LETTERS

Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes

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Calorie restriction extends lifespan and produces a metabolic profile desirable for treating diseases of ageing such as type 2 diabetes[1,2]. SIRT1, an NAD^+-dependent deacetylase, is a principal modulator of pathways downstream of calorie restriction that produce beneficial effects on glucose homeostasis and insulin sensitivity[3–9]. Resveratrol, a polyphenolic SIRT1 activator, mimics the anti-ageing effects of calorie restriction in lower organisms and in mice fed a high-fat diet ameliorates insulin resistance, increases mitochondrial content, and prolongs survival[10–14]. Here we describe the identification and characterization of small molecule activators of SIRT1 that are structurally unrelated to, and 1,000-fold more potent than, resveratrol. These compounds bind to the SIRT1 enzyme–peptide substrate complex at an allosteric site amino-terminal to the catalytic domain and lower the Michaelis constant for acetylated substrates. In diet-induced obese and genetically obese mice, these compounds improve insulin sensitivity, lower plasma glucose, and increase mitochondrial capacity. In Zucker *fa/fa* rats, hyperinsulinaemic-euglycaemic clamp studies demonstrate that SIRT1 activators improve whole-body glucose homeostasis and insulin sensitivity in adipose tissue, skeletal muscle and liver. Thus, SIRT1 activation is a promising new therapeutic approach for treating diseases of ageing such as type 2 diabetes.

To identify activators of human SIRT1 a high-throughput *in vitro* fluorescence polarization assay was developed and used to screen a large collection of small molecules. The hits identified were structurally distinct from resveratrol[11] and subsequently optimized for *in vitro* enzyme activity using a high-throughput mass spectrometry assay. Potency was tracked by determining the concentration of compound required to increase enzyme activity by 50% ($EC_{1.5}$) and the percentage maximum activation achieved at the highest doses of compound tested (resveratrol $EC_{1.5} = 46.2$ μM and maximum activation = 201%; SRT1460 $EC_{1.5} = 2.9$ μM and maximum activation = 447%; SRT2183 $EC_{1.5} = 0.36$ μM and maximum activation = 296%; and SRT1720 $EC_{1.5} = 0.16$ μM and maximum activation = 781%) (Fig. 1a, b). The variation in maximum activation is reminiscent of agonist activity observed with receptors, where compounds can vary in their ability to mediate full receptor activation as compared to an endogenous ligand[15]. These compounds are selective for activation of SIRT1 versus the closest sirtuin homologues, SIRT2 and SIRT3 (SIRT2: resveratrol, SRT1460 and SRT2183 $EC_{1.5} > 300$ μM, SRT1720 $EC_{1.5} = 37$ μM; SIRT3: resveratrol, SRT1460, SRT1720 and SRT2183 $EC_{1.5} > 300$ μM).

The compounds were tested for functional activity in a SIRT1 cell-based (U2OS cells) deacetylation assay (Fig. 1c)[16,17]. SRT2183 was used as a positive control because it is well tolerated in cellular assays. The SIRT1 activators decreased the acetylation state of p53, a known SIRT1 substrate. A selective SIRT1 inhibitor, 6-chloro-2,3,4,9-tetrahydro-1-*H*-carbazole-1-carboxamide[18], abrogated the deacetylation activity, indicating that the effect was dependent on SIRT1.

The *Sir2* gene in *Saccharomyces cerevisiae* and *Drosophila melanogaster* extends lifespan when overexpressed[19,20], and the ability of

