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Functional properties of Claramine: A novel PTP1B inhibitor and insulin-mimetic compound

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ABSTRACT

Protein tyrosine phosphatase 1B (PTP1B) inhibits insulin signaling, interfering with its control of glucose homeostasis and metabolism. PTP1B activity is elevated in obesity and type 2 diabetes and is a major cause of insulin resistance. Trodusquemine (MSI-1436) is a "first-in-class" highly selective inhibitor of PTP1B that can cross the blood–brain barrier to suppress feeding and promote insulin sensitivity and glycemic control. Trodusquemine is a naturally occurring cholestane that can be purified from the liver of the dogfish shark, *Squalus acanthias*, but it can also be manufactured synthetically by a fairly laborious process that requires several weeks. Here, we tested a novel easily and rapidly (2 days) synthesized polyaminosteroid derivative (Claramine) containing a spermino group similar to Trodusquemine for its ability to inhibit PTP1B. Like Trodusquemine, Claramine displayed selective inhibition of PTP1B but not its closest related phosphatase TC-PTP. In cultured neuronal cells, Claramine and Trodusquemine both activated key components of insulin signaling, with increased phosphorylation of insulin receptor-β (IRβ), Akt and GSK3β. Intraperitoneal administration of Claramine or Trodusquemine effectively restored glycemic control in diabetic mice as determined by glucose and insulin tolerance tests. A single intraperitoneal dose of Claramine, like an equivalent dose of Trodusquemine, suppressed feeding and caused weight loss without increasing energy expenditure. In summary, Claramine is an alternative more easily manufactured compound for the treatment of type II diabetes.

1. Introduction

The epidemic of obesity and diabetes is growing rapidly and has become a global problem affecting all age and socio-economic strata. The International Diabetes Federation has indicated that diabetes affects about 285 million people globally. This number is expected to climb to about 438 million by the year 2030 (http://www.idf.org/). Current therapies including Metformin, DPP4 inhibitors and GLP1 mimetics are not without shortcomings [1] and novel therapeutics for the treatment of obesity and diabetes are still in great demand.

Insulin, a hormone secreted by the pancreas after meals, is well known for its function to maintain glucose homeostasis. This is achieved by its actions at the central nervous system as well as at the peripheral tissues. Insulin signaling activates glucose uptake and storage into peripheral target tissues like skeletal muscle to lower blood glucose levels. At the same time, insulin signaling in the brain suppresses hepatic glucose production. This central effect of insulin depends upon activation of ATP-dependent potassium channels by phosphoinositide 3-kinase (PI3K) in orexigenic neurons of the hypothalamus, which in turn regulate efferent vagal innervation of the liver [2,3]. The hypothalamic action of insulin also regulates peripheral fat metabolism by modulating sympathetic innervation of white adipose tissue to inhibit lipolysis. This

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mechanism reduces the availability of gluconeogenic substrates to the liver and further contributes to limit hepatic glucose production [4]. In addition to glycemic control, central insulin signaling also suppresses food intake and body weight [5] by inducing neuropeptide gene expression and altering hypothalamic neuron activity (see review [6]).

The insulin receptor (IRβ) has intrinsic tyrosine kinase activity. Upon binding of insulin, IRβ phosphorylates tyrosyl residues of its own protein and of signaling molecules that elicit its downstream effects, including the insulin receptor substrate (IRS), PI3K and a key serine-threonine kinase Akt [6]. Phosphorylated and activated Akt then increases peripheral glucose uptake by increasing the number of GLUT4 transporters at the cell surface membrane [7]. Akt also phosphorylates and inactivates GSK3β to allow activation of glycogen synthetase and thereby glycogen synthesis, further lowering glucose levels in the blood [8]. In the hypothalamus, GSK3β inactivation also improves peripheral glucose homeostasis and suppresses feeding behavior [9].

An impaired response to insulin is a hallmark of obesity and type 2 diabetes due in large part to the unopposed increased activity of the protein tyrosine phosphatase PTP1B [6,10–13]. PTP1B is a key phosphatase that terminates insulin signaling by dephosphorylating its receptor and downstream signaling molecules IRS1 and PI3K [6,14,15]. Importantly, PTP1B-deficient mice are more sensitive to insulin, have improved glycemic control, and are more resistant to diet-induced obesity than wild-type mice [16]. Mice with ablation of PTP1B in the central nervous system [10] are also resistant to diet-induced obesity and diabetes. These observations point to the importance of PTP1B in the central and peripheral control of insulin signaling. Thus, PTP1B is an attractive target to treat metabolic syndrome.

