 HEPES, 4.7 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 4.05 Na_2HPO_4, 0.95 NaH_2PO_4; pH7.4) including glucose (5 mmol L^{-1}). The cells were treated with RTC-1 or RTB-70 at 10 µmol L^{-1} in DMEM plus 2% (v/v) horse serum overnight or, as indicated, at 37°C. In other experiments, C2C12 cells were treated with Insulin (100 nmol L^{-1}; 30 min, Sigma), cytochalsin B (10 µmol L^{-1}; 30 min, Sigma, Flemington, NJ, USA), or metformin (500 µmol L^{-1}; 6 h, Sigma). In a further experiment,

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Compound C (10 µmol L^{-1}; GE Healthcare) was added to the cells 1 h before the addition of RTC-1 (10 µmol L^{-1} for 6 h). CHO cells (passages 15–25; ECACC) and primary human red blood cells were also treated with RTC-1 (10 µmol L^{-1}; 16 h) before the addition of ^{3}H deoxy-2-glucose.

Glucose uptake

Glucose uptake was monitored using a tritiated version of deoxy-2-glucose, a derivative of glucose that cannot be metabolized (Yun *et al*. 2009). Following treatment, the cells were washed in KRB and ^{3}H deoxy-2-glucose (1 µCi mL^{-1}; specific activity 8 mCi $mmol^{-1}$; PerkinElmer) added for 10 min at 37°C. The cells were washed 3 times in ice-cold KRB, solubilized in 0.1% (w/v) SDS for 30 min, and 500 µl (~250 µg protein) of the extract added to 2 mL scintillation fluid (Ultima Gold, PerkinElmer). The results are expressed as counts per minute per mg protein (c.p.m. mg^{-1}; Wallac MicroBeta, PerkinElmer) as assayed by the BCA method (Smith *et al*. 1985).

SDS–PAGE and Western immunoblotting

Following drug treatments, the cells were washed in PBS and a lysis buffer (in mmol L^{-1}: 50 HEPES (pH 7.5), 150 NaCl, 10 Na_2HPO_4, 50 NaF, 1 EDTA, 1.5 $MgCl_2$, 2 Na_3VO_4, 1 $Na_4P_2O_7$, 1 PMSF, 1X SigmaFAST protease inhibitor cocktail, 10% (v/v) glycerol, and 1% (v/v) Triton X-100) was added. Following incubation for 1 h, the mixture was centrifuged at 12,000 ***g*** and 30 µL supernatant were loaded on 12% SDS–PAGE mini gels. The proteins were transferred to activated PVDF membranes and probed with anti-phospho (Thr172) AMPK alpha (diluted 1/1000; Cell Signalling) for 2 h at RT. Secondary antibody (anti-rabbit; 1/5000, Dako) was added for 1 h at RT. The labeled bands were detected by enhanced chemiluminescence.

PPARγ lance assay

The LanthaScreen TR-FRET PPARγ Co-activator Assay (Life Technologies) was performed according to the manufacturer's protocol. In an agonist mode, the test compounds and agonist control GW1929 (1 µmol L^{-1}; Sigma) were incubated with fluorescein-labeled TRAP220/DRIP-2 coactivator peptide (125 nmol L^{-1}), terbium-labeled anti-GST antibody (5 nmol L^{-1}), and PPARγ LBD (20 nmol L^{-1}) for 1 h at 25°C. The signals at 520 nm were normalized to those obtained from the terbium emission at 495 nm, and the 520 nm/495 nm ratios were used as a measure for the TRAP220/DRIP-2 coactivator recruitment potential of the tested compounds. The ratio of the emission intensity of the acceptor (Fluorescein: λ = 520 nm) divided by the emission intensity of the donor (Tb: λ = 490 nm) was then calculated to determine the degree of nuclear receptor coactivator binding. Each measurement

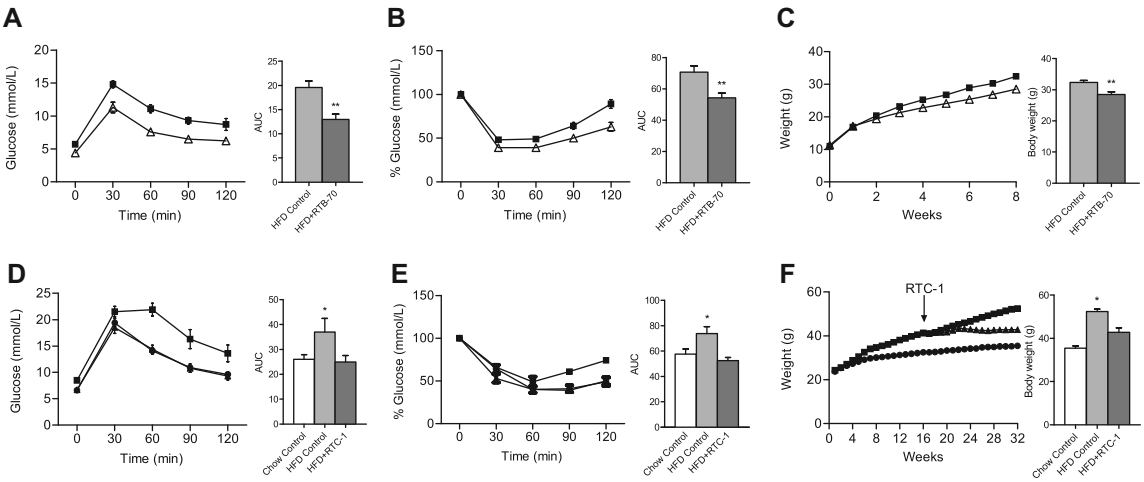

Figure 2
RTB-70 and RTC-1 restore normal glucose handling in high-fat fed mice. Mice fed a high-fat diet and RTB-70 (0.04% (w/w); Δ) for 8 weeks had improved glucose handling abilities compared with mice on high-fat diet alone (■) when assessed by GTT (A) and ITT (B). Mice fed a high-fat diet for 16 weeks and then a high-fat diet and RTC-1 (△) for a further 16 weeks had improved glucose handling abilities compared with mice on high-fat diet alone (■) when assessed by GTT (D) and ITT (E), with levels mimicking control fed mice (●). Total body weights (**c**-RTB-70 and f-RTC-1) are also shown. Bar charts depict area under the curve (AUC) for GTT and ITT, and final body weights. Values are mean ± S.E.M.; $n = 8$ per group; $^*p < 0.05$, $^{**}p < 0.01$; student's *t*-test vs control.

