

Pharmacokinetic and Pharmacodynamic Properties of the Glucokinase Activator MK-0941 in Rodent Models of Type 2 Diabetes and Healthy Dogs

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ABSTRACT

Glucokinase activators (GKAs) are small-molecule agents that enhance glucose sensing by pancreatic β cells and glucose metabolism by hepatocytes. There is strong interest in these agents as potential therapies for type 2 diabetes. Here, we report key pharmacokinetic and pharmacodynamic findings from preclinical studies of the GKA 3-[[6-(ethylsulfonyl)-3-pyridinyl]oxy]-5-[(1S)-2-hydroxy-1-methylethoxy]-N-(1-methyl-1H-pyrazol-3-yl)benzamide (MK-0941). Incubated in vitro with recombinant human glucokinase, 1 μ M MK-0941 lowered the $S_{0.5}$ of this enzyme for glucose from 6.9 to 1.4 mM and increased the maximum velocity of glucose phosphorylation by 1.5-fold. In 2.5 and 10 mM glucose, the EC_{50} values for activation of GK by MK-0941 were 0.240 and 0.065 μ M, respectively. Treatment of isolated rat islets of Langerhans and hepatocytes with 10 μ M MK-0941 increased insulin secretion by

17-fold and glucose uptake up to 18-fold, respectively. MK-0941 exhibited strong glucose-lowering activity in C57BL/6J mice maintained on a high-fat diet (HFD), *db/db* mice, HFD plus low-dose streptozotocin-treated mice, and nondiabetic dogs. In both mice and dogs, oral doses of MK-0941 were rapidly absorbed and rapidly cleared from the blood; plasma levels reached maximum within 1 h and fell thereafter with a half-life of \sim 2 h. During oral glucose tolerance testing in dogs, MK-0941 reduced total area-under-the-curve postchallenge (0–2 h) plasma glucose levels by up to 48% compared with vehicle-treated controls. When administered twice daily to mice for 16 days, and once daily to the dog for 4 days, MK-0941 remained efficacious on successive days. These findings support further investigation of MK-0941 as a potential therapeutic agent for treatment of type 2 diabetes.

Introduction

Glucokinase activators (GKAs) are small-molecule agents that enhance the catalytic activity of glucokinase, a unique hexokinase isoform that plays a pivotal role in glucose-sensing by pancreatic β cells and glucose metabolism by hepatocytes (Grimsby et al., 2003; Matschinsky et al., 2006). Glucokinase activity is allosterically modifiable; binding between a GKA and glucokinase stabilizes the enzyme in a conforma-

tional state with substantially higher affinity for glucose (Kamata et al., 2004). Most GKAs also increase the maximum velocity of glucose phosphorylation (Matschinsky, 2009). Through these actions, GKAs markedly increase insulin release by pancreatic β cells and uptake of glucose by hepatocytes at physiological glucose concentrations (Grimsby et al., 2003, 2008; Matschinsky, 2009). There is thus considerable interest in the development of GKAs as agents that may provide a novel form of pharmacotherapy for type 2 diabetes (Sarabu et al., 2008, 2011; Matschinsky, 2009; Matschinsky and Porte, 2010). Some GKAs have recently progressed to clinical testing in healthy human subjects (Zhai et al., 2008; Migoya et al., 2009) and in patients with

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ABBREVIATIONS: GKA, glucokinase activator; PK, pharmacokinetic; PD, pharmacodynamic; MK-0941, 3-[[6-(ethylsulfonyl)-3-pyridinyl]oxy]-5-[(1S)-2-hydroxy-1-methylethoxy]-N-(1-methyl-1H-pyrazol-3-yl)benzamide; HFD, high-fat diet; HK, hexokinase; DMSO, dimethyl sulfoxide; T-NAD, thio-NAD; ELISA, enzyme-linked immunosorbent assay; HbA_{1c}, glycated hemoglobin; 3-HBA, 3-hydroxybutyric acid; OGTT, oral glucose tolerance test; compound B, 3-[(1S)-2-hydroxy-1-methylethoxy]-5-[4-(methylsulfonyl)phenoxy]-N-1,3-thiazol-2-yl benzamide; STZ, streptozotocin.

type 2 diabetes (Bonadonna et al., 2008, 2010; Zhi et al., 2008; Meininger et al., 2011).

