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PPARγ Antagonist Gleevec Improves Insulin Sensitivity and Promotes the Browning of White Adipose Tissue

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Blocking phosphorylation of peroxisome proliferator–activated receptor (PPAR)γ at Ser^{273} is one of the key mechanisms for antidiabetes drugs to target PPARγ. Using high-throughput phosphorylation screening, we here describe that Gleevec blocks cyclin-dependent kinase 5–mediated PPARγ phosphorylation devoid of classical agonism as a PPARγ antagonist ligand. In high fat–fed mice, Gleevec improved insulin sensitivity without causing severe side effects associated with other PPARγ-targeting drugs. Furthermore, Gleevec reduces lipogenic and gluconeogenic gene expression in liver and ameliorates inflammation in adipose tissues. Interestingly, Gleevec increases browning of white adipose tissue and energy expenditure. Taken together, the results indicate that Gleevec exhibits greater beneficial effects on both glucose/lipid metabolism and energy homeostasis by blocking PPARγ phosphorylation. These data illustrate that Gleevec could be a novel therapeutic agent for use in insulin resistance and type 2 diabetes.

As the prevalence of obesity has exploded over the last several decades, associated metabolic disorders, including type 2 diabetes, dyslipidemia, hypertension, and cardiovascular diseases, have also increased dramatically. As PPARγ agonists, thiazolidinediones (TZDs), which include pioglitazone, represent synthetic insulin-sensitizing drugs that have been widely prescribed for the treatment of type 2 diabetes (1,2). However, the use of TZDs is associated with unwanted side effects, including weight gain, fluid retention, bone fracture, cardiovascular disease, and bladder cancer (3–7). Thus, the U.S. Food and Drug Administration (FDA) recently restricted the use of one TZD, rosiglitazone, for the treatment of type 2 diabetes.

PPARγ is a master regulator of adipocyte differentiation, glucose and lipid metabolism, and inflammation (8–10). Recently, we demonstrated that phosphorylation of PPARγ at Ser^{273} (pS273) is linked to obesity and insulin resistance (11). Phosphorylation does not globally alter its transcriptional activity but dysregulates a specific set of genes with roles in obesity and diabetes (11,12). Moreover, both TZDs and selective PPARγ modulators inhibit cyclin-dependent kinase (CDK)5-mediated PPARγ pS273 (11–13). More specifically, nonagonist PPARγ ligands (SR1664 or UHC1) are antidiabetic and have reduced signals of undesirable side effects caused by TZDs (12,13). These observations indicate that blocking pS273 without classical agonism is an important mechanism to consider in the development of novel antidiabetes drugs targeting PPARγ.

In the current study, we screened a chemical library for compounds that inhibit pS273 in vitro and found that Gleevec, a well-known anticancer drug, blocked PPARγ phosphorylation as a PPARγ ligand without classical agonism. Gleevec improved insulin sensitivity without the commonly observed side effects of TZDs in mice fed a

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high-fat diet (HFD). Furthermore, it negatively regulated proinflammatory responses and glucose production in white adipose tissue (WAT) and liver, respectively. Importantly, it increased energy expenditure by regulating a thermogenic program in subcutaneous WAT (sWAT), resulting in antiobesity effects. Our results demonstrate that Gleevec is a potent therapeutic agent for both diabetes and obesity.

RESEARCH DESIGN AND METHODS

Cell Culture

3T3-L1, human embryonic kidney (HEK)-293, and Raw264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM with 10% FBS. FLAG–wild-type PPARγ (FLAG-PPARγ^{WT}) and FLAG–phosphorylation-deficient PPARγ mutant (FLAG-PPARγ^{S273A}) were subcloned into pMSCV-puro retroviral vector (Agilent Technologies, Santa Clara, CA). Adipocyte differentiation of 3T3-L1 or mouse embryonic fibroblasts (MEFs) expressing PPARγ^{WT} or PPARγ^{S273A} was induced as previously described (11). Fully differentiated 3T3-L1 and MEFs or Raw264.7 cells were preincubated with Gleevec for 24 h and treated with tumor necrosis factor (TNF)-α (50 ng/mL) for 3 h or lipopolysaccharide (LPS) (10 ng/mL) for 6 h, respectively. All chemicals for cell culture were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

In Vitro Kinase Assay

Active Cdk5/p35 or extracellular signal–related kinase (ERK) was purchased from Millipore. In vitro kinase assay was performed as previously described (11). Briefly, 0.5 μg recombinant PPARγ (Cayman Chemical, Ann Arbor, MI) was incubated with active CDK or ERK kinases in kinase assay buffer (25 mmol/L Tris-HCl, pH 7.5; 5 mmol/L β-glycerophosphate; 2 mmol/L dithiothreitol; 0.1 mmol/L Na_3VO_4; and 10 mmol/L $MgCl_2$) containing 10 μmol/L ATP for 15 min at 30°C. Retinoblastoma (Rb) (Cell Signaling Technology, Danvers, MA) was used as a positive control.

Immunoprecipitation and Immunoblotting

HEK-293 cells expressing PPARγ were treated with TNF-α (50 ng/mL), and total cell lysates were incubated with FLAG M2 agarose (Sigma-Aldrich) at 4°C. Immunoprecipitates and total cell lysates were analyzed with phospho-specific antibody against Ser^{273} (11) or anti-PPARγ antibody (Santa Cruz Biotechnology, Inc., Dallas, TX).

Primary Hepatocyte Isolation and Glucose Production Assay

Primary mouse hepatocytes were isolated by the two-step collagenase perfusion method from male C57BL/6 after HFD as previously described (14). Primary hepatocytes were plated and treated with Gleevec for 24 h following to treat forskolin (10 μmol/L) for 6 h. The glucose concentration in the media were measured by glucose assay kit (Sigma-Aldrich).

Gene Expression Analysis

Total RNA was isolated from cells or tissues using Trizol reagents (Invitrogen, Carlsbad, CA). The RNA was reverse transcribed using an ABI reverse transcription kit. Quantitative PCR reactions were performed with SYBR green fluorescent dye using an ABI9300 PCR machine. Relative mRNA expression was determined by the ΔΔ-Ct method normalized to TATA-binding protein levels.

Reporter Gene Assay

HEK-293 cells were transfected with pDR-1 luciferase reporter plasmid, PPARγ, PPARα, or PPARδ, RXRα, or pRL-renillia, respectively (Invitrogen). Reporter gene assay was performed as previously described (11).