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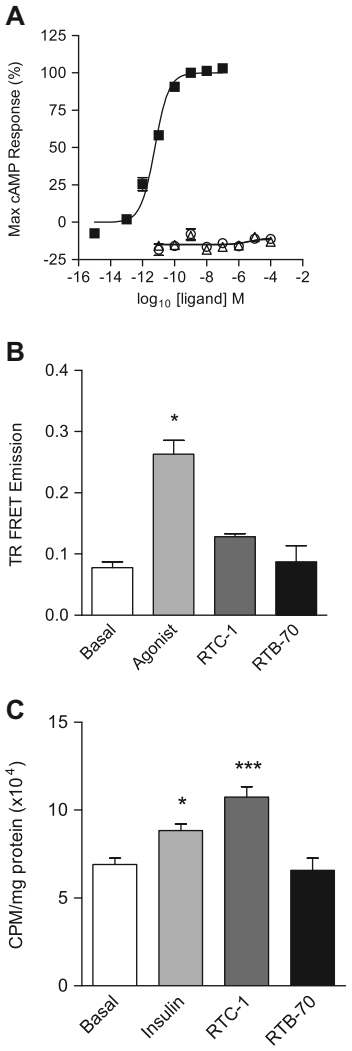

Figure 3
RTC-1 induces glucose uptake in C2C12 mouse muscle cells. The ability of RTC-1 (○) and RTB-70 (Δ) to activate the GLP-1R (A) compared with GLP-1 peptide (■), PPARγ (B), and the known agonist GW1929 (1 μmol L^{-1}) was assessed. Glucose uptake following RTC-1 and RTB-70 (both 10 μmol L^{-1}; 16 h) treatment (C) was also assessed, compared with Insulin (100 nmol L^{-1}; 30 min). Values are mean ± S.E.M.; $n = 6$; *$p < 0.05$, ***$p < 0.001$; student's *t*-test vs basal.

was performed in duplicate. The 520 nm/495 nm ratio was plotted against the test compound concentration.

Homogeneous time-resolved fluorescence (GLP-1R)

The compounds were assayed for GLP-1 activation using a HTRF cAMP detection kit (Cisbio, Bedford, MA, USA). Briefly, 32,000 hGLP1R-expressing Flp-In HEK293 cells were plated in 25 μL/well of PBS with IBMX (Sigma) in 384-well solid bottom white plates and 15 μL/well compound in DMSO solution or controls were added. Following 30 min incubation at RT, 25 μL/well of labeled d2 cAMP and 25 μL/well of anti-cAMP antibody (both diluted 1:20 in lysis buffer) were added to each well. Signals were recorded using the Omega Polarstar (BMG LabTech) with excitation at 330 nm and emissions at 615 nm and 660 nm.

Rat liver mitochondria isolation

Rat liver mitochondria were isolated by the method described by Chappell and Hansford (1972). Immediately after extraction, liver samples were washed free of excess blood, trimmed of any fat and connective tissue, finely chopped, and placed in 50 mL ice-cold (0–4°C) isolation medium (in mmol L^{-1}: 250 sucrose, 5 Tris–HCl, 1 EGTA, pH 7.4). The tissue was homogenized and centrifuged at 750 ***g*** for 5 min at 4°C. The supernatant was filtered through a sieve before being centrifuged at 12,000 ***g*** for 10 min at 4°C. The resulting mitochondrial pellet was resuspended in ice-cold 25 mL isolation medium supplemented with 2% (w/v) defatted BSA and washed twice by centrifugation (12,000 ***g*** for 10 min at 4°C). The final mitochondrial pellet was resuspended in a small volume of isolation medium, and the aliquots were frozen at –20°C.

NADH: ubiquinone oxidoreductase activity analysis

Immediately before the assay, mitochondria were diluted in a hypotonic buffer (25 mmol L^{-1} K_2HPO_4, 5 mmol L^{-1} $MgCl_2$) and permeabilized with three cycles of freeze thawing. The assay was carried at 30°C in a 1 mL cuvette (Shimadzu UV-2550, Milton Keynes, Buckinghamshire, UK). Permeabilized mitochondria were incubated with 50 mmol L^{-1} K_2HPO_4 (pH 7.5), 3 mg mL^{-1} fatty acid-free BSA, 300 μmol L^{-1} KCN and 100 μmol L^{-1} NADH, and baseline activity was measured at 340 nm for 1 min. The reaction was initiated with the addition of 60 μmol L^{-1} ubiquinone and the resulting decrease in absorbance was measured for 3 min. Varying concentrations of RTC-1 or RTB-70 were then added and absorbance was measured for a further 3 min. The effect of the compounds on NADH:ubiquinone oxidoreductase was determined by comparing activity before and after addition, relative to the vehicle control, DMSO. Rotenone (1 μmol L^{-1}) was used as a positive control in this assay.

Oxygen consumption of rat liver mitochondria

Oxygen consumption rates were measured by an Oxygraph Respirometer (Oroboros, Innsbrück, Austria) as previously described (Breen *et al*. 2006). Mitochondria (6.2 μg mL^{-1}) were incubated at 37°C in a respiration

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medium (5 mmol L^{-1} HEPES, 120 mmol L^{-1} KCl, 10 mmol L^{-1} K_2HPO_4, 1 mmol L^{-1} EGTA; pH 7.4) to which 0.1% fatty acid-free bovine serum albumin was added on the day of use. Oxygen consumption rates were measured as the steady-state rates achieved on addition of glutamate (5 mmol L^{-1}) and malate (3 mmol L^{-1}). The sensitivity of these steady-state rates to various concentrations of RTB-70, RTC-1 or the DMSO vehicle control was then determined. Recovery of the steady state was then assessed by the addition of 10 mmol L^{-1} succinate (succinate-KOH, pH 7.4). The Oroboros Oxygraph Respirometer was calibrated according to the procedure of Reynafarje *et al.* (1985), assuming that 406 nmol of oxygen atoms was dissolved in 1 mL of ionic incubation medium at 37°C.