Here, we report key pharmacokinetic (PK) and pharmacodynamic (PD) findings from preclinical studies of MK-0941, a GKA that has recently entered into clinical trials (Meininger et al., 2011). Included in the present report are evaluations of 1) the potency, efficacy, and selectivity of MK-0941 for human glucokinase in vitro, 2) its effect on insulin production by isolated rat islets of Langerhans, 3) its effect on glucose phosphorylation in isolated rat hepatocytes, 4) PK/PD characteristics of MK-0941 in whole-animal rodent models of diabetes, including C57BL/6J mice fed a prolonged high-fat diet (HFD), *db/db* mice, and ICR mice rendered diabetic by a combination of long-term maintenance on a HFD and a single, low-dose treatment with streptozotocin (HFD/STZ mice), and 5) PK/PD characteristics of MK-0941 in the normoglycemic dog.

Materials and Methods

Chemicals. MK-0941 (3-[[6-(ethylsulfonyl)-3-pyridinyl]oxy]-5-[(1*S*)-2-hydroxy-1-methylethoxy]-*N*-(1-methyl-1*H*-pyrazol-3-yl)benzamide; Fig. 1D) was synthesized at the Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd. All chemicals and reagents were purchased from Wako Pure Chemical (Osaka, Japan) or Sigma-Aldrich (St. Louis, MO), except as noted.

Animals. Except as noted, rodents were housed 2 to 3 per cage and maintained under controlled conditions of lighting (12-h light/dark cycle, on at 07:00 AM, off at 7:00 PM), temperature ($23 \pm 2^\circ\text{C}$), and humidity ($55 \pm 15\%$) with access ad libitum to mouse/rat diet (7012 Teklad LM-485; Harlan Laboratories, Indianapolis, IN) and water. Male Sprague-Dawley and Wistar rats were obtained from Charles River Japan, Inc. (Yokohama, Japan). Male C57BL/6J mice were obtained from CLEA Japan, Inc. (Tokyo, Japan) at 5 weeks of age, housed individually, and fed a HFD (total 535 kcal/100 g, 59% of kcal from fat; Oriental Yeast Co., Ltd., Tokyo, Japan) beginning at 6 weeks, and used in experiments beginning at 35 weeks.

For studies in HFD/STZ mice, 4-week-old male ICR mice were obtained from Charles River Japan Inc., housed three per cage, and maintained on a HFD (Mouse Diet, High Fat; Fat Calories (60%), Soft Pellets; Bio-Serv, Frenchtown, NJ). At 7 weeks of age, they were given injections of 90 mg/kg i.p. streptozotocin, a treatment that is

toxic to pancreatic β cells (Mu et al., 2006). Maintenance on the HFD was continued, and experiments were begun at 12 weeks of age.

Male *db/db* and nondiabetic *db/+* (lean) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed seven to nine per cage. Male beagle dogs were obtained from Nossan Corporation (Yokohama, Japan), Institute for Animal Reproduction (Ibaraki, Japan), and Oriental Bio-Service Kanto (Ibaraki, Japan) and housed individually. They were fed laboratory dog chow (CD-5M diet; CLEA Japan Inc., Tokyo, Japan) once each day and had access to water ad libitum. The animal housing facilities met Association for Assessment and Accreditation of Laboratory Animal Care guidelines, and all experimental protocols were approved by an Institutional Animal Care and Use Committee.

In Vitro Assay of Human Glucokinase and Human Hexokinases I, II, and III. Recombinant human liver glucokinase and human hexokinases HK I, II, and III were generated by overexpression in *Escherichia coli* with a flag sequence added to the N terminus of each to facilitate purification by anti-flag M2 affinity column chromatography (Sigma-Aldrich). The purified enzymes were assayed at 30°C using glucose-6-phosphate dehydrogenase as a reporter enzyme. All assay solutions contained 25 mM HEPES, pH 7.2, 2 mM MgCl_2 , 1 mM ATP, 2 U/ml of glucose-6-phosphate dehydrogenase, 1% (v/v) DMSO (the vehicle for MK-0941), and varying levels of glucose (0.31–30 mM) and MK-0941 (0–20 μM). Assays of glucokinase also contained 1 mM dithiothreitol and 0.5 mM thio-NAD (T-NAD); assays of HK I, II, and III also contained 0.5 mM NAD, 10 μM 1-methoxyphenazine methosulfate, and 0.5 mM tetrazolium salt (WST-1; Dojindo Laboratories, Kumamoto, Japan). Product formation was monitored continuously using SpectraMax 190 and 384 microplate readers (Molecular Devices, Sunnyvale, CA) measuring absorbance at 405 nm in glucokinase assays (indicating T-NADH formed by dehydration of T-NAD) and 440 nm in the other hexokinase assays (indicating formation of formazan from WST-1). Reaction velocities were measured over the first 3 to 5 min of incubation.