Animals

All animal experiments were performed according to procedures approved by Ulsan National Institute of Science and Technology's Institutional Animal Care and Use Committee. Five-week-old male C57BL/6J mice (DBL, Eumseong-gun, Korea) were fed an HFD (60% kcal fat, D12492; Research Diets, New Brunswick, NJ) for 10 weeks. After 7 days' injection with Gleevec (25 mg/kg i.p.; the human equivalent dose would be ~1,500 mg/daily) or vehicle mice were injected with D-glucose (1.5 g/kg body weight) after overnight starvation or human insulin (0.75 units/kg body weight) after 6 h starvation for glucose tolerance tests (GTTs) or insulin tolerance tests (ITTs), respectively. For determination of energy expenditure and inflammation, mice were injected daily with 20 mg/kg Gleevec or vehicle for 3 weeks. Oxygen consumption, carbon dioxide production, and food intake were measured by the Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH), and body temperatures were measured rectally using digital thermometer. Blood glucose levels were determined using tail blood and a glucometer. Serum insulin (Crystal Chem, Downers Grove, IL) and serum cholesterol, triglycerides, and free fatty acids were determined by ELISA (Cayman Chemical).

Statistical Analysis

Data are presented as means ± SEM as indicated in the figure legends. Comparisons between two groups were made by unpaired two-tailed Student *t* tests. *P* values of <0.05 were considered statistically significant. Microsoft Excel was used for statistical calculations.

RESULTS

Gleevec Blocks CDK5-Mediated PPARγ pS273

For identification of alternative nonagonist PPARγ ligands that block CDK5-mediated PPARγ pS273, chemical screening was performed using an in vitro kinase assay with 780 FDA-approved drugs. Among the positive candidates, we noted with interest that Gleevec, a potent anticancer drug (Fig. 1*A*), blocked pS273. Although the beneficial effects of Gleevec on glucose metabolism have previously been demonstrated, the molecular and cellular mechanisms remain unclear. Therefore, we focused

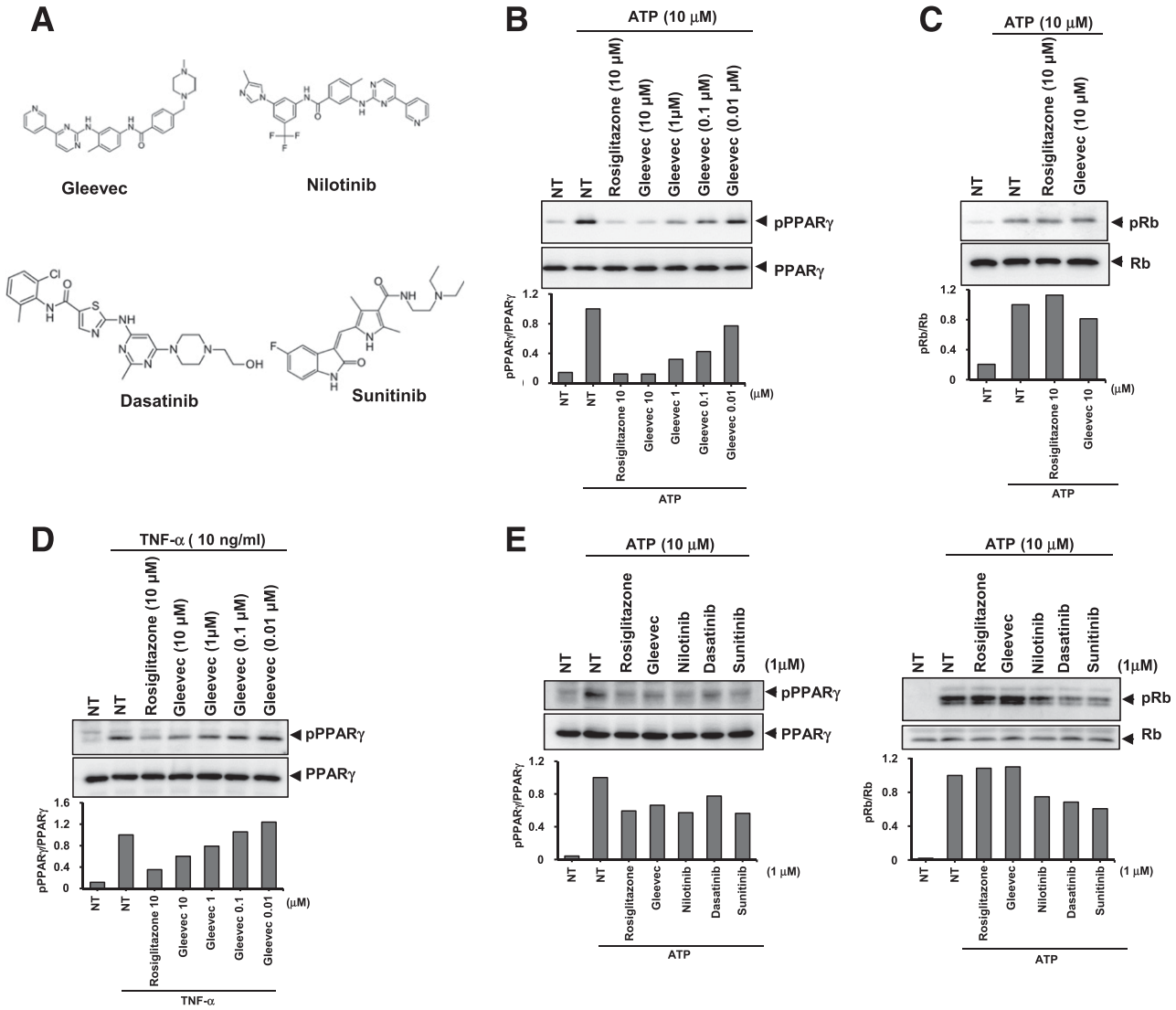

Figure 1—Gleevec blocks CDK5-mediated PPARγ phosphorylation. *A*: Chemical structure of compounds. *B*: In vitro CDK5 assay on full-length PPARγ incubated with rosiglitazone or Gleevec. *C*: Phosphorylation of Rb after treatment with rosiglitazone or Gleevec. *D*: TNF-α–induced phosphorylation of PPARγ in HEK-293 cells expressing PPARγ treated with rosiglitazone or Gleevec. *E*: In vitro CDK5 assay on full-length PPARγ or Rb incubated with rosiglitazone, Gleevec, Nilotinib, Dasatinib, and Sunitinib. NT, not treated.

on the molecular mechanisms of how Gleevec regulates glucose/fat metabolism. As shown in Fig. 1*B*, Gleevec inhibited PPARγ phosphorylation in a dose-dependent manner, and its inhibitory effect was similar to that of rosiglitazone at 10 μmol/L. However, it does not inhibit the CDK5-mediated phosphorylation of retinoblastoma (Rb), another CDK5 substrate (11) (Fig. 1*C*). Furthermore, Gleevec blocked ERK-mediated PPARγ phosphorylation, indicating that Gleevec directly targets PPARγ and inhibits pS273 regardless of the kinases (15) (Supplementary Fig. 1). In HEK-293 cells expressing PPARγ, Gleevec significantly inhibited TNF-α–mediated PPARγ phosphorylation (Fig. 1*D*). Because Gleevec has been widely used for the treatment of chronic myelogenous leukemia (CML) by specifically targeting BCR-Abl Tyr kinase, we tested whether other BCR-Abl Tyr kinase inhibitors block PPARγ phosphorylation. As shown in Fig. 1*E*, while nilotinib, dasatinib, and sunitinib (Fig. 1*A*) inhibited pS273 in a manner similar to that of Gleevec, they also blocked Rb phosphorylation by CDK5, indicating that only Gleevec targets PPARγ. These results suggest that Gleevec specifically blocks pS273, independent of its BCR-Abl targeting.

