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# Inhibition of IKK-β: A new development in the mechanism of the anti-obesity effects of PTP1B inhibitors SA18 and SA32

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ABSTRACT

In a previous study, protein tyrosine phosphatase 1B (PTP1B) inhibitors, **SA18** and **SA32**, exhibited anti-obesity effects in a mouse model by suppressing weight gain and improving blood parameters, including free fatty acid (FFA) levels. In a separate study, depletion of the PTP1B gene in mice suppressed weight gain without significant change in FFA levels. The discrepancy in FFA concentrations between the two studies suggested that the in vivo target of the **SA** compounds might not be limited to PTP1B. In this study, **SA18** and **SA32** were found to be potent inhibitors of IκB Kinase-β (IKK-β). In vivo relevance of the inhibitory activity was evaluated in differentiated adipocytes. Inhibition of IKK-β, in addition to inhibition of PTP1B, in mice treated with the **SA** compounds, could be a possible mechanism of the compound's biological response including the resistance to diet-induced weight gain and improvement in blood parameters. As potent and cell-permeable IKK-β inhibitors, **SA18** and **SA32** could also be valuable in biological experiments.



The prevalence of obesity is rapidly increasing in all societies of the world and is associated with increased health risks, including diabetes mellitus and cardiovascular diseases.[1,2] As such, effective and durable therapeutics are required. Drugs currently approved for the treatment of obesity are sibutramine and orlistat (also known as tetrahydrolipstatin).[3] Sibutramine is a central-acting serotonin–norepinephrine reuptake inhibitor that suppresses appetite. Orlistat is a pancreatic lipase inhibitor that inhibits the digestion and absorption of triglycerides. These drugs, however, are not free from safety concerns. Sibutramine can cause cardiovascular side effects, such as increased blood pressure and heart rate, with less serious adverse effects, including headache, insomnia, and constipation. Orlistat is associated with gastrointestinal side effects such as fecal incontinence and oily stools. Another drug, rimonabant, was recently developed as a type 1 cannabinoid (CB1) receptor antagonist. Tetrahydrocannabinol, a component of marijuana, is known to stimulate the CB1 receptor, causing euphoria and increasing appetite. Blocking the CB1 receptor by rimonabant decrease appetite but, at the same time, increase the risk of adverse psychiatric effects, including serious depression and suicide. Rimonabant, approved in 2006 in the European Union, was suspended in 2008.[4] The safety concerns, as well as unsatisfactory efficacy, of the currently prescribed drugs indicate a strong need for drugs with novel mechanisms of action.

Targeting PTP1B, a cytosolic protein tyrosine phosphatase, provides another opportunity to treat both obesity and diabetes.[5,6] This enzyme, ubiquitously expressed in most tissues, negatively regulates the leptin and insulin signaling pathways by catalyzing the dephosphorylation of Jak2 and the insulin receptor, respectively.[7–10] In accordance with these observations, PTP1B$^{-/-}$ and PTP1B$^{+/-}$ mice were resistant to diet-induced weight gain and retained insulin sensitivity in contrast to the wild-type mice, which gained weight and became insulin resistant.[11,12] The transgenic mice lacking PTP1B were healthy, alleviating the safety concerns over the strategy to inhibit PTP1B as an alternative therapy against diabetes and obesity. Numerous PTP1B inhibitors have been reported as possible lead compounds for a novel drug, but the first-in-class drug targeting PTP1B is yet to be developed.[13] Ertiprotafib was the first PTP1B inhibitor progressed to clinical trials before being stopped in phase II. Recently, another selective PTP1B inhibitor, trodusquemine, proceeded to phase I clinical trials with promising preclinical results as both an appetite suppressant and a hypoglycemic and hypocholesterolemic agent (Fig. 1).[14]

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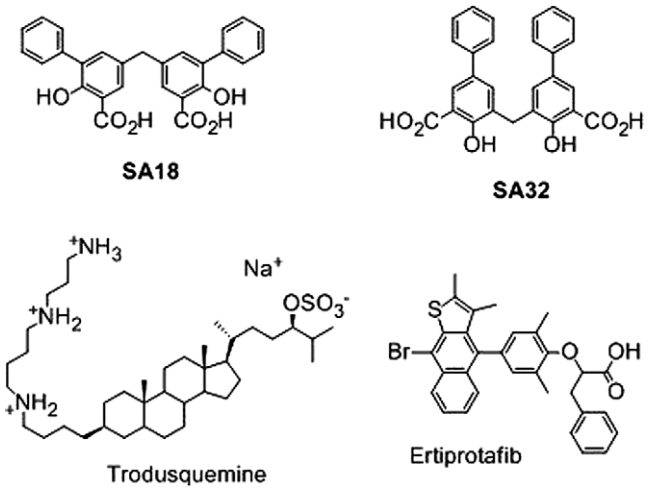