Trodusquemine (MSI-1436), a first-in-class compound, is a highly selective inhibitor of PTP1B that crosses the blood–brain barrier [17–19]. Trodusquemine has a half-life of >1 week *in vivo* [17–19] and selectively blocks PTP1B activity in the brain and neuronal cells [13]. Trodusquemine is a spermine metabolite of cholesterol that was originally isolated from the liver of the dogfish shark, *Squalus acanthias*, during a search for naturally occurring antimicrobial compounds [17]. Trodusquemine has 200-fold selectivity for PTP1B compared to its closest homolog TC-PTP [18]. It promotes insulin and leptin signaling [18,19] and has undergone a phase I clinical trial for the treatment of obesity [18,20]. Although Trodusquemine can be manufactured synthetically, the process is labor-intensive and involves multiple steps over 4 weeks with limited yields [21,22]. Thus, identifying alternative analogs that are easier and less costly to manufacture while retaining the PTP1B-specific inhibitory properties is an important objective.

To this end, we synthesized an analog of Trodusquemine that we named Claramine and evaluated its potential to inhibit PTP1B and its effect on insulin signaling. We found that Claramine is a highly selective inhibitor of PTP1B activity with no apparent effect on its closest related phosphatase TC-PTP. Claramine, like Trodusquemine, activated phosphorylation of components of the insulin-response cascade (IRβ and Akt) required for glucose handling in neuronal cells. In a diabetic mouse model (CaMK2α Cre/LMO4flox mice) [12,13,23] in which LMO4, an endogenous inhibitor of PTP1B, is ablated in the glutamatergic neurons and results in hyperactivated PTP1B is observed in the hypothalamus, we found that both Claramine and Trodusquemine could improve insulin sensitivity on glucose homeostasis. Both compounds cause weight loss by suppressing food intake. Thus, Claramine may represent an improved compound for extended insulin sensitization.

2. Materials and methods

2.1. General procedure for the titanium–mediated reductive amination reaction of Claramine

A mixture of progesterone (123 mg, 0.39 mmol), titanium (IV) isopropoxide (573 μL, 2.02 mmol) and spermine (404 mg, 2 mmol) in absolute methanol (5 mL) was stirred under argon at room temperature for 12 h. Sodium borohydride (38 mg, 1 mmol) was then added at −78 °C and the resulting mixture was stirred for an additional 2 h. The reaction was then quenched by adding water (1 mL) and stirring was maintained at room temperature for 20 min. The resulting inorganic precipitate was filtered off over a pad of Celite and washed with methanol and ethylacetate. The combined organic extracts were dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford the expected crude amino derivative, which was purified by flash chromatography affording the expected amino derivative. Purification by column chromatography (silica gel; CH_2Cl_2/MeOH/NH_4OH (32%), 7:3:1) afforded a pale yellow solid in 45% yield; this compound can be converted subsequently into its hydrochloride salt as white solid $-^1$H NMR (300 MHz, CD_3OD): δ = 1.58 (s, 3H), 1.72 (s, 3H), 1.74 (s, 3H), 1.78–1.86 (m, 8H), 1.90–2.08 (m, 8H), 2.15–2.29 (m, 8H), 2.34–2.61 (m, 17H), 2.70–2.74 (m, 3H), 2.85–2.89 (m, 2H), 3.33–3.39 (m, 2H), 3.46–3.64 (m, 12H), 4.14–4.23 (m, 2H), 4.37–4.44 (m, 1H). $-^{13}$C NMR (75 MHz, CD_3OD): δ = 71.50, 58.96, 56.27, 56.05, 54.78, 49.98, 49.21, 47.99, 47.84, 47.34, 42.62, 40.47, 39.94, 39.49, 39.06, 36.44, 36.14, 35.86, 35.77, 35.63, 33.58, 31.61, 30.45, 28.18, 27.97, 24.37, 23.78, 22.78, 22.52, 21.03, 18.63, 16.30.12.13. $C_{37}H_{72}N_4O$; MS (ESI) m/z = 589.5 $[M+H]^+$.