Gleevec Is a PPARγ Ligand That Lacks Classical Agonism

Next, a LanthaScreen TR-FRET competitive binding assay was performed to assess whether Gleevec directly binds PPARγ. The IC_{50} of Gleevec (1–3 μmol/L) was higher than that of rosiglitazone (0.1 μmol/L) (Fig. 2*A*). Gleevec did not induce transcriptional activity of PPARγ at any concentration tested (Fig. 2*B*). A coactivator recruitment

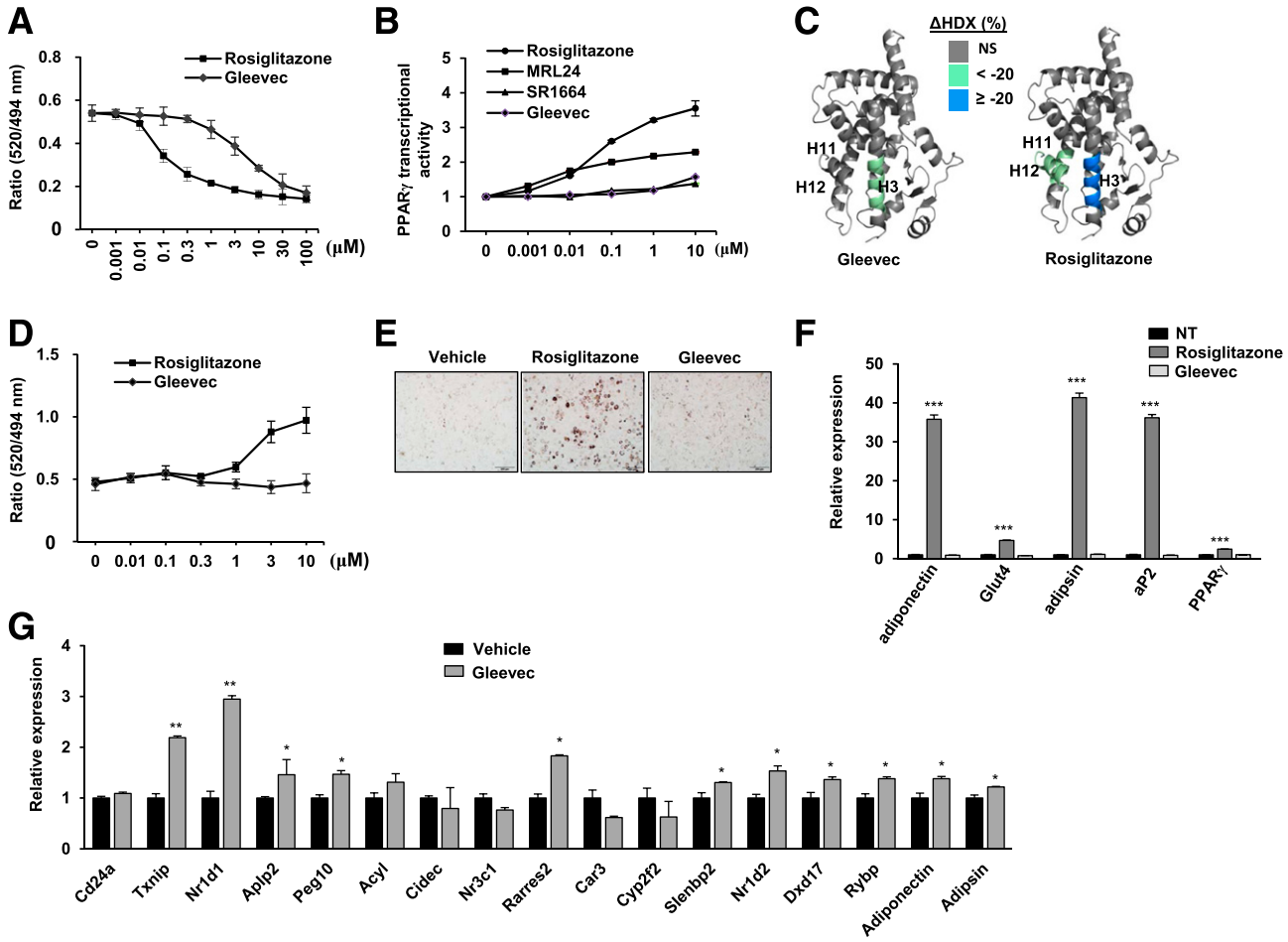

Figure 2—Gleevec is a PPARγ ligand that lacks classical agonism. *A*: Binding affinity of Gleevec or rosiglitazone to the LBD of PPARγ by LanthaScreen assay. *B*: Transcriptional activity of a PPAR-derived reporter gene in HEK-293 cells after treatment with rosiglitazone, MRL24, SR1664, or Gleevec. *C*: Differential hydrogen/deuterium exchange results are mapped onto the PPARγ crystal structure (3PRG) comparing the LBD of PPARγ in the presence of Gleevec and rosiglitazone. *D*: Recruitment of coactivator to PPARγ in the presence of rosiglitazone or Gleevec by LanthaScreen assay. *E*: Lipid accumulation in differentiated 3T3-L1 adipocytes treated with rosiglitazone or Gleevec after Oil red O staining. Expression of adipogenic marker genes (*F*) and gene sets regulated by PPARγ phosphorylation (*G*) in these cells. All error bars represented are SEM. (n = 3.) *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle. HDX, hydrogen/deuterium exchange; H, helix; NS, not significant; NT, not treated.

assay showed that Gleevec impaired recruitment of the CBP coactivator to PPARγ, which was recruited to PPARγ in the presence of rosiglitazone (Fig. 2*D*). Furthermore, Gleevec did not transcriptionally activate either PPARα or PPARδ (Supplementary Fig. 2).

It has been reported that full agonist ligands form a hydrogen bond with Tyr[473] on PPARγ helix 12 (H12); this interaction stabilizes the agonist conformation and allows H12 to serve as the coactivator binding site (activation function-2 surface) (16). Therefore, the stability of the activation function-2 surface is an important determinant for the transactivation of PPARγ. In hydrogen/deuterium exchange analyses of the PPARγ ligand binding domain (LBD), Gleevec binding induced no change in H12 conformational dynamics, in contrast to rosiglitazone, which strongly stabilized the same region (Fig. 2*C* and Supplementary Fig. 3). This suggests that the conformational stability of H12, unaffected by Gleevec, is consistent with the observed absence of transcriptional activity (Fig. 2*B*) and coactivator recruitment (Fig. 2*D*). Next, we compared the in silico docking simulations of Gleevec and SR1664 (Supplementary Fig. 4). The docking score for the LBD of PPARγ revealed that Gleevec fits in a manner similar to that of SR1664 in the proper binding mode.