ATP assay

The Luminescent ATP Detection Assay (Abcam) was performed according to the manufacturer's instructions. C2C12 cells (100 µL) were cultured in a black, clear bottom 96-well plate, and treated with RTC-1 (10 µmol L^{-1}; 1 and 2 h) or rotenone (25 µmol L^{-1}; 1 h). The cells were lysed by addition of detergent solution (50 µL) and incubated for 5 min. Reconstituted substrate buffer (containing D-Luciferin and Luciferase; 50 µL) was added to the cells and incubated for 15 min before luminescence was read. ATP concentration was determined via an ATP standard curve.

Cell cytotoxicity assay

Rat hepatocytes were used to test the cytotoxicity of RTC-1. The cells were plated at 1×10^6 per well and treated with each compound for 4 h at 100 µg mL^{-1}. At the end of the treatment, a Trypan blue exclusion test was carried out and cell viability was assessed using the Countess automated cell counter (Life Technologies). The experiments were repeated three times and carried out by Pharmidex (London, UK).

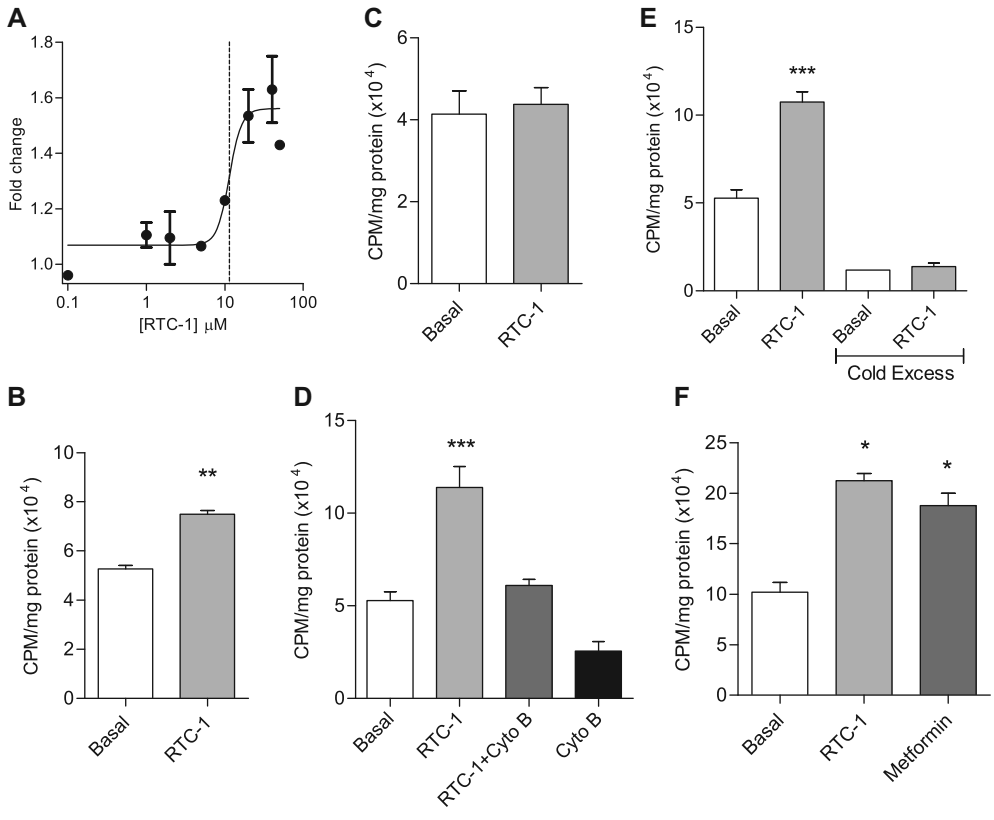

Figure 4
RTC-1 induces glucose uptake in a specific, time-dependent manner. Dose dependency of RTC-1 (16 h) on glucose uptake in C2C12 cells, with an EC_{50} estimated at 11.42 µmol L^{-1} (dashed line; A). Glucose uptake following RTC-1 (10 µmol L^{-1}; 16 h) treatment of CHO cells (B) and primary erythrocytes (C). RTC-1-induced (10 µmol L^{-1}; 16 h) glucose transport inhibited by cytochalasin B (cyto B; 10 µmol L^{-1}; 30 min; D) and co-addition of 5 mmol L^{-1} 'cold' glucose (E). The ability of metformin (500 µmol L^{-1}; 16 h) to increase glucose uptake in C2C12 cells compared with RTC-1 (10 µmol L^{-1}; 16 h; F). The values are mean ± S.E.M.; $n = 4$ per group; $^{**}p < 0.01$, $^{***}p < 0.001$; student's *t*-test vs basal.

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Effects of RTC-1 on the hERG channel

Effects of RTC-1 on the hERG channel currents were determined by the two-electrode voltage clamp technique as described previously (Elliott *et al*. 2009). In brief, cRNA was prepared from the hERG cDNA (acc. No. GI:4557729) clone in the pSP64 vector using the SP6 Megascript Kit (Ambion). Stage V or VI oocytes were isolated from *Xenopus* toads, injected with 50 nL of cRNA (50 pg nL^{-1}), and incubated in ND-96 solution (in mmol L^{-1}: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 2.5 sodium pyruvate, pH 7.5) supplemented with 100 µmol L^{-1} DTT solution at 18°C. After 2–4 days, currents were recorded in Ringer's solution (in mmol L^{-1}: 115 NaCl, 2 KCl, 1.8 $CaCl_2$, 10 HEPES, pH 7.2) using microelectrodes made from borosilicate glass, filled with 3 M KCl, which had resistances between 0.5 and 2.0 M. The effects of compounds on tail currents at –50 mV were examined using a repeated pulse protocol. For this, control currents were first measured in Ringer's solution during repeated depolarizing steps (+30 mV) delivered from a holding potential of –80 mV. The cells were then superfused with RTC-1 (10 µmol L^{-1}) and the current recordings continued until a steady-state effect was achieved. All experiments were repeated at least three times.

