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Inhibition of IκB Kinase β (IKKβ) and Anti-diabetic Effect of SA51

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SA51, a medium potency inhibitor of protein tyrosine phosphatase 1B (PTP1B), was identified to be a potent inhibitor of IκB kinase β (IKKβ). Consistent with this, **SA51** prevented lipopolysaccharide (LPS)-induced breakdown of IκBα in macrophages. The effects of **SA51** in mice were compared with those of structurally related compounds, **SA18** and **SA32**, which were previously reported as inhibitors of both enzymes - less potent against IKKβ but more potent against PTP1B compared to **SA51**. **SA51** improved glucose tolerance and lipid parameters in mice, consistent with the results reported for IKKβ$^{+/-}$ mice. In contrast, **SA18** and **SA32** showed anti-obesity effects without anti-diabetic effects. Collectively, the effects of **SA51** could be due largely to the inhibition of IKKβ, whereas **SA18** and **SA32** may be more likely to inhibit PTP1B, consistent with their relative *in vitro* inhibitory effects.

Key Words : IKKβ Inhibitor, Hypoglycemic agent, PTP1B

Introduction

IKKβ is an enzyme that phosphorylates IκB, an inhibitory subunit of NF-κB.[1] In resting cells, NF-κB is present in the cytoplasm in complex with IκB. When IKKβ is activated by various stimuli, IκB is phosphorylated and subsequently degraded, releasing NF-κB for nuclear translocation. In the nucleus, NF-κB activates the transcription of numerous inflammatory genes. Manipulation of the *IKKβ* gene in mice revealed critical roles of IKKβ in the control of insulin sensitivity. While whole body homozygous deletion of IKKβ (*IKKβ*$^{-/-}$) in mice was embryonic lethal, heterozygous deletion (*IKKβ*$^{+/-}$) which reduced gene dosage by half resulted in normal birth. IKKβ$^{+/-}$ mice were protected from the development of insulin resistance induced by excess dietary fat and leptin deficiency, without beneficial effects on weight gain.[2] The tissue-specific role of IKKβ has also been investigated. Constitutive activation of IKKβ in the hypothalamus caused central insulin and leptin resistance. On the other hand, deletion of IKKβ in the whole brain or in the hypothalamus preserved insulin and leptin signaling in mice fed a high fat diet (HFD).[3] IKKβ ablation in myeloid cells sustained global insulin sensitivity in mice on a HFD.[4] Mice with hepatic deletion of *IKKβ* maintained insulin sensitivity in the liver but developed insulin resistance in muscle and fat upon HFD feeding.[4] Constitutive activation of IKKβ in adipose tissue caused local and systemic inflammation but, interestingly, protected mice from diet-induced weight gain and the development of insulin resistance.[5] No data is available for *IKKβ* deletion in adipose tissue. Collectively, tissue-specific deletion of *IKKβ* in the brain or in myeloid cells protected mice from developing diet-induced insulin resistance.

In addition to IKKβ, PTP1B is also an independently identified target for the control of diabetes and obesity.[6] When the mouse homolog of the *PTP1B* gene was disrupted, both *PTP1B*$^{-/-}$ and *PTP1B*$^{+/-}$ mice were resistant to diet-induced obesity. Excess energy was dissipated as heat, rather than stored as fat. Enhanced insulin sensitivity was also observed in PTP1B-deficient mice.[7,8]

Previously, we reported that methylenedisalicylic acid (MDSA) derivatives (**SA18** and **SA32**) act as PTP1B inhibitors. When **SA18** and **SA32** were tested in a mouse model system, they exhibited anti-obesity effects, as anticipated from their inhibitory activity against PTP1B.[9,10] More recently, **SA18**

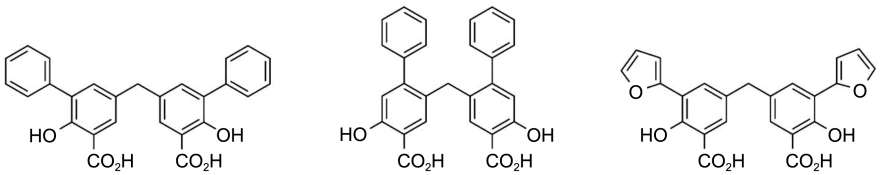

Figure 1. Structures of MDSA derivatives.

and **SA32** were also found to inhibit IKKβ, thus obscuring the *in vivo* target of these compounds.[9] Among the MDSA derivatives previously reported in this laboratory, **SA51** was a less potent inhibitor of PTP1B (IC_{50} = 39 μM) than **SA18** and **SA32**, and was thus depreciated as a potential lead compound for the control of obesity and diabetes. In this study, **SA51** was reconsidered as an inhibitor of IKKβ and its metabolic effects in a mouse model was compared with the effects of **SA18** and **SA32**.

