The novel anti-adipogenic effect and mechanisms of action of SGI-1776, a Pim-specific inhibitor, in 3T3-L1 adipocytes

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Abstract. The proviral integration site for moloney murine leukemia virus (Pim) kinases, consisting of Pim-1, Pim-2 and Pim-3, belongs to a family of serine/threonine kinases that are involved in controlling cell growth and differentiation. Pim kinases are emerging as important mediators of adipocyte differentiation. SGI-1776, an inhibitor of Pim kinases, is widely used to assess the physiological roles of Pim kinases, particularly cell functions. In the present study, we examined the effects of SGI-1776 on adipogenesis. The anti-adipogenic effect of SGI-1776 was measured by Oil Red O staining and AdipoRed assays. The effect of SGI-1776 on the growth of 3T3-L1 adipocytes was determined by cell count analysis. The effects of SGI-1776 on the protein and mRNA expression of adipogenesis-related proteins and adipokines in 3T3-L1 adipocytes were also evaluated by western blot analysis and RT-PCR, respectively. Notably, SGI-1776 markedly inhibited lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes. On a mechanistic level, SGI-1776 inhibited not only the expression of CCAAT/enhancer-binding protein-α (C/EBP-α), peroxisome proliferator-activated receptor-γ (PPAR-γ) and fatty acid synthase (FAS), but also the phosphorylation of signal transducer and activator of transcription-3 (STAT-3). Moreover, SGI-1776 decreased the expression of adipokines, including the expression of leptin and regulated on activation, normal T cell expressed and secreted (RANTES) during adipocyte differentiation. These findings demonstrate that SGI-1776 inhibits adipogenesis by downregulating the expression and/or phosphorylation levels of C/EBP-α, PPAR-γ, FAS and STAT-3.

Introduction

Obesity is a high risk factor for the development of a number of human diseases, including insulin resistance, type 2 diabetes, hyperlipidemia and cancer (1). Obesity is induced by several factors, such as genetic and endocrine abnormalities, certain medicines, a low metabolic rate, nutritional and environmental factors, as well as imbalanced energy homeostasis (2,3). Previous studies have indicated that adipose tissue serves as an energy reservoir and also plays a critical role in the control of energy metabolism by secreting adipokines (3,4). However, there is also strong evidence that the abnormal expansion/accumulation of adipose tissue, which is largely attributable to excessive adipocyte differentiation and an increase in the number and size of fat cells, is closely linked to the development of obesity (5,6). Thus, any compound that inhibits excessive adipocyte differentiation and adipocyte hyperplasia/hypertrophy may have preventive and therapeutic potential against obesity.

It has previously been reported that the differentiation of preadipocytes into mature adipocytes, also termed adipogenesis, is controlled by numerous cellular proteins, transcription factors, adipocyte-specific genes, lipogenic enzymes and signaling proteins. For instance, it has previously been demonstrated that the expression of the family of CCAAT/enhancer-binding proteins (C/EBPs; C/EBP-α, -β and -δ) and peroxisome proliferator-activated receptors (PPARs; PPAR-γ, -α and -β) is critical for adipocyte differentiation (7,8). Moreover, there is evidence indicating that Janus-activated protein kinase-2 (JAK-2)/signal transducer...
and activator of transcription (STAT)-3 and STAT-5 signaling complexes are associated with adipocyte differentiation (9,10). In addition, a number of signaling proteins and factors, including protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated protein kinase-1/2 (ERK-1/2) and adenosine 3',5'-cyclic monophosphate (cAMP), have been found to be of importance for controlling adipocyte differentiation (11-13).