**Figure 1.** Compounds used or mentioned in this study.

In an effort to develop a potent PTP1B inhibitor with hypoglycemic and/or anti-obesity effects, this lab recently reported that compounds **SA18** and **SA32** inhibited PTP1B and suppressed diet-induced obesity.[15,16] When these compounds were fed to diet-induced obese (DIO) mouse models (C57BL/6 J Jms Slc, male) for 4 weeks, significant improvements were observed in obesity-related parameters, including body weight, adipose tissue weight, and related blood parameters—total cholesterol, triglycerides, and FFA (Table 1). In contrast, depletion of the PTP1B gene in mice caused no significant change in the FFA levels; the level tended to increase by deletion of the PTP1B gene.[12] Total cholesterol and triglycerides, in the serum of PTP1B-deleted mice, were not reported. The discrepancy in the FFA concentrations in the serum of the **SA18**- or **SA32**-treated mice and PTP1B-deleted mutant mice suggested that the **SA** compounds might also act on target proteins other than PTP1B.

A decade ago, IKK-β was identified as a target of the anti-inflammatory action of salicylate.[17] Deletion of the IKK-β gene in mice resulted in improved insulin sensitivity and suppression of diet-induced weight gain, implicating the inflammatory process in the pathogenesis of insulin resistance in obesity and diabetes and suggesting IKK-β as a therapeutic target for these diseases.[18] Inspired by these results, as well as the structural feature of **SA18** and **SA32** containing salicylic acid moieties, this lab considered the possibility that these compounds might inhibit IKK-β activity.

The effects of **SA18** and **SA32** on the kinase activity of IKK-β were examined by measuring the catalytic activity of IKK-β to phosphorylate a peptide corresponding to the IκB phosphorylation motif.[19] The phosphorylated peptide product was quantified by an ELISA-type assay employing a secondary antibody complexed with a europium ion ($Eu^{3+}$) in lieu of the amplifying enzyme. For TRF measurement, a $Eu^{3+}$ ion was excited by a laser pulse, with the fluorescence emission measured after a short delay. Use of the TRF technique eliminated the background light level, thus, improving sensitivity compared to classical fluorescence measurements. Both **SA18** and **SA32** were potent inhibitors of IKK-β, with $IC_{50}$ values of 4.7 ± 1.0 and 14 ± 2 μM, respectively, lower concentrations than those required for the half-maximal inhibition of the *p*-nitrophenyl phosphatase activity of PTP1B (Table 2). Aspirin and sodium salicylate, previously known as IKK-β inhibitors, exhibited $IC_{50}$ values of >1 mM under the presented assay conditions, consistent with most of the previous reports.[20] Recently developed IKK-β inhibitors include BMS-345541, PS-1145, SPC-839, ML120B, TPCA1, and Bayer 'compound **A**' with $IC_{50}$ or $K_i$ values of 300, 150, 67, 45, 18, and 2 nM, respectively.[21,22] These are the results of pharmaceutical efforts for the therapeutic intervention of the NF-κB pathway in inflammatory diseases. The **SA** compounds revealed a novel methylenedisalicylate scaffold for IKK-β inhibition and the structural modifications of this scaffold is to be done to achieve the inhibitory potencies comparable to those of the above mentioned inhibitors.

To study whether **SA18** and **SA32** suppressed weight gain and improved blood parameters in mice by inhibition of IKK-β, the effects of these compounds on LPS-inducible NF-κB activation were examined in differentiated adipocytes.[23] Treatment with **SA18** and **SA32** prior to stimulation of the adipocytes with LPS prevented IκB-α degradation. Preadipocytes 3T3-L1 were differentiated into mature adipocytes by inducing adipogenesis with isobutylmethylxanthine, dexamethasone, and insulin. After incubation with **SA18** or **SA32**, the differentiated adipocytes were challenged to induce inflammation with a cytokine mixture containing LPS, TNF-α, interferon-γ, and interleukin-1β. The extent to which **SA18** and **SA32** prevented the degradation of IκB-α in adipocytes was then determined. Western blot analysis of the total cellular extracts using monoclonal antibodies against IκB-α revealed that both **SA18** and **SA32** effectively prevented IκB degradation in adipocytes. Both compounds were effective at 50 μM concentration with an optimum incubation time of 60 min for **SA18** and 30 min for **SA32** (Fig. 2). Similar results were also obtained in cultured murine macrophage Raw 246.7 cells (data not shown).