Experimental

Synthesis of SA51. SA51 was synthesized as previously reported.[9]

IKKβ Assay. The kinase activity of IKKβ was determined with a commercial IKKβ kinase assay kit (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's protocol.[11] The substrate was a biotinylated peptide containing the IκB phosphorylation motif (residues around Ser32 of IκB-α). The enzyme reaction was initiated by addition of the substrate and ATP to a mixture of IKKβ and inhibitor, preincubated for 10 min. The final reaction mixture contained: the IKKβ (10 units, manufacturer's definition); substrate (1.5 μM); ATP (0.4 mM); 25 mM Tris-HCl (pH 7.5); 10 mM $MgCl_2$; 5.0 mM glycerol-2-phosphate; 0.10 mM Na_2VO_4; 2.0 mM DTT; different concentrations of inhibitor (2.5% in DMSO). The reaction was allowed to continue for 30 min and was then quenched by addition of EDTA (50 μL, 50 mM, pH 8). The phosphorylated peptide product was then immobilized on a streptavidin-coated plate and quantified using time-resolved fluorescence (TRF) as previously described.[13] Briefly, The immobilized peptide was treated with a primary antibody against the phosphorylated peptide, followed by Eu^{3+}-labeled secondary antibody. The Eu^{3+} ion was then released to form fluorescent complexes. For TRF measurement, a Eu^{3+} ion was excited by a laser pulse, with the fluorescence emission measured after a short delay.

Effect of SA51 on LPS-induced NF-κB Activation. Raw 264.7 murine macrophage cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated endotoxin-free fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, 100 μg/mL streptomycin, and 100 units/mL penicillin under a humidified 5% CO_2 atmosphere at 37 °C. Raw 264.7 cells were pretreated with **SA51** (100 μM) for 2 h and then treated with LPS (100 ng/mL) for 15 min. Total cell lysates (30 μg) were used for western blot analysis using monoclonal antibodies against IκBα.

Mouse Experiments. Twenty-four C57BL/6J Jms Slc mice (4-wk old, male) were purchased from Japan SLC, Inc., Haruno Breeding branch. The mice were individually housed and maintained on a 12 h light/dark cycle at 22 ± 2 °C. Food and water were available *ad libitum*. The experimental diets, HFD (D12451) and low fat diet (LFD) (D10012G) containing 45% and 16% fat calories, respectively, were obtained from Research Diets (New Brunswick, NJ). After 1 wk of acclimatization (LFD), 8 mice were assigned to a lean control group and maintained on LFD throughout the study. The remaining 16 mice were fed HFD for the first 8 weeks of the study to induce the development of obesity and diabetes. At week 8, the HFD-fed mice were divided into 2 groups containing 8 mice each. One group was fed HFD containing **SA51** (0.12% w/w) for 4 wk. As an obese/diadetic control group, the other group also remained on a HFD throughout the study, but was fed HFD powdered food mixed with 10% H_2O to make dough. For treatment with **SA51**, 240 mg of **SA51** was dissolved in H_2O (20 mL containing 2 equivalent of NaOH), mixed in powdered food (200 g), and kneaded to form dough. Mouse weight was measured every 3 or 4 days during the 4-wk drug feeding period. For GTT, mice were fasted for 6 h starting from the beginning of the light cycle, and glucose (1.0 g/kg of body weight) was injected intraperitoneally. Blood glucose levels were measured from tail bleeds with a glucometer (Accu-Chek Active, Roche Diagnostics, Ireland) at 0 min (prior to glucose administration) and at 20, 40, 60, 90, and 120 min after glucose injection. After 5 days of GTT, the mice were fasted overnight and anesthetized with intraperitoneal injection of secobarbital. Blood samples were taken by cardiac puncture, collected into EDTA tubes, and immediately placed on ice. Blood samples were spun (4000 × g, 10 min, 4 °C), and the plasma was removed and frozen until further analysis. Plasma was analyzed for glucose, triglyceride, total cholesterol, and free fatty acids using diagnostic kits (Glucose C2, TG E, T-Cho E, and NEFA C from Wako Pure chemical Industries, Ltd., Osaka, Japan) following the manufacturer's protocol. Epididymal and retroperitoneal fat pads, and the liver, lungs, and kidneys were excised immediately after blood collection, washed in cold isotonic saline, gently blotted, and then weighed.