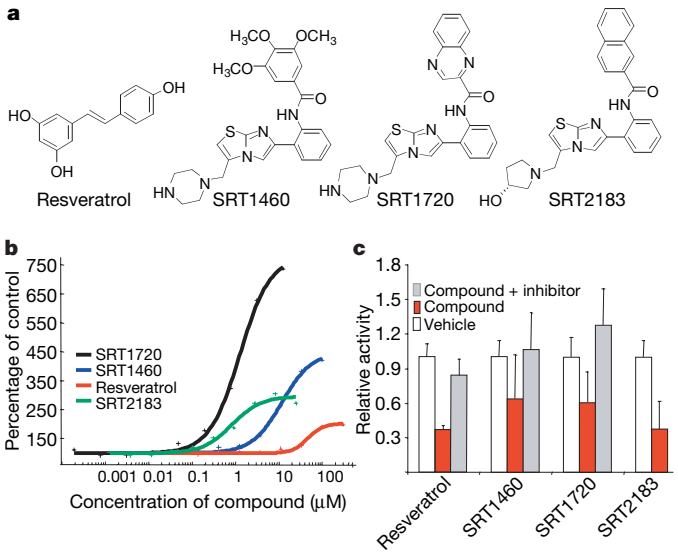

Figure 1 | Identification of potent SIRT1 activators unrelated to resveratrol. **a**, Chemical structures of SIRT1 activators, resveratrol, SRT1460, SRT2183, and SRT1720. **b**, The effect of activators on human SIRT1 enzyme activity measured by mass spectrometry. **c**, Cellular activity was measured using an ICW that monitors the degree of p53 deacetylation in U2OS cells using β-tubulin as a normalization control. Compounds were tested at concentrations of: resveratrol (100 μM), SRT2183 (10 μM), SRT1460 (10 μM), SRT1720 (0.10 μM). Each concentration represents the approximate EC_{50} for each compound. $n = 6$ for all compounds tested except for resveratrol, where $n = 3$. Data are expressed as mean ± s.d. The activation of SIRT1 resulting in p53 deacetylation could be blocked by a SIRT1 inhibitor, 6-chloro-2,3,4,9-tetrahydro-1-*H*-carbazole-1-carboxamide (10 μM). $n = 3$ for all compounds tested.

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calorie restriction to extend lifespan is abrogated when the gene is deleted[20–22]. We have observed lifespan extension in yeast with analogues structurally related to SRT1460 that activate the yeast SIR2 enzyme (data not shown).

To address the mechanism of activation of the human SIRT1 enzyme, we determined the effect of compound on the V_{max} (velocity of enzyme-catalysed reaction at infinite concentration of substrate) and the K_m (Michaelis constant) of SIRT1 for its two substrates, NAD^+ and acetylated peptide. None of the compounds affected the K_m for NAD^+ or the V_{max} (data not shown); however, all compounds decreased the K_m of SIRT1 for acetylated peptide substrate (Fig. 2a). The magnitude of the K_m effect positively correlated with the $EC_{1.5}$ values for the compounds. A similar K_m-type activation mechanism has been proposed for small molecule activators that bind to an allosteric site of glucokinase[23].

The energetics of binding of SRT1460 to purified SIRT1 enzyme were studied by isothermal titration calorimetry. The titration of SRT1460 against purified human SIRT1-C did not result in detectable binding (data not shown). In the presence of acetylated peptide substrate SRT1460 exhibited signs of protein-binding-site saturation best fitting a one site binding model (Fig. 2b). The dissociation constant (K_d) for SRT1460 was 16.2 μM and the enthalpy (ΔH) was −6.1 kcal mol^{-1}. The data suggest that SRT1460, and other related SIRT1 activators, bind to a SIRT1–peptide substrate complex and promote a more productive conformation that enhances catalytic activity. Binding of substrate may induce a conformational change that leads to the exposure of an allosteric binding site. In support of this hypothesis, it has been demonstrated that NAD^+ does not bind in the absence of acetylated peptide substrate to a closely related sirtuin, SIRT2 (ref. 24). A K_m-type mechanism is supported by isothermal titration calorimetry experiments that show a roughly twofold increase in the affinity for peptide substrate binding to SIRT1-C in the presence of SRT1460 ($K_{d(-SRT1460)} = 25.7$ μM and $K_{d(+SRT1460)} = 14.1$ μM; data not shown).

To determine whether SRT1460 and SRT1720 bind and activate the enzyme at the same molecular site as resveratrol, an isobologram analysis was performed. A concentration matrix of two compounds, resveratrol versus SRT1720 and SRT1720 versus SRT1460, was examined to determine whether the combination was antagonistic, additive or synergistic. In both cases the compound combination resulted in additivity consistent with the hypothesis that a single allosteric site exists on the SIRT1–substrate complex to which structurally diverse compounds can bind (Fig. 2c).