To further determine whether Gleevec lacks classical agonism, we tested its effects on adipocyte differentiation (8,9). As shown in Fig. 2*E*, rosiglitazone dramatically stimulated adipocyte differentiation of fibroblasts (preadipocytes), whereas Gleevec did not increase lipid accumulation. Moreover, the expression of adipogenic markers was also increased by rosiglitazone but not by Gleevec (Fig. 2*F*). Then, we tested the ability of Gleevec to regulate gene expression in fully differentiated adipocytes (Fig. 2*G*). As shown in Fig. 2*G*, Gleevec upregulated the expression of 11 of 17 (64%) diabetes genes

dysregulated by pS273 in fully differentiated adipocytes (11) (Fig. 2*G*). These data suggest that Gleevec is not a classical transcriptional agonist of PPARγ but specifically regulates pS273.

Gleevec Improves Insulin Sensitivity in Mice With HFD-Induced Obesity

Next, we determined whether Gleevec exerts antidiabetes properties in vivo. After wild-type C57BL/6 mice were fed an HFD for 10 weeks, GTTs and ITTs were performed after treatment with 25 mg/kg/day Gleevec for 7 days. Both GTTs and ITTs were markedly improved without affecting body weight (Fig. 3*A*, *B*, and *E*). Treatment with Gleevec in HFD-fed mice significantly decreased pS273 (Fig. 3*C*). Furthermore, the potency of phosphorylation inhibition was positively correlated with improved glucose tolerance (Fig. 3*D*). Control mice that received vehicle only remained hyperinsulinemic, but Gleevec substantially reduced insulin levels in these mice (Fig. 3*F*), and the level of fed blood glucose was significantly reduced (Supplementary Fig. 5*A*). Insulin resistance, as computed by HOMA of insulin resistance, showed a clear improvement after treatment with Gleevec (Fig. 3*G*). In addition, serum triglyceride and free fatty acid levels significantly decreased in Gleevec-treated mice (Supplementary Fig. 5*B* and *C*). Treatment with Gleevec also altered the expression of 9 of 17 (52.9%) diabetes genes dysregulated by pS273, all in the direction predicted for inhibition of pS273 (11) (Fig. 3*H*). The expression of adiponectin seemed to be unaffected by treatment with Gleevec for

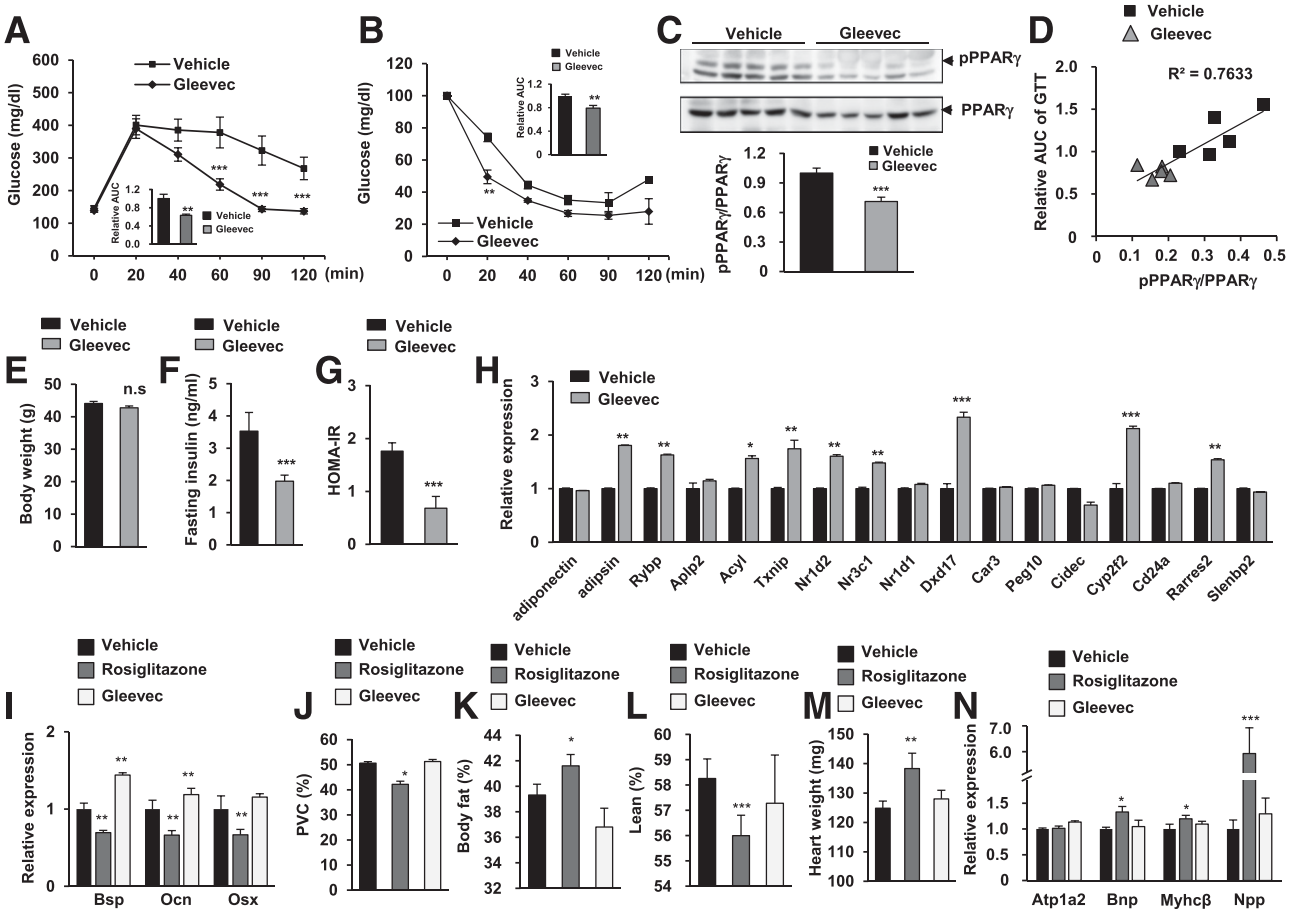

Figure 3—Gleevec has potent insulin-sensitizing effects in mice with high fat–induced obesity without severe side effects that TZDs have. Intraperitoneal GTT (*A*) and intraperitoneal ITT (*B*) after 7 days of treatment with vehicle or Gleevec (25 mg/kg) in HFD-fed mice treated (n = 5). Inset, area under the curve (AUC). *C*: Phosphorylation of PPARγ in WAT. Quantification of PPARγ phosphorylation compared with total PPARγ was performed (n = 5). *D*: Correlation between the levels of PPARγ phosphorylation normalized to the total PPARγ protein and the changes of AUC by GTT. Pearson correlation coefficient and P value are shown. Fasting body weight (*E*), fasting insulin (*F*), and HOMA of insulin resistance (HOMA-IR) (*G*) were determined in these mice (n = 5). *H*: Expression of gene sets regulated by PPARγ phosphorylation in WAT (n = 5). *I*: Human mesenchymal stem cells were differentiated with 10 mmol/L β-glycerophosphate, 50 μmol/L ascorbate-2-phosphate, or 100 nmol/L dexamethasone treated with Gleevec or rosiglitazone for 2 weeks. The expression of osteoblast marker genes was determined by quantitative PCR (n = 3). Packed cell volume (PVC) in whole blood (*J*), the percent of body fat mass (*K*), the percent of lean mass (*L*), and heart weight (*M*) were measured, and the expression of marker genes for heart failure and cardiac hypertrophy in heart (*N*) was determined in HFD-fed mice treated with rosiglitazone or Gleevec for 14 days (n = 6). All represented error bars are SEM. (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle.

