Adipose tissue-targeted 11β-hydroxysteroid dehydrogenase type 1 inhibitor protects against diet-induced obesity

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Abstract. Current pharmacological treatments for obesity and metabolic syndrome have various limitations. Recently, adipose tissue 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) has been proposed as a novel therapeutic target for the treatment of obesity and metabolic syndrome. Nevertheless, there is no adipose tissue-targeted 11β-HSD1 inhibitor available now. We sought to develop a new 11β-HSD1 pharmacological inhibitor that homes specifically to the white adipose tissue and aimed to investigate whether adipose tissue-targeted 11β-HSD1 inhibitor might decrease body weight gain and improve glucose tolerance in diet-induced obesity mice. BVT.2733, an 11β-HSD1 selective inhibitor was connected with a peptide CKGGRAKDC that homes to white fat vasculature. CKGGRAKDC-BVT.2733 (T-BVT) or an equimolar mixture of CKGGRAKDC and BVT.2733 (NT-BVT) was given to diet-induced obesity mice for two weeks through subcutaneous injection. T-BVT decreased body weight gain, improved glucose tolerance and decreased adipocyte size compared with vehicle treated mice. In adipose tissue T-BVT administration significantly increased adiponectin, vaspin mRNA levels; In liver T-BVT administration decreased the mRNA level of phosphoenolpyruvate carboxykinase (PEPCK), increased the mRNA levels of mitochondrial carnitine palmitoyltransferase-I (mCPT-I) and peroxisome proliferator-activated receptor α (PPARα). No significant differences in adipocyte size and hepatic gene expression were observed after treatment with NT-BVT compared with vehicle treated mice, though NT-BVT also decreased body weight gain, improved glucose tolerance, and increased uncoupling protein-2 (UCP-2) mRNA levels in muscle. These results suggest that an adipose tissue-targeted pharmacological inhibitor of 11β-HSD1 may prove to be a new approach for the treatment of obesity and metabolic syndrome.

Key words: Adipose tissue, 11β-hydroxysteroid dehydrogenase type 1, Blood glucose, Metabolic syndrome
ameliorate metabolic syndrome in many mouse models of diabetes and obesity [7-9]. However, 11β-HSD1 is widely expressed in many tissues [10-12], the regulation and function of 11β-HSD1 may differ in different tissues. In fact, dysregulation of 11β-HSD1 in obesity is tissue-specific. 11β-HSD1 is decreased in liver and enhanced in adipose tissue both in rodent obesity and human obesity [13-23]. Future more, several transgenic mice models have supported that adipose tissue 11β-HSD1 plays a key role in the pathogenesis of visceral obesity and the metabolic syndrome [5, 15, 22, 24].

As such, adipose tissue 11β-HSD1 has been proposed as a novel therapeutic target for the treatment of obesity and metabolic syndrome. Nevertheless, there is no adipose tissue-targeted 11β-HSD1 inhibitor available now. So we sought to develop a new 11β-HSD1 pharmacological inhibitor that homes specifically to the white adipose tissue and aimed to investigate whether and how adipose tissue-targeted 11β-HSD1 inhibitor decreases body weight gain and improves glucose tolerance in diet-induced obesity (DIO) mice.

### Materials and Methods

#### Reagents

BVT.2733, an 11β-HSD1 selective inhibitors were synthesized according to the patent information. CKGGRKDC was synthesized according to Mikhail [2]. T-BVT was synthesized by GL Biochem (Shanghai) Ltd (Shanghai, China). Anti-F4/80 antibody were purchased from Serotec (Oxford, UK). M-MLV, dNTP, RNase inhibitor and other reverse transcription reagents were purchased from Promega Corp (Madison, WI, USA). Trizol were pure hased from Invitrogen (Carlsbad, CA, USA).