To define the compound binding site a series of SIRT1 truncation mutants was generated (Fig. 2d). SIRT1-A (156–664) and SIRT1-B (172–664) retain similar activity and activation to the parent construct (SIRT1-C), whereas SIRT1-D (219–664) exhibits a marked decrease in enzyme activity. SIRT1-D expresses poorly in *Escherichia coli* and may lose activity simply due to misfolding. The SIRT1-E (225–664), SIRT1-F (230–664), SIRT1-G (235–664), and SIRT1-H (240–664) constructs exhibit enzymatic activity comparable to SIRT1-C, but the ability to be activated by SRT1720 and resveratrol is significantly decreased. The SIRT1 truncation data suggest that amino acids 240–664 comprise the core catalytic domain, and that the amino acids 183–225 N-terminal to the core domain are important in defining the compound binding site.

The therapeutic potential of SIRT1 activators to treat insulin resistance and diabetes was tested in three *in vivo* models of type 2 diabetes. SRT1720 exhibited a pharmacokinetic profile (Fig. 3a) suitable for *in vivo* evaluation in both mouse (bioavailability = 50%, terminal $t_{1/2} = \sim 5$ h, Area Under the Curve (AUC) = 7,892 ng h^{-1} ml^{-1}) and rat (bioavailability = 25%, terminal $t_{1/2} = \sim 8.4$ h, AUC = 3,714 ng h^{-1} ml^{-1}). SRT501, a reformulated version of resveratrol with improved bioavailability (11% bioavailability, terminal $t_{1/2}$ of ~ 1 h and an AUC of 10,524 ng h^{-1} ml^{-1}), was also examined in genetically obese mice ($Lep^{ob/ob}$) and diet-induced obesity (DIO) mice. SRT501 (500 or 1,000 mg per kg (body weight)) and SRT1720 (100 mg per kg (body weight)) were dosed in all efficacy studies once daily by oral gavage. During these studies we did not measure food intake or metabolic rates so there may be uncertainty about how the compound is working. We did measure body weight during these studies and this did not change.

In DIO mice, fasting blood glucose levels are elevated (120–150 mg dl^{-1} range) after being placed on a high-fat diet. Administration of SRT1720 reduced fed glucose levels after 1 week of treatment with further reduction after 3 weeks of treatment (Fig. 3b) that continued through 10 weeks of dosing. Glucose excursion during an intraperitoneal glucose tolerance test was also significantly reduced in the SRT1720 group, and comparable to rosiglitazone, a PPARγ activator that has been used to treat type 2 diabetes (Fig. 3c; glucose AUC: control, 603 ± 32 mg h^{-1} dl^{-1}; SRT1720, 462 ± 25 mg h^{-1} dl^{-1}; rosiglitazone, 496 ± 20 mg h^{-1} dl^{-1}). SRT1720 did not have an effect on fasting glucose in chow-fed mice (data not shown), indicating that pharmacological SIRT1 activation is unlikely to induce hypoglycaemia.

DIO mice are insulin resistant and hyperinsulinaemic (3.9 ± 0.7 ng dl^{-1}) compared to normal chow fed controls (0.4 ± 0.1 ng dl^{-1}). SRT1720 significantly reduced the hyperinsulinaemia after 4 weeks, partially normalizing elevated insulin levels similar to rosiglitazone treatment (Fig. 3d). Consistent with the effects on improved glucose tolerance and fasting insulin levels, a reduction in glucose levels during an insulin tolerance test was greater in SRT1720 or rosiglitazone treated animals (Fig. 3e). SRT1720 did not reduce insulin levels in