Compound synthesis

Details of compound synthesis for this study are given in the Supplementary Materials and methods (see section on supplementary data given at the end of this article).

Statistical analysis

The values are expressed as the mean ± S.E.M. Statistical comparisons are made using two-tailed student's *t*-test compared with basal levels, unless otherwise stated.

Results

Effects on glucose handling

The initial compound (RTB-70) and one of its derivatives, RTC-1, were assessed for their ability to improve glucose

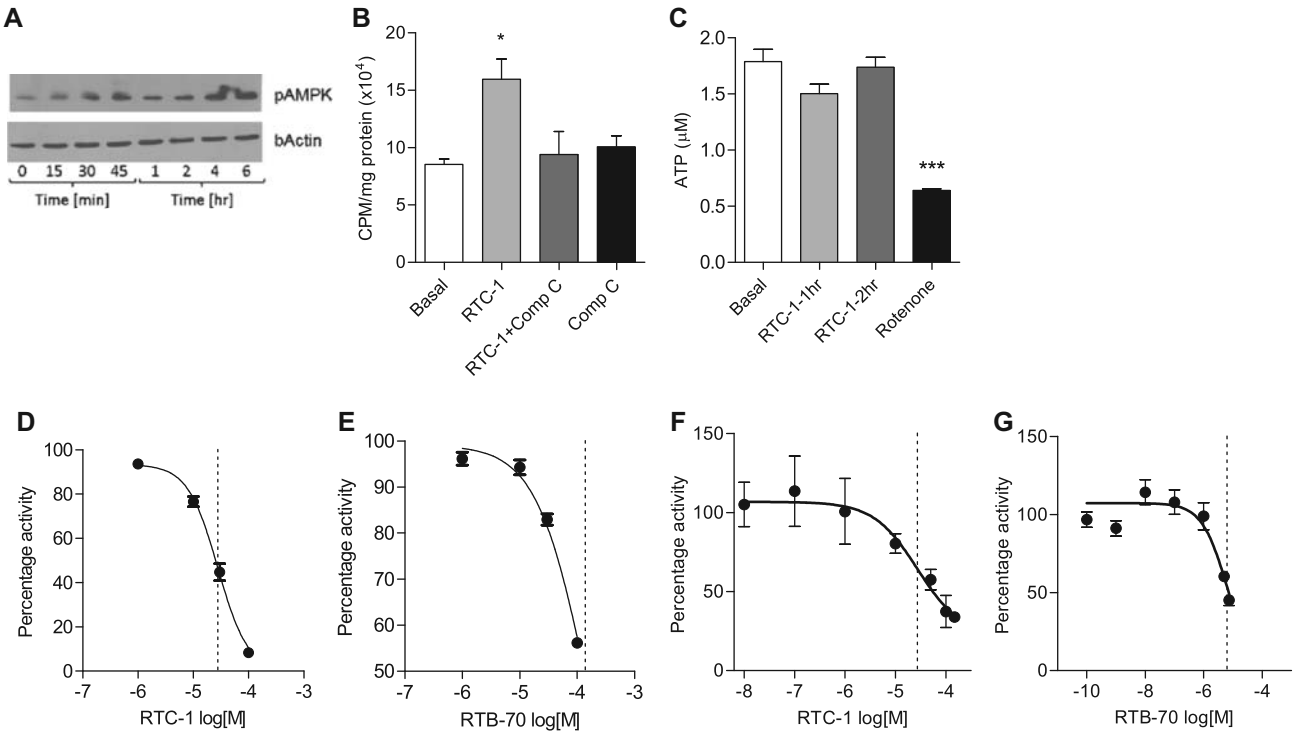

Figure 5
RTC-1 induces an increase in AMPK phosphorylation via the inhibition of NADH oxidation. RTC-1-induced (10 µmol L^{-1}) time-dependent increase in the phosphorylation of AMPK in C2C12 cells (A). The AMPK inhibitor, Compound C (1 h before addition of RTC-1; 10 µmol L^{-1}), blocks the RTC-1-induced (6 h) glucose uptake (B). RTC-1 effect on ATP levels (C). RTC-1- (D) and RTB-70-induced (E) inhibition of oxygen consumption in intact mitochondria. IC_{50} was calculated at 27 µmol L^{-1} for RTC-1 and 138 µmol L^{-1} for RTB-70 (indicated by the dashed lines). RTC-1- (F) and RTB-70-induced (G) inhibition of NADH oxidation in disrupted mitochondria. IC_{50} was calculated at 27 µmol L^{-1} for RTC-1 and 6 µmol L^{-1} for RTB-70 (indicated by the dashed line). Values are mean ± S.E.M.; $n = 3$ per group; *$p < 0.05$, ***$p < 0.001$; student's *t*-test vs basal.

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handling in mice subjected to a high-fat diet. In the case of RTB-70, the compound was added to the diet for 8 weeks from the start of the study. With RTC-1, the compound was administered for 16 weeks after a preceding 16 week period on the diet alone when insulin resistance became evident. Fig. 2A and B reveal that RTB-70 significantly improved glucose handling in both glucose and insulin tolerance tests. This was also true in the intervention study with RTC-1 (Fig. 2D and E). Comparisons with an untreated chow-fed group indicated that RTC-1 restored glucose handling to levels not significantly different to controls. Both compounds also reduced the weight gain exhibited by the control groups of animals (Fig. 2C and F). There were no significant changes in food intake between the control and treated groups. The mice exhibited no adverse reaction to the compounds and no hyperactivity was observed as well as no deaths occurred.