2.2. Cell culture

F11 neuronal cells (a chimeric cell line of the mouse neuroblastoma cell line N18TG-2 fused with embryonic rat dorsal-root ganglion neurons) [24] can be easily cultured without special coating on the plates [25,26]. F11 cells were grown and maintained as described previously [27,28].

2.3. Phosphatase activity assays

PTP1B phosphatase activity was measured with the PhosphoSeek PTP1B Assay Kit (BioVision) in extracts from F11 cells according to the instructions of the manufacturer with PTP1B enzyme and phosphatase inhibitor as positive and negative controls, respectively [12,13]. T-cell protein tyrosine phosphatase (TC-PTP) activity was measured using the Human/Mouse/Rat Active TC-PTP DuoSet IC ELISA kit (Cat #DYC2468, R&D Systems) according to the manufacturer's instructions with appropriate controls [13].

2.4. Immunoblot analyses

Protein extraction and immunoblot analysis were performed as described previously [29–33]. All antibodies were obtained from Cell Signaling Inc. including primary antibodies specific to phosphorylated IRβ (pY1345), Akt (pS473), GSK3β (pS9). For total protein detection, antibodies specific to IRβ, Akt, GSK3β, β-actin were used.

2.5. Animal

CamK2αCre/LMO4flox (KO) and age-matched LMO4flox (WT) littermate mice were genotyped and maintained on a CD1 background under a regular chow, as described [13,32,34,35]. All

procedures were approved according to guidelines of the Canadian Council on Animal Care.

2.6. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

3.5-month-old male mice received intraperitoneal injection of Claramine or Trodusquemine dissolved in saline (5 mg/kg body weight) 24 h or 48 h prior to GTT or ITT, respectively. Mice were fasted overnight (~16 h) with access to water prior to GTT at 10:00. Basal blood glucose sampled from the saphenous vein was measured using a standard glucometer prior to and after mice received a glucose bolus (2 g/kg body weight of 20% D-glucose) by intraperitoneal injection as described previously [13]. A separate cohort of mice was fasted for 4 h prior to ITT, performed between 14:00 and 17:00. Human recombinant insulin (Sigma; Cat. #91077C), diluted in sterile saline was administered by intraperitoneal injection at 0.75 U/kg and blood glucose levels were monitored as above.

2.7. Food intake

Mice were transferred to individual housing and habituated for 2 days. Food intake was measured after intraperitoneal injection of saline or Claramine or Trodusquemine (both at 5 mg/kg body weight).

2.8. Indirect calorimetry

Mice were single-housed in metabolic chambers. After 2 h of habituation, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured for 24 h using an Oxymax system with automatic temperature and light controls (Columbus Instruments). Temperature was maintained at 24 °C with 12 h light/dark cycle. Heat production and locomotor activity were also ascertained, as described previously [35].

2.9. Statistical analyses

All results are expressed as mean ± SEM and analyzed using SPSS Software (IBM). For between-group comparisons, a two-tailed Student's t test was used. For body weight change, ANOVA with post hoc least significant difference test was used for between-group comparisons. $p < 0.05$ was considered significant.

3. Results

3.1. Claramine blocks PTP1B activity both in-vitro and in cultured neuronal cells

Claramine (Fig. 1A) is a novel compound related to Trodusquemine (Fig. 1B) that can be readily synthesized within 2 days. In

