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Identification of a novel 11β-HSD1 inhibitor from a high-throughput screen of natural product extracts

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ABSTRACT

Selective inhibitors of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) have considerable potential as a treatment for metabolic syndrome including type 2 diabetes mellitus and obesity. To identify 11β-HSD1 inhibitors, we conducted high-throughput screening (HTS) of active natural product extracts from the Korea Chemical Bank, including Tanshinone I, Tanshinone IIA, and flavanone derivatives, and 2- and 3-phenyl-4H-chromen-4-one. Then Tanshinone IIA and its derivatives were targeted for the development of a lead compound according to the HTS results. However, the mechanism for anti-adipogenic effect through 11β-HSD1 enzyme inhibition by Tanshinone IIA is not clear. Tanshinone IIA (**2a**) concentration-dependently inhibited 11β-HSD1 activity in human and mouse 11β-HSD1 overexpressed cells and 3T3-L1 adipocytes. Tanshinone IIA (**2a**) also inhibited 11β-HSD1 enzyme activities in murine liver and fats. Furthermore, Tanshinone IIA (**2a**)-suppressed adipocyte differentiation of cortisone-induced adipogenesis in 3T3-L1 cells was associated with the suppression of the cortisone-induced adipogenesis-specific markers mRNA and protein expression. In 3T3-L1 preadipocytes, Tanshinone IIA (**2a**)-inhibited cortisone induced reactive oxygen species formation in a concentration-dependent manner. Thus, these results support the therapeutic potential of Tanshinone IIA (**2a**) as a 11β-HSD1 inhibitor in metabolic syndrome patients.

1. Introduction

Glucocorticoids are steroid hormones that regulate several physiological processes. Modulation of glucocorticoid action has been implicated as a potential treatment for a variety of diseases, including metabolic syndrome, inflammation, osteoporosis, glaucoma and age-related cognitive disorder. 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) catalyzes the conversion of inactive cortisone to its active form, cortisol, during glucocorticoid

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synthesis. Transgenic mice overexpressing 11β-HSD1 in adipose tissue develop visceral obesity, insulin resistance, hyperlipidemia and hypertension without alterations in circulating glucocorticoids [1,2], while mice with 11β-HSD1 overexpression in the liver develop MS without obesity [3]. Conversely, global deletion of 11β-HSD1 causes reduced visceral fat accumulation and improved insulin sensitivity when fed a high-fat diet [4,5].

Adipogenesis involves multi-processes, which include preadipocyte proliferation, differentiation, and fatty acid oxidation and synthesis, and is controlled by a number of molecular factors [6]. Recently, Bujalska et al. (2008) reported that 11β-HSD1 inhibitors inhibit adipogenesis and may give beneficial effect to treat obesity in diabetic patients [7], since glucocorticoids are essential for terminal adipogenesis [8]. Also there are enormous evidences that mature adipocytes express late differentiation genes related to lipid metabolism and lipid transport such as glycerol-3-phosphate dehydrogenase (G3PD) [9,10], peroxisome proliferator-activated receptor γ (PPARγ) [9], fatty acid-binding protein 4 (FABP4) [11], glucose transporter 4 (GLUT4) [12] and fatty acid synthase (FAS) [13], which is regulated by glucocorticoids [14,15].

Oxidative stress has been implicated in various diseases including insulin resistance, obesity, cardiovascular disease, and type 2 diabetes [16–18]. The increased oxidative stress in fat is an important pathogenic mechanism of obesity-associated metabolic syndrome [16]. Furthermore, obese adipose tissue is relevant to chronic inflammation [19]. Julien et al. [20] found that insulin resistance is associated with induction of differentiating preadipocytes and inhibition of differentiation marker genes expression, and the ratio of differentiating preadipocytes was higher in obese individuals compared with control group. This suggests that adipocyte differentiation may contribute to obesity-associated insulin resistance [21]. As exemplified from 11β-HSD1 overexpression, high glucocorticoid stress is known to regulate adipose tissue differentiation, function, and distribution and cause visceral adiposity, insulin resistance [1,22] and 11β-HSD1 activities at the interface of inflammation and obesity [23]. Ishii-Yonemoto et al. [24] showed that inflammatory stimuli-induced 11β-HSD1 intensifies NF-κB and MAPK signaling pathways and results in further induction of proinflammatory molecules in activated preadipocytes. This suggests that a 11β-HSD1 inhibitor will provide protection from the progression of insulin resistance in obese people.