#### Animals

C57BL/6J mice (male, 2 weeks) were purchased from Slac Laboratories (Shanghai, China). The mice were housed three or four per cage in a room kept at 23 ± 1 °C with a 12-h light, 12-h dark cycle and were allowed free access to water and food. For DIO studies, starting at 3 weeks of age, male mice received a normal fat diet containing 10% calory from fat (Collaborative Bio-Engineering Corporation, Nanjing, China) or a high fat diet (HFD) containing 50% calory from fat (Collaborative Bio-Engineering Corporation, Nanjing, China) for 24 weeks. During the last two weeks diet-induced obese C57BL/6J mice were subcutaneously injected with 10 μg T-BVT or an equimolar mixture of NT-BVT twice a day. Body weight and food intake were determined weekly. At the time the mice were killed, trunk blood was collected, centrifuged, and stored at -20 °C. Tissues were weighed, frozen in liquid nitrogen, and stored at -80 °C. All animal studies were approved and experimental design approved and followed as per the Animal Care and Use Committee of Nanjing Medical University.

#### Intraperitoneal glucose tolerance test

The mice were fasted overnight and then injected Intraperitoneally with 2 mg/g D-glucose (25% stock solution in saline). Blood samples were taken by tail venesection at 0 min (before injection and within 1 min of disturbing the cage) and at 15-, 30-, 60- and 120-min intervals after the glucose load. Glucose was measured with the Accu-Chek Aviva system (Roche, Germany) at 0-, 15-, 30-, 60-min. Insulin was measured with the Ultra Sensitive Mouse Insulin ELISA kits (Millipore Corporation, MA) at 0-, 15-, 30-, 60-min.

#### Immunohistochemistry and adipocyte size

Mice were killed epididymal adipose tissue were weighed and fixed in 10% formalin, embedded in a random orientation in paraffin and cut into 5μm sections. The sections were incubated with anti-F4/80 antibody (dilution, 1:100) overnight at 4 °C and were incubated with a secondary antibody of Histofine Simple Stain Max PO (rat) for 30 min. Negative control studies were also performed without using these first antibodies. All sections were counterstained with hematoxylin. The number of adipocyte was counted in 10 different high-power fields from each section and a total of 4 or 5 mice per group were used.

#### Serum adiponectin and leptin

Serum levels of adiponectin were measured using a commercially ELISA kits from Millipore (Millipore Corporation, MA). Serum levels of leptin were measured using a commercially available kit from Linco (LINCO Research Inc., St. Charles, MO).

#### RNA preparation and quantitative real-time PCR

Total RNA was extracted from homogenized tissues using TRIZOL (Invitrogen) according to the manufacturer’s instructions. Two micrograms of total RNA were reverse-transcribed with 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV, Promega,
BVT.2733, an 11β-HSD1 selective inhibitor were synthesized according to the patent information. Next, we produced a synthetic peptide composed of two functional domains: one is the white fat vasculature homing motif CKGGRAKDC and the other is the selective inhibitor for the 11β-HSD1 enzyme, BVT.2733. The resulting chimeric, fat-targeted, 11β-HSD1 selective inhibitor termed CKGGRAKDC/BVT.2733 was synthesized according to the instructions published by Kolonin MG et al [2]. To further confirm if the CKGGRAKDC motif could target specifically to the fat vasculature, the soluble CKGGRAKDC peptide were linked to 5-carboxyfluorescein (FITC) at its carboxy terminus and administrated intravenously in C57BL/6J mice for an in vivo distribution study. In C57BL/6J mice, CKGGRAKDC–FITC specifically localized to the blood vessels of white fat (Fig. 1a). No CKGGRAKDC–FITC homing was observed in blood vessels of the brown fat (Fig. 1b), the liver (Fig. 1c), or muscle (d). Homing of the CKGGRAKDC-FITC peptide to white fat vasculature (arrows) was indicated. Scale bar, 50μm.