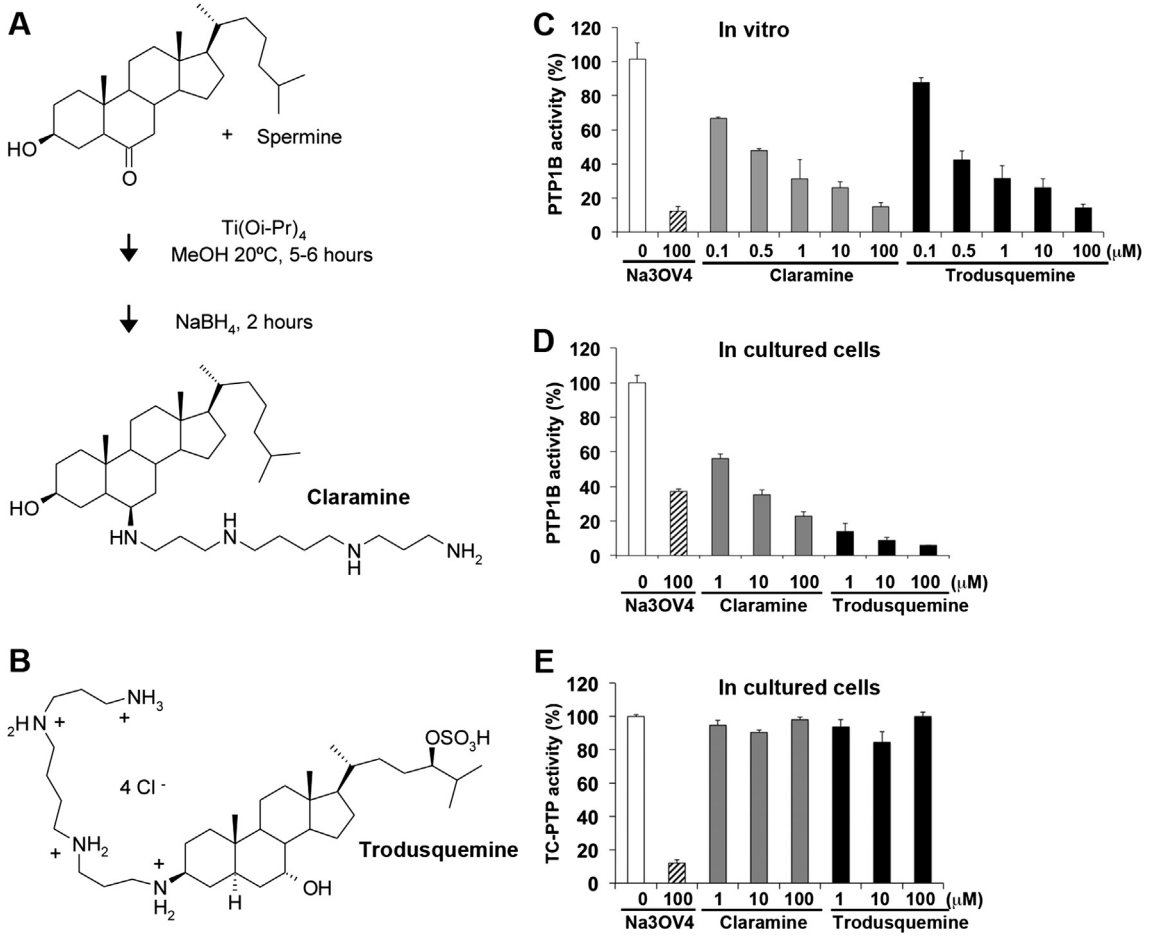

Fig. 1. Like Trodusquemine, Claramine inhibits PTP1B but not TC-PTP activity. Structure of Claramine (A) and Trodusquemine (B). For PTP1B activity studies, confluent neuronal F11 cells were serum-starved for 6 h prior to harvest. (C) Cell lysates were incubated with Claramine, Trodusquemine or the pan-phosphatase inhibitor sodium orthovanadate (Na_3OV_4) for 30 min at indicated doses in test tubes and then subjected to the PTP1B activity assay. (D,E) Serum-starved cells were treated with Claramine, Trodusquemine or Na_3OV_4 for 30 min and then cell lysates were harvested and subjected to the PTP1B (D) or TC-PTP (E) activity assays. Values are mean% ± SEM. n = 3 experiments per conditions.

