

Discovery of Novel Forkhead Box O1 Inhibitors for Treating Type 2 Diabetes: Improvement of Fasting Glycemia in Diabetic *db/db* Mice^[S]

Takeyuki Nagashima, Nobuharu Shigematsu, Riyo Maruki, Yasuharu Urano, Hirotugu Tanaka, Akiyoshi Shimaya, Teruhiko Shimokawa, and Masayuki Shibasaki

Pharmacology Research Labs, Drug Discovery Research, Astellas Pharma Inc., Ibaraki, Japan (T.N., H.T., A.S., T.S., M.S.); Astellas Research Technologies Co., Ltd., Ibaraki, Japan (N.S., R.M.); and Chemistry Research Labs, Drug Discovery Research, Astellas Pharma Inc., Ibaraki, Japan (Y.U.)

Received April 21, 2010; accepted August 16, 2010

ABSTRACT

Excessive hepatic glucose production through the gluconeogenesis pathway is partially responsible for the elevated glucose levels observed in patients with type 2 diabetes mellitus (T2DM). The forkhead transcription factor forkhead box O1 (Foxo1) plays a crucial role in mediating the effect of insulin on hepatic gluconeogenesis. Here, using a *db/db* mouse model, we demonstrate the effectiveness of Foxo1 inhibitor, an orally active small-molecule compound, as a therapeutic drug for treating T2DM. Using mass spectrometric affinity screening, we discovered a series of compounds that bind to Foxo1, identifying among them the compound, 5-amino-7-(cyclohexylamino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (AS1842856), which potently inhibits human Foxo1

transactivation and reduces glucose production through the inhibition of glucose-6 phosphatase and phosphoenolpyruvate carboxykinase mRNA levels in a rat hepatic cell line. Oral administration of AS1842856 to diabetic *db/db* mice led to a drastic decrease in fasting plasma glucose level via the inhibition of hepatic gluconeogenic genes, whereas administration to normal mice had no effect on the fasting plasma glucose level. Treatment with AS1842856 also suppressed an increase in plasma glucose level caused by pyruvate injection in both normal and *db/db* mice. Taken together, these findings indicate that the Foxo1 inhibitor represents a new class of drugs for use in treating T2DM.

Introduction

Endogenous glucose production is excessive not only under fasted but also fed conditions, contributing to the long-term elevated glucose level observed in patients with mild (Perriello et al., 1997) and advanced (Boden et al., 2001) type 2 diabetes mellitus (T2DM). The liver is the major site of endogenous glucose production, producing glucose by either

gluconeogenesis or glycogenolysis. Recent studies have suggested that gluconeogenesis is largely responsible for the overproduction of glucose in patients with T2DM, whereas glycogenolysis is either unchanged or even reduced (Wajngot et al., 2001), suggesting that the gluconeogenesis pathway may be an extremely effective drug target for pharmacological intervention in patients with T2DM.

The Forkhead box class O subfamily of forkhead transcription factors consists of the functionally related proteins Foxo1, Foxo3a, and Foxo4 in mammals and is regulated by phosphorylation via the phosphoinositide 3-kinase-Akt pathway (Hall et al., 2000; Nakae et al., 2001). Foxo1 in particular has been shown to play an important role in the control of key

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.

doi:10.1124/mol.110.065714.

[S] The online version of this article (available at <http://molpharm.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: T2DM, type 2 diabetes mellitus; Foxo1, forkhead box O1; AS1842856, 5-amino-7-(cyclohexylamino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; G6Pase, glucose-6 phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; EF1 α , elongation factor 1 α ; AUC, area under the curve; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; CBP, cAMP response element-binding protein-binding protein; AS1841674, 1-cyclopentyl-6-fluoro-4-oxo-7-(tetrahydro-2H-pyran-3-ylamino)-1,4-dihydroquinoline-3-carboxylic acid; AS1838489, 7-(cyclohexylamino)-6-fluoro-4-oxo-1-(prop-1-en-2-yl)-1,4-dihydroquinoline-3-carboxylic acid; AS1837976, 7-(cyclohexylamino)-6-fluoro-1-(3-fluoroprop-1-en-2-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; AS1805469, 7-(cyclohexylamino)-1-(cyclopent-3-en-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid; AS1846102, 7-(cyclohexylamino)-6-fluoro-5-methyl-4-oxo-1-(pentan-3-yl)-1,4-dihydroquinoline-3-carboxylic acid; 4 \times IRE, four-repeated insulin responsive element.

gluconeogenic enzymes, namely G6Pase and PEPCK. Insulin suppresses Foxo1 activity via Akt phosphorylation-dependent nuclear exclusion (Jackson et al., 2000), ubiquitination, and degradation (Matsuzaki et al., 2003), and transgenic expression of a constitutively active form of Foxo1 has been shown in turn to impair glucose tolerance and insulin sensitivity (Nakae et al., 2002). In addition, the liver-specific expression of the dominant-negative form of Foxo1 has demonstrated that inactivation of Foxo1 subsequently reduces the expression levels of PEPCK and G6Pase, thereby decreasing gluconeogenesis in H4IIE cells and diabetic *db/db* mice (Altomonte et al., 2003). A liver-specific deletion of Foxo1 resulted in a significant reduction in hepatic glucose production (Matsumoto et al., 2007). Furthermore, antisense oligonucleotide-mediated inhibition of Foxo1 activity improved hepatic and peripheral insulin actions in high-fat diet-induced obese mice (Samuel et al., 2006). Taken together, these previous findings suggest that inhibition of Foxo1 activity leads to a reduction in hepatic gluconeogenesis, thereby improving hepatic insulin action and enhancing peripheral insulin-stimulated glucose metabolism. We therefore considered that alteration of Foxo1 activity might represent a new therapeutic approach to treating T2DM with hepatic insulin resistance. However, no study has yet reported on a small-molecule inhibitor directly inhibiting Foxo1 activity. Here, we present our discovery of a series of

potent and selective small-molecule inhibitors of Foxo1, which we identified by affinity selection using mass spectrometry, and we describe the in vitro and in vivo inhibiting ability of AS1842856.

Materials and Methods

Foxo1 Inhibitor Compounds. The Foxo1 inhibitors used in the present study [5-amino-7-(cyclohexylamino)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (AS1842856), 1-cyclopentyl-6-fluoro-4-oxo-7-(tetrahydro-2H-pyran-3-ylamino)-1,4-dihydroquinoline-3-carboxylic acid (AS1841674), 7-(cyclohexylamino)-6-fluoro-4-oxo-1-(prop-1-en-2-yl)-1,4-dihydroquinoline-3-carboxylic acid (AS1838489), 7-(cyclohexylamino)-6-fluoro-1-(3-fluoroprop-1-en-2-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (AS1837976), 7-(cyclohexylamino)-1-(cyclopent-3-en-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (AS1805469), and 7-(cyclohexylamino)-6-fluoro-5-methyl-4-oxo-1-(pentan-3-yl)-1,4-dihydroquinoline-3-carboxylic acid (AS1846102)] were synthesized by Astellas Pharma Inc. (Ibaraki, Japan). AS1842856 was dissolved in 6% cyclodextrin (Sigma-Aldrich, St. Louis, MO) for oral administration.

Vectors. Full-length cDNA for human Foxo1, human Foxo3a, and human Foxo4 were inserted into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) to generate pcDNA-Foxo1, pcDNA-Foxo3a, and pcDNA-Foxo4, respectively. For the protein expression experiment, FLAG-tag was inserted into pcDNA-Foxo1 to generate pcDNA-FLAG-Foxo1. The reporter plasmid pGL3-4xIRE-Luc was constructed by inserting four copies of a 22-base pair

TABLE 1

Primers and probes

Probes are FAM-5'→3'-TAMRA; primers are 5'→3'.