1 week, but we observed the expressions of both adiponectin and adipsin were significantly increased by treatment with Gleevec for 3 weeks in WAT (data not shown). This difference could be caused by other environmental factors such as the communications with immune cells because other diabetes genes dysregulated by phosphorylation of PPARγ did not exactly match in between adipocytes and adipose tissue after treatment with Gleevec. Taken together, these results indicate that Gleevec has a potent insulin-sensitizing effect with preferential regulation of diabetes genes sensitive to pS273.

Gleevec Results in Significantly Reduced Common TZD Side Effects

TZDs such as rosiglitazone can cause weight gain and fluid retention, all of which contribute to increased cardiac dysfunction (3). They also increase the risk for bone fracture by reducing bone formation and bone mineral density (4). As shown in Fig. 3*I*, treatment of human mesenchymal stem cells with rosiglitazone reduced the expression of genes involved in bone formation, including bone sialoprotein (*Bsp*), osteocalcin (*Ocn*), and osterix (*Osx*). Importantly, Gleevec did not affect the expression of these gene sets (Fig. 3*I*). An increase in body fat was also observed after treatment with rosiglitazone, but Gleevec treatment did not cause any changes in either body fat or lean mass percentage (Fig. 3*K* and *L*). As indicated in Fig. 3*J*, Gleevec had no detectable effect on hemodilution compared with either vehicle or rosiglitazone treatment.

Next, we analyzed the expression of cardiac genes associated with heart failure or hypertrophy. Cardiac hypertrophy is characterized by increased protein synthesis and enlarged cardiomyocytes, leading to increased cardiac muscle mass (17). As shown in Fig. 3*N*, the expression of natriuretic peptide type B (*Bnp*), myosin heavy chain β (*β-Mhc*), and nandrolone phenylpropionate (*Npp*) was increased in rosiglitazone-treated mice. However, Gleevec did not alter their expression. Consistent with these results, HFD-fed mice treated with rosiglitazone, but not Gleevec, showed increased heart weight (Fig. 3*M*). These results strongly suggest that Gleevec greatly improves insulin sensitivity without the associated adverse effects observed after treatment with most TZDs in vivo.

Gleevec Ameliorates Hepatic Steatosis and Reduces Hepatic Glucose Production

Obesity-induced insulin resistance is associated with fatty liver disease, and dysregulation of hepatic glucose output greatly contributes to hyperglycemia in both humans and mice. Histological observations revealed that Gleevec

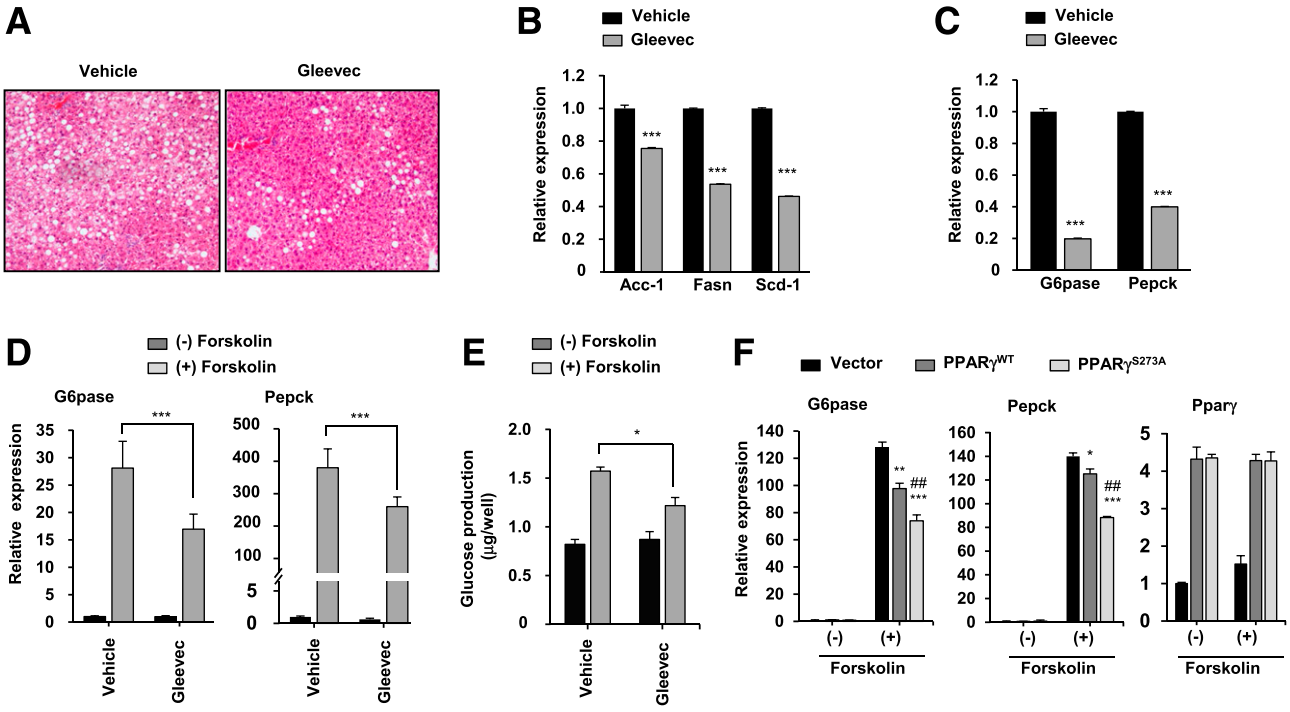

Figure 4—Gleevec reduces hepatic steatosis and glucose production in liver. *A*: Histological analysis by H-E staining of liver in HFD-fed mice treated with Gleevec for 7 days. Expression of lipogenic (*B*) and gluconeogenic (*C*) genes in liver (n = 5). *D*: Expression of gluconeogenic genes in primary hepatocytes isolated from HFD-fed mice for 10 weeks after treatment with Gleevec (n = 3). *E*: Glucose concentration in the primary hepatocyte media (n = 3). *F*: Expression of gluconeogenic genes and PPARγ in the primary hepatocytes expressing PPARγ^{WT} or PPARγ^{S273A} (n = 3). All represented error bars are SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with vector; ##P < 0.01 compared between PPARγ^{WT} and PPARγ^{S273A}.

remarkably reduced hepatic steatosis in HFD-induced obese mice (Fig. 4*A*). Consistent with hematoxylin and eosin (H-E) staining, it also decreased the expression of hepatic lipogenic genes, including acetyl-CoA carboxylase 1 (*Acc-1*), fatty acid synthase (*Fasn*), and stearoyl-CoA desaturase-1 (*Scd-1*) (Fig. 4*B*). Furthermore, Gleevec reduced the expression of gluconeogenic genes, such as glucose-6-phosphatase (*G6pase*) and *Pepck* in the livers of HFD-fed mice (Fig. 4*B* and *C*).