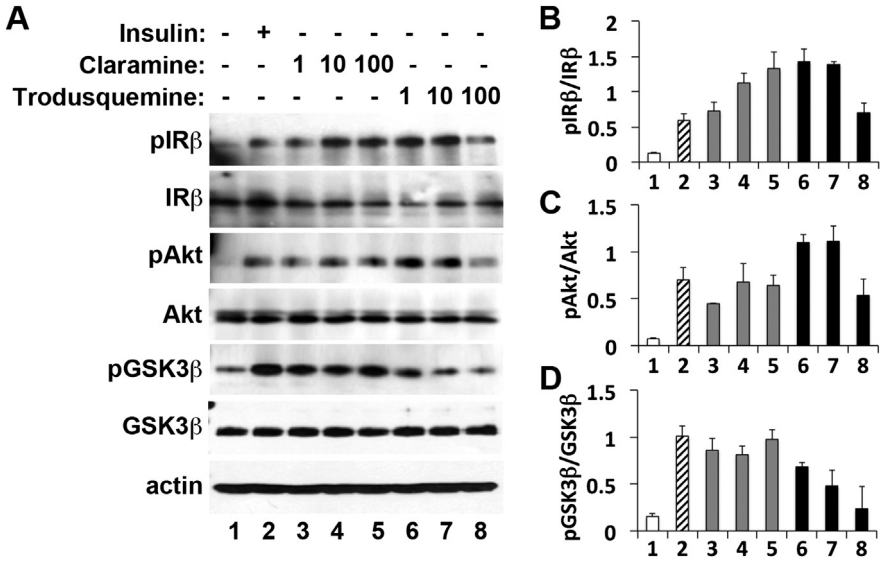

Fig. 2. Claramine and Trodusquemine have insulin-mimetic effects in neuronal cells. Confluent neuronal F11 cells were serum-starved for 6 h and then incubated with or without Claramine or Trodusquemine (1–100 μM as indicated) for 30 min at indicated doses. Controls cells were treated with or without insulin (100 nM) for 5 min prior to harvest (lanes 1 and 2). Equal amounts of protein were separated by SDS-PAGE and total or phosphorylated proteins were detected by immunoblot analysis (A) and quantitated using Image J software (B–D). Abbreviations are described in the Results.

contrast, Trodusquemine requires up to 4 weeks to be synthesized. To test whether Claramine had a similar effect on PTP1B activity as Trodusquemine, we conducted *in vitro* assays and experiments using cultured neuronal cells. We found that Claramine blocked PTP1B activity in whole cell lysates *in vitro* and in cultured cells. Both Claramine and Trodusquemine effectively blocked PTP1B activity in a dose-dependent manner when applied to whole cell lysates *in vitro*. Claramine achieved 50% PTP1B inhibition at a concentration of 0.5 μM, similar to Trodusquemine (Fig. 1C). However, when compounds were applied to intact living cells, Trodusquemine appeared to be more effective than Claramine at blocking PTP1B activity (Fig. 1D), suggesting a greater cellular permeability of Trodusquemine in the 30-min treatment window relative to Claramine.

3.2. Claramine has no effect on TC-PTP activity

TC-PTP is a closely related protein phosphatase to PTP1B [36]. We further evaluated whether Claramine has any effect on TC-PTP1B activity. F11 cells were incubated with 1, 10 or 100 μM of