Species & Gene	GenBank Accession No.	Probe/Primers	Sequence
Rat	<i>G6Pase</i>	Probe	TGGGTGGCAGTGGTTGGAGACT
		Forward	TTCAAGAGACTGTGGGCATCAAT
	<i>PEPCK</i>	Reverse	ATCCACTTAAAGACGAGGTTG
		Probe	CTATGTGGTGGCCAGCATCG
	<i>Foxo1</i>	Forward	AAGATTGGTATTGAGCTGACAGAC
		Reverse	AGAGGTCCCATCCGTGT
Mouse	<i>G6Pase</i>	Probe	ATGGCCGTGCCCTGCCCC
		Forward	CATGCACAGCAAACCTCTTCAGT
		Reverse	TGTGTGAGGCATGGTGTTC
		Probe	TGGGTGGCAGTGGTCGGAGACT
		Forward	TTAAAGAGACTGTGGGCATCAAT
	<i>PEPCK</i>	Reverse	ATCCACTTGAAGACGAGGTTG
		Probe	CTATGTGGTGGCCAGCATCG
		Forward	GGTATTGAAGTACAGACTCGC
		Reverse	CACAGATATGCCCATCCGA
	<i>Foxo1</i>	Probe	CCCTTTGCCCCAGATGCCTATG
		Forward	AAGTACAGATACGGCCAATCC
		Reverse	CGTAACCTGATTTGCTGTCTCTG
	<i>apoCIII</i>	Probe	CTGACAAAGTTCACCGCTTCTGGGA
		Forward	TGAAAGGCTACTGGAGCAAAGTTT
		Reverse	TGGTTGGTCCTCAGGGTTAGA
	<i>ABCG5</i>	Probe	TGATTGGCAGCTATAATTTTGGG
		Forward	CCACGTGGCGGACCAA
		Reverse	CGCTCGCCACTGGAATTT
Mouse/rat	<i>ABCG8</i>	Probe	TTTTCCACCCTGATCCGTCG
		Forward	GCTGCCCGGATGATAGAG
		Reverse	CCGGAAGTCATTGGAATCTG
	<i>IL-1β</i>	Probe	CATGGCACATTCTGTTCAAAGAGAGCCTG
		Forward	TCGCTCAGGGTCACAAAGAAA
		Reverse	CCATCAGAGGCAAGGAGGAA
	<i>GK</i>	Probe	AGGACGCCATGACGGCACT
		Forward	GAAGACGAAACACCAGATGTATTC
		Reverse	GATGTAGTCAAAGAGCATCTCCG
	<i>EF1α</i>	Probe	TTTGCTGTTCTGTGACATGAGGCAG
		Forward	GACTACCCTCCACTTGGTCG
		Reverse	GATGACACCCACAGCAACTG

insulin-responsive element identical with that of rat PEPCK into the pGL3 vector (Promega, Madison, WI) containing firefly luciferase cDNA. pGL4.75 (Promega) containing *Renilla reniformis* luciferase cDNA driven by the cytomegalovirus promoter was used as a control transfection plasmid.

Protein Expression, Phosphorylation, and Purification. FreeStyle 293-F cells were transiently transfected with pcDNA-FLAG-Foxo1 using 293fectin (Invitrogen). Forty-eight h after transfection treatment, the cells were centrifuged at 1000g and then stored at -20°C until use. After thawing, lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) were administered to the collected cells on ice for 30 min. Solution clarification was achieved by centrifugation at 8000g for 20 min at 4°C . Anti-FLAG M2-Agarose affinity gel (Sigma-Aldrich) equilibrated in Tris-buffered saline (50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) was added to the clarified sample and rotated at 4°C for 2 h. The sample was then washed with buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100), and then with buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.1% Zwittergent 3-12) (EMD Chemicals, Gibbstown, NJ). After subsequent washing with buffer C (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Zwittergent 3-12), Foxo1 protein was eluted using 250 $\mu\text{g}/\text{ml}$ FLAG peptide in buffer C, and protein presence was confirmed using SDS-PAGE. To dephosphorylate the FLAG-tagged Foxo1 protein, λ -phosphatase (1 U/ μl) was added to the proteins along with buffer D (50 mM HEPES, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 2 mM dithiothreitol, 2 mM MnCl_2 , and protease inhibitor cocktail) and incubated at 37°C for 2 h. Dephosphorylated Foxo1 proteins were subsequently identified by Western blotting with anti-phospho-Foxo1 as described below, dialyzed in buffer E (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1% Zwittergent 3-12) at 4°C overnight, and then applied to a DEAE-cellulose equilibrated with buffer F (50 mM HEPES, pH 7.5, and 1% Zwittergent 3-12). Elution was performed with a linear gradient of 0 to 0.5 M NaCl in the equilibrated buffer. Peak fractions containing Foxo1 were pooled and the purity of the protein sample was confirmed by SDS-PAGE.

Affinity Screening Using Mass Spectrometry. In the present study, affinity selection using mass spectrometry to identify small-molecule ligands as Foxo1 inhibitors was conducted in accordance with a protocol described previously (Annis et al., 2007). In brief, combining a soluble protein and a mass-encoded small-molecule library (our compound library, which consists of molecules with a molecular mass of 250–500 kDa) in a physiologically relevant buffer led to the formation of a complex of the protein with any suitable library member. The complexes were then separated from nonbound library members and immediately transferred to a reversed-phase chromatography column. The column was maintained at 60°C and pH < 2 to promote the dissociation of ligands from the complex, after which the dissociated ligands were eluted into a high-resolution mass spectrometer for analysis. Further details regarding the selection method are presently being prepared for publication.

Reporter Gene Assay. HepG2 cells maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) in high-glucose solution (25 mM) and 10% fetal bovine serum (FBS) were transfected using reagents from Bio-Rad (Hercules, CA). Transfection efficiency was controlled by including pGL4.75 in each transfection experiment. To ensure equal amounts of DNA, empty plasmids were included for each transfection. Cells were cultured in DMEM supplemented with 10% FBS for 5 h after transfection, after which medium was replaced with DMEM supplemented with 1% FBS with or without the addition of Foxo1 inhibitor AS1842856 at the indicated concentrations. Cells were then incubated a further 20 h. Luciferase activity was measured using a Wallac 1420 ARVOsx (PerkinElmer Life and Analytical Sciences, Waltham, MA) and normalized for *R. reniformis* luciferase activity for each sample.

Glucose Production Assay. Rat hepatoma Fao cells possess a complete gluconeogenic enzyme system that allows the cells to survive and grow in low-glucose (5.5 mM) or glucose-free medium. Cells were cultured in DMEM with 5.5 mM glucose and 10% FBS. Just before the start of any procedures, the medium was replaced with the same medium described above without FBS. Glucose production rate was measured using glucose CII-test reagent (Wako Pure Chemical Industries, Osaka, Japan). In brief, after 18 h of treatment with either insulin or AS1842856 at the indicated concentrations, the cells were washed three times with PBS. The cells were then incubated for 3 h at 37°C in 5% CO_2 in a glucose production buffer (glucose-free DMEM, pH 7.4, containing 20 mM sodium pyruvate, without phenol red).

RNA Extraction and Quantitative RT-PCR. Total RNA was prepared from aliquots of liver tissue or plated Fao cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Probes and primers used are described in Table 1. TaqMan PCR was performed using ABI Prism 7900 Sequence Detection System in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA). The mRNA level was normalized to the elongation factor 1 α (EF1 α) mRNA level.

Western Blot Analysis. Fao cells were homogenized in CellLytic M Cell Lysis Reagent (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitor cocktail (Sigma-Aldrich). Each protein concentration was quantified using a standard Bradford assay (protein assay; Bio-Rad). Equal amounts of protein lysates were resolved on 5 to 15% SDS-polyacrylamide gels (DRC, Tokyo, Japan) and then transferred to a polyvinylidene difluoride membrane. After blocking with Blocking One solution (Nacalai Tesque, Kyoto, Japan), the membrane was blotted with the primary antibody [anti-Foxo1 antibody (H-128; Santa Cruz Biotechnology, Santa Cruz, CA)], anti-phospho-[Ser256] Foxo1, anti-Akt, anti-phospho-[Ser407] Akt, anti-ERK, anti-phospho-[Thr202/

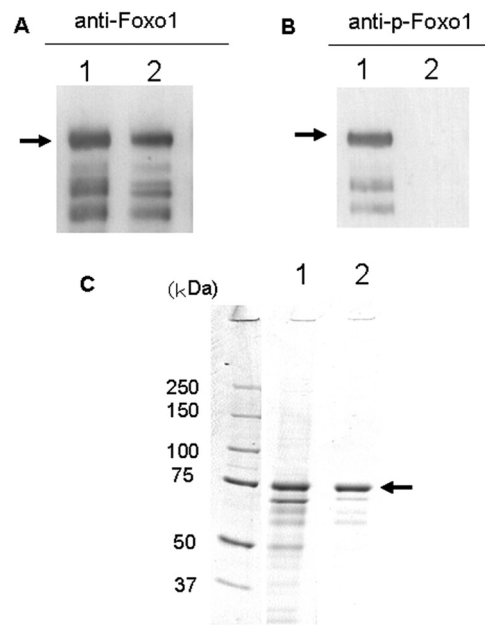


Fig. 1. Expression, dephosphorylation, and purification of human Foxo1 protein from 293-F cells expressing wild-type Foxo1. Free-Style 293-F was transiently transfected with FLAG-Foxo1 expression plasmid. Samples were resolved by 5 to 15% SDS-PAGE and subjected to Western blotting using anti-Foxo1 (A) or anti-phospho-Foxo1 (B). Human Foxo1 is indicated with an arrow. Lane 1, purified Foxo1 protein using a FLAG-tagged gel. Lane 2, purified Foxo1 protein with 30 min of λ -phosphatase treatment. C, dephosphorylated Foxo1 protein described above was subjected to DEAE-cellulose chromatography for further purification. Human Foxo1 is indicated with an arrow. Dephosphorylated Foxo1 protein before (lane 1) and after (lane 2) DEAE-cellulose chromatography.