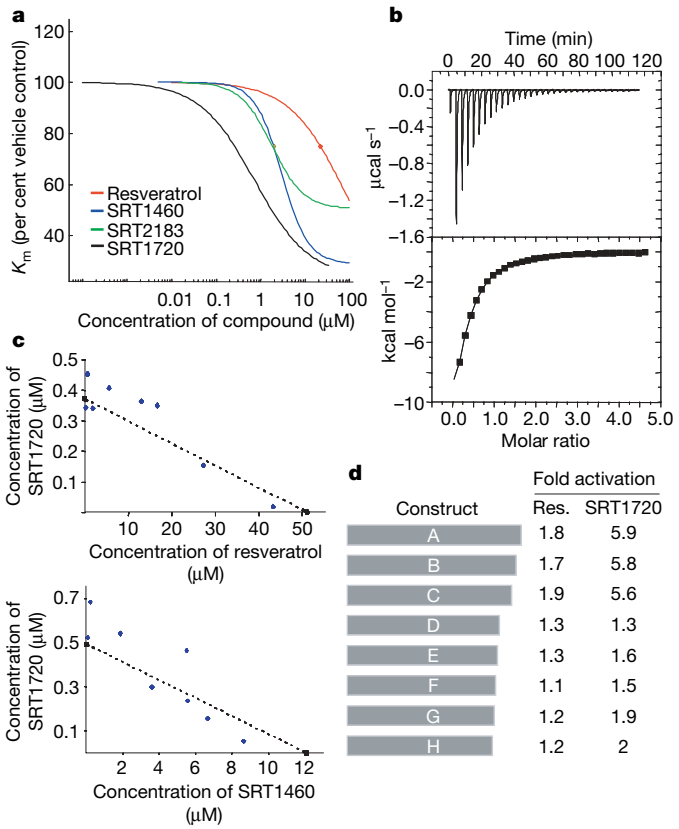

Figure 2 | *In vitro* characterization of activators of human SIRT1. **a**, The effect of SIRT1 activators on peptide substrate K_m. **b**, Calorimetric titrations of SIRT1-C–peptide substrate complex with the activator SRT1460. Top panel: heat of binding SRT1460 to enzyme–peptide complex. Bottom panel: integrated fit with a one-site binding model. **c**, Isobologram analysis of resveratrol versus SRT1720 and SRT1720 versus SRT1460. The experimental data are best fit to the theoretical line of additivity (dashed line). **d**, SIRT1 N-terminal truncations define the allosteric compound binding site. The ability of resveratrol and SRT1720 to activate SIRT1 was examined against a series of N-terminal deletions in the mass spectrometry assay.

mice fed a normal chow diet that are not insulin resistant (Fig. 3d). The effect of SRT1720 on blood glucose and insulin levels is not secondary to weight loss as there was no difference in the weight of mice treated with SRT1720 compared to vehicle-treated DIO mice (data not shown).

As observed in calorie restriction[6,25], SRT1720 treatment (10 weeks) increases mitochondrial capacity by 15% in gastrocnemius muscle as measured by citrate synthase activity (Fig. 3f). In DIO mice SRT1720 mimics several of the effects observed after calorie restriction including improved insulin sensitivity, normalized glucose and insulin levels, and elevated mitochondrial capacity.

To study SRT1720 in a genetic model of type 2 diabetes, *Lep*$^{ob/ob}$ mice were placed on a high-fat diet to induce a diabetic state and then dosed with SRT1720. SRT1720 treatment significantly reduced fasting blood glucose to near normal levels (Fig. 3g) after 1 week. SRT501, a much less potent SIRT1 activator, also lowered fasting blood glucose in *Lep*$^{ob/ob}$ mice after 3 weeks (1,000 mg per kg (body weight)) and in DIO mice after 4 weeks (500 mg per kg (body-weight)) of oral dosing (Fig. 3h, i). SRT501 also reduces hyperinsulinaemia in DIO mice measured as fed insulin levels (data not shown). As observed with SRT1720, no difference in body weight was evident after treatment with SRT501 in *Lep*$^{ob/ob}$ mice.