SGI-1776, N-[(1-methylpiperidin-4-yl)methyl]-3-[3-(trifluoromethoxy)phenyl]imidazo[1,2-b]pyridazin-6-amine, has been shown to inhibit three members of the Pim kinase family (Pim-1, Pim-2 and Pim-3) (14). Pim kinases are constitutively active serine/threonine kinases that are known to be over-expressed in hematological malignancies, such as acute myeloid leukemia and multiple myeloma (15). Pim kinases have multiple substrates that are involved in transcription, protein translation, cell proliferation and apoptosis. Due to its ability to inhibit Pim kinase, SGI-1776 has been tested as a possible treatment for a number of hematological malignancies (16). Of note, it has been previously suggested that Pim-2 is substantially expressed in adipocytes (17) and the expression of Pim-1 in adipose tissue may be used as a marker of adipocytic differentiation (18). There is also compelling evidence indicating that Pim kinases are downstream target genes of STAT-3 (19).

In a recent studies of ours, we demonstrated that a meridianin C derivative, which inhibits protein kinases, including Pim kinases (20), markedly inhibited adipogenesis (21). This research led us to further hypothesize that SGI-1776, a Pim-specific inhibitor would suppress adipogenesis to a greater extent than a meridianin C derivative.

In the present study, we investigated the anti-adipogenic effects of SGI-1776 on 3T3-L1 adipocytes. To the best of our knowledge, this is the first study which demonstrates that SGI-1776 exerts a prominent anti-adipogenic effect without affecting the viability of adipocytes and that the anti-adipogenic effect of SGI-1776 is largely associated with the decreased expression and/or phosphorylation levels of C/EBP-α, PPAR-γ, fatty acid synthase (FAS) and STAT-3.

**Materials and methods**

**Materials.** Polyclonal C/EBP-α (sc-61), monoclonal PPAR-γ (sc-7273), monoclonal STAT-3 (sc-8019), monoclonal phosphorylated (p-)STAT-3 (p-STAT-3; sc-8059), polyclonal STAT-5 (sc-835) and polyclonal p-STAT-5 (sc-101806) antibodies were all purchased from Santa Cruz Biotechnology, Inc. (Delaware, CA, USA). Monoclonal β-actin (A5441) antibody, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and insulin were all purchased from Sigma (St. Louis, MO, USA). SGI-1776 was purchased from Merck (Darmstadt, Germany). Dexamethasone and insulin were all purchased from Sigma (St. Louis, MO, USA). SGI-1776 was purchased from Merck (Darmstadt, Germany).

**Cell culture and differentiation.** As previously described (27), 3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were grown to the contact inhibition stage and remained in the post-confluent stage for 2 days in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and penicillin-streptomycin (Wegene, Daegu, Korea). Differentiation was then induced by changing the medium to DMEM supplemented with 10% FBS (Wegene, Daegu, Korea) plus a cocktail of hormones (MDI), containing 0.5 mM IBMX (M), 0.5 µM dexamethasone (D) and 5 µg/ml insulin (I) in the presence or absence of SGI-1776 or butein (Sigma) at the indicated concentrations. Following 48 h MDI induction, the differentiation medium was replaced with DMEM supplemented with 10% FBS and 5 µg/ml insulin in the presence or absence of SGI-1776 at the indicated concentrations. The cells were then fed every other day with DMEM containing 10% FBS in the presence or absence of SGI-1776 or butein at the indicated concentrations until day 8. On day 8, the preadipocytes became mature adipocytes that were rounded and filled with many oil droplets.

**Oil Red O staining.** On day 8 of differentiation, the mock- (not treated with SGI-1776 or butein) or SGI-1776- or butein-treated 3T3-L1 cells were washed twice with phosphate-buffered saline (PBS), fixed with 10% formaldehyde for 2 h at room temperature, washed with 60% isopropanol and dried completely. The fixed cells were then stained with Oil Red O (Sigma, St. Louis, MO, USA) working solution for 1 h at room temperature and were then washed twice with distilled water. Lipid droplets were observed under a light microscope (TS100; Nikon, Tokyo, Japan).

**Cell count assay.** The 3T3-L1 preadipocytes seeded in 24-well plates were similarly grown under the above-mentioned differentiation conditions. On day 8 of differentiation, the mock- or SGI-1776-treated 3T3-L1 cells, which cannot be stained with trypan blue dye, were counted under a microscope. The cell count assay was carried out in triplicate. Data are presented as the means ± standard error of 3 independent experiments.

