Pharmacological characterization of a small molecule inhibitor of c-Jun kinase

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JNK is therefore a potential target for the reduction of fatty acids, resulting from increased adiposity and nutrient production, endoplasmic reticulum stress, and saturated fatty acids, resulting from increased adiposity and nutrient intake, stimulate JNK activity and play a central role in the suppression of insulin receptor mediated signaling pathways (2, 14, 19). JNK is therefore a potential target for the reduction of insulin resistance and treatment of type 2 diabetes.

Dysregulation of cytokine production in metabolically active tissues such as adipose and muscle tissue, combined with inflammation resulting from macrophage infiltration into adipose tissue, has been associated with a decrease in insulin action. JNK activation via phosphorylation of c-Jun plays a key role in activating the activator protein-1 transcription factor comprised of Jun, Fos, activating transcription factor (ATF), and the Maf subfamily. Of the three JNK isoforms, JNK1 is the central player in modulating insulin action, phosphorylating IRS-1 on serine residues including Ser307, which reduces IRS-1 tyrosine phosphorylation and signaling capacity through the insulin receptor (1). JNK1 activity is elevated in liver, muscle, and adipose tissue in obese mice (13) and in skeletal muscle from obese and type 2 diabetic humans where it is correlated with increased phosphorylation of IRS-1 on Ser307 (5). Moreover, JNK1 deficiency has also been shown to protect mice from the development of insulin resistance by enhancing insulin signaling and reducing adiposity in diet-induced and genetic models of obesity (13).

Here we describe for the first time the characterization of compound A, a novel small molecule, selective JNK inhibitor, and demonstrate its ability to improve insulin resistance and reduce weight gain in high-fat fed mice.

MATERIALS AND METHODS

Materials. Escherichia coli LPS, palmitate (P0500), oleate (O1008), fatty acid free BSA (A9205), insulin (I-5523), dexamethasone (D-1756), and IBMX (I-5879) were obtained from Sigma-Aldrich (St. Louis, MO). Kits for analysis of phosphorylated c-Jun (Ser63), insulin, phosphorylated IRS-1 (Ser307), and cytokines, in addition to Tris lysis buffer, and protease and phosphatase inhibitors were from Mesoscale Discovery (MD; Gaithersburg, MD). DMEM, RPMI 1640, and HEPES-buffered solution were from Invitrogen (Carlsbad, CA; catalog no. 11885, 11995, 11875-093 and 15630-1136, respectively). The cytokines TNFα, IL-1β, and IFNγ were obtained from R&D Systems (Minneapolis, MN). The EGM-2 BulletKit was from Cambrex Bioproducts (CC-3162, Walkersville, MD).

BSA-conjugated free fatty acid. Oleate and palmitate were dissolved in 5 ml of 99% (vol/vol) ethanol. A precipitate was formed by addition of 40 µl of 10N NaOH to the shaking fatty acid solution that was dried in a ventillation hood for overnight. A hot soap was created by adding 5 ml of distilled water to the dried fatty acids on a hot plate, which was combined with an ice-cold BSA solution [13.3 ml of 30% (wt/vol) BSA and 20 ml of distilled water]. The volume was made up to 40 ml with water to make a 10 mM oleate/palmitate solution, and the solution was filtered through a 0.45-µm filter.

Kinase enzymatic assays. Kinase inhibition assays were conducted radiometrically using [γ-32P]ATP similarly as described previously.

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(4, 10). Inhibitor constant \( (K_i) \) assays for JNK isoforms were performed as described previously (7). All other kinases were tested for IC\(_{50}\) through the Upstate KinaseProfiler service (Upstate, Dundee, UK) at the experimentally determined \( K_m \) concentration of ATP for the relevant kinase, except for insulin receptor, which was tested with 200 \( \mu \)M ATP (below the \( K_m \)) and MEK1, MEK4, MKK6, and \( \text{MKK7} \) that were tested with 10 \( \mu \)M ATP (\( K_m \) not determined) in linked assays MEK1-inactive MAPK2-myelin basic protein, MKK4-inactive JNK1-1-peptide substrate, MKK7-inactive JNK1-1-peptide substrate, and MKK6-inactive SAPK2a-myelin basic protein, respectively. All kinases were of human origin except for AMPK (rat) and MKK4 (mouse). JNK1 (rat), JNK2 (mouse), GST-JNK3, and the phosphoacceptor substrate GST-ATF2 (aa 19–96) were obtained from the University of Dundee Division of Signal Transduction Therapy (Dundee, UK).