In order to ascertain the mechanism by which these compounds exert their anti-diabetic effects, their ability to activate well-known type 2 diabetes targets was assessed. Neither RTB-70 nor RTC-1 was agonist of GLP-1R (Fig. 3A) or PPARγ (Fig. 3B). The compounds were also tested for their effects on glucose uptake in a mouse muscle cell line, C2C12. Glucose uptake was assayed by using ^{3}H-deoxy-2-glucose, a derivative of glucose which cannot be metabolized. RTC-1 induced a significant increase in glucose uptake compared with vehicle-treated cells (Fig. 3C), which was more pronounced than the normal response to the positive control, insulin (100 nmol L^{-1}; 30 min). Perhaps meaningfully, RTB-70 did not affect glucose uptake significantly. Furthermore, the derivatives of RTC-1 that passed the Eli Lilly 'Open Innovation Drug Discovery' novelty screen were assessed for their efficacy in their anti-diabetic screenings. These compounds did not induce the secretion of insulin or GLP-1 from INS-1e and NCI cell lines, respectively (data not shown).

RTC-1 induced a concentration-dependent increase in glucose uptake when incubated with muscle cells for 16 h, with an EC_{50} calculated at ~11 µmol L^{-1} (Fig. 4A). This action of RTC-1 is not restricted to muscle cells as treatment of CHO cells with RTC-1 (10 µmol L^{-1}; 16 h) also induced a significant increase in glucose uptake compared with vehicle-treated controls (Fig. 4B). RTC-1 is not exerting its effect by directly activating the glucose transporter, as no glucose uptake was stimulated in erythrocytes (Fig. 4C). However, the ability of cytochalasin B (10 µmol L^{-1}; an inhibitor of the glucose transporter) to prevent this compound-induced uptake (Fig. 4D) indicated that the uptake was facilitated by a glucose transporter. This conclusion was reinforced by the observation that the addition of excess 'cold' glucose (5 mmol L^{-1}) along with the ^{3}H deoxy-2-glucose inhibited the amount of detectable tritium in the cells (Fig. 4E).

Mechanism of action

AMPK, being the major metabolic biosensor of the cell, was the logical protein to investigate. Phosphorylation of AMPK at threonine 172 is an indicator of AMPK activation. Using C2C12 cells, Western immunoblotting

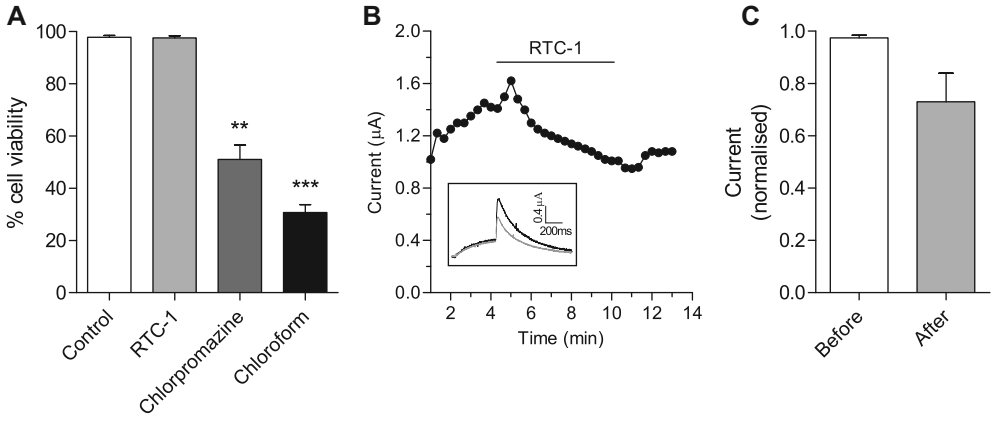

Figure 6

RTC-1 is not cytotoxic and does not activate hERG significantly. Assay of RTC-1 (100 µg/mL; 4 h) cytotoxicity as measured by Trypan Blue exclusion in rat hepatocytes compared with the known toxic agents Chlorpromazine and Chloroform (both 100 µg mL^{-1}; A). Representative peak of the effect of RTC-1 (10 µmol L^{-1}) on tail currents of hERG-expressing oocytes prepared from *Xenopus* toads, as determined by a repeated pulse protocol (insert: representative current traces before (black trace) and after (gray trace) the application of RTC-1; B). Normalized data (n = 3; C), before and after RTC-1 application. The values are mean ± S.E.M.; n = 3 per group; $^{**}p < 0.01$, $^{***}p < 0.001$; student's t-test vs control.

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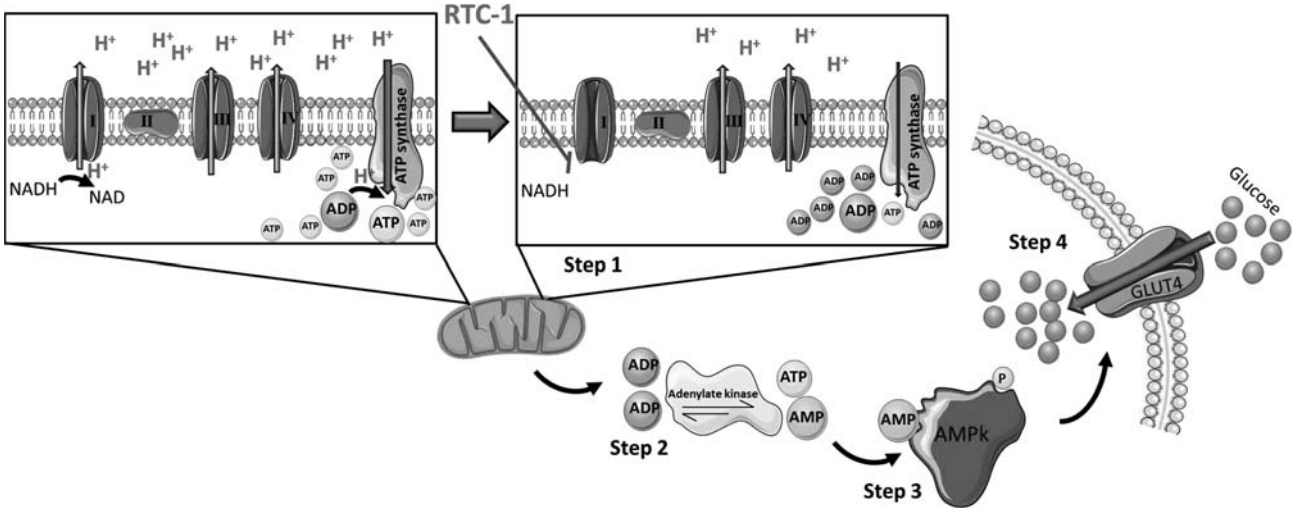