Fig. 1 In vivo fat homing of the CKGGRAKDC motif in C57BL/6J mice. FITC-CKGGRAKDC peptide was administrated intravenously in C57BL/6J mice, and 5 minutes post-injection, different tissues were collected and processed for distribution assay. The green immunofluorescence in formalin-fixed paraffin section is visible only in white fat (a), but not detectable in brown fat (b), liver (c), or muscle (d). Homing of the CKGGRAKDC-FITC peptide to white fat vasculature (arrows) was indicated. Scale bar, 50μm.
The body weight loss was similar between T-BVT and NT-BVT treatment. At the time the mice were killed subcutaneous and epididymal adipose tissues were collected and weighed, we found that T-BVT treatment reduced subcutaneous adipose tissue weight (Fig. 2c) and NT-BVT decreased the weight of epididymal adipose tissues (Fig. 2d). Additionally, T-BVT treatment did not change the energy intake (Fig. 2a).

T-BVT improves glucose tolerance in DIO mice

There is no significant difference in fasting plasma glucose and fasting plasma insulin between HFD and treated mice (Fig. 3a, 3b). However, glucose tolerance test showed that both treatments can reduce the plasma glucose at 15 minutes point. The glucose level in the

BVT.2733 (T-BVT) was synthesized by GL Biochem (Shanghai) Ltd (Shanghai, China).

T-BVT decreases body weight gain in DIO mice

C57BL/6J mice were fed a normal fat diet or HFD for 24 weeks. Mice on HFD showed a significantly higher body weight gain compared with mice on a normal fat diet (data not shown). Glucose tolerance was evaluated by the glucose tolerance test and HFD-fed mice had an impaired glucose tolerance test in comparison to the normal fat diet mice (data not shown). And then the DIO mice received two weeks of T-BVT or an equimolar mixture of NT-BVT treatment. All mice were fed with the HFD during the treatment. The treatment was not only able to prevent the development of obesity, but also caused rapid weight loss (Fig. 2b), and the body weight loss was similar between T-BVT and NT-BVT treatment. At the time the mice were killed subcutaneous and epididymal adipose tissues were collected and weighed, we found that T-BVT treatment reduced subcutaneous adipose tissue weight (Fig. 2c) and NT-BVT decreased the weight of epididymal adipose tissues (Fig. 2d). Additionally, T-BVT treatment did not change the energy intake T-BVT during the course of the treatment (Fig. 2a).
T-BVT protects against DIO

BVT.2733 improved metabolic profile, expression of genes known to play an important role in adipose tissue metabolism were evaluated. In epididymal adipose tissue, compared with HFD mice, T-BVT administration had significantly increased adiponectin (Fig. 5a), leptin (Fig. 5b) and vaspin (Fig. 5c) mRNA levels. However, no significant differences in adipose tissue gene expression were observed for NT-BVT treatment. As expected, T-BVT treatment had also significantly increased adiponectin and leptin mRNA expression compared with the mixture treatment. On analyzing resistin and visfatin expression, there was no significant difference among the three groups (Fig. 5d, 5e). In subcutaneous adipose tissue compared with HFD mice, T-BVT administration had significantly increased adiponectin (Fig. 6a), vaspin (Fig. 6c) and visfatin (Fig. 6d). The peptide-BVT.2733 treatment caused the adipocytes to appear smaller than treatment with the mixture (p < 0.05).

T-BVT decreases adipocyte size

An overall change in the size of the adipocytes was observed after the treatment, resulting in the appearance of smaller adipocytes because there were more adipocytes in the fixed area (Fig. 4). The peptide-BVT.2733 treatment caused the adipocytes to appear smaller than treatment with the mixture (p < 0.05).

The effect of T-BVT mediated adipose tissue-targeted 11β-HSD1 inhibition on adipose tissue gene expression

To evaluate the molecular mechanism underlying BVT.2733 improved metabolic profile, expression of genes known to play an important role in adipose tissue metabolism were evaluated. In epididymal adipose tissue, compared with HFD mice, T-BVT administration had significantly increased adiponectin (Fig. 5a), leptin (Fig. 5b) and vaspin (Fig. 5c) mRNA levels. However, no significant differences in adipose tissue gene expression were observed for NT-BVT treatment. As expected, T-BVT treatment had also significantly increased adiponectin and leptin mRNA expression compared with the mixture treatment. On analyzing resistin and visfatin expression, there was no significant difference among the three groups (Fig. 5d, 5e). In subcutaneous adipose tissue compared with HFD mice, T-BVT administration had significantly increased adiponectin (Fig. 6a), vaspin (Fig. 6c) and visfatin (Fig. 6d).
of uncoupling protein-2 (UCP-2) (Fig. 7c), a protein implicated in elimination of oxidants produced during fat metabolism in muscle [25]. Compared with T-BVT administration mice, NT-BVT also increased the gene expression of Glucose transporter type 4 (GLUT4) (Fig. 7b). However, the expression of pyruvate dehydrogenase kinase-4 (PDK4) (Fig. 7a), a protein has proven to be particularly important for muscle glucose oxidation [26], GLUT4 (Fig. 7b) and UCP-2 (Fig. 7c) were not changed after two weeks' T-BVT treatment.