Glucose uptake in differentiated 3T3L1 cells. Glucose uptake was measured from differentiated 3T3L1 cells. Briefly, the 3T3-L1 cells were seeded in 12-well plates and grown to confluence in culture medium composed of DMEM with 10% (vol/vol) calf serum, 10 U/ml penicillin, and 10 \( \mu \)g/ml streptomycin for 3 days in a humidified atmosphere of 5% CO\(_2\) at 37°C. Subsequently, cells were differentiated into mature adipocytes by incubating them with culture medium supplemented with 1.7 \( \mu \)M insulin, 0.5 mM IBMX, and 0.25 \( \mu \)M dexamethasone for 3 days. Media were then replaced with culture medium containing 10 nM insulin for an additional 2 days and in medium without insulin for 3 more days. Before the glucose uptake assay, the differentiated cells were starved overnight in low glucose-DMEM without FCS in the presence or absence of free fatty acids (FFAs; 300 \( \mu \)M for inhibition of basal glucose uptake and 100 \( \mu \)M for inhibition of insulin-stimulated glucose uptake) conjugated to BSA (0.86 mg/ml palmitate and 1.88 mg/ml oleate in 10% BSA solution; see above for details) and compound A (0.3 or 0.5 \( \mu \)M) as indicated. Subsequently, cells were washed once with Dulbecco’s PBS and incubated in FFA solution in the presence or absence of compound A for a further 2 h at 37°C. 2-Deoxy-D-[\(^{14}\)C]glucose (Amersham, Piscataway, NJ) and cold 2-deoxy-D-glucose solution were added to the cell media for 30 min at room temperature. 2-Deoxy-D-glucose uptake was terminated by washing the cells twice with ice-cold PBS. Subsequently, cells were lysed in 1 N NaOH. The incorporated radioactivity was measured from the neutralized lysates by liquid scintillation spectrometry.

IRS-1 Western blots. 3T3L1 cells were differentiated in six-well plates for generation of protein lysates for Western blotting. Cells starved in low glucose-DMEM without calf serum were preincubated with compound A for 15 min where indicated and then treated with 3 nM insulin for 15 min or 1 h. Cells were washed once and lysed in lysis buffer [1% (vol/vol) Triton-X-100, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 20 mM Tris pH 7.5, 100 mM NaCl, 50 mM NaF, 3 mM sodium pyrophosphate, and 1 mM EDTA pH 8.0 with protease inhibitor cocktail (Roche, 11697498001) and phosphatase inhibitors (Calbiochem, 52462536) for 10 min on ice. Debris was removed from the lysate by centrifugation at 12,000 \( g \) for 10 min at 4°C, and 75 \( \mu \)g of lysate was loaded onto 7% (vol/vol) NuPAGE (Invitrogen) for Western blot analysis.

Luminex assay for phosphorylated c-Jun. U936 cells (2.25 \( \times \) 10\(^6\) cells/well) were seeded into 96-well Millipore 1.22-um filter plates in starvation medium [RPMI supplemented with MEM sodium pyruvate solution, L-glutamine, and 0.1% (wt/vol) BSA] and incubated at 37°C and 5% (vol/vol) CO\(_2\) overnight. Starved cells were pretreated with compound A for 15 min, followed by 1-h incubation with 10 ng/ml TNF\(_\alpha\) to stimulate JNK activity as measured by c-Jun phosphorylation. Cells were lysed in lysis buffer (Upstate Biotech/Millipore, Charlottesville, VA), and lysates were centrifuged. The total lysates from each well were incubated with beads coated with antibody against c-Jun phosphorylated on Ser63, and the bound phosphorylated protein was detected by Bio-Plex systems from Bio-Rad (Hercules, CA).

