

## Inhibition of I $\kappa$ B Kinase $\beta$ (IKK $\beta$ ) 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 I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ). Consistent with this, **SA51** prevented lipopolysaccharide (LPS)-induced breakdown of I $\kappa$ B $\alpha$  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 IKK $\beta$  but more potent against PTP1B compared to **SA51**. **SA51** improved glucose tolerance and lipid parameters in mice, consistent with the results reported for IKK $\beta$ <sup>+/-</sup> 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 IKK $\beta$ , whereas **SA18** and **SA32** may be more likely to inhibit PTP1B, consistent with their relative *in vitro* inhibitory effects.

**Key Words** : IKK $\beta$  Inhibitor, Hypoglycemic agent, PTP1B

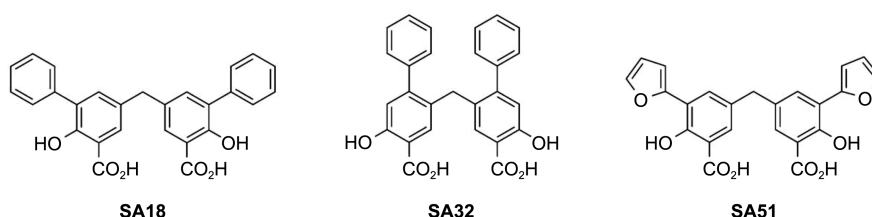
### Introduction

IKK $\beta$  is an enzyme that phosphorylates I $\kappa$ B, an inhibitory subunit of NF- $\kappa$ B.<sup>1</sup> In resting cells, NF- $\kappa$ B is present in the cytoplasm in complex with I $\kappa$ B. When IKK $\beta$  is activated by various stimuli, I $\kappa$ B is phosphorylated and subsequently degraded, releasing NF- $\kappa$ B for nuclear translocation. In the nucleus, NF- $\kappa$ B activates the transcription of numerous inflammatory genes. Manipulation of the *IKK $\beta$*  gene in mice revealed critical roles of IKK $\beta$  in the control of insulin sensitivity. While whole body homozygous deletion of IKK $\beta$  (*IKK $\beta$ <sup>-/-</sup>*) in mice was embryonic lethal, heterozygous deletion (*IKK $\beta$ <sup>+/-</sup>*) which reduced gene dosage by half resulted in normal birth. IKK $\beta$ <sup>+/-</sup> mice were protected from the development of insulin resistance induced by excess dietary fat and leptin deficiency, without beneficial effects on weight gain.<sup>2</sup> The tissue-specific role of IKK $\beta$  has also been investigated. Constitutive activation of IKK $\beta$  in the hypothalamus caused central insulin and leptin resistance. On the other hand, deletion of IKK $\beta$  in the whole brain or in the hypothalamus preserved insulin and leptin signaling in mice fed a high fat diet (HFD).<sup>3</sup> IKK $\beta$  ablation in myeloid cells sus-

tained global insulin sensitivity in mice on a HFD.<sup>4</sup> Mice with hepatic deletion of *IKK $\beta$*  maintained insulin sensitivity in the liver but developed insulin resistance in muscle and fat upon HFD feeding.<sup>4</sup> Constitutive activation of IKK $\beta$  in adipose tissue caused local and systemic inflammation but, interestingly, protected mice from diet-induced weight gain and the development of insulin resistance.<sup>5</sup> No data is available for *IKK $\beta$*  deletion in adipose tissue. Collectively, tissue-specific deletion of *IKK $\beta$*  in the brain or in myeloid cells protected mice from developing diet-induced insulin resistance.

In addition to IKK $\beta$ , PTP1B is also an independently identified target for the control of diabetes and obesity.<sup>6</sup> When the mouse homolog of the *PTP1B* gene was disrupted, both *PTP1B<sup>-/-</sup>* and *PTP1B<sup>+/-</sup>* 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.<sup>7,8</sup>

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.<sup>9,10</sup> More recently, **SA18**



**Figure 1.** Structures of MDSA derivatives.

and **SA32** were also found to inhibit IKK $\beta$ , thus obscuring the *in vivo* target of these compounds.<sup>9</sup> Among the MDSA derivatives previously reported in this laboratory, **SA51** was a less potent inhibitor of PTP1B ( $IC_{50} = 39 \mu\text{M}$ ) 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 IKK $\beta$  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.<sup>9</sup>

**IKK $\beta$  Assay.** The kinase activity of IKK $\beta$  was determined with a commercial IKK $\beta$  kinase assay kit (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's protocol.<sup>11</sup> The substrate was a biotinylated peptide containing the I $\kappa$ B phosphorylation motif (residues around Ser32 of I $\kappa$ B- $\alpha$ ). The enzyme reaction was initiated by addition of the substrate and ATP to a mixture of IKK $\beta$  and inhibitor, preincubated for 10 min. The final reaction mixture contained: the IKK $\beta$  (10 units, manufacturer's definition); substrate (1.5  $\mu\text{M}$ ); ATP (0.4 mM); 25 mM Tris-HCl (pH 7.5); 10 mM MgCl<sub>2</sub>; 5.0 mM glycerol-2-phosphate; 0.10 mM Na<sub>2</sub>VO<sub>4</sub>; 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  $\mu\text{L}$ , 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.<sup>13</sup> Briefly, The immobilized peptide was treated with a primary antibody against the phosphorylated peptide, followed by Eu<sup>3+</sup>-labeled secondary antibody. The Eu<sup>3+</sup> ion was then released to form fluorescent complexes. For TRF measurement, a Eu<sup>3+</sup> ion was excited by a laser pulse, with the fluorescence emission measured after a short delay.