**Quantification of intracellular triglyceride (TG) content by AdipoRed assay.** On day 8 of differentiation, the lipid content in the mock- or SGI-1776- or butein-treated 3T3-L1 cells was measured using a commercially available AdipoRed Assay Reagent kit according to the manufacturer’s instructions (Lonza, Basel, Switzerland). Following 10 min of incubation, fluorescence was measured using a multilabel reader (Victor3; PerkinElmer, Waltham, MA, USA) with the excitation set at 485 nm and the emission at 572 nm.

**Preparation of whole cell lysates.** As previously described (27), at a designated time point, the 3T3-L1 cells were washed twice with PBS and exposed to modified RIPA buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1X)]. The cell lysates were collected and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was saved, and the protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA, USA).

**Western blot analysis.** Proteins (50 µg) were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were washed with Tris-buffered saline (10 mM Tris-Cl, 150 mM NaCl, pH 7.5), supplemented with 0.05% (v/v) Tween-20 (TBST), followed by blocking with TBST containing 5% (v/v) non-fat dried milk. The membranes were incubated
overnight with antibodies specific for C/EBP-α, PPAR-γ, STAT-3, p-STAT-3, STAT-5, p-STAT-5, FAS or β-actin at 4°C. The membranes were then exposed to secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature and further washed 3 times with TBST. Immunoreactivity was detected using ECL reagents. Equal protein loading was assessed by the expression levels of actin.

Reverse transcription-polymerase chain reaction (RT-PCR).

At a designated time point, total cellular RNA in the mock- or SGI-1776-treated 3T3-L1 cells was isolated using RNAzol-B (Tel-Test, Inc., Friendswood, TX, USA). Three micrograms of total RNA were reverse transcribed using a random hexadecoxynucleotide primer and reverse transcriptase. Single-stranded cDNA was amplified by PCR using specific primers. The primer sequences used for amplification were as follows: C/EBP-α sense, 5'-TTGAGATGCTTCCGC-3' and antisense, 5'-GTTAGCCAGTTCC-3'; adiponectin sense, 5'-GGAGATGCAGGTCTTCTTGGT-3' and antisense, 5'-GACC-3'; leptin sense, 5'-CCAAACCCTCATCAA GACC-3' and antisense, 5'-CTGAAGGGCCACCACCTCTG-3'; FAS sense, 5'-TTGCTTGCCAC TACAGAATGC-3' and antisense, 5'-AAGAGCCTCAGAG CGACAAT-3'; and β-actin sense, 5'-TCACATCTCTGAGTCATCCAA GACC-3' and antisense, 5'-GTGGTGCATCTTTGCTTGAATGAA-3'; regulated on activation, normal T cell expressed and secreted (RANTES) sense, 5'-TTGAGATGCTTCCGC-3' and antisense, 5'-GTTAGCCAGTTCC-3'; monocyte chemoattractant protein-1 (MCP-1) sense, 5'-CTCAATCTCTGAGTCATCCAA GACC-3' and antisense, 5'-CTGCTTTGCGAACACATTG-3'; and the phosphorylation levels of adipogenic transcription factors, including the families of C/EBPs, PPARs and STATs. Western blot analysis revealed that SGI-1776 markedly inhibited the phosphorylation levels of STAT-3 during adipocyte differentiation.

Statistical analysis. Cell count analysis was completed in triplicate and repeated 3 times. Data are expressed as the means ± standard error (SE). The significant differences between groups were determined by one-way ANOVA (Laerd Statistics, Chicago, IL, USA). All significance testing was based upon a P-value <0.05, indicating a statistically significant difference.