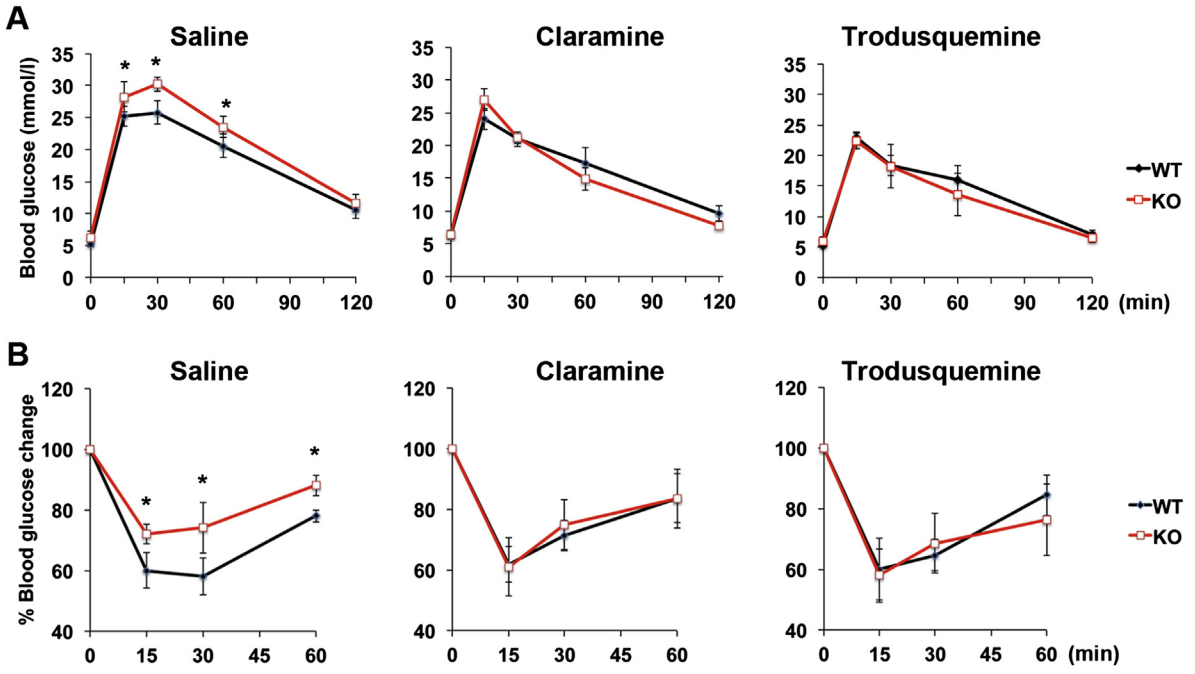

Fig. 3. Claramine, like Trodusquemine, has an anti-diabetic effect. (A) Glucose handling and (B) insulin sensitivity in diabetic mice (KO, Camk2αCre/LMO4flox mice) were restored after intraperitoneal injection of Claramine (5 mg/kg) or Trodusquemine (5 mg/kg). Age-matched littermate LMO4flox mice were used as controls (WT). Values are mean ± SEM. n = 6–8 mice per group. *, $p < 0.05$.

Claramine or Trodusquemine for 30 min and TC-PTP activity was performed in harvested cells. As indicated in Fig. 1E neither compound had an effect on TC-PTP activity. Together with Fig. 1D, our studies show that both compounds are highly specific inhibitors of PTP1B.

3.3. Claramine activates components of insulin signaling in neuronal cells

Upon insulin stimulation, phosphorylation and activation of a linear signaling cascade IR/IRS1/PI3K/Akt leads to the phosphorylation of GSK3β. Insulin signaling also induces transient oxidation and inactivation of its inhibitor PTP1B [37], but this inactivation is defective in insulin resistance and type 2 diabetes, where PTP1B activity is elevated [12,13]. Thus, blocking PTP1B activity would prevent dephosphorylation of IRβ, IRS1 and PI3K and thereby augment insulin signaling [14,38]. Previously, we reported that inhibition of PTP1B by Trodusquemine leads to phosphorylation of IRβ, Akt, and GSK3β [12]. Here, we asked whether Claramine, like Trodusquemine, could increase phosphorylation of these key components of the insulin-signaling cascade. As evident in Fig. 2, both Claramine and Trodusquemine can activate phosphorylation of IRβ, Akt and GSK3β like insulin (compare lanes 3–5 and 6–8 to lanes 2 and 1). Unlike Trodusquemine that showed attenuation of insulin pathway activation at higher doses (compared lane 8 to 6), Claramine elicited insulin pathway activation at all doses tested in F11 neuronal cells.