**Effect of SA51 on LPS-induced NF- $\kappa$ B 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  $\mu\text{g}/\text{mL}$  streptomycin, and 100 units/mL penicillin under a humidified 5% CO<sub>2</sub> atmosphere at 37 °C. Raw 264.7 cells were pretreated with **SA51** (100  $\mu\text{M}$ ) for 2 h and then treated with LPS (100 ng/mL) for 15 min. Total cell lysates (30  $\mu\text{g}$ ) were used for western blot analysis using monoclonal antibodies against I $\kappa$ B $\alpha$ .

**Mouse Experiments.** Twenty-four C57BL/6J Mice 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  $\pm$  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 con-

trol 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/diabetic control group, the other group also remained on a HFD throughout the study, but was fed HFD powdered food mixed with 10% H<sub>2</sub>O to make dough. For treatment with **SA51**, 240 mg of **SA51** was dissolved in H<sub>2</sub>O (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  $\times$  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.

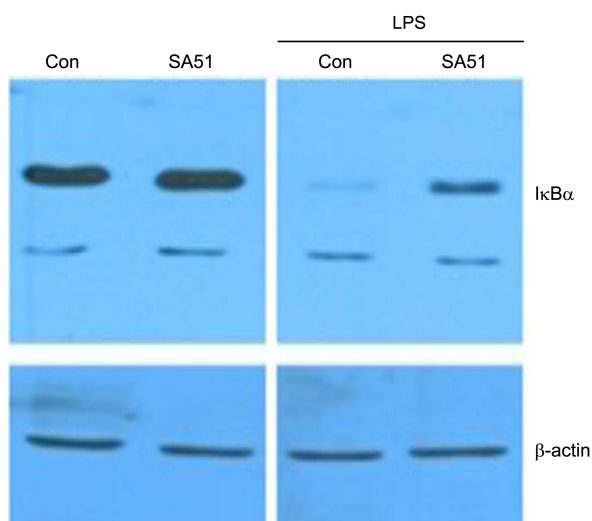
### Results and Discussion

**SA51** was examined for its inhibitory activity against IKK $\beta$  and shown to inhibit IKK $\beta$  with an  $IC_{50}$  value of 3.2  $\mu\text{M}$ , which was lower than that of **SA18** and **SA32** (Table 1). To investigate whether **SA51** inhibits IKK $\beta$  on a cellular level, the effect of **SA51** on LPS-induced I $\kappa$ B $\alpha$  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 I $\kappa$ B $\alpha$  in macrophages was determined. Western blot analysis revealed that LPS caused I $\kappa$ B $\alpha$  degradation as previously described, and **SA51** effec-

**Table 1.**  $IC_{50}$  values of MDSA derivatives against PTP1B and IKK $\beta$ <sup>a</sup>

Compound	PTP1B ( $\mu\text{M}$ )	IKK $\beta$ ( $\mu\text{M}$ )
<b>SA51</b>	39 $\pm$ 7	3.2 $\pm$ 0.9
<b>SA18</b>	20 $\pm$ 1 <sup>b</sup>	4.7 $\pm$ 1.0 <sup>b</sup>
<b>SA32</b>	19 $\pm$ 7 <sup>b</sup>	14 $\pm$ 0.2 <sup>b</sup>

<sup>a</sup>Values are the means  $\pm$  standard deviation of three experiments. The kinetic data were analyzed using the GraFit 5.0 program (Erithacus Software). <sup>b</sup>Data reproduced from our previous publications.<sup>11</sup>

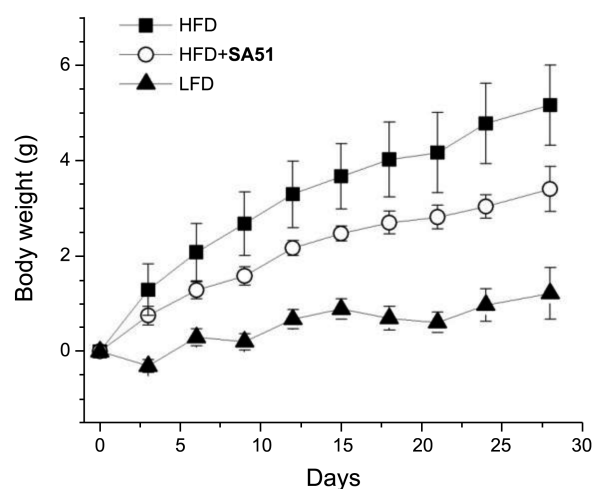


**Figure 2.** Effect of SA51 on LPS-induced NF- $\kappa$ B activation. Western blot analysis of I $\kappa$ B $\alpha$  in macrophages (Raw 264.7) preincubated with or without SA51 (100  $\mu$ M) for 2 h and challenged with LPS (100 ng/mL) for 15 min. Total cellular extracts were used for western blot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

tively prevented I $\kappa$ B $\alpha$  degradation (Fig. 2). These observations confirmed that SA51 inhibits IKK $\beta$  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 IKK $\beta$  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 administered 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 differences in food consumption were observed between the control and drug-fed groups (data not shown).