Animal experiments. All animal procedures described in this study complied with the Pfizer Institutional Animal Care and Committee, National Institutes of Health, and animal care guidelines. To investigate the effects of compound A on insulin sensitivity, male C57BL/6 mice (7–8 wk old) obtained from the Jackson Laboratory (Bar Harbor, ME) were housed in groups of three per cage in a temperature-controlled room with a light-dark cycle from (6:30 AM–6:30 PM). Mice had access to food and water ad libitum. For high-fat diet (HFD) studies, C57BL/6 mice were placed on a diet containing 60% kcal% fat (Research Diets #D12492, New Brunswick, NJ) or control diet 10.5 kcal% fat (Research Diets #D12450B) for a period of 10 wk. Four weeks before the study start mice were single housed. Mice were sham-dosed twice a day for 4 days before the study commenced and were randomized to treatment groups based on glucose, insulin, and triglyceride levels determined 1 day before the study start. All mice were dosed twice a day either with compound A (30 mg/kg), rosiglitazone (1.5 mg/kg), or vehicle (0.5% methylcellulose) as indicated. Body weights were taken every 3–5 days. Tail bleeds for nonfasting glucose, insulin, and triglycerides were taken at the start of the study and on day 13 of the study. The insulin tolerance test was performed on day 15 of the study. On day 21 of the study, blood samples for plasma levels of compound A were taken via cardiac puncture while the animals were under isoflurane anesthesia, and samples of fat tissue were taken for analysis of JNK activity. For insulin tolerance tests, food was removed ~4 h before the test. Glucose was determined via tail nicking of mice using a glucometer (OneTouch; Lifespan, Milpitas, CA), and human insulin (Humulin; Eli Lilly, Indianapolis, IN) was administered intraperitoneally at 0.3 U/kg in 0.9% saline containing 3% BSA. Blood glucose was measured at the indicated times. Changes in insulin sensitivity after the bolus of insulin were examined by calculating the linear slope of the fall in glucose [\( \text{KGlu} \)] and the area under the curve (AUC) subsequent to insulin administration.

To monitor the effects of compound A on body weight, male C57BL/6 mice (Charles River; ~20 g upon arrival) were started on 45% high-fat diet (Research Diets, 12451) 1 wk after arrival in house, and studies were started after mice had been on the diet for 10 wk. Mice were weighed and sorted according to body weight and randomly assigned to four groups using Graphpad software. Mice were sham-dosed twice a day for a total of 7 days before experiment start upon which vehicle or compound A (30 mg/kg) or rimonabant (3 mg/kg) was administered orally twice a day for 24 consecutive days. After cessation of treatment, mice were monitored for 18 days without dosing. Food intake and body weight were measured daily throughout the study. Body composition was measured using PIXImus on days 3, 24, and 42, where day 0 was the first day of treatment.

For LPS studies, mice were fed with normal chow (Formulab Diet #5001; PMI Nutrition, Brentwood, MO) and acclimated for ~1 wk before study start. On the day of study, mice were randomly assigned to treatment groups and food was removed at 9 AM. LPS in 0.9% saline solution was administered intraperitoneally at 11 AM, and control mice were injected with 0.9% saline. Compound A (30 mg/kg) or vehicle (0.5% methylcellulose) was administered by oral gavage 30 min before or 60 min subsequent to the LPS injection. Two hours after LPS administration, mice were anesthetized using isoflurane, blood was sampled via cardiac-puncture, and tissue samples were removed from epididymal fat. Tissues were placed in 1.5 ml lysing matrix D tubes (MPBio Solon) and immediately frozen in liquid nitrogen.

Analysis of tissue phosphorylated c-Jun and plasma cytokines. Blood was collected in heparinized tubes for generation of plasma for analysis of cytokines, glucose, and insulin. Glucose was measured using a clinical analyzer (ALFA Wassermann, West Caldwell, NJ). For tissue analysis, 1 ml of modified Tris lysis buffer (containing per 10 ml: 200 \( \mu \)l MS3 protease inhibitor, 200 \( \mu \)l phosphatase inhibitor I and II, 1 mM PMSF, and 0.5 M NaCl) was added to the liver or fat samples, and homogenization was performed using a Fast-Prep homogenizer (Q-biogene, Irvine, CA). Samples were subsequently analyzed by Luminex assay for phosphorylated c-Jun.
centrifuged for 10 min at 4°C at 13,000 rpm. The protein content of the resulting supernatant was determined using the BCA kit (Pierce Biotechnology, Rockford, IL). Fifty micrograms of tissue protein were used for determination of JNK activity. JNK activity was measured by quantitation of phosphorylated (Ser63) c-Jun (Pc-Jun) per manufacturer’s instructions.

Statistics. Data are means ± SD or ± SE, where noted. Statistical differences between multiple treatment groups were compared using the one-way ANOVA followed by the Tukey’s test for least significant differences (PRISM) or by Student’s t-test. Results were considered significant at \( P \leq 0.05 \).

RESULTS

Biochemical characterization of compound A. Compound A is a reversible, ATP-competitive aminopyridine inhibitor of JNK (Fig. 1A). It was synthesized after a structure-based modification of a small molecule inhibitor from a MAP kinase project (unpublished data). It is a potent pan-JNK inhibitor, with \( K_i \) for JNK1, JNK2, and JNK3 of 9, 17, and 16 nM, respectively (Table 1). Screening a diverse set of protein and lipid kinases showed that compound A has a high degree of selectivity for JNK, with an estimated margin of at least 100-fold over other MAP kinases (MKK1, MKK4, MKK7β, and p38MAPKα/β), >27-fold over glycogen synthase kinase-3β, and 65-fold over cyclin-dependent kinase-2 (Table 1).