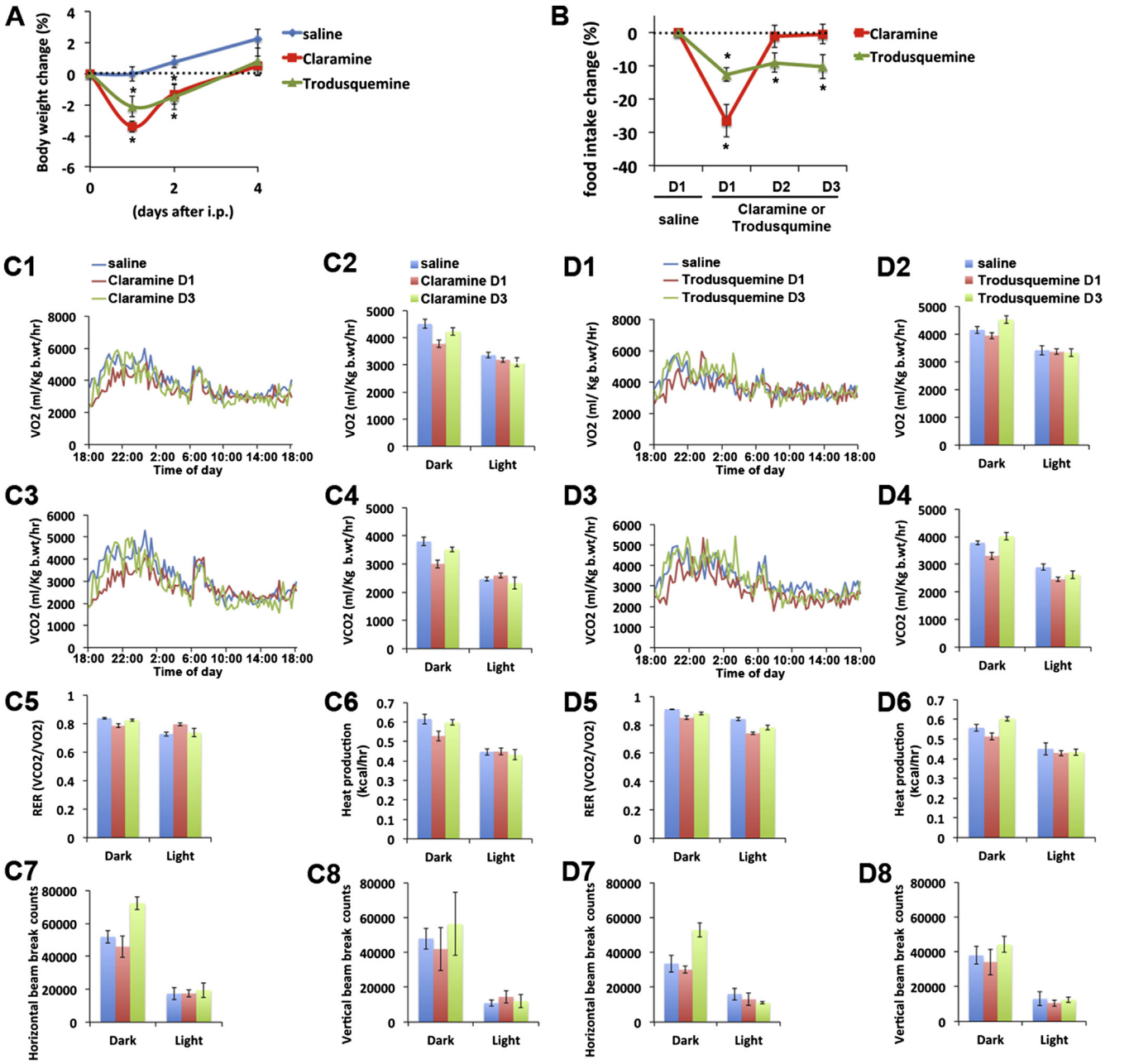

Fig. 4. Claramine causes weight loss by transient suppression of food intake rather than by altering energy expenditure. The effect of a single dose intraperitoneal (i.p.) injection of saline, Claramine or Trodusquemine (5 mg/kg) on body weight progression (A), food intake (B) and energy expenditure (C1-8, D1-8) was measured in 2-month old male CD1 wild type mice. D1, Day 1 and D3, Day 3. n = 8 mice per group. Values are mean ± SEM. *, $p < 0.05$, compared to saline treatment on Day 1 (A, B). Dark, from 18:00–6:00; Light, from 6:00–18:00.

3.4. Claramine restores glycemic control in a diabetic mouse model

We next compared the effect of Claramine versus Trodusquemine on glycemic control in a Type 2 diabetes mouse model, the CaMK2αCre/LMO4flox mice. Our previous studies show the LIM domain only 4 gene (LMO4) is an endogenous inhibitor of PTP1B and that CaMK2αCre/LMO4flox mice with LMO4 ablated in glutamatergic neurons have elevated PTP1B activity in the hypothalamus and exhibit insulin resistance when fed normal chow [13,23]. We also showed that Trodusquemine restores insulin signaling in these mice [12]. Here, we found that a single intraperitoneal injection of Claramine at an equivalent dose to Trodusquemine could restore insulin sensitivity in these diabetic mice, as determined by administration of glucose and insulin tolerance tests (Fig. 3A and B).