Tyr204] ERK, anti-S6K, or anti-phospho-[Thr389] S6K (Cell Signaling Technology, Danvers, MA). The membrane was then washed and blotted again with horseradish peroxidase-conjugated anti-rabbit IgG antibody (The Jackson Laboratory, Bar Harbor, ME). The per-

oxidase activity was visualized with ECL-Plus (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Animal Study. *db/db* mice aged 6 weeks were purchased from Charles River (Yokohama, Japan), and ICR mice aged 6 weeks were purchased from CREA (Tokyo, Japan). The mice were fed standard rodent chow and water ad libitum in sterile cages with a 12-h light/dark cycle. Pyruvate or glucose tolerance tests were performed in male mice aged 7 to 9 weeks ($n = 6$). Mice were orally administered either AS1842856 dissolved in 6% cyclodextrin or vehicle (6% cyclodextrin only) at three time points (8 AM, 6 PM, and 8 AM on the second day). Food was removed after initial dosing and withheld throughout the study (26-h fasting). For glucose or pyruvate tolerance tests, glucose or pyruvate at 2 g/kg was administered intraperitoneally 2 h after final dosing, and blood was collected for plasma preparation from the retro-orbital sinus into heparinized capillary tubes (Chase Scientific Glass, Rockwood, TN) at baseline and 30, 60, and 120 min after dosing. Plasma glucose level was measured using glucose CII-test reagent (Wako Pure Chemical Industries), and area under the curve (AUC) was calculated using the SAS software package (SAS Institute Japan, Ltd., Tokyo, Japan). Fasting plasma insulin level was determined using a murine insulin ELISA kit (Shibayagi, Gunma, Japan). To obtain liver tissue for protein and mRNA analyses, animals were sacrificed after 26-h fasting. All animal experiments were conducted in accordance with the guidelines of the Animal Experiment Committee of Astellas Pharma Inc.

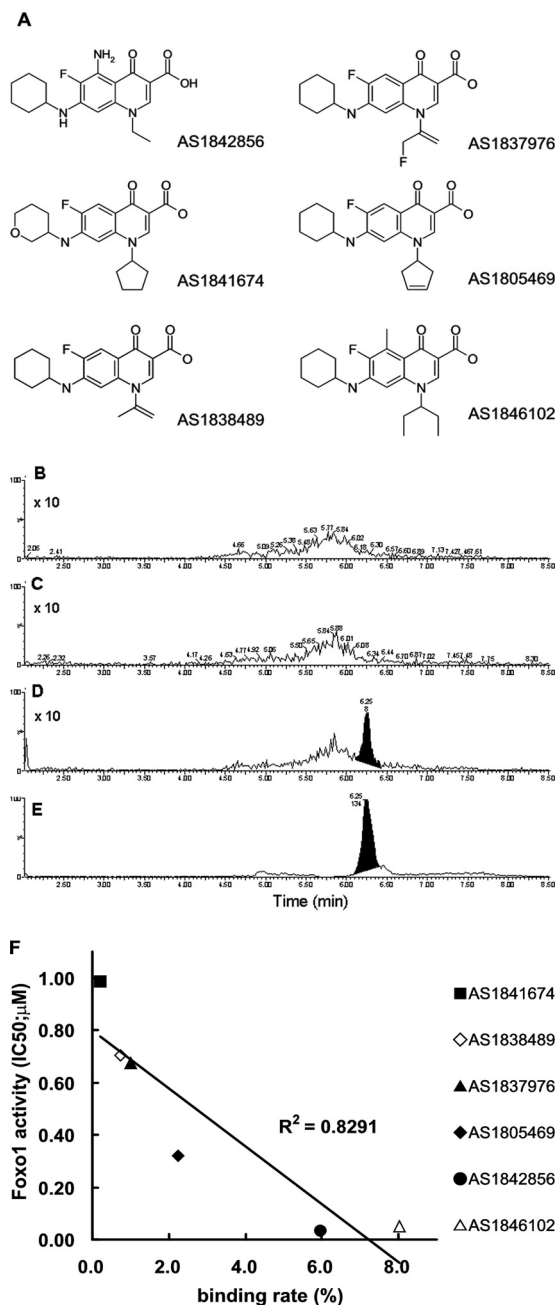


Fig. 2. Discovery of AS1842856 as novel Foxo1 inhibitors by mass spectrometric affinity. A, structures of Foxo1 inhibitors. B to E, exact ion chromatogram of m/z 348.1 (M+H)⁺ from affinity selection-mass spectrometry. B, control (no protein); C, complex of phosphorylated Foxo1 with AS1842856 (after B/F separation); D, complex of dephosphorylated Foxo1 with AS1842856 (after B/F separation). The black peak shows AS1842856 bound to the Foxo1 protein. E, complex of dephosphorylated Foxo1 with AS1842856 (no B/F separation). The black peak (set as 100%) describes the amount of input AS1842856. F, the correlation of binding strength and inhibitory activity against Foxo1-mediated transcription. The x-axis shows the binding rate, which is the percentage that each compound bound to the Foxo1 protein (in the case of AS1842856, as shown in Fig. 2D) to the amount of the input each compound (in the case of AS1842856, as shown in Fig. 2E). The y-axis shows the IC₅₀ value that the compounds inhibit Foxo1-mediated transactivation by conducting reporter assays (as described in Fig. 3).

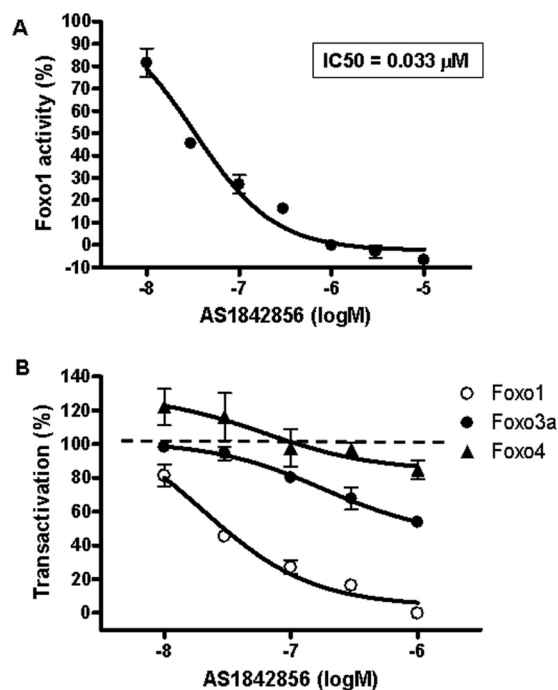


Fig. 3. Dose-dependent and selective inhibition of Foxo1-mediated transactivation with AS1842856. A, to establish basal activity, HepG2 cells were transiently cotransfected with 4×IRE luciferase plasmid, pGL4.75 control plasmid, and Foxo1 expression plasmid. After transfection, cells were serum-starved for 18 h in the presence or absence (set as 100%) of AS1842856 at various concentrations, as indicated. To determine basal activity, HepG2 cells were cotransfected with a 4×IRE luciferase plasmid, pGL4.75, and pcDNA3.1. After transfections, cells were serum-starved for 18 h in dimethyl sulfoxide (set as 0%). Luciferase activity was determined and normalized to the coexpressed *R. reniformis* luciferase. The mean \pm S.E. of four independent experiments is shown. B, HepG2 cells were cotransfected with 4×IRE luciferase plasmids, *R. reniformis* luciferase control plasmids, and Foxo protein plasmids (Foxo1, Foxo3a, or Foxo4). After transfections, cells were serum-starved for 18 h in the presence or absence (set as 100%) of increasing concentrations of AS1842856. Basal activity and relative luciferase activity were calculated as described above. The mean \pm S.E. of four independent experiment is shown.