On the other hand, in liver the expressions of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme for gluconeogenesis, and glucose-6-

6d) mRNA levels. On the other hand, NT-BVT treatment had also significantly increased visfatin (Fig. 6d) mRNA levels. The expression of leptin (Fig. 6b) and resistin (Fig. 6e) were not changed after two weeks treatment.

**The effect of T-BVT and NT-BVT administration on the gene expression of muscle and liver**

To investigate whether T-BVT and NT-BVT administration effected the gene expression of muscle and liver, expression of genes known to play an important role in energy dissipation were evaluated. Compared with HFD mice NT-BVT increased the gene expression of uncoupling protein-2 (UCP-2) (Fig. 7c), a protein implicated in elimination of oxidants produced during fat metabolism in muscle [25]. Compared with T-BVT administration mice, NT-BVT also increased the gene expression of Glucose transporter type 4 (GLUT4) (Fig. 7b). However, the expression of pyruvate dehydrogenase kinase-4 (PDK4) (Fig. 7a), a protein has proven to be particularly important for muscle glucose oxidation [26], GLUT4 (Fig. 7b) and UCP-2 (Fig. 7c) were not changed after two weeks' T-BVT treatment. On the other hand, in liver the expressions of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme for gluconeogenesis, and glucose-6-
phosphatase (G6Pase), which regulates the outflow of glucose originating from either gluconeogenesis or glycogen degradation [27, 28] were evaluated. Compared with HFD mice T-BVT decreased the expression of PEPCK (Fig. 8a). Since the glucocorticoid not only play a crucial role in the glucose metabolic but also in the expression of hepatic genes involved in lipid metabolism, so the hepatic mRNA levels encoding genes for lipid catabolism mitochondrial carnitine palmitoyl-transferase-I (mCPT-I), a rate-limiting enzyme in the mitochondrial β-oxidation pathway [29], peroxisomal acyl-CoA oxidase (ACO), another enzyme of fatty acid oxidation [30], and UCP-2 were evaluated. Moreover, peroxisome proliferator-activated receptor α (PPARα), the key hepatic transcription factor that promotes expression of genes for lipid catabolism [31] was also evaluated. Interestingly, we fond that T-BVT treatment also increased the expression of mCPT-I (Fig. 8c) and
to determine if T-BVT affected circulating adipokines. As expected, T-BVT administration increased the level of adiponectin compared with the HFD group and the mixture treatment group (Fig. 9a). Surprisingly, the leptin levels were not significantly changed (Fig. 9b).

### Discussion

Recently many 11β-HSD1 inhibitors have been...
under development due to the promising therapeutic effects on obesity and type 2 diabetes. However, there has been no report so far that 11β-HSD1 inhibitor can act on adipose tissue specifically. In this study, we described a novel adipose tissue-targeted 11β-HSD1 inhibitor T-BVT. This novel drug improved glucose tolerance and was able to resist dietary weight gain in DIO C57BL/6J mice model system.