We further analyzed the activity of compound A on JNK kinase activity in a cell-based assay utilizing U937 cells. TNFα activates JNK through a signal cascade that results in the phosphorylation of Thr183/Tyr185 residues. The activation of JNK results in the increase of cJun phosphorylation (18). As shown in the Western blot (Fig. 1B), we observed an increase in phosphorylation of JNK after TNFα treatment of U937 cells (bottom left), which coincided with the induction of cJun phosphorylation (bottom right) compared with that in vehicle-treated cells. Pretreatment with compound A had no effect alone on phosphorylation of c-Jun of JNK in mock-stimulated cells. However, cJun phosphorylation was specifically and completely abolished when cells were pre-
Phosphorylation of c-Jun with an IC50 of 102 nM (Fig. 1). Compounds A blocked the phosphorylation of JNK by ELISA. Compound A dose dependently inhibited TNFα-stimulated phosphorylation of c-Jun, as determined by the reduction in phosphorylated c-Jun in fat tissue (Fig. 1). This suggests that the binding and activation by upstream kinases are not inhibited by compound A but may be enhanced through an intrinsic feedback regulation mechanism. Using similar conditions, we determined that compound A dose dependently inhibited TNFα-stimulated JNK kinase activity, as determined by the reduction in phosphorylation of c-Jun by ELISA. Compounds A blocked the phosphorylation of c-Jun in fat compared with vehicle control.

**Compound A inhibits LPS-stimulated phosphorylated c-Jun in C57Bl/6 mice.** LPS, an acute inflammatory mediator, has previously been demonstrated to elevate cytokines and JNK activity (8, 21). We administered compound A (30 mg/kg) to C57Bl/6 mice before and subsequent to LPS injection and examined the changes in circulating cytokines IL-12p40, MCP-1, TNFα, and IL-6, in addition to the levels of phosphorylated c-Jun in fat tissue (Fig. 2). Plasma levels of compound A were determined at the end of the study for the mice that received compound A before or after LPS dosing. Mice that received compound A before or after saline injection tended to have higher plasma levels of compound A compared with mice that received LPS, although this was only significant for groups that received compound A after the intraperitoneal injections [means ± SE: 1.096 ± 0.17 μM (2.5 h after dosing no LPS), 0.686 ± 0.21 μM (2.5 h after dosing with LPS), 3.362 ± 0.27 μM (1.5 h after dosing no LPS), and 1.29 ± 0.43 μM (1.5 h after dosing with LPS; P < 0.01] compared with mice treated with compound A after saline injection. These data suggested that LPS may affect the pharmacokinetic parameters of compound A, and thus comparisons of effects of the JNK inhibitor were made within LPS-treated groups. LPS-stimulated phosphorylated c-Jun in fat compared with vehicle control was elevated by 3.6 ± 0.6-fold compared with a 1.4 ± 0.18-fold increase when compound A was administered 30 min before LPS injection (P < 0.01, compared with LPS control group; Fig. 2A). In contrast, the LPS stimulation of phosphorylated c-Jun levels was not significantly altered when compound A was administered 60 min after LPS injection (fold increase in phosphorylated c-Jun compared with control, LPS with vehicle 3.9 ± 0.1-fold, and LPS with compound A 3.6 ± 0.2-fold). Mice dosed with compound A before LPS had a significant decrease in plasma levels of IL-12p40, IL-6, MCP-1, and TNFα (all P < 0.01 vs. LPS control; means ± SE %decrease from mice treated with LPS and vehicle: 64 ± 6, 74 ± 6, 27 ± 12, and 70 ± 9, respectively; Fig. 2B). However, no significant changes in cytokines were observed in mice dosed with compound A subsequent to the LPS injection. This suggests that inhibition of JNK pharmacologically is necessary before administration of the LPS inflammatory stimulus to reduce the

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**Table 1. Kinase selectivity of compound A**