Statistical Analysis. Statistical analyses were conducted using the SAS 8.2 software package (SAS Institute Japan, Ltd., Tokyo, Japan). The IC_{50} was calculated using regression analysis. Data were expressed as the mean \pm S.E.M. Statistically significant differences between pairs of groups were determined using Student's *t* test, whereas those between multiple groups were assessed using Dunnett's multiple range test. A value of $p < 0.05$ was taken as significant.

Results

Expression, Dephosphorylation, and Purification of Foxo1. The mammalian cell line Free-Style 293-F was transiently transfected with an expression plasmid encoding human Foxo1 protein fused with a FLAG tag at its N terminus. Expression of FLAG-tagged Foxo1 was confirmed by Western blot analysis (Fig. 1A, lane 1), and we noted that more than half of the purified Foxo1 was detected by the Foxo1 antibody, which recognizes Ser256-phosphorylated proteins (Fig. 1B, lane 1). Phosphorylation of Ser256 has been reported to suppress Foxo1 transactivation, with the phosphorylated Foxo1 binding to 14-3-3 proteins (Zhang et al., 2002). We confirmed by fragment analysis using mass spectrometry that the purified Foxo1 does indeed bind to 14-3-3 proteins (data not shown). To convert the phosphorylated Foxo1 to its active form, we treated the purified protein with a λ -phosphatase and obtained completely dephosphorylated protein (Fig. 1B, lane 2). The dephosphorylated protein was then subjected to DEAE-cellulose chromatography for further purification and purified for compound screening as shown in Fig. 1C, lane 2.

Screening of Foxo1 Inhibitors by Mass Spectrometric Affinity. We conducted high-throughput screening by incubating dephosphorylated Foxo1 protein with our mass-

encoded compound libraries, followed by separation of bound and free compounds (B/F separation). Compounds bound to the Foxo1 protein were dissociated from the enzyme by reverse-phase high-performance liquid chromatography, and then analyzed by mass spectroscopy and identified according to the appropriate mass information embedded in each library. This screening process led to the identification of the compounds (Fig. 2A) against the dephosphorylated active form of Foxo1 protein (Fig. 1C, lane 2). In these compounds, AS1842856 bound the dephosphorylated Foxo1 at approximately 6% to the amount of the input compound (Fig. 2, D and E). We confirmed that AS1842856 does not bind to the phosphorylated inactive form (Fig. 2C). Furthermore, we confirmed that the percentage that each compound bound to the Foxo1 protein is correlated to the activity of Foxo1-mediated transactivation (Fig. 2F). AS1842856 was also found to dose-dependently bind to the dephosphorylated Foxo1 protein (Supplemental Figure S1).

Effects of AS1842856 on Foxo1-Mediated Transactivation. We tested the effect of AS1842856 on Foxo1-mediated transactivation by conducting reporter assays using a four-repeated insulin responsive element (4 \times IRE) promoter construct containing cognate Foxo1 binding elements (Hall et al., 2000). In HepG2 cells transiently transfected with a Foxo1 expression vector, AS1842856 potently repressed Foxo1-mediated promoter activity in a dose-dependent manner similar to that seen in insulin treatment (Fig. 3A and Supplemental Figure S2). In mammals, other members of the Forkhead box O family, including Foxo3a and Foxo4, possess a forkhead DNA binding domain and a transactivation domain in the C terminus (Furuyama et al., 2000). The Foxo family binds to consensus Foxo binding sites (including IRE)

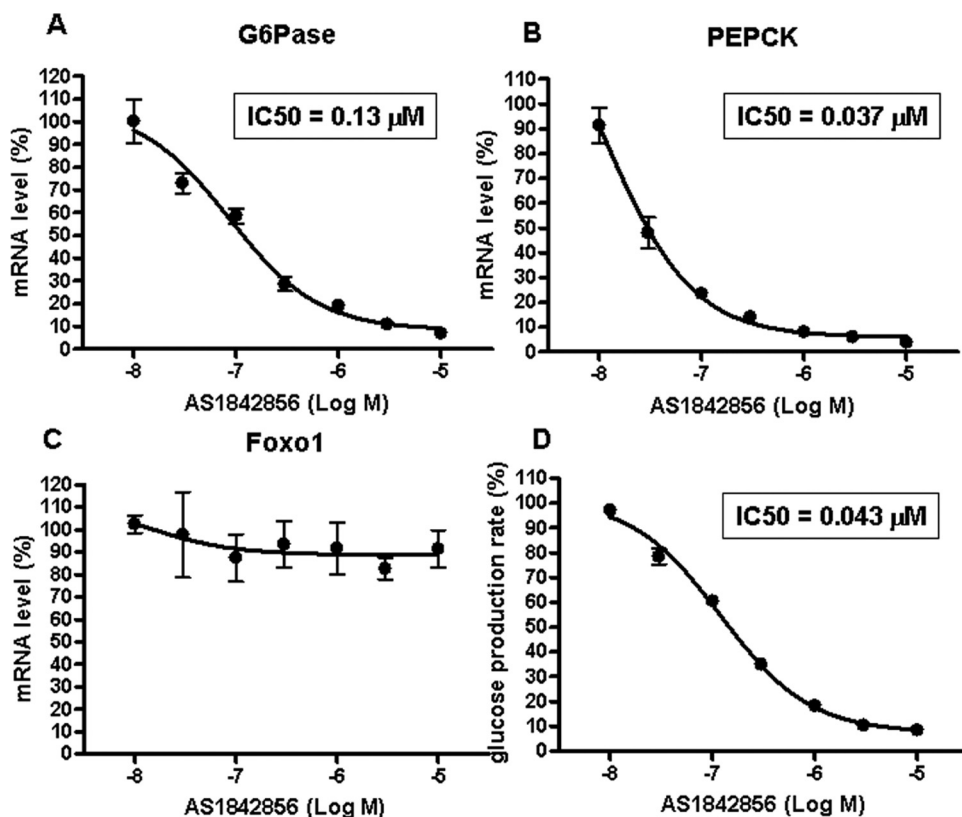


Fig. 4. Dose-dependent inhibition of gluconeogenesis of AS1842856 in Fao cells. Fao cells were incubated with AS1842856 for 18 h. G6Pase (A), PEPCK (B), and Foxo1 (C) gene expression relative to rat EF1 α mRNA were measured using quantitative RT-PCR. The mean \pm S.E. of three independent experiments is shown. D, glucose concentration in the medium was measured after incubation for 3 h in glucose production buffer. The mean \pm S.E. of three independent experiments is shown.

in the promoter region of the target genes, subsequently activating gene expression (Onuma et al., 2006).

In the present study, AS1842856 administered at 0.1 μM inhibited Foxo3a- and Foxo4-mediated promoter activity by 3 and 20%, respectively. In contrast, Foxo1-mediated promoter activity was decreased by 70% (Fig. 3B). These findings indicate that AS1842856 predominantly suppresses Foxo1-mediated transactivation by directly binding to Foxo1.

Effects of AS1842856 on Gluconeogenesis in Fao Cells. To examine the effect of Foxo1 inhibitors on gluconeogenesis, we analyzed the gene expression levels of endogenous G6Pase and PEPCK using quantitative RT-PCR analysis after 18-h treatment with AS1842856. Rat EF1 α was measured as an internal control. Treatment of Fao cells with AS1842856 resulted in a dose-dependent inhibition of mRNA levels of both G6Pase (Fig. 4A) and PEPCK (Fig. 4B). On the other hand, no differences were noted in Foxo1 mRNA levels in the relative concentrations of the compound (Fig. 4C). AS1842856 also inhibited glucose production in Fao cells (Fig. 4D) without causing cell damage on comparison of total protein levels with those after administration of dimethyl sulfoxide control in Fao cells (data not shown). These results suggest that the Foxo1 inhibitor AS1842856 may suppress endogenous G6Pase and PEPCK activities by decreasing their mRNA levels, which may lead to inhibition of glucose production in Fao cells.