Figure 7
A proposed mechanism of action of these compounds on muscle cells initially involves the inhibition of the NADH dehydrogenase portion of complex I in mitochondria (Step 1). This leads to a build-up of ADP, which is subject to the action of adenylate kinase converting two ADP molecules into one ATP and one AMP (Step 2). The rise in AMP levels is detected by AMPK, which is activated by phosphorylation (Step 3). One of the downstream effects of this is an increase in glucose uptake via the GLUT4 transporter in the plasma membrane (Step 4). In the whole animal, the action of inducing more glucose uptake into the cell not only keeps intracellular ATP at normal levels but importantly reduces circulating glucose levels, thereby reducing the harmful effects of glucose intolerance and insulin resistance. A full colour version of this figure is available at http://dx.doi.org/10.1530/JME-15-0225.

showed that RTC-1 (10 µmol L^{-1}) induced an increase in Thr172 phosphorylation of AMPK in a time-dependent manner, the first increase occurring at 45 min (Fig. 5A). In order to confirm the sequence of events through which glucose enters the cell, pre-treatment with an inhibitor of AMPK activation, Compound C (1 h; 10 µmol L^{-1}), before RTC-1 (10 µmol L^{-1}; 6 h) was seen to attenuate the RTC-1-induced increase in glucose uptake (Fig. 5B). Therefore indicating that AMPK is a major intermediate in the effect of these compounds.

The mode of action of these compounds could be a direct effect on the enzyme or through the normal biological mechanism, namely an increase in the AMP to ATP ratio. RTC-1 induced a decrease (not statistically different from basal levels) in ATP levels 1 h after addition to cells; there was no discernible difference observed at 2 h (Fig. 5C) and thereafter. Rotenone (25 µmol L^{-1}), a known complex I inhibitor, acted as a positive control. Interestingly, RTC-1 (IC_{50} 27 µmol L^{-1}), and to a lesser extent RTB-70 (IC_{50} 138 µmol L^{-1}), both inhibited oxygen consumption by mitochondria, (Fig. 5D and E, respectively). The addition of succinate restored the oxygen consuming ability of these mitochondria (Supplementary Fig. 1). These observations led to the identification of the target protein. Assays of NADH oxidation using purified disrupted mitochondria in the presence of ubiquinone indicated that RTC-1 and RTB-70 (Fig. 5F and G, respectively) inhibit the NADH dehydrogenase in complex I of the electron transport chain of mitochondria (IC_{50} for RTC-1 is 27 µmol L^{-1}). The data illustrated in Fig. 4F indicate that metformin, which has previously been shown to inhibit complex I in muscle cells (Brunmair *et al*. 2004), has a similar effect on glucose uptake in C2C12 mouse muscle cells to that of RTC-1. The response is very modest compared with RTC-1, despite a 50-fold higher dose.

The cell toxicity profile of RTC-1 confirmed the lack of adverse reactions observed in the animal studies. RTC-1 did not induce cell death in rat hepatocytes following 4 h treatment at 100 µg mL^{-1} compared with the known cytotoxic agents chlorpromazine and chloroform (Fig. 6A). The ability of druggable compounds to effect hERG, a voltage-gated potassium channel involved in cardiac action potential repolarization, is a recognized preclinical safety test (Bowlby *et al*. 2008, Priest *et al*. 2008). RTC-1 (10 µmol L^{-1}) did not significantly affect hERG tail current amplitudes as measured by voltage clamp in oocytes from *Xenopus* toads (Fig. 6B and C).

Discussion

The results above present a potential new class of anti-diabetic compounds which possess a targeted effect on the NADH dehydrogenase of complex I of the mitochondrial

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respiratory chain, the result of which is to activate AMPK. This enzyme, in turn, activates the pathway by which the GLUT4 transporter is mobilized to the plasma membrane of most mammalian cells with the consequent increase in glucose uptake by the cell. In this respect, the mechanism has elements in common with physical exercise (Winder & Hardie 1996). In both cases, there is a requirement for increased ATP production, probably partly effected through an increase in the AMP to ATP ratio through the action of adenylate kinase, which is met by elevated glucose mobilization/influx and utilization by the cell. At the levels studied, cellular ATP levels remain constant, though there may be a non-significant transient decrease on first exposure of cells to the compounds. This contrasts strongly with the effects of rotenone, an insecticide which produces long-lasting significant decreases in ATP levels (Fendel *et al*. 2008). These compounds appear to have a similar mode of action to metformin (Bridges *et al*. 2014, Brunmair *et al*. 2004, El-Mir *et al*. 2000, Fontaine 2014, Owen *et al*. 2000, Zhou *et al*. 2001), the drug of choice for treating type 2 diabetes. Although metformin has some major effects on the liver cells (Bailey & Turner 1996, Madiraju *et al*. 2014, Miller *et al*. 2013), it also affects xmuscle cells to inhibit complex I activity (Brunmair *et al*. 2004, Wessels *et al*. 2014), activate AMPK (Brunmair *et al*. 2004, Li *et al*. 2015, Musi *et al*. 2002) and induce glucose uptake (Kumar & Dey 2002), which are its other important anti-diabetic effects. RTC-1 appears to be more potent in inhibiting mictochondrial complex I, with an IC_{50} calculated at 27 $\mu mol\,L^{-1}$ compared with previously stated IC_{50} values of between 1.2 and 27 $mmol\,L^{-1}$ for metformin (Jenkins *et al*. 2013, Piel *et al*. 2015). One possible drug-dependent side effect of complex I inhibition is reported to be lactic acidosis (Brown *et al*. 1998, Misbin 1977, Wang *et al*. 2003). Although this was not specifically tested for in these mice, the characteristic symptoms of lactic acidosis, such as nausea, vomiting, muscle weakening and rapid breathing, were not evident in the mice that were treated with the drug for up to 16 weeks. The gastrointestinal side effects associated with metformin treatment, such as diarrhoea, retching and abdominal pain (Florez *et al*. 2010), were also absent. There are no effects on other potential drug targets for type 2 diabetes such as the GLP-1 receptor and PPARγ. Based on the toxicology screenings, there is also no evident toxicity at the doses tested.