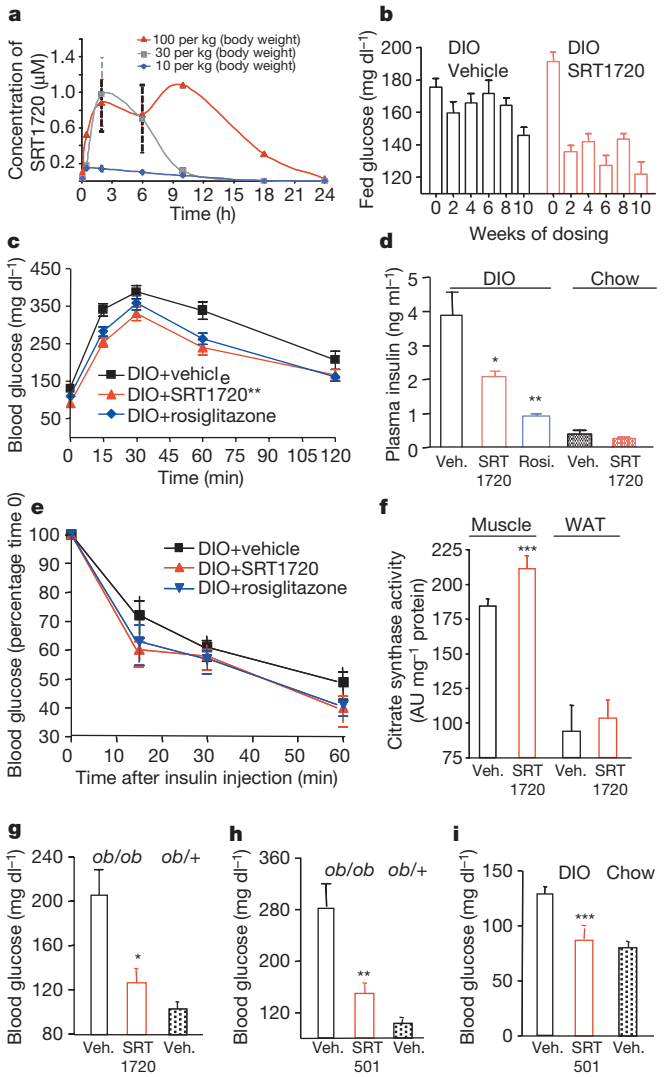

Figure 3 | SIRT1 activators in mouse models of type 2 diabetes. **a**, Plasma levels of SRT1720 after administration by oral gavage. **b**, Effect of SRT1720 treatment over 10 weeks on fed plasma glucose levels in DIO mice. **c**, Glucose excursion during an intraperitoneal glucose tolerance test (5 weeks) in DIO mice treated with the indicated compounds. **d**, Plasma insulin levels after 10 weeks treatment with indicated compounds. **e**, Glucose response in DIO mice during an insulin tolerance test after 10 weeks treatment with the indicated compounds. **f**, Skeletal muscle citrate synthase activity (V_{max} mg^{-1} protein, 11 weeks, $n = 5$). **g**, Effect of SRT1720 treatment in diabetic *Lep*$^{ob/ob}$ mice (1 week). **h**, Effect of SRT501 treatment (1,000 mg per kg (body weight)) in diabetic *Lep*$^{ob/ob}$ mice after 2 weeks. **i**, Effect of SRT501 treatment (500 mg per kg (body weight), 4 weeks) in DIO mice. All studies consisted of ten mice per group unless noted. Statistics were conducted as an ANOVA; asterisk $P < 0.05$, double asterisk $P < 0.01$ and triple asterisk $P < 0.001$. Data are expressed as mean ± s.e.m.

We also performed *in vivo* studies in the Zucker *fa/fa* rat, which is a genetically obese rodent model for studying insulin resistance. Rats were treated with vehicle or SRT1720 for 4 weeks. Body weight was not different between the two groups throughout the study. After 3 weeks treatment, fed blood glucose was significantly lower in *fa/fa* rats (Fig. 4a) treated with SRT1720 compared to vehicle-treated (140 ± 5 versus 110 ± 3 mg dl^{-1}, vehicle versus SRT1720, $P < 0.001$). In support of improved glucose metabolism after SIRT1 activation, both the glucose (AUC: 4,550 ± 463 versus 2,490 ± 236 mg min^{-1}dl^{-1}, vehicle versus SRT1720, $P < 0.01$) and insulin (AUC: 277 ± 32 vs. 127 ± 25 ng min^{-1}dl^{-1}, vehicle versus SRT1720, $P < 0.01$) responses during an oral glucose tolerance test were significantly improved in the SRT1720-treated group (Fig. 4b, c). The decrease in glucose in the face of reduced hyperinsulinaemia indicates improved insulin sensitivity. To assess more definitively insulin action after SRT1720 treatment, hyperinsulinaemic-euglycaemic clamps were performed on *fa/fa* rats after 4 weeks of treatment. Consistent with improved glucose tolerance, the glucose infusion rate required to maintain euglycaemia was ~35% higher in SRT1720-treated *fa/fa* rats (Fig. 4d, $P < 0.01$), and the total glucose disposal rate was increased by ~20% (Fig. 4e, $P < 0.05$). The insulin-stimulated glucose disposal rate, which primarily represents glucose disposal into skeletal muscle, was 55% higher in the SRT1720-treated group (Fig. 4f, $P < 0.05$). Along with the increased glucose disposal rate and glucose infusion rate, liver insulin sensitivity was significantly enhanced in the SRT1720 group, as evidenced by a greater suppression of hepatic glucose production during the clamp (Fig. 4g, $P < 0.05$). Adipose tissue insulin sensitivity was also improved after SIRT1 activation (Fig. 4h), as indicated by lower (~40%) plasma free fatty acid concentration during the clamp in the SRT1720-treated group (0.48 ± 0.03 versus 0.82 ± 0.10 mmol l^{-1}, SRT1720 versus vehicle, $P < 0.05$).