Effects of AS1842856 on Foxo1 Phosphorylation and Expression and Insulin Signaling. Foxo1-transactivation activity is regulated by its Ser256 phosphorylation in response to insulin (Guo et al., 1999; Zhang et al., 2002). To investigate the action mechanisms of an inhibitor compound, we studied the alteration in phosphorylation of Foxo1 by treatment with a Foxo1 inhibitor. Fao cell lysates were prepared from cells treated with either insulin or AS1842856, and relative concentration of phosphorylated Foxo1 protein was determined by Western blot analysis. In insulin-treated cells, Foxo1 protein was phosphorylated on Ser256 in response to insulin in a dose-dependent manner. In contrast, Foxo1 remained unchanged in AS1842856-treated cells (Fig. 5A).

We then investigated the effect of Foxo1 inhibitor AS1842856 on insulin signaling. Single treatment with AS1842856 had no effect on the phosphorylation state of Akt, ERK, or S6K in Fao cells (Fig. 5B). To determine whether AS1842856 synergizes with insulin to induce phosphorylation of various insulin signaling-related molecules, we examined the effect of AS1842856 in 0.1 or 1 nM insulin. No effect was noted on treatment with AS1842856 and insulin on the insulin signaling (Fig. 5C).

Effects of AS1842856 on Glucose Metabolism in Normoglycemic Mice. To investigate the molecular mechanism of Foxo1 inhibitor in vivo, we evaluated the effect of AS1842856 using normoglycemic ICR mice. Pharmacokinetic analysis of exposure to AS1842856 revealed that although the maximum plasma concentration (C_{max}) was sufficient (0.3 μM at a dose of 100 mg/kg), plasma concentration of AS1842856 could not be detected ($<0.1 \mu\text{M}$) 2 h after oral administration; data not shown). These results indicated that a single dose (100 mg/kg) of AS1842856 had no effect on hepatic G6Pase and PEPCK gene expression levels in ICR mice (data not shown), and we therefore opted to orally administer the compound three times within 26 h.

Total liver RNA was prepared individually from mice after oral administration of AS1842856 (100 mg/kg) at three time points during 26-h fasting and was subjected to real-time quantitative RT-PCR analysis. The hepatic G6Pase and PEPCK mRNA levels were measured using mouse EF1 α mRNA as an internal control. As shown in Fig. 6, B and C, the hepatic relative levels of both G6Pase and PEPCK mRNAs were significantly reduced in the AS1842856-treated group compared with the vehicle-control group. However, no significant differences were noted in 26-h fasting plasma glucose level in AS1842856-treated mice at any of the three postdosing time points (Fig. 6A). In contrast, the plasma glucose level was significantly reduced in mice treated with AS1842856 after injection of pyruvate (Fig. 6, D and E).

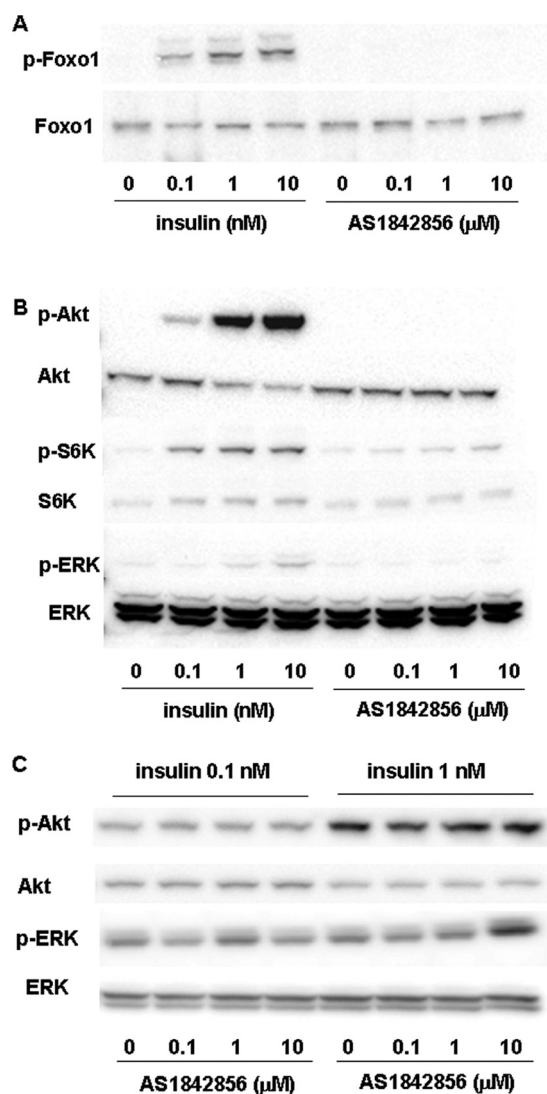


Fig. 5. The phosphorylation of Foxo1 and the insulin signaling remain unchanged with AS1842856. A and B, Fao cells were serum-starved (1 h) and incubated for 30 min with either insulin or AS1842856 at the indicated concentration. Protein lysates from Fao cells were then immunoblotted with anti-phosphorylated Foxo1 or anti-Foxo1 antibody (A), anti-phosphorylated Akt, anti-Akt, anti-phosphorylated S6K, anti-S6K, and either anti-phosphorylated ERK or anti-ERK (B). C, Fao cells were serum-starved and incubated for 30 min with 0.1 or 1 nM insulin and AS1842856 at the indicated concentration. Protein lysates from Fao cells were then immunoblotted with indicated antibodies.

Effects of AS1842856 on Glucose Metabolism in Diabetic *db/db* Mice. *db/db* mice are an animal model of human T2DM. In the present study, hepatic Foxo1 mRNA expression level in these mice was significantly increased compared with the nondiabetic control mice (Altomonte et al., 2003). We also examined the effect of Foxo1 inhibitor on hepatic gluconeogenesis in the diabetic *db/db* mouse model. Mice were administered AS1842856 at three time points during 26-h fasting, after which liver tissue was collected for preparation of total RNA and subsequently analyzed with real-time RT-PCR. As shown in Fig. 6, B and C, the expression levels of G6Pase and PEPCK mRNA normalized with the EF1 α mRNA level were significantly decreased in the AS1842856-treated mice compared with the vehicle-treated ones, which correlated with the reduction rate of fasting glucose level in AS1842856-treated mice at dosages of both 30 and 100 mg/kg (Fig. 7A). No significant difference in gene expression level of Foxo1 was noted between AS1842856-treated and vehicle-treated mice at any of the three time points after dosing (Fig. 7D). In pyruvate challenge tests, a glucose-lowering effect was observed in AS1842856-treated mice (Fig. 7, E and F). Taken together, these results indicate

that the Foxo1 inhibitor compound AS1842856 can regulate gluconeogenesis both in vitro and in vivo.

After pyruvate loading, plasma glucose levels at each time point were higher in *db/db* mice than in normoglycemic ICR mice (Fig. 7B), indicating that hepatic gluconeogenesis was activated in *db/db* mice. Furthermore, we found that AS1842856 improved impaired glucose tolerance independent of plasma insulin levels in *db/db* mice (Fig. 7, G and H). These data suggest that Foxo1 inhibitor administration may improve insulin sensitivity through suppression of hepatic gluconeogenesis in patients with T2DM.