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50, μM</th>
<th>Kinase</th>
<th>IC50, μM</th>
<th>Kinase</th>
<th>IC50, μM</th>
</tr>
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<tr>
<td>JNK1</td>
<td>0.009</td>
<td>GSK3β</td>
<td>1.9</td>
<td>IR</td>
<td>&gt;30</td>
</tr>
<tr>
<td>JNK2</td>
<td>0.017</td>
<td>SAPK3</td>
<td>6.2</td>
<td>MAPKAP-K2</td>
<td>&gt;30</td>
</tr>
<tr>
<td>JNK3</td>
<td>0.016</td>
<td>IKKβ</td>
<td>13</td>
<td>MEK1</td>
<td>&gt;30</td>
</tr>
<tr>
<td>CDK2/cyclinA</td>
<td>0.59</td>
<td>MAPK2</td>
<td>13</td>
<td>p70S6K</td>
<td>&gt;30</td>
</tr>
<tr>
<td>MKK7β</td>
<td>0.86</td>
<td>MKK6</td>
<td>15</td>
<td>PDK1</td>
<td>&gt;30</td>
</tr>
<tr>
<td>MKK4</td>
<td>0.92</td>
<td>SAPK2b</td>
<td>15</td>
<td>PKC9</td>
<td>&gt;30</td>
</tr>
<tr>
<td>SAPK4</td>
<td>1.2</td>
<td>AMPK</td>
<td>&gt;30</td>
<td>SAPK2a</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

*Values are inhibitor constant \( K_i \).
extent to which LPS induces increases in cytokines and JNK activity.

**Compound A reduces food intake and weight in diet-induced obese mice.** JNK1 knockout mice fed a HFD exhibit less adiposity than wild-type mice (13). Since a reduction in weight and fat content per se would be expected to improve insulin resistance, the effect of compound A on weight, food intake, and body composition of C57BL/6 mice fed a HFD were examined and compared with changes observed with the CB1 antagonist rimonabant. The change in body weight in mice dosed for 24 days with compound A was significantly greater than vehicle-treated mice \( (P < 0.01) \) and pair-fed mice \( (P < 0.05) \), weight after 24 days of dosing; compound A treatment 34.2 ± 0.7 g (13.1 ± 1% decrease), vehicle 42.2 ± 0.8 g (6.6 ± 2.1% increase), and mice pair fed to compound A-treated mice 36.8 ± 0.8 g (7 ± 1% decrease); Fig. 3A]. Mice treated with 3 mg/kg rimonabant weighed 38.7 ± 1.4 g after 24-day dosing (4.1 ± 1.8% decrease; \( P < 0.01 \) vs. vehicle). There was a clear and immediate change in the body weight of animals treated with rimonabant and compound A, with the rate of body weight loss of compound A treated mice mirroring that of the matched pair-fed cohort, suggesting primarily an anorectic mechanism. Although the weight loss of the rimonabant treated mice reached a plateau within the first week of study, the compound A treated mice continued to lose weight throughout the 24-day dosing period. The body weight of compound A treated mice returned to original levels within the 18-day washout period, albeit at a slower rate than pair-fed mice. The relative degree and kinetics of weight loss were paralleled by changes in food intake (Fig. 3, B and C). Reductions in food-intake in rimonabant-treated mice were of a lesser magnitude than those in compound A-treated mice and returned to baseline levels within a week, whereas that in compound A treated mice remained suppressed yet constant throughout the dosing period (spikes at days 8 and 14 correspond to delivery of fresh food). Interestingly, once dosing ceased, there was no immediate spike in food consumption in the compound A treated mice as observed in the pair-fed mice, although in general the food-intake during the rebound period exceeded baseline levels (Fig. 3B). After 24 days of dosing, fat mass was significantly decreased in mice treated with compound A and in mice pair fed to compound A treated mice, compared with that in vehicle-treated mice [Fig. 3D; %fat: vehicle 44.0 ± 0.6; compound A 30.1 ± 0.9 (\( P < 0.01 \) vs. vehicle)].

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![Graph A](#) Changes in body weight expressed as percentage of initial weight.

![Graph B](#) Changes in average 24-h food intake.

![Graph C](#) Cumulative food intake.

![Graph D](#) Changes in lean and fat mass in HFD mice treated for 24 days with vehicle (■), compound A (x), rimonabant (○), and mice pair fed to compound A treated animals (▲). DIO, diet-induced obese mice; FI, food intake; mc, methylcellulose. Changes in body weight, food intake, and body composition during the 18 days after cessation of treatment are also shown. *\( P < 0.01 \); **\( P < 0.01 \), compared with vehicle-treated mice.