Results

SGI-1776 exerts potent anti-adipogenic effects. Primarily, we wished to determine whether the Pim-selective inhibitor, SGI-1776, inhibits intracellular lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes by Oil Red O staining. The timescale of 3T3-L1 preadipocyte differentiation is illustrated in Fig. 1A. Many lipid droplets were formed in the differentiated 3T3-L1 preadipocytes cultured in the induction medium on day 8 of cell differentiation, compared with the undifferentiated cells (preadipocytes), in which no lipid droplets had formed (Fig. 1B, upper panels). However, treatment with SGI-1776 for 8 days markedly reduced the amount of lipid droplets in a concentration-dependent manner, as compared with the mock-treated cells. Evidently, treatment with 10 µM SGI-1776 completely blocked the accumulation of lipid droplets. The SGI-1776-mediated lipid-reducing effect was also confirmed by light microscopy (Fig. 1B, lower panels).

In addition, we noted that SGI-1776 decreased the levels of TG during adipocyte differentiation in a dose-dependent manner, with maximum reduction being achieved with an inhibitor concentration of 10 µM (Fig. 1C). However, using a cell count assay, it was observed that SGI-1776 induced a slight decrease in viability at 10 µM, but not at 5 µM (Fig. 1D). Certain compounds, such as butein (40 µM), have also previously been shown to exert anti-adipogenic effects on 3T3-L1 adipocytes (22). In this study, we thus compared the anti-adipogenic effect of SGI-1776 (5 µM) with that of butein (40 µM) used as a positive control. In agreement with the results of previous studies, treatment with butein at 40 µM markedly inhibited both lipid droplet formation (Fig. 1E) and TG synthesis (Fig. 1F) during adipocyte differentiation. Of note, treatment with SGI-1776 at 5 µM exerted more potent inhibitory effects on both lipid droplet formation and TG synthesis than butein (40 µM). Therefore, the concentration of 5 µM of SGI-1776 was selected for use in further experiments due to the potent inhibitory effects it exerted on both lipid accumulation and TG synthesis without affecting the number of adipocytes.

Inhibition of the early adipogenic process is critical for the SGI-1776-mediated anti-adipogenic effects. We subsequently sought to determine which stage(s) of adipocyte differentiation is inhibited by SGI-1776. To this end, the 3T3-L1 preadipocytes were incubated with induction medium (MDI, insulin and FBS) in the presence or absence of SGI-1776 (5 µM) for the indicated periods of time, as illustrated in Fig. 2A. As expected, the mock-treated cells incubated with MDI, insulin and FBS for 2, 5 and 8 days exhibited high lipid accumulation (treatment a). The 3T3-L1 preadipocytes treated with SGI-1776 from day 0 to 2 (treatment b), or from day 0 to 5 (treatment c) showed a comparatively low lipid content. The preadipocytes treated with SGI-1776 from day 0 to 8 exhibited the highest inhibition of lipid droplet accumulation (treatment d). Of note, differentiating 3T3-L1 cells treated with SGI-1776 from day 2 to 5, from day 2 to 8 or from day 5 to 8 (treatment e, f and g) exhibited a much weaker inhibition of adipogenic differentiation, compared with treatment b, c and d (Fig. 2B). The results of the AdipoRed assay further demonstrated that treatment b, c and d resulted in the highest reduction of TG during adipocyte differentiation (Fig. 2C). Treatment f also substantially reduced the TG content during adipocyte differentiation, whereas treatment e and g led to a slight reduction in the TG content. These results collectively suggest that SGI-1776 inhibits adipogenesis particularly at an early phase of differentiation.