Hepatic Gene Expression by Foxo1 Inhibitor in *db/db* Mice. In the liver, Foxo1 directly controls not only G6Pase and PEPCK but also apolipoprotein CIII (apoCIII) (Altomonte et al., 2004), glucokinase (Ganjam et al., 2009), and ABCG5/ABCG8 (Biddinger et al., 2008). Given the recent finding that Foxo1 was able to promote IL-1 β production in macrophages (Su et al., 2009), we tested in the present study whether AS1842856 administration affects hepatic expression levels of these genes in *db/db* mice. After administration, expression was found to be significantly altered in the AS1842856-treated mice compared with the vehicle-

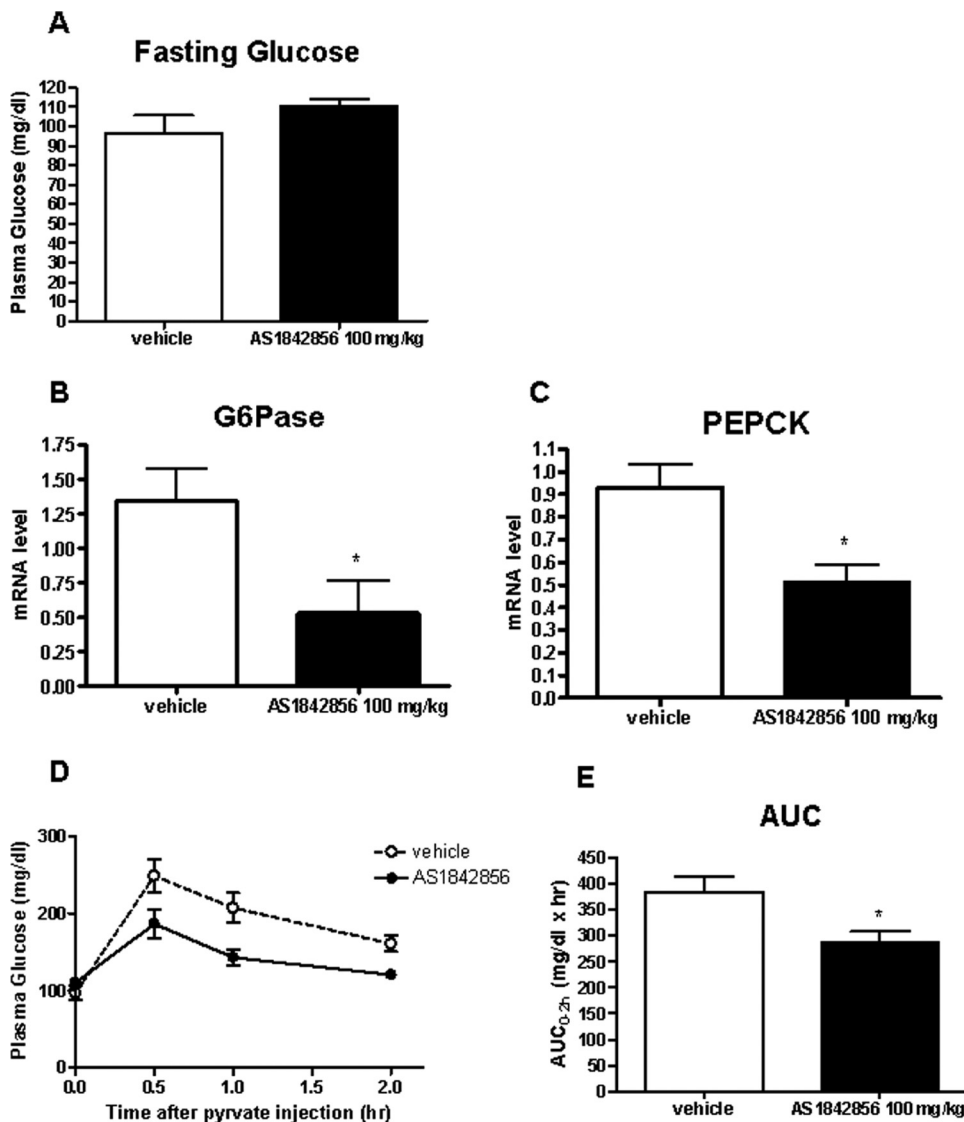


Fig. 6. Effects of AS1842856 on gluconeogenesis in normal ICR mice. AS1842856 (100 mg/kg) was orally administered to ICR mice at three time points during 26-h fasting. A, blood glucose levels after 26-h fasting. B, hepatic G6Pase mRNA (B) and PEPCK mRNA levels (C) relative to mouse EF1 α mRNA. D and E, pyruvate (2 g/kg) was intraperitoneally injected into ICR mice 2 h after the final dose of AS1842856. D, time course of changes in the blood glucose levels during the pyruvate challenge. E, the AUC during the pyruvate challenge. The values are the mean \pm S.E. ($n = 5$). Asterisks indicate significant differences: *, $p < 0.05$ versus vehicle, using Student's t test.

treated controls (Figs. 8, A–E), indicating that AS1842856 does indeed exert control over Foxo1-related gene expression levels in vivo.

Discussion

Here, to discover safe and efficacious gluconeogenesis inhibitors, we identified the small-molecule Foxo1 inhibitor AS1842856 using affinity selection-mass spectrometry. Results showed that AS1842856 reduced fasting plasma glucose levels in a dose-dependent manner, improving glucose tolerance independently of the insulin level.

Foxo1 protein has been shown to activate glucose production after directly binding to promoters of several gluconeogenesis-related genes, thereby contributing to fasting and postprandial hyperglycemia. Although several reports thus far have suggested that reducing Foxo1 activity may represent a potential strategy for treating type 2 diabetes (Nakae et al., 2001; Altomonte et al., 2003; Qu et al., 2006; Biddinger et al., 2008), the pharmacological effects of Foxo1 inhibition on

insulin signaling and glucose metabolism have yet to be fully elucidated. In the present study, we identified AS1842856 as a potent Foxo1 inhibitor and demonstrated its ability to inhibit Foxo1-transactivation activity and decrease gluconeogenesis through inhibition of G6Pase and PEPCK mRNA levels in a rat hepatic cell line and a diabetic *db/db* mouse model. To our knowledge, ours is the first successful identification of a Foxo1 inhibitor demonstrating inhibition of Foxo1-mediated activation both in vitro and in vivo. Our study also provides a novel tool for exploring the chemical biology of Foxo1 function and signaling mechanisms.

We showed that recombinant Foxo1 obtained from FreeStyle 293-F cells is phosphorylated, potentially resulting in a conformational change that may promote nuclear exclusion after phosphorylation of Thr24 and Ser319 (Zhang et al., 2002). We noted that AS1842856 bound only to the dephosphorylated Foxo1, suggesting that AS1842856 selectively inhibited activated Foxo1. In our reporter gene assay, AS1842856 potently inhibited Foxo1 ($IC_{50} = 0.03 \mu M$),