Insulin Secretion by Isolated Rat Islets of Langerhans. Islets of Langerhans were prepared from 9-week-old male Wistar rats by collagenase digestion (Okeda et al., 1979; Gotoh et al., 1985). In brief, the animals were anesthetized by injection of sodium pentobarbital (50 mg/kg i.p.). The abdomen was opened, and the common bile duct was ligated near its attachment to the duodenum. It was cannulated and collagenase solution (Hanks' balanced salt solution supplemented with 20 mM HEPES, pH 7.2, 50 U/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, and 960 U/ml collagenase) was infused into the pancreas in situ. The engorged pancreas was then excised and incu-

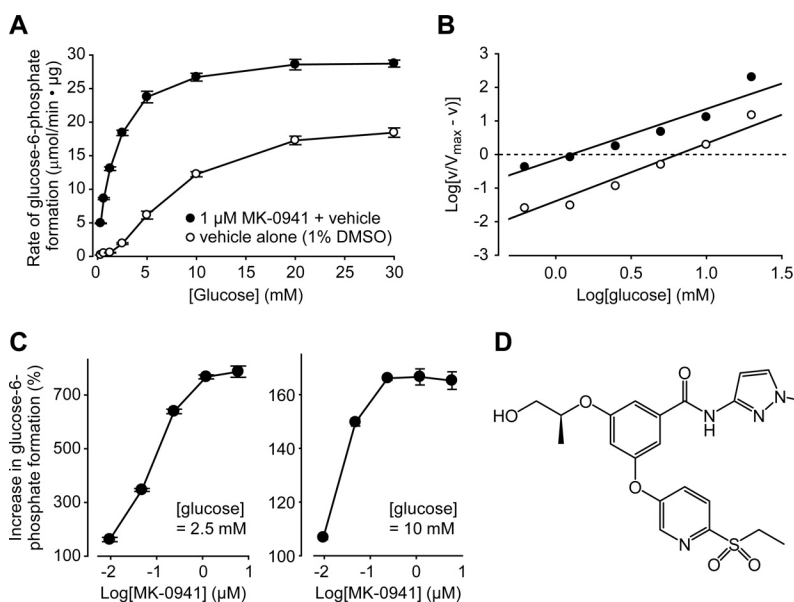


Fig. 1. Modulation of human glucokinase kinetics by MK-0941. Glucose concentration-versus-velocity relationships (A) and Hill plots in the presence of 1 μM MK-0941 (●) or vehicle alone [1% (v/v) DMSO; ○] (B). C, MK-0941 concentration-versus-effect relationships compared at the glucose concentrations of 2.5 mM (left) and 10 mM (right). The data are means \pm S.E. from three separate experiments. D, the chemical structure of MK-0941.

bated for ~40 min at 37°C, after which it was triturated through the tip of a Pasteur pipette and filtered through layered gauze to remove undigested tissue fragments. The filtrate was resuspended in ice-cold Hanks' balanced saline solution containing 5% fetal bovine serum, 20 mM HEPES, pH 7.2, 50 U/ml penicillin, and 50 µg/ml streptomycin, and centrifuged in a Ficoll-Conray density gradient. The band enriched for islets was resuspended in buffer and spread onto plastic dishes, and intact islets were selected by microscopic examination. These were incubated overnight in RPMI 1640 culture media (Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum, 11.1 mM glucose, 20 mM HEPES, 50 U/ml penicillin, 50 µg/ml streptomycin, and 1 mM adenosine at 37°C. They were then divided into treatment groups with nine islets per group and incubated for 1.5 h in 12-well plates, each well containing 1.5 ml of modified Krebs-Ringer-bicarbonate buffer with 2.5 or 10 mM glucose and vehicle alone [DMSO at a final concentration of 0.1% (v/v)] or vehicle plus MK-0941 at final concentrations of 0.1 to 10 µM (Okamoto et al., 1992). At the end of the incubation, insulin concentration in the buffer was determined by ELISA (Rat Insulin ELISA Kit; Morinaga Institute of Biological Science, Yokohama, Japan).