Recent studies have suggested that SIRT1 might be important in regulating hepatic gluconeogenesis, through deacetylation and activation of PGC-1α[26,27]. However, transgenic mice that overexpress SIRT1 exhibit improved insulin sensitivity and are not hyperglycaemic[28]. To investigate if SRT1720 treatment alters hepatic gluconeogenesis, *fa/fa* rats were subjected to a pyruvate tolerance test (PTT) after 3 weeks of treatment. The glucose response during the PTT was markedly blunted in SRT1720-treated *fa/fa* rats relative to vehicle (Fig. 4i), demonstrating decreased hepatic gluconeogenesis. These results show that the overall *in vivo* effect of SIRT1 activation is to ameliorate the heightened gluconeogenic capacity of Zucker *fa/fa* rats and not to stimulate hepatic glucose production. In addition, because Zucker *fa/fa* rats exhibit hepatic insulin resistance, the decreased gluconeogenesis after SRT1720 treatment is further evidence of improved hepatic insulin sensitivity.

To address the therapeutic potential of SIRT1 activation for the improvement of age-related diseases, we have identified and characterized novel small molecule activators of SIRT1 both *in vitro* and *in vivo*. These compounds are 1,000-fold more potent activators than, and structurally unrelated to, resveratrol. Similar to resveratrol, these compounds bind directly to the SIRT1-acetylated peptide complex at the same site and lower the K_m for peptide substrate resulting in a more productive catalytic complex. SIRT1 deletion experiments indicate that amino acids 183–225 are critical for maintaining activation of SIRT1 by these compounds and define the allosteric binding site. It is possible that acetylated peptide binding to SIRT1 induces a