SGI-1776 decreases the expression of C/EBP-α and PPAR-γ and the phosphorylation levels of STAT-3 during adipocyte differentiation. To elucidate the mechanisms underlying the SGI-1776-mediated anti-adipogenic effect, we examined the effects of SGI-1776 (5 µM) on the expression and/or activity (phosphorylation) levels of adipogenic transcription factors, including the families of C/EBPs, PPARs and STATs. Western blot analysis revealed that SGI-1776 markedly inhibited the adipogenesis-dependent protein expression of C/EBP-α and PPAR-γ during adipocyte differentiation, particularly on day 5 and 8 (Fig. 3A and E). The results from RT-PCR also revealed that SGI-1776 suppressed the mRNA expression of C/EBP-α and PPAR-γ during adipocyte differentiation (Fig. 3B and F).
These results suggest that SGI-1776 downregulates PPAR-γ and C/EBP-α expression at the transcriptional level. Moreover, SGI-1776 markedly decreased the phosphorylation levels of STAT-3 on day 2 during adipocyte differentiation (Fig. 3C and G). By contrast, SGI-1776 did not markedly affect the phosphorylation levels of STAT-5. Notably, SGI-1776 also had the capacity to suppress STAT-3 phosphorylation during the early stages of adipogenesis induced by MDI at the treatment...
times of 2, 4, 8 and 24 h (Fig. 3D and H). SGI-1776 did not markedly alter the total protein levels of STAT-3 and STAT-5 during adipocyte differentiation at the designated time points. SGI-1776 downregulates the protein and/or mRNA expression of FAS, leptin and RANTES during adipocyte differentiation. The expression and secretion of adipokines occur during preadipocyte differentiation (28-30). Therefore, we examined whether SGI-1776 (5 µM) regulates the expression of adipocyte-specific genes and/or adipokines during adipocyte differentiation. SGI-1776 markedly suppressed the protein and mRNA expression levels of FAS on days 5 and 8 during adipocyte differentiation (Fig. 4A-C). Furthermore, the results from RT-PCR demonstrated that SGI-1776 markedly reduced the insulin- and FBS-induced mRNA expression of leptin and RANTES on days 5 and 8 (Fig. 4B-D). However, SGI-1776 did not greatly affect the mRNA levels of adiponectin or MCP-1 on days 5 and 8 (Fig. 4B and D).

Discussion

Excessive adipocyte differentiation causes the abnormal expansion/accumulation of adipose tissue, leading to the high secretion of adipokines, which have been implicated in inflammation, insulin resistance and metabolic disorders (5,6). Thus, inhibitors of adipocyte differentiation likely have therapeutic potential as anti-obesity drugs. Recently, we demonstrated that a meridianin C derivative, which inhibits protein kinases including Pim kinases (20), markedly blocks adipocytic differentiation (21). In the present study, we focused on the role of Pim kinases in 3T3-L1 adipogenesis. We noted that when the 3T3-L1 cells differentiated in the presence of a potent and selective Pim inhibitor, such as SGI-1776, adipogenesis was severely inhibited.

Mature adipocytes have a spherical shape and are filled with a high number of intracellular lipid droplets, and these can be distinguished from fibroblast-like preadipocytes (23). The present study demonstrated that SGI-1776 (5 µM) markedly inhibited lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes by 8 days of differentiation (Fig. 1B and C), suggesting that it exerts a potent anti-adipogenic effect. Cell count analysis further revealed that treatment with SGI-1776 (5 µM) for 8 days did not markedly affect the viability of 3T3-L1 adipocytes (Fig. 1D).

As previously mentioned, the induction of adipocyte differentiation is largely dependent on the expression and activity of