Glucose Phosphorylation in Isolated Rat Hepatocytes.

Hepatocytes were harvested from 8- to 9-week-old male Wistar rats as described previously (Seglen, 1976). In brief, the animals were fasted overnight and anesthetized by intraperitoneal injection of sodium pentobarbital. The abdomen was opened and the liver perfused with collagenase solution (10 mM HEPES, pH 7.5, 137 mM NaCl, 5.4 mM KCl, 0.5 mM NaH₂PO₄, 0.4 mM Na₂HPO₄, 5 mM CaCl₂, 0.5 mM 0.5 g/l collagenase, and 0.05 g/l trypsin inhibitor type IIS) via a cannula inserted into the portal vein. The partially digested liver was then excised and passed through a mesh filter, and isolated cells were collected from the filtrate by centrifugation in a buffer composed of 12.5 mM HEPES, pH 7.3, 118 mM NaCl, 4.8 mM KCl, 24 mM NaHCO₃, 2.5 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄, and 5 mM glucose. The cells were suspended at a density of 3.5×10^5 cells/ml in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 25 mM glucose, 10% fetal bovine serum, 100 nM dexamethazone, 100 nM insulin, 100 U/ml penicillin, and 100 µg/ml streptomycin; 2-ml aliquots were then transferred to collagen-coated six-well plates (7.0×10^5 cells/well), where they were incubated for 3 h at 37°C under humidified 95% air/5% CO₂. After exchanging with fresh medium, the cells were further incubated overnight while the hepatocytes formed a monolayer.

Glucose phosphorylation was measured by modification of methods described previously (Agius, 1997; Futamura et al., 2006). In brief, hepatocytes were incubated in 0.1 to 30 µM MK-0941 dissolved in DMSO [final concentration, 0.3% (v/v)] or vehicle alone for 3 h in glucose-free Dulbecco's modified Eagle's medium supplemented with 2.5 mM glucose and 5 µCi/well [2-³H]glucose. During incubations, phosphorylation of glucose liberated ³H₂O into the medium; [2-³H]glucose was subsequently separated from ³H₂O on a ion-exchange mini-column (Amberlite IRA-400; Organo Corp., Tokyo, Japan), eluted with H₂O, and quantified using a liquid scintillation analyzer (TRI-CARB 2500; PerkinElmer Life and Analytical Sciences, Waltham, MA).

Levels of MK-0941 in Plasma, Liver, and Pancreas after a Single Oral Dose in the Rat. Male Sprague-Dawley rats (7 weeks of age) were fasted overnight and administered 3 mg/kg MK-0941 by gavage in 5 ml/kg 1% (w/v) aqueous methylcellulose. Separate groups of three animals each were euthanized for necropsy at 0.5 h and 3 h after dosing by anesthetizing with 50 mg/kg pentobarbital. Blood was drawn from the abdominal vein and the liver and pancreas were removed, weighed, and homogenized. To obtain plasma, the blood was centrifuged at 3000 rpm, 4°C, for 10 min. Plasma and tissue MK-0941 concentrations were determined by liquid chromatography (Alliance HT 2790; Waters, Milford, MA) coupled to API-3000 mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) with an electrospray ionization source in the positive mode. Chromatographic separation was achieved on an Atlantis dC18 column (150 ×

2.1 mm i.d., 3.5 µm; Waters, Milford, MA), the mobile phase consisting of 0.1% formic acid in 50% acetonitrile at a flow rate of 0.3 ml/min.

Pharmacokinetic and Pharmacodynamic Evaluation of MK-0941 in HFD Mice. After 29 weeks on a HFD, C57BL/6J HFD mice were assigned to blood glucose-matched treatment groups (9–12 per group) that received vehicle alone [1% (w/v) aqueous methylcellulose in 5 ml/kg body weight] or vehicle plus 1, 3, 10, or 30 mg/kg MK-0941 by gavage twice daily (at ~09:00 AM and ~5:00 PM) for 14 days. The PD analysis of blood glucose levels included nine animals from each group; three additional animals were included in each MK-0941-treated group for PK analysis. On days 1, 7, and 14, blood was obtained from tail bleeds before dosing and at 1, 2, 4, 6, 8, and 12 h after dosing. The evening dose was postponed on these days until after the last sample had been obtained. In the PD analysis, 10 µl of blood was collected and analyzed immediately with a glucometer (Antsense II; Bayer Medical, Osaka, Japan). In the PK analysis, 50 µl of blood was collected into a heparinized capillary and centrifuged for 5 min at 12,000 rpm (at room temperature) to isolate plasma. MK-0941 concentrations were then determined as described above.