Results and Discussion

SA51 was examined for its inhibitory activity against IKKβ and shown to inhibit IKKβ with an IC_{50} value of 3.2 μM, which was lower than that of **SA18** and **SA32** (Table 1). To investigate whether **SA51** inhibits IKKβ on a cellular level, the effect of **SA51** on LPS-induced IκBα degradation was examined in Raw264.7 murine macrophage cells. After incubation with **SA51**, the macrophages were challenged with LPS to induce inflammation, and the extent to which **SA51** prevented the degradation of IκBα in macrophages was determined. Western blot analysis revealed that LPS caused IκBα degradation as previously described, and **SA51** effec-

Table 1. IC_{50} values of MDSA derivatives against PTP1B and IKKβ[a]

Compound	PTP1B (μM)	IKKβ (μM)
SA51	39 ± 7	3.2 ± 0.9
SA18	20 ± 1[b]	4.7 ± 1.0[b]
SA32	19 ± 7[b]	14 ± 0.2[b]

[a]Values are the means ± standard deviation of three experiments. The kinetic data were analyzed using the GraFit 5.0 program (Erithacus Software). [b]Data reproduced from our previous publications.[11]

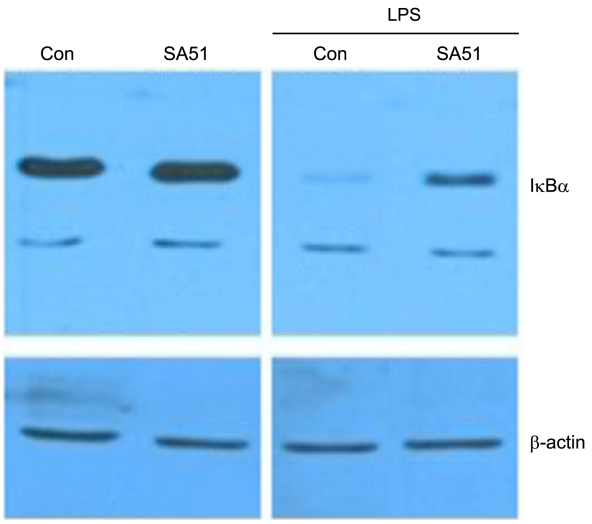

Figure 2. Effect of **SA51** on LPS-induced NF-κB activation. Western blot analysis of IκBα in macrophages (Raw 264.7) preincubated with or without **SA51** (100 μM) for 2 h and challenged with LPS (100 ng/mL) for 15 min. Total cellular extracts were used for western blot analysis using monoclonal antibodies against IκBα. (Each of the lanes was rearranged next to each other photographically from a single gel without modification.)

tively prevented IκBα degradation (Fig. 2). These observations confirmed that **SA51** inhibits IKKβ in macrophage cells.

The *in vivo* effect of **SA51** was also examined in a mouse model system (C57BL/6J) to determine whether **SA51** ameliorates diet-induced insulin resistance, as anticipated from its inhibition of IKKβ enzyme activity. Mice were raised on a HFD for the obese/diabetic group or on a low fat diet (LFD) for the lean control group for 8 wk. Then, HFD-fed mice were divided into obese/diabetic control and test groups. The test group was fed HFD + **SA51** for an additional 4 wk. In parallel, the obese/diabetic and the lean control groups were fed HFD and LFD, respectively, throughout the same period. **SA51** was provided as a mixture with the food (1.2 g **SA51** per kg of diet). The estimated amount of **SA51** administrated was 90 mg/day/kg of mouse weight, based on the average values of mouse weight and daily food consumption. During the drug-feeding period, no significant diffrences in food consumption were observed between the control and drug-fed groups (data not shown).