3.5. Claramine suppresses food intake

A single intraperitoneal injection of Trodusquemine (10 mg/kg) suppresses food intake and causes weight loss in C57BL6 and AKR/J mice [17,18]. We found that a single half-dose (5 mg/kg) intraperitoneal injection of Trodusquemine was sufficient to suppress food intake and cause weight loss in CD1 mice (Fig. 4A). Claramine at an equivalent dose (5 mg/kg) has a stronger but more transient effect on feeding suppression for the first 24 h than Trodusquemine (Fig. 4B). Overall weight loss was not significantly different between mice treated with Claramine or Trodusquemine (Fig. 4A). It should be noted that neither treatment at this low dose (5 mg/kg) altered energy expenditure, as shown by calorimetry analysis (Fig. 4C1-4C8, 4D1-4D8); oxygen consumption (Fig. 4C1, 4C2, 4D1, 4D2), CO_2 production (Fig. 4C3, 4C4, 4D3, 4D4) and heat production (Fig. 4C6, 4D6) was similar before and after treatment. Metabolic substrate utilization as determined by the VO_2/VCO_2 ratio (i.e., carbohydrate near 1 and fat near 0.7) was also not altered by either treatment (Fig. 4C5, 4D5). Also, beam-break analysis showed no effect on locomotor activity by either Claramine or Trodusquemine (Fig. 4C7, 4C8, 4D7, 4D8). Thus, food intake suppression rather altered energy expenditure accounts for much of the weight loss by the single low dose of Claramine or Trodusquemine.

4. Discussion

In search of new selective inhibitors of PTP1B to improve insulin signaling and glycemic control for type II diabetes, we tested an analog of Trodusquemine, a known selective inhibitor of PTP1B. Here, we report that a novel and easily manufactured compound Claramine is also a selective inhibitor of PTP1B. Like Trodusquemine, Claramine had a strong insulin-mimetic action in neuronal cells and rapidly restored glycemic control and insulin sensitivity in diabetic mice. In addition, a single dose of Claramine transiently suppressed food intake causing weight loss in mice.

The observation that a single intraperitoneal administration of Claramine suppresses feeding behavior indicates that Claramine can cross the blood brain barrier (BBB). Claramine likely acts on the hypothalamus, the brain region that orchestrates metabolic homeostasis by regulating feeding behavior and sympathetic tone to peripheral tissues [6,39,40]. This is consistent with the action of its close analog Trodusquemine that is known to cross the BBB and act on the hypothalamus to suppress appetite [12,13,17–19].

By using neuronal F11 cells we showed that Claramine has an insulin-like effect, increasing IRβ and Akt phosphorylation. Beyond this effect of Claramine on cultured neuronal cells, Claramine could also effectively correct insulin resistance *in vivo* by blocking PTP1B activity. Although we did not test the effect of Claramine in hepatocytes or skeletal muscle, a single intraperitoneal injection of Claramine effectively restored insulin sensitivity and glycemic control in diabetic mice similar to the observed effect of Trodusquemine. It seems likely that insulin signaling in these tissues is also restored by Claramine, as has been reported for Trodusquemine [12,13,18].

Finally, the mechanism whereby Claramine inhibits PTP1B activity remains unclear. Recent evidence has demonstrated that Trodusquemine acts as an allosteric inhibitor that binds to two targets within PTP1B, one near the catalytic domain and another in the C-terminal, non-catalytic segment of PTP1B [41]. Both targets were shown to be important since point mutations at both sites (Ser372 to proline and Leu192 to alanine), preserved catalytic activity but rendered PTP1B insensitive to Trodusquemine [41]. Claramine has a spermine structure quasi equivalent to Trodusquemine but lacks the sulfate present in Trodusquemine. Given that we observe a similar inhibition of PTP1B activity by Claramine, this suggests that the sulfate group of Trodusquemine is dispensable for it to block PTP1B enzymatic activity. On the other hand, we suspect that the OH moiety present in both Claramine and Trodusquemine may be critical for PTP1B inhibition. Future studies testing 3-amino and polyaminosterol derivatives (compound 6i or 8i) described in our previous publications [21,22] that share a similar structural backbone to Trodusquemine and Claramine but without the OH moiety will allow us to further test this hypothesis. Together, these compounds will help to shed further light on how PTP1B activity can be blocked by this polyaminosterol class of inhibitors with the goal of designing a more potent and specific inhibitor analog.

In conclusion, Claramine is an easy to manufacture compound that selectively blocks PTP1B activity and activates an insulin-like response through IRβ, Akt and GSK3β phosphorylation. Thus, Claramine may be a potential lead compound for developing new agents for the treatment of type 2 diabetes.

Conflict of interest

None.

Acknowledgments

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Transparency document

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