Next, we examined whether the reduced expression of gluconeogenic genes by Gleevec in vivo is a direct cell-autonomous effect. As shown in Fig. 4*D* and *E*, Gleevec directly decreased the expression of *G6pase* and *Pepck* (Fig. 4*D*) and glucose production (Fig. 4*E*) in primary hepatocytes. We also determined whether pS273 plays a role in hepatic gluconeogenesis. When PPARγ^{WT} or a PPARγ^{S273A} was expressed in primary hepatocytes, PPARγ^{S273A} suppressed the expression of *G6pase* and *Pepck* genes more efficiently than PPARγ^{WT} without altering the expression of PPARγ itself (Fig. 4*F*). These results strongly suggest that Gleevec improves hepatic steatosis and directly regulates hepatic glucose production in a pS273-dependent manner.

Gleevec Ameliorates Adipose Tissue Inflammation

Many studies have reported that obesity is associated with a state of chronic low-grade inflammation that facilitates the development of insulin resistance (18,19). Therefore, suppression of inflammation in adipose tissue may have therapeutic potential for the treatment of enhanced inflammatory responses in obesity. Therefore, we determined whether Gleevec suppresses chronic inflammation in the obese state. As shown in Fig. 5*A*, histological sections of WAT in HFD-fed mice treated with Gleevec for 21 days had smaller adipocytes than those of vehicle-treated mice; they also had fewer crown-like structures formed by aggregated macrophages in adipose tissue (20) (Fig. 5*A*). To further investigate the effects of Gleevec on inflammation in WAT, we assessed the expression of proinflammatory and macrophage marker genes. As shown in Fig. 5*B* and *C*, proinflammatory genes such as interleukin-6 (*Il-6*), *Mcp-1*, and *Tnf-α* were significantly reduced in WAT after treatment with Gleevec, whereas *IL-1β* was not changed. Moreover, the expression of macrophage marker genes (*F4/80*, *cd68*, and *cd11b*) and the M1 macrophage marker gene, *cd11c*, was also downregulated by Gleevec. Interestingly, Gleevec-treated HFD-fed mice showed significantly increased expression of M2 macrophage markers, including arginase (*Arg*) and mannose receptor (*Mrc-1*) in WAT (Fig. 5*D*).

In a previous study, we reported that pS273 of PPARγ directly affects suppressing M1 macrophage activation (13). Thus, we next investigated whether pS273 of PPARγ is directly involved in anti-inflammatory activity in adipocytes and M2 polarization in macrophages. Overexpression of PPARγ^{S273A} in PPARγ-deficient MEF cells significantly blocked proinflammatory gene expression compared with that of PPARγ^{WT} (Fig. 5*E*). In macrophages, overexpression

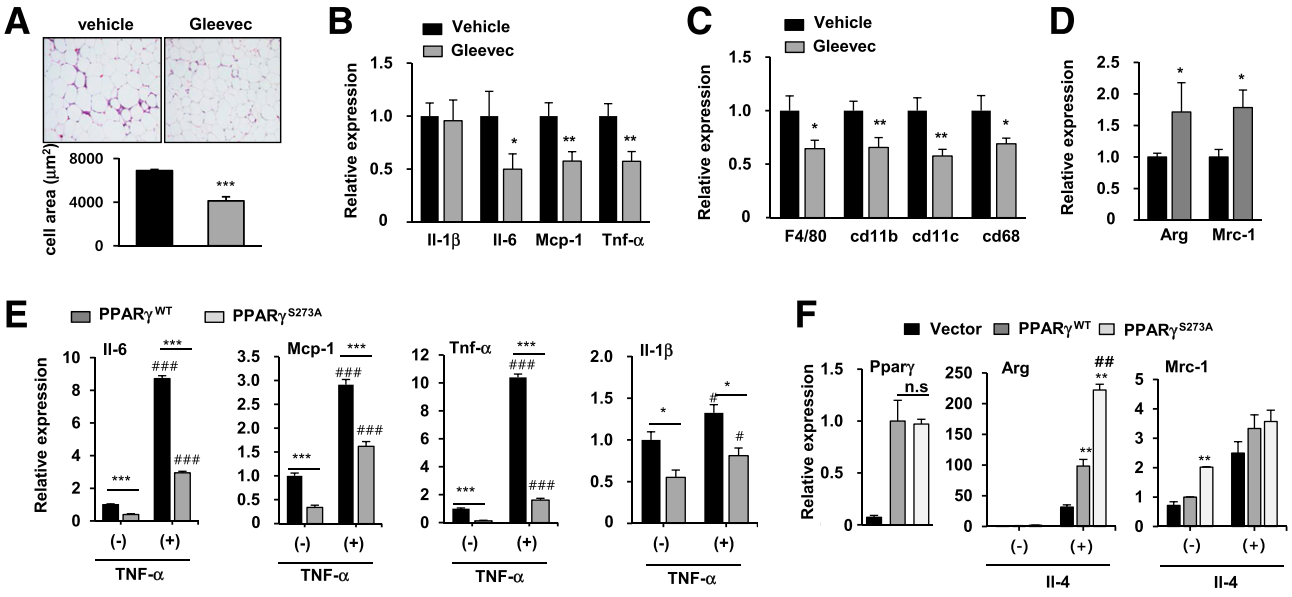

Figure 5—Gleevec ameliorates adipose tissue inflammation. *A*: Histological analysis by H-E staining of WAT in HFD-fed mice treated with Gleevec for 21 days. Adipocyte size was calculated on histological sections of WAT (n = 6). Expression of marker genes for M1 macrophage (*B*), total macrophage (*C*), and M2 macrophage (*D*) (n = 6). All represented error bars are SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with vehicle. *E*: Expression of proinflammatory genes in MEFs expressing PPARγ^{WT} or PPARγ^{S273A} treated with TNF-α (50 ng/mL) (n = 3). All represented error bars are SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with PPARγ^{WT}; #P < 0.05, ##P < 0.01, ###P < 0.001 compared with TNF-α–treated cells. n.s, not significant. *F*: Expression of proinflammatory genes in Raw264.7 macrophages expressing PPARγ^{WT} or PPARγ^{S273A} treated with LPS (10 ng/mL) (n = 3). All represented error bars are SEM. **P < 0.01 compared with vector and ##P < 0.01 compared with PPARγ^{WT}. n.s, not significant.

of PPARγ^{S273A} promoted IL-4–mediated M2 macrophage activation (Fig. 5*F*). Consistent with these results, Gleevec significantly suppressed TNF-α–induced proinflammatory gene expression in both 3T3-L1 adipocytes and primary macrophages (Supplementary Fig. 6*A* and *B*). Furthermore, in vitro transwell chemotaxis assay showed that macrophages had lower chemotactic capacity toward conditioned media from Gleevec-treated 3T3-L1 cells compared with vehicle-treated conditioned media (Supplementary Fig. 6*C*). Taken together, these data indicate that blocking pS273 by Gleevec causes decreased macrophage inflammation and infiltration to WAT by substantially suppressing the proinflammatory responses in adipocytes, thus ameliorating adipose inflammation.