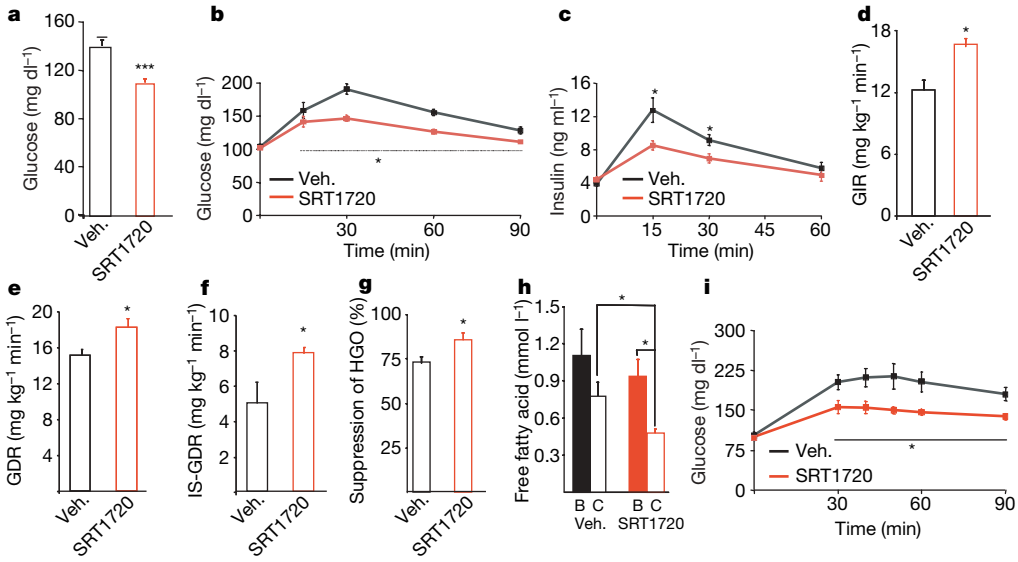

Figure 4 | SIRT1 activator SRT1720 in the Zucker *fa/fa* rat model. **a**, Post-absorptive blood glucose (3 weeks). **b**, **c**, Glucose and insulin responses during an oral glucose tolerance test (3.5 weeks, ANOVA). After 4 weeks a hyperinsulinaemic-euglycaemic clamp study was conducted. **(d–f)** Glucose infusion rate (GIR), glucose disposal rate (GDR) and insulin-stimulated glucose disposal rate (IS-GDR) were significantly enhanced. **g**, Hepatic glucose output (HGO) suppression (%). **h**, Plasma fatty acid concentration during the clamp was significantly lower in SRT1720-treated animals (ANOVA). B, basal conditions. C, clamp conditions. **i**, Glucose excursion during a PTT was reduced, indicating reduced gluconeogenic capacity. All studies consisted of $n \geq 5$ rats per group. Statistics were conducted as student *t*-test unless noted otherwise; asterisk $P < 0.05$, double asterisk $P < 0.01$, and triple asterisk $P < 0.001$. Data are expressed as mean ± s.e.m.

conformational change that exposes an allosteric site in this region of the enzyme. An endogenous regulator of SIRT1 has yet to be identified, and it is tempting to speculate that an endogenous activator of SIRT1 exists and may be increased after calorie restriction and other mild physiological stresses. Selective SIRT1 activators ablate insulin resistance and diabetes in DIO mice fed a high-fat diet and in diabetic $Lep^{ob/ob}$ mice. In addition, these new SIRT1 activators ameliorate the metabolic disturbances in Zucker *fa/fa* rats. SIRT1 activators improve glucose homeostasis and insulin sensitivity in key metabolic tissues including liver, muscle and fat. These compounds seem to mimic the beneficial effects of calorie restriction on mitochondrial and metabolic function in mammals *in vivo* and hold promise for treating diseases of ageing such as type 2 diabetes.

METHODS SUMMARY

The full details of the materials and methods are presented in the Supplementary Information. The compounds, SRT1460, SRT1720, and SRT2183, were synthesized at Sirtris Pharmaceuticals. SIRT1 constructs were expressed in *E. coli* and purified by affinity, size exclusion, and/or ion exchange chromatography. The SIRT1 fluorescence polarization assay used a peptide based on p53 linked to biotin at the N-terminal end and to a fluorescent tag at the C-terminal end. The reaction was a coupled enzyme assay where the first reaction was the deacetylation reaction and the second was cleavage by trypsin at the exposed lysine. The mass spectrometry assay was used for all SAR studies and employs the same peptide as the HTS assay. For the in cell western assay (ICW) the acetylation state of p53 was determined using a polyclonal antibody against the acetylated lysine residue at position 382 in p53. The effect of test compounds on the K_m of SIRT1 for peptide substrate was examined using the mass spectrometry assay. Isothermal titration calorimetry was performed using a VP-ITC (MicroCal, Inc.). For the isobologram analysis a concentration matrix of two compounds was created and tested against SIRT1 using the mass spectrometry assay. For pharmacokinetic studies compounds were administered by oral gavage or intravenously to C57BL/6 male mice at the doses indicated. For all *in vivo* efficacy studies vehicle or test compound was administered once daily by oral gavage. For the DIO studies, C57BL/6 male mice were fed a high-fat diet until their mean body weight reached approximately 40 g. $Lep^{ob/ob}$ mice and heterozygous Lep^{ob+} mice were placed on a high-fat diet for a minimum of 1 week before the start of a study and remained on the high-fat diet for the duration of the study. Male fatty (*fa/fa*) Zucker (ZF) rats (Harlan Sprague–Dawley, Inc.) were used for the studies.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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