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**Fig. 3.** Compound A treatment of high-fat diet (HFD) mice results in reduction of body weight and food intake and decrease in percent body fat over 24 days of dosing. A: changes in body weight expressed as percentage of initial weight. B: changes in average 24-h food intake. C: cumulative food intake. D: changes in lean and fat mass in HFD mice treated for 24 days with vehicle (■), compound A (x), rimonabant (○), and mice pair fed to compound A treated animals (▲). DIO, diet-induced obese mice; FI, food intake; mc, methylcellulose. Changes in body weight, food intake, and body composition during the 18 days after cessation of treatment are also shown. *\( P < 0.01 \); **\( P < 0.01 \), compared with vehicle-treated mice.
mice. The negative slope of the linear drop in glucose observed in any of the treated HFD-fed mice or low fat diet fed glucose initially increased after the insulin injection in vehicle- and 20 min for the HFD mice (Fig. 4 rapidly between 8 and 12 min in lean animals and between 8 and 214 HFD rosiglitazone 214 H11006 performed on day 15 changes did not reach significance.

Changes in biomarkers following compound A dosing in diet-induced mice

Table 2. Changes in biomarkers following compound A dosing in diet-induced mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Body Weight, g</th>
<th>Glucose, mg/dl</th>
<th>Insulin, ng/ml</th>
<th>Triglycerides, mg/dl</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td>Day 19</td>
<td>Day 0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>30.2 ± 0.5</td>
<td>29.5 ± 0.4</td>
<td>29.6 ± 0.4</td>
<td>184 ± 9</td>
</tr>
<tr>
<td>HFD, C57BL/6</td>
<td>37.8 ± 1.1§</td>
<td>36.7 ± 1.3§</td>
<td>37.2 ± 1.3§</td>
<td>212 ± 6§</td>
</tr>
<tr>
<td>C57BL/6 HFD, cmpd. A</td>
<td>38.1 ± 1.3§</td>
<td>33.2 ± 1.1</td>
<td>32.4 ± 1.1</td>
<td>200 ± 10</td>
</tr>
<tr>
<td>C57BL/6, HFD, Rosi</td>
<td>38.1 ± 1.1§</td>
<td>38.4 ± 1.2§</td>
<td>38.9 ± 1.1§</td>
<td>203 ± 10</td>
</tr>
</tbody>
</table>

*P < 0.05, †P < 0.01 compared to vehicle-treated mice on high-fat diet (HFD). ‡P < 0.05, §P < 0.01 compared with vehicle-treated mice on the low-fat diet (LFD). Rosi, rosiglitazone; cmpd. A, compound A.
3T3L1 adipocytes with insulin, tyrosine phosphorylation of IRS-1 appeared 5 min poststimulation (Fig. 5A, top and middle) and remained associated with the faster hypo-phosphorylated form of IRS-1. This phosphorylation was followed, 15 min post-stimulation, by the negative phosphorylation of IRS-1 on Ser307, which only comigrated with the hyper-phosphorylated form of IRS-1 (Fig. 5A, top and middle), judged by the retarded mobility and intensity of the protein bands. The comigration of serine phosphorylated IRS-1 with the slower-migrating form of IRS-1 is illustrated most clearly in reference to a nonspecific band shown in Fig. 5B. The pretreatment of 3T3L1 adipocytes with compound A prevented the phosphorylation of IRS-1Ser307 in response to insulin as shown by Western blot (Fig. 5B, top) and quantitatively using the MSD assay (Fig. 5B, bottom). Compound A reduced the phosphorylation of IRS-1Ser307 as well as cJun to ~40% of the insulin-stimulated levels (P < 0.05; Fig. 5, B and C). These results are consistent with the proposed role of JNK activation in the negative regulation of insulin signaling.

Glucose uptake in differentiated 3T3L1 cells. Previous studies (11) showed that FFA treatment of differentiated 3T3-L1 adipocytes leads to activation of a number of signaling pathways including JNK and IKKβ, which in turn regulate the phosphorylation of IRS-1Ser307. Accordingly, we measured the effects of compound A on glucose uptake in differentiated adipocytes. Pretreatment of adipocytes with up to 0.5 μM compound A had no effect on basal glucose uptake (Fig. 6B). However, compound A (0.3 μM) completely reversed the inhibitory effect of FFAs on basal glucose uptake (Fig. 6A). Insulin stimulated glucose uptake in 3T3L1 adipocytes by fivefold (Fig. 6B) but was almost completely inhibited by pretreatment with 100 μM FFA (Fig. 6B). However, preincubation of adipocytes with FFAs in addition to 0.3 μM compound A partially attenuated the effects of FFA on insulin-stimulated glucose uptake (Fig. 6B; P < 0.05, compared with FFA alone).