To evaluate the molecular mechanisms underlying the novel drug in being able to improve metabolic profile, expression of genes known to play an important role in adipose tissue metabolism were evaluated. In adipose tissue T-BVT administration had significantly increased adiponectin, leptin, visfatin and vaspin mRNA levels. Given the fact that adiponectin [32], vaspin [33] and visfatin [34] can improve insulin sensitivity, leptin can reduce caloric intake and increase energy expenditure [35, 36], improved metabolic profile was partly due to the improved adipokines. In addition, adipose tissue is a potential player in the alteration of hepatic metabolism [37], the adipose tissue 11β-HSD1 overexpression causes adipose tissue insulin resistance resulting in increased lipolysis and serum free fatty acids (FFA) flux, alteration of adipokine secretion and consequently hepatic fat accumulation and insulin resistance [15, 38]. So immediately we wanted to know whether T-BVT action in adipose tissue was sufficient to improve the glucose and fat metabolism of liver. The mRNA levels of PEPCK, the rate-limiting enzyme for gluconeogenesis, G6Pase, which regulates the outflow of glucose originating from either gluconeogenesis or glycogen degradation [27, 28], the mCPT-1, a rate-limiting enzyme in the mitochondrial β-oxidation pathway [29], ACO, another enzyme of fatty acid oxidation [30], UCP-2, a protein implicated in elimination of oxidants produced during fat metabolism [25] and PPARα, the key hepatic transcription factor that promotes expression of genes for lipid catabolism [31] were evaluated. As a result, our data supported that T-BVT mediated adipose tissue-targeted 11β-HSD1 inhibition not only increased adiponectin, visfatin and vaspin in adipose tissue but also decreased the expression of PEPCK, increased the expression of mCPT-1 and PPARα in liver. So we concluded that T-BVT mediated adipose tissue-targeted 11β-HSD1 inhibition also improved the glucose and fat metabolism of liver.

On the other hand, NT-BVT also decreased body weight gain, improved glucose tolerance and increased the expression of vaspin and visfatin in subcutaneous adipose tissue. However, NT-BVT not only had effected on adipose tissue but also had effected on other tissues such as liver and muscle. So the molecular mechanisms of NT-BVT improve metabolic profile may differ from that of T-BVT. The mRNA levels of PDK4, a protein has proven to be particularly important for muscle glucose oxidation [26], GLUT4, a recycling membrane protein, is required for dietary glucose uptake into muscle [39] and UCP-2 were also evaluated in muscle. The data supported that NT-BVT also increased UCP-2 expression in muscle. However, the genes expressions for gluconeogenesis and lipid catabolism in liver were not changed after NT-BVT treatment.

Previously, several transgenic mice models have also supported that adipose tissue 11β-HSD1 plays a key role in the pathogenesis of visceral obesity and the metabolic syndrome. In this article we found that the phenotypic characterization of T-BVT treated DIO mice revealed several similarities with transgenic ap2-h11β-HSD2 mice [5] and 11β-HSD1-deficient mice [40]. These mice models demonstrate an improved metabolic response to HFD characterized by resistance to diet-induced obesity, reduced fat mass, and improved glucose tolerance. In addition, all these models have increased expression of adiponectin and reduced expression of resistin in adipose tissue. Given the fact that adiponectin can improve insulin sensitivity, these similarities suggest that altered glucocorticoid metabolism in adipose tissue is a major contributor to these aspects of the metabolic phenotype.

Nevertheless, there are several important differences between T-BVT treated DIO mice and the transgenic mice. The major difference is that T-BVT is an adipose tissue-specific 11β-HSD1 pharmacological inhibitor, the genotype of the mice is not changed. As we are all well aware that transgenic mouse model systems cannot be used to study human obesity. What’s more, T-BVT mediated adipose tissue-targeted 11β-HSD1 inhibition not only improved the expression of adiponectin but also improved the glucose and fat metabolism of liver. So the novel drug has more clinical meanings, since it may be used in human compared with transgenic method in the future.

In summary, we developed and tested a novel adipose tissue-specific 11β-HSD1 pharmacological inhibitor T-BVT in vivo. The novel drug improves glucose tolerance and resists to dietary weight gain in DIO C57BL/6J mice. This metabolic phenotype is associated with increased adiponectin, visfatin and
vaspin mRNA levels in adipose tissue and decreased the expression of PEPCK, increased the expression of mCPT-I and PPARα in liver.

Duality of interest

The authors declare that they have no conflict of interest.

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