Blood Glucose in db/db Diabetic Mice Given Single Doses of MK-0941. Male db/db and db/+ (lean) mice (9–10 weeks of age, weighing 43–45 g) were administered single doses by gavage of either vehicle alone (0.5% methylcellulose, 10 ml/kg) or vehicle plus 3 or 10 mg/kg MK-0941. The doses were administered at ~9:00 AM, and animals were fasted afterward so that blood glucose levels would not be affected by possible treatment effects on feeding behavior. Using a glucometer (One Touch Basic; Lifescan, Inc., Milpitas, CA), glucose was measured before dosing and at 0, 1, 2, 3, and 4 h after dosing in blood from tail bleeds.

Pharmacokinetic and Pharmacodynamic Evaluation of MK-0941 in HFD/STZ Mice. When the HFD/STZ mice were 11 weeks of age, blood samples (15 µl) were collected from the tail vein into heparinized capillaries and analyzed for content of glycated hemoglobin (HbA_{1c}) using an HbA_{1c} analyzer (DCA2000 or DCA2000+; Bayer-Sankyo, Tokyo, Japan). At 12 weeks of age, the mice were assigned to treatment groups (10–13 per group) that received vehicle alone [5 ml/kg of 1% (w/v) aqueous methylcellulose] or vehicle plus 10 mg/kg MK-0941 twice daily by gavage for 16 days. The PD analysis included 10 animals from each group; three additional animals were included in each MK-0941-treated group for PK analysis. Blood glucose was measured as described for HFD mice.

On day 16, the mice were euthanized by isoflurane inhalation 4 h after the morning dosing. The abdomen was opened, and blood was drawn from the abdominal vein into a chilled, heparinized syringe; 50 µl of this was analyzed for HbA_{1c} as described above. The rest was centrifuged at 7500 rpm, 4°C, for 10 min to obtain plasma. The liver was removed, weighed, and homogenized. Its lipids were extracted by the Folch method (Folch et al., 1957) and analyzed for triglycerides and total cholesterol content using the Determiner-L TG II and Determiner-L TC II enzymatic assay kits (Kyowa Medex Co., Ltd., Tokyo, Japan). Plasma free fatty acids, triglycerides, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, lactic acid, and 3-hydroxybutyric acid (3-HBA) levels were determined using a Hitachi AutoAnalyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). Plasma drug concentrations were determined as described under *Levels of MK-0941 in Plasma, Liver, and Pancreas after a Single Oral Dose in the Rat*.

Pharmacokinetic and Pharmacodynamic Evaluation of MK-0941 in Healthy Dogs. Healthy male beagle dogs (ages 11–30 months) were assigned to treatment groups, fasted overnight, and administered by gavage single doses of vehicle alone [2 ml/kg of 60% (v/v) aqueous polyethylene glycol] or vehicle plus 0.1, 0.3, 1, or 3 mg/kg MK-0941 ($n = 3–6$ per group). An oral glucose challenge (2 g/kg in 10 ml/kg saline) followed 30 min later. Before dosing with the study drug, before the glucose challenge, and at 1.0, 1.5, 2.0, 2.5, 4, 6, 8, and 24 h after dosing, blood samples (1 ml) were drawn from the

cephalic vein into heparinized tubes and immediately centrifuged for 10 min at 3000 rpm and 4°C to obtain plasma. Plasma glucose concentrations were determined using the Determiner GL-E kit (Kyowa Medex Co., Ltd., Tokyo, Japan) according to the procedure described in the manufacturer's protocol but with modification of scale to accommodate analysis of smaller sample volumes. Plasma insulin levels were determined by ELISA as described for our assay of isolated rat islets (see *Glucose Phosphorylation in Isolated Rat Hepatocytes*), except that dog insulin (Morinaga Institute of Biological Science) was used as the standard. Plasma drug concentrations were determined as described under *Levels of MK-0941 in Plasma, Liver, and Pancreas after a Single Oral Dose in the Rat*. Food was provided to the animals beginning 3.5 h after oral glucose dosing.