Studies on mice fed a high-fat diet reflect this mechanism of action. Given either at the start of the diet or once insulin resistance/type 2 diabetes has been established, the compounds effected a very significant restoration of the normal GTT and ITT parameters. Again there were no indicators of toxicity: no deaths, no lethargy or hyperactivity, no diarrhoea, and no change in food intake, even on continuous treatment for up to 16 weeks. The weight gain in the mice was normalized, this was particularly evident in the epididymal fat pad (C Redondo, personal communication), which was greatly increased in the diabetic untreated animals but very similar to chow-fed controls in the compound-treated groups. The ability of these compounds to improve glucose handling in high-fat fed mice is similar to studies carried out with metformin (Matsui *et al*. 2010) and other compounds that target complex I (Jenkins *et al*. 2013).

One unexplained result was the success of RTB-70 *in vivo* and in the NADH dehydrogenase assay, as measured in disrupted mitochondria (IC_{50} 6.3 $\mu mol\,L^{-1}$), but it showed reduced efficacy on the inhibition of oxygen consumption as measured in intact mitochondria (IC_{50} 138 $\mu mol\,L^{-1}$) and no significant effect on glucose uptake as measured in whole cells. These results suggest that the compound may not penetrate membrane lipids very efficiently and therefore not influence the AMP to ATP ratio over the time course of the cellular experiments. This inconsistency could, however, be the result of the nature and time differences between the two kinds of experiment. It may well be that RTB-70 has a longer residence time in the animals and is therefore able to partition successfully into cells to produce similar responses over a time frame of weeks rather than hours. There is also the theoretical possibility that the compound may be metabolized *in vivo* to a more permeable/active derivative.

All the parameters studied thus far showed that these compounds have the potential to have much more effective anti-diabetic effect at a very much lower dose than metformin. The lower doses may prevent some of the intestinal problems associated with the higher doses required with metformin treatment (Florez *et al*. 2010, Kirpichnikov *et al*. 2002); however, both preclinical and clinical testing would need to be carried out to confirm this. The mechanism of action (schematic: Fig. 7) appears not to be as an insulin sensitizer but rather as an insulin substitute, and in that respect may also be of value to individuals with type 1 diabetes. Since there also promises to be a positive effect on weight, that in itself might attenuate the deleterious effects of excess visceral adipose tissue. It is possible in the future that these compounds can become an important tool in the treatment of type 2 diabetes.

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Supplementary data
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Declaration of interest
The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have declared that no competing interests exist.

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Author contribution statement
JBCF, JCS, RKP, AS, DSDM and CJB conceived and designed the experiments; DSDM, SL, RD, CR, CJB, GKK, TSM, and MFR performed the experiments; DSDM, SL, RD, CR, and GKK analyzed the data and DSDM, SL, GKK, JCS and JBCF wrote the paper.

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References

Bailey CJ & Turner RC 1996 Metformin. *New England Journal of Medicine* **334** 574–579.

Bowlby MR, Peri R, Zhang H & Dunlop J 2008 hERG (KCNH2 or Kv11.1) K + channels: Screening for cardiac arrhythmia risk. *Current Drug Metabolism* **9** 965–970. (doi:10.2174/138920008786485083)

Breen EP, Gouin SG, Murphy AF, Haines LR, Jackson AM, Pearson TW, Murphy PV & Porter RK 2006 On the mechanism of mitochondrial uncoupling protein 1 function. *Journal of Biological Chemistry* **281** 2114–2119. (doi:10.1074/jbc.M511575200)

Bridges HR, Jones AJ, Pollak MN & Hirst J 2014 Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochemical Journal* **462** 475–487. (doi:10.1042/BJ20140620)

Brown JB, Pedula K, Barzilay J, Herson MK & Latare P 1998 Lactic acidosis rates in type 2 diabetes. *Diabetes Care* **21** 1659–1663. (doi:10.2337/diacare.21.10.1659)

Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R, Roden M, Gnaiger E, Nohl H, Waldhäusl W, *et al*. 2004 Thiazolidinediones, like metformin, inhibit respiratory complex I: A common mechanism contributing to their antidiabetic actions? *Diabetes* **53** 1052–1059. (doi:10.2337/diabetes.53.4.1052)

Chappell J & Hansford R 1972 Preparation of mitochondria from animal tissues and yeasts. In *Subcellular components: Preparation and fractionation*, Second Edition, pp 77–91. Ed G Birnie. Baltimore, MD, USA: University Park Press.

Donnan PT, MacDonald TM & Morris AD 2002 Adherence to prescribed oral hypoglycaemic medication in a population of patients with Type 2 diabetes: A retrospective cohort study. *Diabetic Medicine* **19** 279–284. (doi:10.1046/j.1464-5491.2002.00689.x)

El-Mir MY, Nogueira V, Fontaine E, Avéret N, Rigoulet M & Leverve X 2000 Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *Journal of Biological Chemistry* **275** 223–228. (doi:10.1074/jbc.275.1.223)

Elliott DJ, Dondas NY, Munsey TS & Sivaprasadarao A 2009 Movement of the S4 segment in the hERG potassium channel during membrane depolarization. *Molecular Membrane Biology* **26** 435–447. (doi:10.3109/09687680903321081)

Fendel U, Tocilescu MA, Kerscher S & Brandt U 2008 Exploring the inhibitor binding pocket of respiratory complex I. *Biochimica et Biophysica Acta* **1777** 660–665. (doi:10.1016/j.bbabio.2008.04.033)

Florez H, Luo J, Castillo-Florez S, Mitsi G, Hanna J, Tamariz L, Palacio A, Nagendran S & Hagan M 2010 Impact of metformin-induced gastrointestinal symptoms on quality of life and adherence in patients with type 2 diabetes. *Postgraduate Medicine* **122** 112–120. (doi:10.3810/pgm.2010.03.2128)