The effects of **SA51** on weight control were marginal. The **SA51**-fed group tended to gain less weight than the obese control group, but the difference was not statistically significant (p = 0.144) (Figure 3). The reduction of the epididymal and retroperitoneal fat weights was not significant either

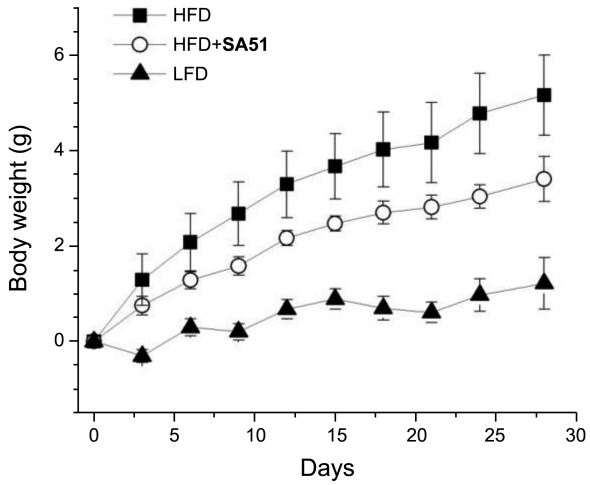

Figure 3. Effect of LFD, HFD and HFD + **SA51** on the body weight of DIO mice (C57BL/6J Jms Slc male, Japan SLC, Inc. Haruno Breeding branch). Data points represent the mean ± SEM; n = 8/group. Mice fed LFD served as a lean control reference group.

(data not shown). On the other hand, some of the obesity-related parameters reached statistical significance. **SA51** significantly reduced feed efficiency, which is the ratio of feed consumed to live-weight gain in a given period (Figure 4).[12] Significantly improvement was also observed in the serum concentrations of triglycerides, total cholesterol, and non-esterified fatty acids (NEFAs) (Table 2).

At the end of the 4-wk drug-feeding period, mice were subject to a glucose tolerance test (GTT). Mice were fasted

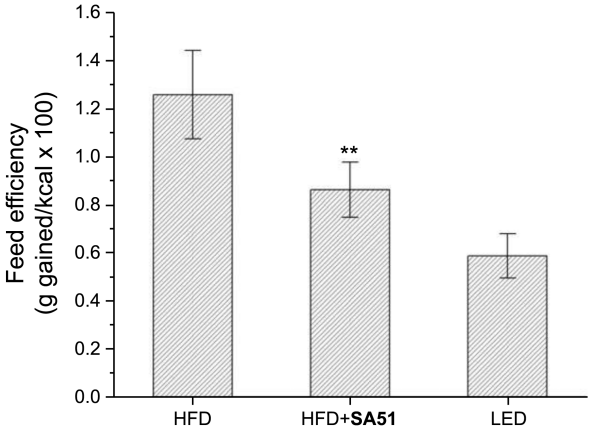

Figure 4. Effect of 4-wk treatment with **SA51** on the feeding efficiency (body weight gain per calories consumed) of mice. All values are the means ± SEM; n = 8/group. **p < 0.005 for comparisons against the obese control group. Mice fed LFD served as a lean control reference group.

Table 2. Effect of 4-wk treatment with **SA51** on body weight, adipose tissue weight, and related blood parameters

	Body Weight Gain (g)	Adipose Tissue Weight (g)		Triglyceride (mg/dL)	Total Cholesterol (mg/dL)	NEFA (mEq/L)
		Epididymal	Retroperitoneal			
LFD	1.22 ± 0.54*	0.49 ± 0.04*	0.11 ± 0.02*	65 ± 5*	108 ± 5*	0.18 ± 0.01*
HFD	5.17 ± 0.84	1.94 ± 0.16	0.63 ± 0.06	93 ± 11	150 ± 8	0.72 ± 0.03
HFD + **SA51**	3.41 ± 0.47 (p = 0.144)	1.62 ± 0.23	0.55 ± 0.07	62 ± 5*	109 ± 9*	0.17 ± 0.05*

*p < 0.05 for comparisons against obese control group (HFD).