In a subsequent study, healthy male beagle dogs ($n = 2-4$ per group) were assigned to treatment groups given daily doses of vehicle (the same as above), 1, or 10 mg/kg MK-0941 by gavage for 4 days ($n = 2-4$ per group). On each day before dosing and at 0.5, 1, 2, 4, 6, 8, and 24 h after dosing, blood samples were collected and analyzed for plasma glucose, MK-0941, and insulin content as described above. Before the first dose, the animals were fasted overnight. Food was provided to the animals beginning 4 h after daily doses.

Statistical Methods. Values for EC_{50} (concentrations that produced half-maximal effects), $S_{0.5}$, and V_{max} were calculated using Prism 3.03 and 4.00 (GraphPad Software, Inc., San Diego, CA) with a four-parameter logistic equation. In the analysis of insulin secretion by isolated islets of Langerhans, differences between outcomes in the drug-treated and vehicle-treated groups were evaluated using the Dunnett test (SAS version 5.0; SAS Institute Inc., Cary, NC). In the analysis of blood glucose levels in *db/db* mice, statistical significance was tested using Student's *t* test. Blood glucose concentrations in HFD mice were analyzed for significance using Tukey's multiple-comparison test (SAS version 5.0). Data from the dog and from HFD/STZ mice were analyzed using Dunnett's multiple comparison procedure (SAS version 5.0). Statistical significance was defined as $p < 0.05$ in comparisons between actively treated and vehicle-treated groups.

Results

Effects of MK-0941 on the Kinetics of Recombinant Human Glucokinase In Vitro. Addition of 1 μ M MK-0941 to assays of glucokinase decreased the enzyme's $S_{0.5}$ for glucose (increased its affinity) from 6.9 ± 0.47 to 1.4 ± 0.20 mM and increased the V_{max} for glucose phosphorylation from 20.1 ± 1.0 to 30.8 ± 1.5 nmol \cdot min $^{-1}$ \cdot μ g $^{-1}$ protein (Fig. 1, A and B). The EC_{50} for activation of glucokinase by MK-0941 depended on glucose concentration. In 2.5 and 10 mM glucose, it was 0.240 ± 0.009 and 0.065 ± 0.005 μ M, respectively (Fig. 1C).

Selectivity of MK-0941 for Glucokinase. Based on the EC_{50} values determined in the above analysis, we selected 20 μ M as the concentration of MK-0941 to use when testing its selectivity for glucokinase over other hexokinases; this concentration was considerably in excess of that needed for maximal glucokinase activation. When added to incubations of human hexokinases HK I, II, and III, 20 μ M MK-0941 had no significant effects on either K_m or V_{max} (Table 1).

Insulin Secretion from Isolated Rat Islets of Langerhans. Under control conditions (i.e., in the absence of study drug), isolated rat islets of Langerhans responded to physiologically relevant increases in ambient glucose concentration by increasing their secretion of insulin. In 16.7 mM glucose, the islets secreted insulin at a rate approximately 14-fold that observed in 2.5 mM glucose (Fig. 2A).

Treatment of islets with MK-0941 increased insulin secre-

TABLE 1

Kinetic analysis of the activity of human hexokinase isoforms HK I, HK II, and HK III, in the presence and absence of 20 μ M MK-0941. The data are means \pm S.E. from three separate experiments.

	V_{max}		K_m for Glucose	
	20 μ M MK-0941	Vehicle Alone	20 μ M MK-0941	Vehicle Alone
	nmol \cdot μ g $^{-1}$ \cdot min $^{-1}$		mM	
HK I	4.14 ± 0.07	4.44 ± 0.11	0.038 ± 0.009	0.039 ± 0.010
HK II	9.73 ± 0.39	9.92 ± 0.58	0.103 ± 0.004	0.101 ± 0.001
HK III	8.19 ± 0.07	7.83 ± 0.10	0.018 ± 0.001	0.017 ± 0.001