Fontaine E 2014 Metformin and respiratory chain complex I: The last piece of the puzzle? *Biochemical Journal* **463** e3–e5. (doi:10.1042/BJ20141020)

Jenkins Y, Sun TQ, Markovtsov V, Foretz M, Li W, Nguyen H, Li Y, Pan A, Uy G, Gross L *et al*. 2013 AMPK activation through mitochondrial regulation results in increased substrate oxidation and improved metabolic parameters in models of diabetes. *PLoS One* **8** e81870. (doi:10.1371/journal.pone.0081870)

Jørgensen SB, Nielsen JN, Birk JB, Olsen GS, Viollet B, Andreelli F, Schjerling P, Vaulont S, Hardie DG, Hansen BF *et al*. 2004 The alpha2–5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. *Diabetes* **53** 3074–3081. (doi:10.2337/diabetes.53.12.3074)

Kirpichnikov D, McFarlane SI & Sowers JR 2002 Metformin: An update. *Annals of Internal Medicine* **137** 25–33. (doi:10.7326/0003-4819-137-1-200207020-00009)

Kumar N & Dey CS 2002 Metformin enhances insulin signalling in insulin-dependent and-independent pathways in insulin resistant muscle cells. *British Journal of Pharmacology* **137** 329–336. (doi:10.1038/sj.bjp.0704878)

Li DJ, Huang F, Lu WJ, Jiang GJ, Deng YP & Shen FM 2015 Metformin promotes irisin release from murine skeletal muscle independently of AMP-activated protein kinase activation. *Acta Physiologica* **213** 711–721. (doi:10.1111/apha.12421)

Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, Prigaro BJ, Wood JL, Bhanot S, MacDonald MJ *et al*. 2014 Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* **510** 542–546. (doi:10.1038/nature13270)

Marsin AS, Bouzin C, Bertrand L & Hue L 2002 The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. *Journal of Biological Chemistry* **277** 30778–30783. (doi:10.1074/jbc.M205213200)

Matsui Y, Hirasawa Y, Sugiura T, Toyoshi T, Kyuki K & Ito M 2010 Metformin reduces body weight gain and improves glucose intolerance in high-fat diet-fed C57BL/6 J mice. *Biological and Pharmaceutical Bulletin* **33** 963–970. (doi:10.1248/bpb.33.963)

Matsuzaki S & Humphries KM 2015 Selective inhibition of deactivated mitochondrial complex I by biguanides. *Biochemistry* **54** 2011–2021. (doi:10.1021/bi501473h)

Miller RA, Chu Q, Xie J, Foretz M, Viollet B & Birnbaum MJ 2013 Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* **494** 256–260. (doi:10.1038/nature11808)

Misbin RI 1977 Phenformin-associated lactic acidosis: Pathogenesis and treatment. *Annals of Internal Medicine* **87** 591–595. (doi:10.7326/0003-4819-87-5-591)

Munday MR, Campbell DG, Carling D & Hardie DG 1988 Identification by amino acid sequencing of three major regulatory phosphorylation sites on rat acetyl-CoA carboxylase. *European Journal of Biochemistry* **175** 331–338. (doi:10.1111/j.1432-1033.1988.tb14201.x)

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DOI: 10.1530/JME-15-0225

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Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, Zhou G, Williamson JM, Ljunqvist O, Efendic S *et al*. 2002 Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes* **51** 2074–2081. (doi:10.2337/diabetes.51.7.2074)

Owen MR, Doran E & Halestrap AP 2000 Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochemical Journal* **348** Pt 3 607–614. (doi:10.1042/bj3480607)

Pehmøller C, Treebak JT, Birk JB, Chen S, Mackintosh C, Hardie DG, Richter EA & Wojtaszewski JF 2009 Genetic disruption of AMPK signaling abolishes both contraction- and insulin-stimulated TBC1D1 phosphorylation and 14–3-3 binding in mouse skeletal muscle. *Acta Physiologica* **297** E665–E675. (doi:10.1152/ajpendo.00115.2009)

Piel S, Ehinger JK, Elmér E & Hansson MJ 2015 Metformin induces lactate production in peripheral blood mononuclear cells and platelets through specific mitochondrial complex I inhibition. *Acta Physiologica* **213** 171–180. (doi:10.1111/apha.12311)

Priest BT, Bell IM & Garcia ML 2008 Role of hERG potassium channel assays in drug development. *Channels* **2** 87–93. (doi:10.4161/chan.2.2.6004)

Reynafarje B, Costa LE & Lehninger AL 1985 O2 solubility in aqueous media determined by a kinetic method. *Analytical Biochemistry* **145** 406–418. (doi:10.1016/0003-2697(85)90381-1)

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ & Klenk DC 1985 Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150** 76–85. (doi:10.1016/0003-2697(85)90442-7)

Wang DS, Kusuhara H, Kato Y, Jonker JW, Schinkel AH & Sugiyama Y 2003 Involvement of organic cation transporter 1 in the lactic acidosis caused by metformin. *Molecular Pharmacology* **63** 844–848. (doi:10.1124/mol.63.4.844)

Wessels B, Ciapaite J, van den Broek NM, Nicolay K & Prompers JJ 2014 Metformin impairs mitochondrial function in skeletal muscle of both lean and diabetic rats in a dose-dependent manner. *PLoS One* **9** e100525. (doi:10.1371/journal.pone.0100525)

Winder WW & Hardie DG 1996 Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. *American Journal of Physiology* **270** E299–E304.

Winder WW, Wilson HA, Hardie DG, Rasmussen BB, Hutber CA, Call GB, Clayton RD, Conley LM, Yoon S & Zhou B 1997 Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *Journal of Applied Physiology* **82** 219–225.

Yun J, Rago C, Cheong I, Pagliarini R, Angenendt P, Rajagopalan H, Schmidt K, Willson JK, Markowitz S, Zhou S *et al*. 2009 Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. *Science* **325** 1555–1559. (doi:10.1126/science.1174229)

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N *et al*. 2001 Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation* **108** 1167–1174. (doi:10.1172/JCI13505)

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