Discussion

Compound A is a reversible ATP-competitive JNK inhibitor with a Ki of 9 nM and a cell-based IC50 of 102 nM (21). Cytokines and FFA, elevated during obesity and type 2 diabetes, activate a number of kinases including JNK (6, 9). Activated JNK phosphorylates Ser307 (16) of IRS-1 and IRS-2 (19), directly obstructing insulin signaling and hampering the uptake of glucose into peripheral tissues. JNK1 and JNK2 are broadly distributed, whereas JNK3 is expressed predominantly in neurons; however, JNK1 is the main target for type 2 diabetes. Compound A is nonselective for JNK1 and JNK2, which may have additional benefits over a JNK1 selective inhibitor, since studies (21) demonstrated that JNK2 may play a role in improvements in metabolic regulation when JNK1 activity is partially reduced.

Consistent with the role of JNK in inflammatory-mediated signaling, treatment of C57BL/6 mice with compound A before administration of LPS resulted in a significant inhibition of LPS-induced JNK activity and cytokines (Fig. 2). The observed decrease in exposure of compound A in mice treated with LPS compared with that in control mice may be related to previously reported changes in hepatic microsomal cytochrome P-450 enzymes and gastrointestinal absorption after LPS exposure (3, 17).

Reduction in JNK activity both genetically and pharmacologically has been demonstrated to improve insulin resistance in a number of diabetic animal models. JNK1 knockout mice fed a HFD or ob/ob mice deficient in JNK1 had a significant
reduction in insulin resistance compared with wild-type counterparts (13). Treatment of C57BL/KLs-db/db mice or C57BL/6 mice fed a high-fat, high-sucrose diet with a cell-permeable JNK-inhibitory peptide (JIP) resulted in improved insulin sensitivity and glucose tolerance, accompanied by elevated IRS-1 tyrosine phosphorylation, reduced phosphorylation of IRS-1Ser307, and increased serine and threonine phosphorylation of Akt in liver, fat and muscle, suggestive of an improvement in insulin sensitivity.

Fig. 5. Relief of insulin receptor substrate-1 (IRS-1) Ser307 phosphorylation induced by the negative feedback of insulin stimulated by compound A. A: Western blots show that insulin IRS-1 Ser307 phosphorylation (middle) as a response to the negative feedback of insulin stimulation follows IRS-1 Tyr phosphorylation (bottom). B: Western blot and quantitation by ELISA of IRS-1 serine phosphorylation is inhibition by JNK inhibitor compound A after 1 h insulin treatment. #Progressive mobility shift of IRS-1 upon phosphorylation compared with the nonspecific band. C: quantitation Ser73 of c-Jun from the extracts in B by ELISA (see MATERIALS AND METHODS). *P < 0.05.

Fig. 6. Compound A relieves the inhibition of insulin stimulated glucose uptake by FFA in differentiated 3T3L1 cells. A: in differentiated 3T3L1 cells, free fatty acid (FFA)-mediated repression of basal glucose uptake is completely relieved by compound A, measured by cellular 2-deoxy-D-[14C]glucose uptake. B: FFA-mediated repression of insulin stimulated glucose uptake is partially relieved by compound A. *P < 0.05 between the bracketed 2 groups.
signaling (15). Overexpression of dominant negative JNK in the liver of db/db mice or in mice on a high-fat/high-sucrose diet also improved insulin sensitivity, which was associated with reductions in expression of gluconeogenesis enzymes and in endogenous hepatic glucose production (15). Moreover, the JNK inhibitor SP600125 was reported to lower blood glucose, increase insulin, and improve glucose tolerance after 17 days of dosing in db/db mice (7).

We provide in this set of studies the first description of a small molecule inhibitor of JNK, having an effect on food intake and weight. Compound A resulted in a greater degree of weight loss and adiposity compared with pair-fed mice after 24 days of dosing in high-fat fed mice, suggesting that the anorectic effect of the JNK inhibitor was not the only contributing factor to the weight reduction. Although it remains a formal possibility that some of the effects of compound A on weight and fat mass are due to toxicity, we observed no significant alterations in liver enzymes after the 4 wk of dosing. Furthermore, lean mass was not decreased during the weight loss study and weight and fat mass were completely restored to baseline levels during the rebound period. Changes in other metabolic parameters such as alterations in energy expenditure or lipid metabolism may have contributed to the weight loss, although these were not investigated in the present study. Notably, Hirosumi et al. (13) suggested that the increased insulin sensitivity and decrease in weight gain in JNK-deficient mice was primarily due to a decrease in adipocyte size, adiposity, and alterations in adipose tissue redistribution, although they did observe a small reduction in food intake and increase in core body temperature. It remains unclear to what contribution central inhibition of JNK by compound A contributes to the effects on weight. Identification of a more soluble JNK1 inhibitor would allow intracerebroventricular studies to be performed to shed light on this question.