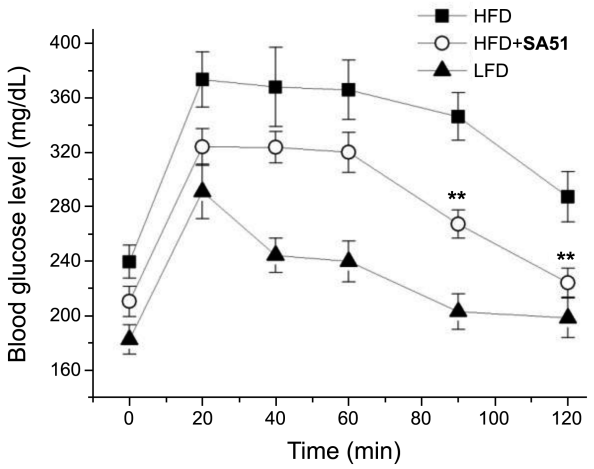

Figure 5. Glucose Tolerance Test. Mice in the HFD + **SA51** group were maintained on HFD for 8 wk followed by HFD + **SA51** for 4 wk. HFD and LFD groups were maintained on HFD or LFD, respectively, for the 12-wk period. Mice fasted for 6 h were injected intraperitoneally with glucose (1.0 g glucose/kg body weight). Blood glucose levels were measured at the indicated times (mean ± standard deviation; n = 8/group). The significance of the difference between the HFD group and **SA51**-fed group was calculated by one-way ANOVA. **represents $p < 0.05$.

for 6 h and then injected with glucose (1.0 g glucose/kg of body weight) intraperitoneally. Blood glucose levels were measured every 20 or 30 min after the injection of glucose. At 90 and 120 min after glucose challenge, blood glucose concentrations in the **SA51**-fed group were significantly lower than those in the HFD-fed obese/diabetic control group, which indicates the potential of **SA51** for the control of diabetes (Figure 5).

SA51-related toxicity was not observed in the test group of mice. Examination of the weights of the liver, kidney, and lungs revealed no significant differences between the drug-treated group and the HFD- and LFD-fed control groups. No observable indications of toxicity were detected in the outer appearance of these organs in the **SA51**-treated group of mice.

Previous studies showed that *PTP1B*-knockout in mice improved insulin sensitivity and suppressed weight gain without significant changes in free fatty acid (FFA) levels.[9,10] Heterozygous deletion of *IKKβ* in mice also enhanced insulin sensitivity but reduced serum FFA concentrations without protecting from weight gain.[2] In our study, treatment of mice with **SA51** improved glucose tolerance and reduced FFA levels in serum without significantly suppressing weight gain. These results are consistent with the effects of heterozygous deletion of *IKKβ*. In our previous study, feeding mice **SA18** or **SA32** (400 mg/day/kg of body weight) suppressed diet-induced weight gain without improving glucose tolerance.[11] As shown in Table 1, **SA** series of compounds inhibited both IKKβ and PTP1B, **SA51** most potent against IKKβ and least potent against PTP1B. The inhibitory effects of **SA51** in mice might therefore affect IKKβ more than PTP1B. Importantly, the effect of **SA51** in mice was consistent with the effects of genetic deletion of *IKKβ*. On the other hand, the effects of **SA18** and **SA32** in mice were inconsistent with *IKKβ* deletion, but also partly consistent with PTP1B deletion. Our current results suggest that the effects of **SA51** could be due largely to the inhibition of IKKβ, whereas **SA18** and **SA32** may be more likely to inhibit PTP1B. The possibility that **SA51** inhibits PTP1B in addition to IKKβ cannot be ruled out because **SA51** treatment in mice tended to reduce weight gain, albeit not a statistically significant level. Additional studies are required to explain unambiguously the molecular basis of the *in vivo* effects of **SA51**, as well as **SA18** and **SA32**.

Conclusions

SA51, a medium potency inhibitor of PTP1B, was found to be a potent inhibitor of IKKβ. Structurally related compounds, **SA18** and **SA32**, were also known to inhibit both of the enzymes with IC_{50} values higher against IKKβ and lower against PTP1B, compared to **SA51**. In a mouse model system, **SA51** exhibited anti-diabetic effects without significant effects in weight control, in contrast to **SA18** and **SA32** which showed anti-obesity effects without anti-diabetic effects. The *in vivo* effects of **SA51** were consistent with *IKKβ* deletion in mice; those of **SA18** and **SA32** partly with PTP1B ablation in mice. The differences in *in vivo* effects of **SA** compounds could be due to differential inhibition of IKKβ and PTP1B by these compounds.

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