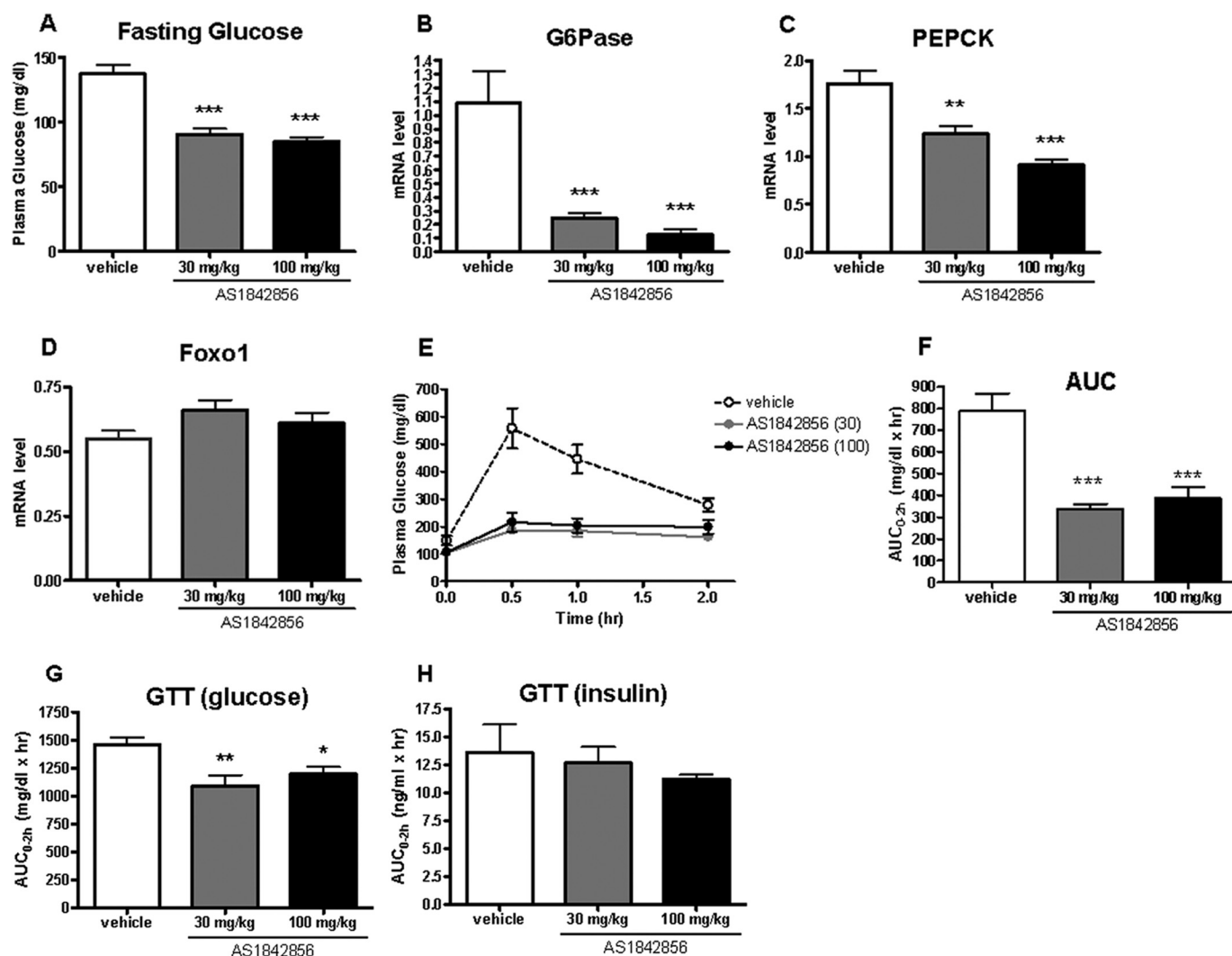


Fig. 7. Fasting glucose lowering effects in diabetic *db/db* mice treated with AS1842856. AS1842856 (30 and 100 mg/kg) was orally administered to *db/db* mice at three time points during 26-h fasting. A, fasting plasma glucose levels. B, hepatic G6Pase mRNA (B), PEPCK mRNA levels (C), and Foxo1 mRNA levels (D) relative to mouse EF1 α mRNA. E and F, pyruvate (2 g/kg) was intraperitoneally injected into *db/db* mice 2 h after the final dose of AS1842856. E, time course of changes in the plasma glucose levels during the pyruvate challenge. F, the AUC during the pyruvate challenge. G and H, glucose (2 g/kg) was intraperitoneally injected into *db/db* mice 2 h after the final dose of AS1842856. G, plasma glucose AUC_{0-2h} during the glucose tolerance test (GTT). H, plasma insulin AUC_{0-2h} during the GTT. The values are the mean \pm S.E. ($n = 6$). Asterisks indicate significant differences: *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ versus vehicle using Dunnett's multiple range test.

whereas inhibition of Foxo3a and Foxo4, also members of the Forkhead box O family, was significant lower ($IC_{50} > 1 \mu M$), indicating that the inhibitor compound is potent and selective for Foxo1.

Regarding how AS1842856 modulates Foxo1 activity and gluconeogenic gene expression in the liver, treatment of insulin leads to Foxo1 phosphorylation via Akt, Foxo1 nuclear exclusion, and degradation, subsequently resulting in the inhibition of Foxo1 target gene expression (Matsuzaki et al., 2003). Such an effect may be achieved by one of three potential routes, the first being that AS1842856 may induce Foxo1 phosphorylation and nuclear exclusion. However, given that AS1842856 did not alter the phosphorylation of Foxo1 or insulin signaling, this mechanism may be ruled out (Fig. 5, A–C). Another potential mechanism is that AS1842856 down-regulates hepatic Foxo1 expression, thereby resulting in the inhibition of Foxo1-mediated transactivation. However, given that we observed no change in the gene expres-

sion level of Foxo1 in either the hepatoma cell line or *db/db* mice model after administration of AS1842856 (Fig. 4, C and D), we believe this compound is unlikely to induce down-regulation at the gene level. The final possible mechanism is that AS1842856 binds to the dephosphorylated Foxo1, interfering with Foxo1 protein interacting with IRE and thereby resulting in inhibition of Foxo1-mediated transactivation and gluconeogenic gene expression. Yamagata et al. (2004) demonstrated that a small heterodimer partner antagonized the transactivation function of Foxo1 through direct interaction and dissociation of coactivator cAMP response element-binding protein-binding protein (CBP) from Foxo1 (Yamagata et al., 2004). Given these previous findings, we may discern that AS1842856 probably interferes with Foxo1 binding to endogenous CBP, although further studies are needed to fully elucidate the interaction between Foxo1 and CBP proteins.

Although direct inhibitors of gluconeogenesis are indeed effective in reducing glucose levels, concerns remain regard-

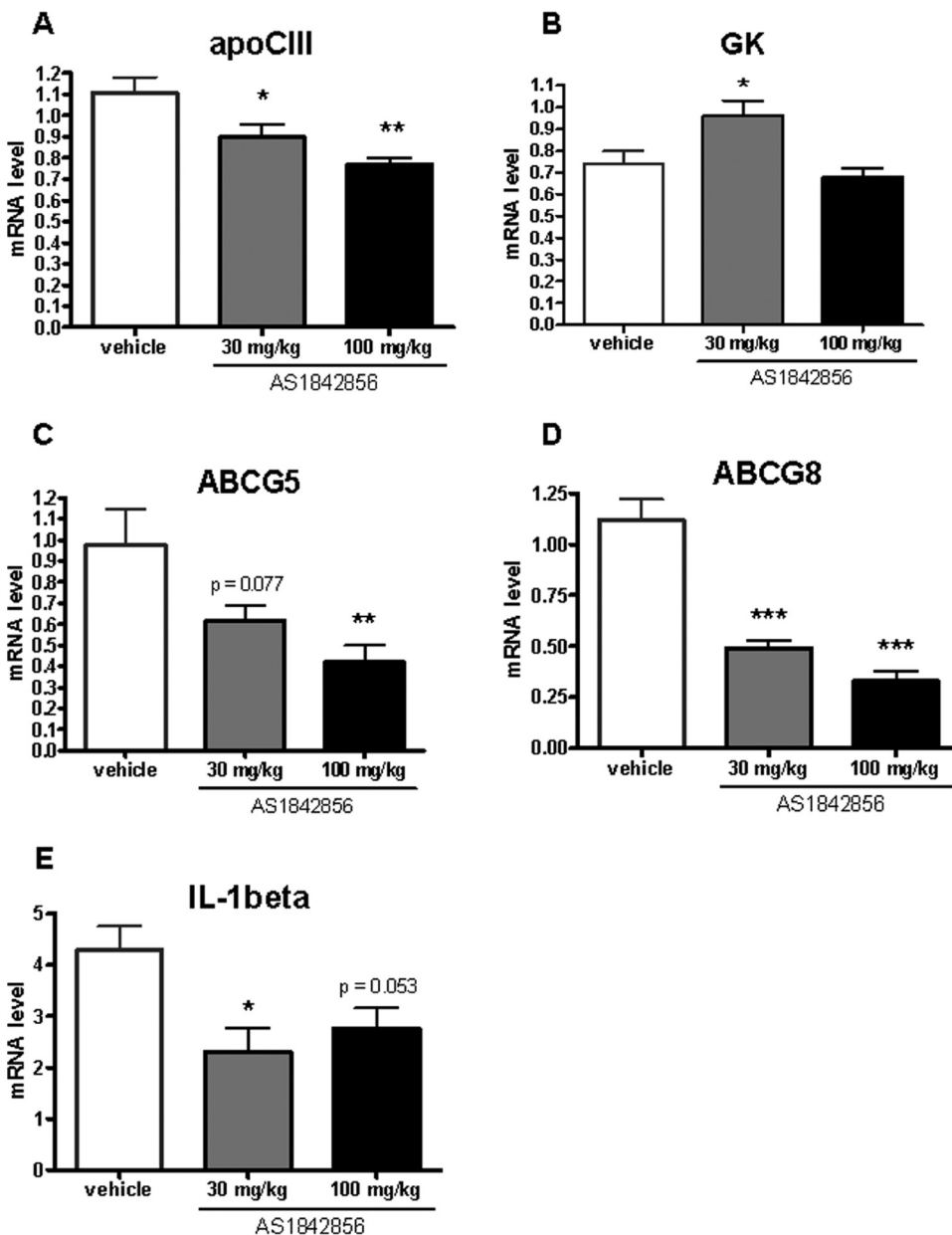


Fig. 8. Effects of AS1842856 on hepatic gene expression regulated Foxo1. Total liver RNA was prepared after oral administration of AS1842856 (30 and 100 mg/kg) at three time points during 26-h fasting. Hepatic apoCIII mRNA (A), glucokinase (GK) mRNA (B), ABCG5 mRNA (C), ABCG8 mRNA (D), and interleukin (IL)-1 β mRNA (E) levels relative to mouse EF1 α mRNA. The values are the mean \pm S.E. ($n = 6$). Asterisks indicate significant differences: *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ versus vehicle using Dunnett's multiple range test.