A further study designed to investigate the insulin-sensitizing effects of compound A in a high-fat fed mouse demonstrated that the normalization in body weight was accompanied by improvements in insulin sensitivity to levels comparable with those in the lean cohort and significant reduction of glucose and triglycerides compared with vehicle-treated HFD mice (P < 0.01; Table 2 and Fig. 4, A and B). A significant decrease in phosphorylated c-Jun as an indication of inhibition of JNK activity was observed in fat homogenates from high-fat fed mice treated with compound A compared with vehicle-treated mice (Fig. 4C), indicating that compound A was acting at the desired target. We determined JNK activity in adipose tissue, as preliminary studies suggested that this tissue gave the best signal using our detection system. An elevation in JNK activity has been previously described in the liver, fat, and muscle of high-fat fed mice compared with lean counterparts (13, 19). At the time of the study, with compound A was terminated, the HFD mice did not exhibit a significant increase in phosphorylated c-Jun in fat compared with the lean mice. Our observations suggest that whereas at the start of the study HFD mice were clearly hyperinsulinemic and elevated JNK activity would be anticipated, the stress of twice daily dosing may have contributed to the reduction in hyperinsulinemia and lack of overt elevation in JNK activity observed at the end of the study. These findings do not preclude the fact that compound A mediated inhibition of JNK in HFD mice earlier in the study improved metabolic parameters, including weight, compared with vehicle-treated HFD mice. Future inclusion of a satellite group of mice where JNK activity was determined at the start of the study would support this point.

We did not investigate the activity of other kinases, aside from JNK, that have been suggested to play a role in the reduction of insulin signaling such as NK-κB (23) or PKCθ (12). Moreover studies to examine the level of IRS-1Ser307 from in vivo samples were unsuccessful and would be a valuable addition in future studies to support the mechanistic hypothesis behind JNK inhibition. Free plasma levels of compound A at the end of the study, taken 1 h after the final dose, were ~1.5 μM; although it is possible that some of the effects observed in the HFD mice may have been due to off-target effects, this would not be predicted if the in vitro selectivity window is reflected in the in vivo setting.

Reports (20) suggest that deletion of JNK1 in a nonhematopoietic cell type results in resistance to diet-induced obesity through an increase in metabolic rate without any effect on food intake, whereas removal of JNK1 from hematopoietic cells protects against diet-induced insulin resistance without affecting obesity. The effect of compound A on weight would have played a major role in the improvement in metabolic parameters. However, since compound A would be expected to inhibit JNK in all cell types, it would be anticipated based on studies from Solinas et al. (19, 20) that both the effects on metabolic rate and the direct effects on insulin resistance would be manifest. Studies in vitro supported the possibility that compound A could directly improve insulin signaling and glucose uptake in 3T3-L1 adipocytes. We observed that compound A improved insulin signaling by dampening the insulin-stimulated phosphorylation of IRS-1Ser307 in differentiated 3T3-L1 adipocytes, which was associated with a decrease in phosphorylated c-Jun levels (Fig. 5, B and C). The partial inhibition of insulin-stimulated IRS-1Ser307 after compound A exposure most likely reflects the contribution of other kinases that are involved in IRS-1 phosphorylation, such as IKKβ (23) and PKCθ (12). Notably while compound A completely reversed the FAA-mediated inhibition of basal glucose uptake, there was only a partial reversal of the inhibitory effects of FFAs on insulin-stimulated glucose uptake. These findings may suggest that FAA inhibit glucose uptake via different signaling mechanisms under basal and insulin-stimulated conditions and merit further research. Although we did not obtain information regarding tissue levels of serine or tyrosine phosphorylated IRS-1 from our in vivo studies, our in vitro studies in adipocytes support the possibility of a direct role for compound A as an enhancer of insulin signaling, through a JNK-dependent mechanism, which may have contributed to improvements in insulin sensitivity in addition to those resulting from changes in weight. Examination of insulin sensitivity in mice pair fed to those treated with compound A would provide further enlightenment on the relative contribution of weight loss to the insulin sensitization effect in HFD mice.

In summary, we have demonstrated that a potent, selective JNK inhibitor is able to attenuate the effects of elevated fatty acids on induction of insulin resistance both in vitro and in vivo. Although we are unable to dissociate the effects of compound A in vivo on weight-reduction from any direct effects on inflammatory-mediated attenuation of insulin signaling, our studies support a hypothesis that inhibition of JNK may have beneficial effects on weight, adiposity, and insulin...
sensitivity and strongly support JNK as a target for type 2 diabetes and obesity.

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