These observations demonstrate that **SA18** and **SA32** behaved as IKK-β inhibitors in adipose cells. In a previous study, heterozy-

**Table 2**
$IC_{50}$ values of compounds **SA18** and **SA32** against IKK-β and PTP1B

| | IKK-β (μM)[19] | PTP1B (μM) |
|---|---|---|
| **SA18** | 4.7 ± 1.0[a] | 20 ± 1[b] |
| **SA32** | 14 ± 0.2[a] | 19 ± 1[b] |
| Salicylic acid | >1000 | - |

[a] Data expressed as the mean ± SEM of two experiments.[19] Compounds **SA18** and **SA32** were synthesized as previously described.[15,16]
[b] Data reproduced from our previous publications.[15,16]

**Table 1**
Effect of 28 days of treatment with **SA18** and **SA32** on body weight, adipose tissue weight, and related blood parameters[a]

| | Body weight (g) | Adipose tissue weight (g) | | Triglyceride (mg/dL) | Total cholesterol (mg/dL) | NEFA (mEq/L) |
|---|---|---|---|---|---|---|
| | | Epididymal | Retroperitoneal | | | |
| LFD | 26.2 ± 1.4* | 0.52 ± 0.09* | 0.15 ± 0.04* | 47 ± 8 | 104 ± 11* | 0.23 ± 0.07* |
| HFD | 33.4 ± 2.8 | 1.80 ± 0.49 | 0.51 ± 0.11 | 63 ± 32 | 141 ± 10 | 0.35 ± 0.09 |
| HFD+**SA18** | 29.2 ± 2.5* | 0.86 ± 0.24* | 0.26 ± 0.08* | 42 ± 7* | 129 ± 8* | 0.24 ± 0.09* |
| HFD+**SA32** | 28.9 ± 1.7* | 0.83 ± 0.34* | 0.26 ± 0.07* | 48 ± 8 | 133 ± 14 | 0.14 ± 0.06* |

[a] Data reproduced from the previous publications in our laboratory with minor corrections in SEM of NEFA data considering significant figures.[15,16] High-fat-diet (HFD) was fed to mice (C57BL/6 J Jms Slc, male, 5 weeks old) ad libitum for 8 weeks to induce obesity and then **SA18** and **SA32** were fed mixed with HFD for 4 weeks. For the obese and lean control groups, HFD or low-fat-diet (LFD) was fed all through the 12 weeks period. Data expressed as the mean ± SEM.
* Significantly different from the HFD group ($p$ <0.005).

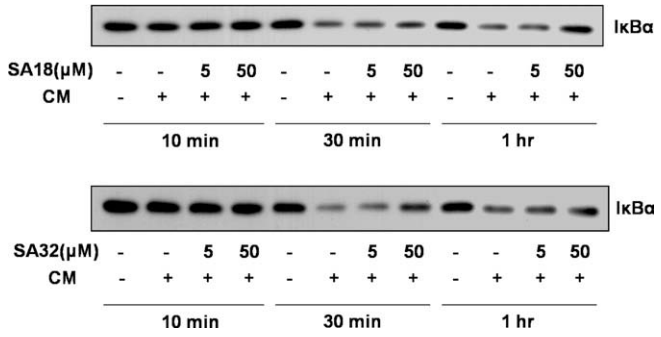


**Figure 2.** Effect of **SA18** and **SA32** on LPS-inducible NF-κB activation.[21] Western blot analysis of the IκB-α in differentiated adipocytes preincubated with and without **SA18** and **SA32**, for 30 min, and then challenged with cytokine mixture (LPS 1 μg/mL + TNF-α 1 ng/mL + interferon-γ 10 ng/mL + interleukin-1β 1 ng/mL) for indicated time periods. Total cellular extracts were used for Western blot analysis using monoclonal antibodies against IκB-α.

gous deletion of IKK-β in mice resulted in suppression of diet-induced weight gain and reduction of serum FFA levels, consistent with the response of the mice treated with **SA18** and **SA32**.[18] Taken together, these observations indicate that the biological effect of the compounds in mice could be, at least in part, a result of inhibition of IKK-β.

Similar examples of the complex mechanism of action were recently found in ertiprotafib, a PTP1B inhibitor stopped in phase II clinical trials. Observation of discrepancies in blood parameters between PTP1B-deficient and ertiprotafib-treated mice groups led to the finding of additional mechanisms of ertiprotafib action including inhibition of IKK-β and activation of PPARα and PPARγ.[20,24]

In conclusion, compounds **SA18** and **SA32** were identified as IKK-β inhibitors with medium potencies. The novel scaffold could be used for intensive medicinal chemistry efforts to develop more potent IKK-β inhibitors and this is in progress. Also, inhibition of IKK-β, in addition to inhibition of PTP1B, in mice treated with **SA18** and **SA32**, could be a possible mechanism of the compound's biological response including the resistance to diet-induced weight gain and improvement in blood parameters.

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