Gleevec Increases Energy Expenditure and Adaptive Thermogenesis

Recent studies have shown that PPARγ ligands affect energy balance by promoting browning of WAT (21). Therefore, we investigated whether Gleevec regulates energy expenditure using CLAMS. As shown in Fig. 6*A* and *B*, we observed a highly significant increase in energy expenditure in Gleevec-treated mice compared with vehicle-treated controls. Importantly, there was no change in respiratory exchange ratio, indicating that Gleevec did not stimulate any substantial shift from carbohydrate to fat-based fuels (Supplementary Fig. 7). Importantly, these changes in energy expenditure in Gleevec-treated mice were not dependent on food intake or physical activity (Fig. 6*B*). At the molecular level, Gleevec produced a significant increase in broad brown/beige fat thermogenic and mitochondrial genes, including *Ucp-1*, *Pgc-1α*, and *cox-5b* in WAT and interscapular brown adipose tissue (BAT) (Fig. 6*C* and Supplementary Fig. 8*A*). In addition, Gleevec stimulated the expression of beige adipocyte marker (*Cd137* and *Tmem26*) and β-oxidation genes (carnitine palmitoyltransferase 1b [*Cpt1b*]) (Fig. 6*C*).

To further examine the effects of Gleevec on cold-induced thermogenesis in vivo, mice were challenged with cold exposure at 4°C. Acute exposure (9 h) to cold significantly dropped rectal temperatures (Fig. 6*D*). As expected, Gleevec-treated mice showed a more resistant phenotype (decreased rectal temperature) against cold (Fig. 6*D*). WAT morphology was also analyzed under these conditions by H-E staining. As shown in Fig. 6*E*, multilocular adipocytes were observed in the sWAT of Gleevec-treated mice. Furthermore, the expression of thermogenic and mitochondrial genes, including *Ucp-1*, *Cidea*, *Cox5b*, *Atp5b*, and *Cpt1b*, was significantly increased in sWAT and BAT of Gleevec-treated mice after cold exposure (Fig. 6*F* and Supplementary Fig. 8*B*).

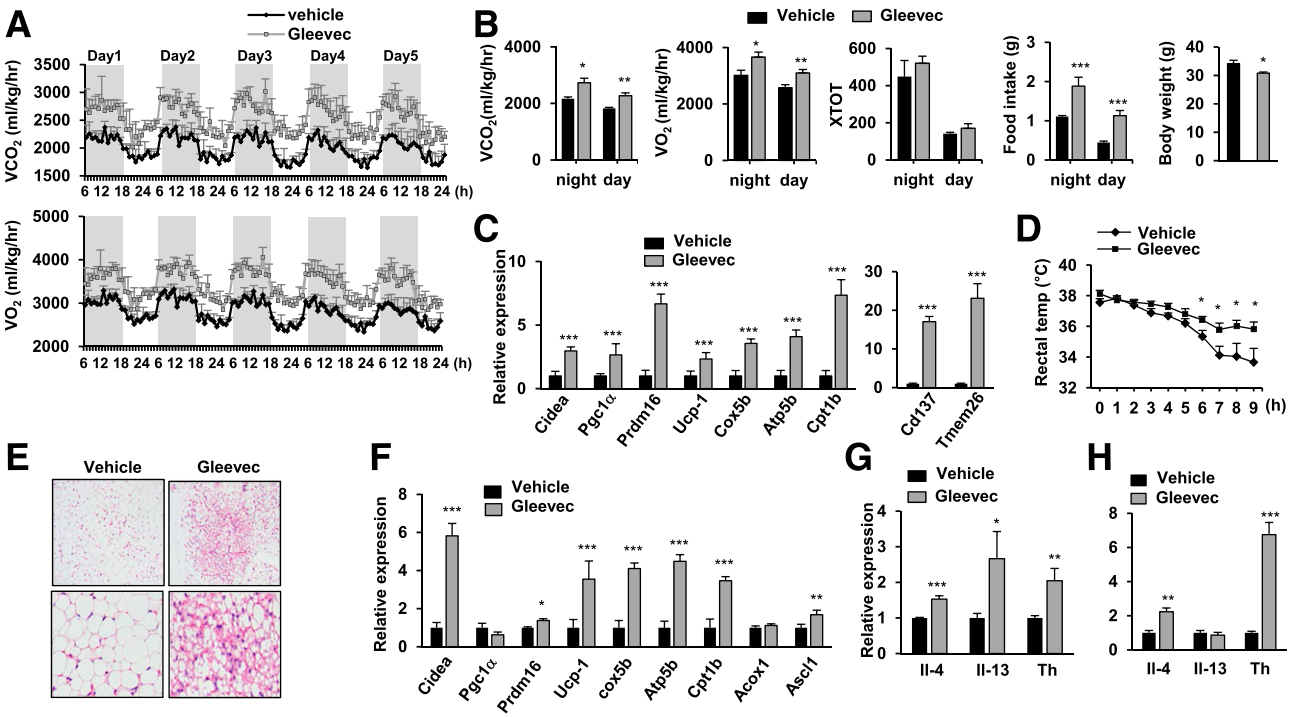

Figure 6—Gleevec increases energy expenditure and adaptive thermogenesis. *A* and *B*: VO_2, VCO_2, food intake, and locomotor activity (total beam breaks on the *x*-axis [XTOT]) were measured by CLAMS in HFD-fed mice treated with Gleevec for 21 days ($n = 4$). *C*: Expression of thermogenic, β-oxidation, and beige marker genes in sWAT ($n = 6$). *D*: Rectal temperature was measured at the indicated time points for mice placed in a cold room ($n = 4$). *E*: H-E staining of sWAT in these mice. *F*: Expression of genes for thermogenesis and β-oxidation ($n = 4$). Expression of *Il-4*, *Il-13*, and *Th* in subcutaneous WAT was determined by quantitative PCR with (*G*) or without (*H*) cold exposure ($n = 4$–6). All represented error bars are SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle. hr, hour; temp, temperature.

For determination of whether the browning effect of Gleevec is cell autonomous, Gleevec was administered to the stromal vascular fraction of inguinal WAT during differentiation in vitro. There were no detectable differences in the expression of *Ucp-1* or *Pgc-1α* in fully differentiated adipocytes (Supplementary Fig. 9), indicating that Gleevec may induce adipose tissue thermogenesis in vivo independent of its direct action on adipocytes.