ing the potential induction of hypoglycemia (Arion et al., 1997). Here, we demonstrated that fasting glucose level was reduced in *db/db* mice treated with a low dose (30 mg/kg) of AS1842856 but not in ICR mice treated with a high dose (100 mg/kg). Several previous reports have shown that hepatic expression of Foxo1 is significantly elevated, and localization of Foxo1 protein in the nucleus is increased in *db/db* mice (Altomonte et al., 2003) and diet-induced obesity mice (Qu et al., 2006; Samuel et al., 2006). These data imply that diabetic mice with significantly elevated hepatic Foxo1 expression are more susceptible to Foxo1 inhibitors than normal mice. Foxo1 contributes to the pathogenesis of hyperglycemia in diabetes, and in a diabetic or obese state, hepatic Foxo1 expression can become deregulated, resulting in elevated production and increased nuclear localization of Foxo1, which is attributable to its enhanced transactivation activity in stimulated gluconeogenic gene expression after deterioration of degree of fasting hyperglycemia in patients with T2DM.

In summary, we identified the small-molecule Foxo1 inhibitor AS1842856 using affinity selection-mass spectrometry. AS1842856 exerted a fasting glucose-lowering effect in a dose-dependent manner and improved glucose tolerance. Further characterization of this Foxo1 inhibitor compound may facilitate understanding the physiological and pathophysiological functions of the Foxo1 protein.

Acknowledgments

We thank Drs. Seitaro Mutoh, Hiroshi Kayakiri, Hiroyuki Arakawa, Nobuya Nishio, Yuji Koga, Daisuke Kaga, Tsuchiya Kazuyuki, Hiroyuki Sakuma, Ryosuke Nakano, and Takao Fujimura (Astellas Pharma Inc.) for their helpful advice and experimental support.

References

- Altomonte J, Cong L, Harbaran S, Richter A, Xu J, Meseck M, and Dong HH (2004) Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. *J Clin Invest* **114**:1493–1503.
- Altomonte J, Richter A, Harbaran S, Suriawinata J, Nakae J, Thung SN, Meseck M, Accili D, and Dong H (2003) Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. *Am J Physiol Endocrinol Metab* **285**:E718–E728.
- Annis DA, Nickbarg E, Yang X, Ziebell MR, and Whitehurst CE (2007) Affinity selection-mass spectrometry screening techniques for small molecule drug discovery. *Curr Opin Chem Biol* **11**:518–526.
- Arion WJ, Canfield WK, Ramos FC, Schindler PW, Burger HJ, Hemmerle H, Schubert G, Below P, and Herling AW (1997) Chlorogenic acid and hydroxynitrobenzaldehyde: new inhibitors of hepatic glucose 6-phosphatase. *Arch Biochem Biophys* **339**:315–322.
- Biddinger SB, Haas JT, Yu BB, Bezy O, Jing E, Zhang W, Unterman TG, Carey MC, and Kahn CR (2008) Hepatic insulin resistance directly promotes formation of cholesterol gallstones. *Nat Med* **14**:778–782.
- Boden G, Chen X, and Stein TP (2001) Gluconeogenesis in moderately and severely hyperglycemic patients with type 2 diabetes mellitus. *Am J Physiol Endocrinol Metab* **280**:E23–E30.
- Furuyama T, Nakazawa T, Nakano I, and Mori N (2000) Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* **349**:629–634.
- Ganjam GK, Dimova EY, Unterman TG, and Kietzmann T (2009) FoxO1 and HNF-4 are involved in regulation of hepatic glucokinase gene expression by resveratrol. *J Biol Chem* **284**:30783–30797.
- Guo S, Rena G, Cichy S, He X, Cohen P, and Unterman T (1999) Phosphorylation of serine 256 by protein kinase B disrupts transactivation by FKHR and mediates effects of insulin on insulin-like growth factor-binding protein-1 promoter activity through a conserved insulin response sequence. *J Biol Chem* **274**:17184–17192.
- Hall RK, Yamasaki T, Kucera T, Waltner-Law M, O'Brien R, and Granner DK (2000) Regulation of phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein-1 gene expression by insulin. The role of winged helix/forkhead proteins. *J Biol Chem* **275**:30169–30175.
- Jackson JG, Kreisberg JI, Koterba AP, Yee D, and Brattain MG (2000) Phosphorylation and nuclear exclusion of the forkhead transcription factor FKHR after epidermal growth factor treatment in human breast cancer cells. *Oncogene* **19**:4574–4581.
- Matsumoto M, Pocai A, Rossetti L, Depinho RA, and Accili D (2007) Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. *Cell Metab* **6**:208–216.
- Matsuzaki H, Daitoku H, Hatta M, Tanaka K, and Fukamizu A (2003) Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc Natl Acad Sci USA* **100**:11285–11290.
- Nakae J, Biggs WH, 3rd, Kitamura T, Caveness WK, Wright CV, Arden KC, and Accili D (2002) Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat Genet* **32**:245–253.
- Nakae J, Kitamura T, Silver DL, and Accili D (2001) The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest* **108**:1359–1367.
- Onuma H, Vander Kooi BT, Boustead JN, Oeser JK, and O'Brien RM (2006) Correlation between FOXO1a (FKHR) and FOXO3a (FKHRL1) binding and the inhibition of basal glucose-6-phosphatase catalytic subunit gene transcription by insulin. *Mol Endocrinol* **20**:2831–2847.
- Perriello G, Pampanelli S, Del Sindaco P, Lalli C, Ciofetta M, Volpi E, Santeusano F, Brunetti P, and Bolli GB (1997) Evidence of increased systemic glucose production and gluconeogenesis in an early stage of NIDDM. *Diabetes* **46**:1010–1016.
- Qu S, Altomonte J, Perdomo G, He J, Fan Y, Kamagata A, Meseck M, and Dong HH (2006) Aberrant Forkhead box O1 function is associated with impaired hepatic metabolism. *Endocrinology* **147**:5641–5652.
- Samuel VT, Choi CS, Phillips TG, Romanelli AJ, Geisler JG, Bhanot S, McKay R, Monia B, Shutter JR, Lindberg RA, Shulman GI, and Veniant MM (2006) Targeting foxo1 in mice using antisense oligonucleotide improves hepatic and peripheral insulin action. *Diabetes* **55**:2042–2050.
- Su D, Coudriet GM, Hyun Kim D, Lu Y, Perdomo G, Qu S, Slusher S, Tse HM, Piganelli J, Giannoukakis N, Zhang J, and Henry Dong H (2009) FoxO1 links insulin resistance to proinflammatory cytokine IL-1beta production in macrophages. *Diabetes* **58**:2624–2633.
- Wajngot A, Chandramouli V, Schumann WC, Ekberg K, Jones PK, Efendic S, and Landau BR (2001) Quantitative contributions of gluconeogenesis to glucose production during fasting in type 2 diabetes mellitus. *Metab Clin Exp* **50**:47–52.
- Yamagata K, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J, and Fukamizu A (2004) Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1. *J Biol Chem* **279**:23158–23165.
- Zhang X, Gan L, Pan H, Guo S, He X, Olson ST, Mesecar A, Adam S, and Unterman TG (2002) Phosphorylation of serine 256 suppresses transactivation by FKHR (FOXO1) by multiple mechanisms. Direct and indirect effects on nuclear/cytoplasmic shuttling and DNA binding. *J Biol Chem* **277**:45276–45284.

Address correspondence to: Dr. Takeyuki Nagashima, Department of Metabolic Diseases, Drug Discovery Research, Astellas Pharma Inc., 21 Miyukigaoka, Tsumakubara, Ibaraki, 305-8585, Japan. E-mail: takeyuki.nagashima@jp.astellas.com