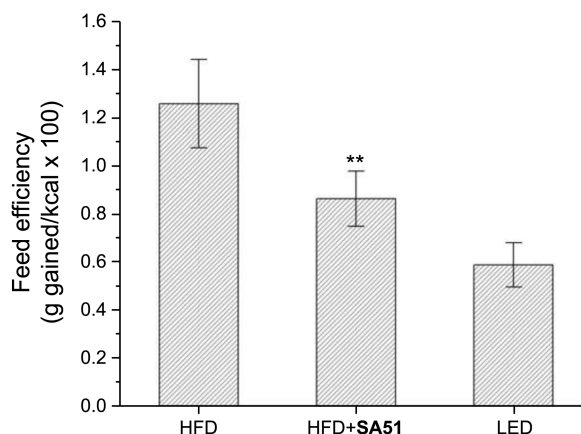
The effects of SA51 on weight control were marginal. The SA51-fed group tended to gain less weight than the obese control group, but the difference was not statistically significant ( $p = 0.144$ ) (Figure 3). The reduction of the epididymal and retroperitoneal fat weights was not significant either



**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  $\pm$  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).<sup>12</sup> 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

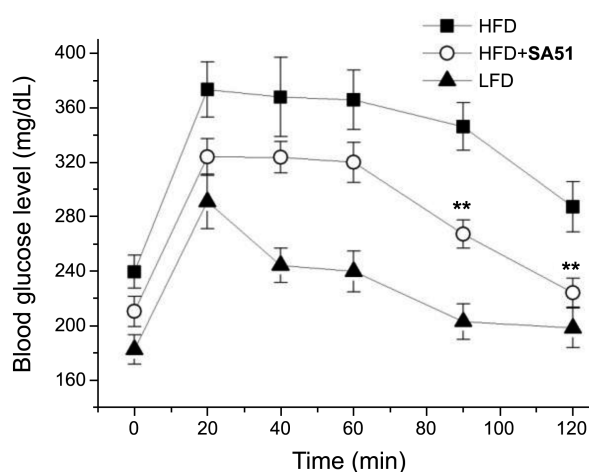


**Figure 4.** Effect of 4-wk treatment with SA51 on the feeding efficiency (body weight gain per calories consumed) of mice. All values are the means  $\pm$  SEM;  $n = 8$ /group. \*\* $p < 0.005$  for comparisons against the obese control group. Mice fed LFD served as a lean control reference group.

**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 $\pm$ 0.54*	0.49 $\pm$ 0.04*	0.11 $\pm$ 0.02*	65 $\pm$ 5*	108 $\pm$ 5*	0.18 $\pm$ 0.01*
HFD	5.17 $\pm$ 0.84	1.94 $\pm$ 0.16	0.63 $\pm$ 0.06	93 $\pm$ 11	150 $\pm$ 8	0.72 $\pm$ 0.03
HFD + SA51	3.41 $\pm$ 0.47 ( $p = 0.144$ )	1.62 $\pm$ 0.23	0.55 $\pm$ 0.07	62 $\pm$ 5*	109 $\pm$ 9*	0.17 $\pm$ 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  $\pm$  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.<sup>9,10</sup> Heterozygous deletion of *IKK $\beta$*  in mice also enhanced insulin sensitivity but reduced serum FFA concentrations without protecting from weight gain.<sup>2</sup> 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 *IKK $\beta$* . 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.<sup>11</sup> As shown in Table 1, SA series of compounds inhibited both *IKK $\beta$*  and *PTP1B*, SA51 most potent against *IKK $\beta$*  and least potent against *PTP1B*. The inhibitory effects of SA51 in mice might therefore affect *IKK $\beta$*  more than *PTP1B*. Importantly, the effect of SA51 in mice was consistent with the effects of genetic deletion of *IKK $\beta$* . On the other hand, the effects of SA18 and SA32 in mice were

inconsistent with *IKK $\beta$*  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 *IKK $\beta$* , whereas SA18 and SA32 may be more likely to inhibit *PTP1B*. The possibility that SA51 inhibits *PTP1B* in addition to *IKK $\beta$*  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 *IKK $\beta$* . Structurally related compounds, SA18 and SA32, were also known to inhibit both of the enzymes with  $IC_{50}$  values higher against *IKK $\beta$*  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 *IKK $\beta$*  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 *IKK $\beta$*  and *PTP1B* by these compounds.

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