Recent studies have demonstrated that IL-4 activates M2 macrophage polarization and, alternatively, activated macrophage produces catecholamine, to be important for induction of thermogesis in WAT and BAT (22). As Gleevec promoted the expression of M2 marker genes, including *Arg* and *Mrc-1* in adipose tissue (Fig. 5*C*), we next examined the expression of *IL-4*, *IL-13*, and Tyr hydroxylase (*Th*), the rate-limiting enzyme to synthesize catecholamines in WAT. As shown in Fig. 6*G* and *H*, treatment with Gleevec resulted in significantly increased expression of *IL-4*, *IL-13*, and *Th* either during cold exposure (Fig. 6*G*) or in normal housing temperature (22°C) (Fig. 6*H*). Taken together, these results indicate that Gleevec increases energy expenditure by upregulating beige/brown fat thermogenic genes in vivo. And these effects were induced by a phenotypic switch in adipose tissue macrophages, along with increased expression of *Th*, possibly via inducing expression of IL-4 and IL-13.

DISCUSSION

Full agonist PPARγ ligands, including TZDs, have been widely used to treat type 2 diabetes (2,8,9). However, patients treated with these drugs have exhibited higher incidence of serious adverse effects, such as weight gain, bone fracture, and congestive heart failure, compared with other oral hypoglycemic agents. Recent studies have shown that the insulin-sensitizing effects of PPARγ ligands are not dependent on classical agonism but, rather, are a consequence of ligand-dependent inhibition of pS273 by CDK5/ERK kinases (11,12,15). More specifically, nonagonist PPARγ ligands, such as SR1664 and UHC1, have illustrated glucose-lowering effects similar to those of TZDs while lacking the commonly observed side effects. These studies have allowed us the opportunity to find a potential compound for the treatment of type 2 diabetes. Thus, we aimed to discover compounds that block pS273 and lack classical agonism with high binding affinity to PPARγ. Drug repositioning is the application of known drugs and compounds to new indications, which can save time and costs because they have already been tested in humans and detailed information is available on their pharmacology, formulation, and potential toxicity (23). Thus, we screened PPARγ ligands that block pS273 with an FDA-approved drug library, and Gleevec was determined to fit these criteria.

Gleevec, a specific BCR-Abl kinase inhibitor, is a well-known anticancer drug that exhibits dramatic effects for the treatment of CML and gastrointestinal stromal tumors (24,25). In addition to its antineoplastic activity, several studies have reported that Gleevec has a blood glucose-lowering effect in patients suffering from both CML and type 2 diabetes (26,27) or gastrointestinal stromal tumors (28). Restoration of insulin sensitivity or amelioration of hyperlipidemia has also been noted in CML patients without diabetes who show significant insulin resistance at diagnosis (29,30). Although the beneficial effects of Gleevec on glucose and lipid metabolism have previously been demonstrated, the molecular and cellular mechanisms remain unclear. Previous studies have demonstrated that Gleevec controls hyperglycemia by preserving pancreatic β-cell mass or reducing endoplasmic reticulum stress in rats with diet-induced obesity or diabetic *db/db* mice (31–34), while the current study clearly demonstrates that the potent insulin-sensitizing action of Gleevec is derived from a distinct molecular mechanism. Gleevec acts as a PPARγ antagonist ligand that directly blocks pS273 while lacking classical agonism (Fig. 2 and Supplementary Figs. 1–4). Several lines of evidence have shown improved insulin sensitivity through blocking pS273 by Gleevec: Gleevec ameliorates hepatic steatosis and glucose production in liver (Fig. 4), attenuates proinflammatory responses in WAT (Fig. 5), and promotes browning and energy expenditure in WAT (Fig. 6), all of which contribute to increased insulin sensitivity (Fig. 3).

In our previous study, we demonstrated that blocking pS273 inhibits LPS-induced inflammation in macrophages (13). Furthermore, Gleevec reportedly suppresses LPS- or TNF-α–induced inflammatory cytokine production in macrophages by blocking inhibitor of κB phosphorylation and subsequent DNA binding of nuclear factor-κB, thus inhibiting nuclear factor-κB activation (35). Thus, we speculate that inhibition of pS273 by Gleevec regulates adipose tissue inflammation in the obese state. Indeed, we observed that Gleevec redirects adipose tissue macrophages from an M1 to an M2 polarization state (Fig. 5), suggesting that Gleevec reduces macrophage infiltration by suppressing the proinflammatory response in adipocytes, as well as regulating M1/M2 polarization in macrophages, thus ameliorating adipose tissue inflammation.

In the present results, we observed that Gleevec induces adipose tissue thermogenesis in vivo independent of its direct action on adipocytes. How does Gleevec promote browning of WAT? According to recent evidence, an increase in M2 macrophages produces catecholamine in the induction of thermogenic and β-oxidation gene expression in WAT and BAT in cold-exposed mice (22). Furthermore, this effect is accompanied by increased eosinophil-derived IL-4 in WAT (36). Accordingly, genetic deletion of *Il4ra* or *Th* in myeloid cells significantly impairs the development of thermogenic beige fat in mice (36,37). Recently, Lee et al. (38) reported that IL-4, derived from type 2 innate lymphoid, stimulated eosinophils and directly promoted the proliferation and commitment of platelet-derived growth factor receptor α^+ (*Pdgfrα*$^+$) adipocyte precursors, which are bipotential cells that

differentiate into either beige or white adipocytes within sWAT and enhance differentiation into beige adipocytes. Interestingly, we have shown that Gleevec treatment increased the proportion of M2 macrophages (Fig. 5) and the expression of beige maker genes in sWAT (Fig. 6). Furthermore, we observed that PPARγ^{S273A} mutant increased IL-4–induced M2 polarization of macrophages (Fig. 5). Together, induction of *IL-4/IL-13* expression and alternative macrophage activation by inhibition of PPARγ phosphorylation might promote regulation of expression of genes associated with brown/beige adipose thermogenesis.

In conclusion, we found that Gleevec has potent beneficial effects on insulin sensitivity to relieve metabolic disorders by regulating glucose and lipid metabolism without causing several adverse effects, including body weight gain, fatty liver, fluid retention, bone fracture, and cardiac hypertrophy, which have been observed after treatment with most TZDs. Notably, Gleevec regulates energy expenditure by promoting the development of beige fat in WAT. These results establish an important role for Gleevec in regulating adipose tissue thermogenesis and white adipose plasticity toward BAT and shed light on the development of novel therapeutic drugs for the treatment of obesity and type 2 diabetes.

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Author Contributions. S.-S.C. and J.H.C. conceived the hypothesis, designed and researched data, and wrote the manuscript. E.-S.K., J.-E.J., D.P.M., A.J., J.Y.K., S.Y.C., Y.R.Y., H.-J.J., E.-K.K., J.P., H.M.K., I.H.L., S.B.P., K.-J.M., P.-G.S., and P.R.G. researched data and assisted with data interpretation. J.H.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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