Figure 2. Effect of SGI-1776 on different processes of 3T3-L1 adipocyte differentiation. (A) Schematic outline of the experimental setup: the double-headed arrows indicate the length of treatment. 3T3-L1 cells were treated with or without SGI-1776 for the indicated periods of time. (B) 3T3-L1 preadipocytes were induced to differentiate with induction medium containing MDI, insulin and fetal bovine serum (FBS) in the presence or absence of SGI-1776, as described in Materials and methods (a-g) at the indicated time points. The cellular lipid content at each time-point was assessed by Oil Red O staining (left panels). Phase-contrast images of the cells were also acquired following treatment (right panels). Each images is representative of 3 independent experiments. (C) Quantification of triglyceride (TG) content in control or SGI-1776-treated 3T3-L1 adipocytes on day 8 by AdipoRed assay. Values represent the means ± SE of data from 3 independent experiments with 3 replicates. *P<0.05 vs. control (no treatment).
adipogenesis-related transcription factors, such as C/EBP-α and PPAR-γ. It has been previously demonstrated that blocking their expression and/or activity with either pharmacological inhibitors or small interfering RNA leads to the inhibition of lipid accumulation during adipocyte differentiation, and that the knockdown of C/EBP-α or PPAR-γ decreases or impairs the differentiation of white adipose tissue in mice (7,8,24). The present study demonstrated that SGI-1776 inhibited the protein and mRNA expression of C/EBP-α and PPAR-γ during adipocyte differentiation (Fig. 3A, B, E and F). Thus,
the SGI-1776-mediated anti-adipogenic effect appears to be closely associated with the reduced expression of C/EBP-α and PPAR-γ. Previously, it has also been reported that the family of STATs, including STAT-1, STAT-3, STAT-5 and STAT-6, is expressed in both 3T3-L1 preadipocytes and adipocytes (9), and that several members of the STAT family, including STAT-3 and STAT-5, are critical for 3T3-L1 adipocyte differentiation (10,21,25-27). Of note, previous studies have further demonstrated that STAT-3 is rapidly (within hours) activated during adipocyte differentiation, and that active STAT-3 plays an important role in the early stages of adipogenesis (10,21,27). In the present study, we noted that SGI-1776 blocked not only the early adipogenic process (Fig. 2B and C), but also MDI (but neither insulin nor FBS) signaling to activate STAT-3 during the early stage of adipogenesis (10,21,27). Of note, previous studies have further demonstrated that STAT-3 is rapidly (within hours) activated during adipocyte differentiation, and that active STAT-3 plays an important role in the early stages of adipogenesis (10,21,27). In the present study, we noted that SGI-1776 blocked not only the early adipogenic process (Fig. 2B and C), but also MDI (but neither insulin nor FBS) signaling to activate STAT-3 during the early stage of adipogenesis (Fig. 2B and C). It is thus likely that the STAT-3-dependent blockage of early adipogenesis is also crucial for the SGI-1776-mediated anti-adipogenic effect. Previous studies have indicated that STAT-3 regulates adipocyte differentiation via PPAR-γ, and that STAT-3 activity is also important for the adipogenesis-induced expression of C/EBP-α and PPAR-γ (26,27). Hence, it is suggested in this study that SGI-1776 inhibits adipogenesis primarily through the inhibition of STAT-3 at the early adipogenic process, which subsequently downregulates the expression of C/EBP-α and PPAR-γ.

Previous research has demonstrated that the increased expression and activity of C/EBPs and PPARs is necessary for the expression of adipocyte-specific genes and adipokines, including FAS, leptin, adiponectin, MCP-1 and RANTES (28-30). Of those, FAS is a lipogenic enzyme involved in fatty acid synthesis, and its expression is highly upregulated in cells or tissues with high rates of fatty acid synthesis (31). In addition, it has previously been shown that FAS mRNA and protein expression are substantially increased during 3T3-L1 preadipocyte differentiation (21,27,32). In this study, we noted that SGI-1776 also had the ability to markedly inhibit the insulin- and FBS-mediated induction of FAS at both the protein and mRNA level in 3T3-L1 adipocytes (Fig. 4). These results suggest that SGI-1776 exerts an anti-lipogenic effect by suppressing FAS expression, which further contributes to the SGI-1776-mediated anti-adipogenic
effect. It has been well documented that the maturation of adipocyte differentiation is characterized by the capacity of mature adipocytes to express and secrete adipokines, which are involved in the endocrine control of energy homeostasis (33,34). Therefore, considering that SGI-1776 inhibits the mRNA expression of leptin and RANTES, but not adiponectin, during the adipocyte differentiation process (Fig. 4B and D), we suggest that SGI-1776 further blocks the adipocyte differentiation maturation process.

In conclusion, in this study, we demonstrate firstly that a pan-Pim kinase inhibitor, SGI-1776, exerts potent anti-adipogenic effects in 3T3-L1 cells that are related to the reduced expression and/or phosphorylation levels of PPAR-γ, C/EBP-α, FAS and STAT-3. The findings of this study thus suggest that the Pim-specific inhibitor, SGI-1776, be considered a potential therapeutic agent for the prevention and treatment of obesity in which excessive adipogenesis plays pathological roles.

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