In the present study, we conducted high-throughput screening (HTS) of active natural product extracts from the Korea Chemical Bank, including Tanshinone I and II derivatives (**1** and **2**), 1-hydroxylanthra-9,10-quinones (**3**), plumbaeins (**4**), 3-phenyl-4-chromen-4-ones (**5**), flavones and flavanones (**6** and **7**), and 6,6-dimethylbicyclo[3.1.1]hept-2-enes (**8**). Among these groups, a series of Tanshinone IIA (**2a**) and its derivatives (**2b-e**) were investigated for the development of a lead compound. Furthermore, we elucidated the anti-adipogenic effect of Tanshinone IIA (**2a**) in cortisone-treated preadipocytes through inhibition of 11β-HSD1 enzyme activity, adipogenesis-related gene expression and reactive oxygen species (ROS) formation.

2. Materials and methods

2.1. Cell culture and differentiation

For the human and mouse 11β-HSD1 activity assays, a pCMV6-recombinant human or mouse 11β-HSD1 plasmid was transfected for 24 h in CHO-K1 cells (ATCC, Manassas, VA, USA, #CCL-61, hamster ovary ancestral cells). After transfection of the plasmid, human and mouse 11β-HSD1 expression were analyzed by western blot or RT-PCR using whole cell extracts. To establish a human or mouse 11β-HSD1 overexpressed stable cell line, cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum and 200 μg/ml G-418 in a 5% CO_2 environment for 4 weeks.

3T3-L1 cells (ATCC #CL-173, mouse adipocyte) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. The 3T3-L1 cells differentiated to mature adipocytes using the same medium containing 20 μg/ml insulin, 0.5 mM isobutylmethylxanthine and 1 μM dexamethasone for 3 days, and then were replaced with a medium containing 20 μg/ml insulin for 2 days. The cells were cultured for 1 day in the maintained medium.

2.2. In vitro assay for 11β-HSD1 activity

We examined the cellular 11β-HSD1 enzyme activity according to the assay of Park and collaborators [25]. Human and mouse 11β-HSD1 overexpressing CHO-K1 cells or adipocytes were seeded at 2×10^4 cells/well (11β-HSD1 overexpressing CHO-K1 cells) or 5×10^4 cells/well (adipocytes) onto 96 or 24-well plates and were incubated in a medium containing 160 nM cortisone in the presence or absence of compounds for 3 h (human and mouse 11β-HSD1 overexpressing CHO-K1 cells) or 24 h (adipocytes). Small aliquots (10 μl) of the reaction mixtures were removed and subjected to a homogeneous time-resolved fluorescence (HTRF) cortisol assay in accordance with the manufacturer's instructions (Nihon Schering, Tokyo, Japan). The HTRF assay is based on the competition between free cortisol and XL665-conjugated cortisol for binding to an anti-cortisol antibody labeled with europium (Eu^{3+}) cryptate. Eu^{3+} cryptate and XL665 act as a donor and acceptor, respectively. If the two fluorophores are in close proximity, fluorescence resonance energy transfer (FRET) occurs upon excitation. The specific signal is expressed as the percentage of Delta F, which is the value calculated from the ratio of 665 nm/615 nm $[(R_{sample} - R_{negative})/R_{negative} \times 100]$, and is inversely proportional to the concentration of cortisol in the sample or the calibrator. The cortisol concentration was calculated from the calibration curve obtained from Delta F versus the standard solution. Negative control group is no cell medium, and positive control group is medium of cortisone treated 11β-HSD1 overexpressed cells. The IC_{50} values of the compounds were determined from concentration-dependent inhibition curves obtained using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA). Carbenoxolone was used as the reference compound.

2.3. Ex vivo assay for 11β-HSD1 activity

Male C57BL/6 mice, 9 weeks old, were intraperitoneally injected with vehicle (dimethyl sulfoxide (DMSO/PEG400 in H_2O) or Tanshinone IIA (**2a**) at 50 mg/kg and sacrificed 1 h post dose ($n = 4$ per group). The liver and fat pads were sectioned into three 30–40 mg samples and placed into 48-well Falcon plates containing prewarmed assay media that consisted of 1 μM cortisone and 100 nM NADPH in DMEM. The plates were incubated for 3 h at 37 °C. Then the cortisol product in the media was quantitated using a cortisol ELISA kit (Assay Designs Inc., Ann Arbor, MI, USA). The enzyme activity is expressed as the pg/mL of product formed per mg wet tissue weight. The inhibition of 11β-HSD1 enzyme activity is expressed as the percentage of cortisol concentration, which is the value calculated from following mathematical expression;

$$\%\ \text{Inhibition of } 11\beta - \text{HSD1 enzyme activity in liver and fats} = 100 - \frac{\text{Cortisol concentration}_{sample}}{\text{Cortisol concentration}_{DMSO}} \times 100$$