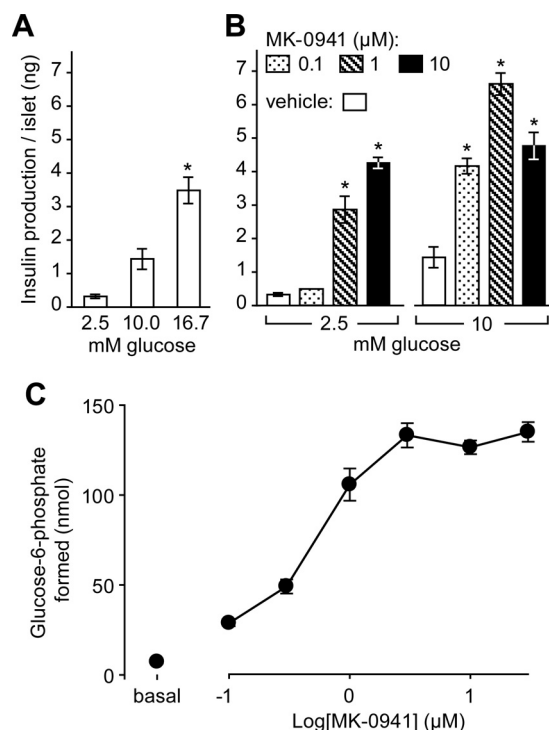


Fig. 2. Enhancement by MK-0941 of insulin production by isolated rat islets (A and B) and glucose uptake by isolated hepatocytes (C). A, insulin production (mean \pm S.E. per islet) during incubations in 2.5, 10.0, or 16.7 mM glucose (without MK-0941 or vehicle) for 1.5 h. B, insulin production during incubations in 2.5 or 10 mM glucose with vehicle alone [DMSO; final concentration, 0.1% (v/v)] or vehicle plus MK-0941 (final concentrations, 0.1, 1, or 10 μ M). C, mean (\pm S.E.) quantities of 3 H $_2$ O released from [3 H]glucose (2.5 mM) in triplicate incubations of 7×10^5 hepatocytes for 3 h. *, $p < 0.05$ for comparisons with 2.5 mM (A) and vehicle alone (B).

tion, and the size of the effect depended on both MK-0941 concentration and glucose concentration. In 2.5 mM glucose, the rate of insulin secretion elicited by 10 μ M MK-0941 was comparable with that elicited by incubation in 16.7 mM glucose without MK-0941 (Fig. 2B). There was also a strong effect of 1.0 μ M MK-0941 on insulin secretion in 2.5 mM glucose, but 0.1 μ M MK-0941 had no effect on insulin secretion at this glucose concentration. This was consistent with the fact that 0.1 μ M is well below the EC_{50} observed for MK-0941 in kinetic studies of glucokinase in 2.5 mM glucose (0.240 ± 0.009 μ M). When islets were incubated in 10 mM glucose, MK-0941 enhanced insulin secretion significantly at all concentrations tested (0.1, 1.0, and 10 μ M). This was consistent with the observation that the EC_{50} for glucokinase activation by MK-0941 in 10 mM glucose is less than 0.1 μ M.

The concentration-versus-effect data were consistent with a relationship of occlusion between the effects of glucose and

MK-0941 concentration. Combining 10 mM glucose with 10 μ M MK-0941 (the highest glucose and MK-0941 levels tested) did not elicit greater insulin secretion than exposure of islets to 16.7 mM glucose alone or a combination of 2.5 mM glucose with 10 μ M MK-0941.

MK-0941 and Glucose Phosphorylation in Rat Primary Hepatocyte Cultures. Addition of MK-0941 to primary cultures of rat hepatocytes incubated in 2.5 mM glucose increased the rate of glucose phosphorylation in a concentration-dependent manner. At the highest concentration of MK-0941 tested (30 μ M), phosphorylation was increased by approximately 18-fold over the basal rate (Fig. 2C). The relationship between concentration and effect exhibited a sigmoidal saturation curve that fit well to an E_{\max} model with an EC_{50} of 0.44 ± 0.03 μ M (mean \pm S.E. of three separate experiments). This value was consistent with the EC_{50} determined in enzyme assays of recombinant human glucokinase incubated in 2.5 mM glucose (0.240 ± 0.009 μ M).

Levels of MK-0941 in Plasma, Liver, and Pancreas after a Single Oral Dose in the Rat. At 0.5 h after dosing in normal rats given 3 mg/kg MK-0941 by gavage, the mean (\pm S.D.) plasma concentration of MK-0941 was 0.89 ± 0.20 μ M; the tissue levels in liver and pancreas were 3.5 ± 0.5 and 0.2 ± 0.1 nmol/g of tissue (wet weight), respectively ($n = 3$ animals). At 3 h after dosing, the mean plasma concentration was 0.21 ± 0.17 μ M; the mean concentration in liver was 1.1 ± 1.1 nmol/g ($n = 3$). The concentration in pancreas was 0.2 nmol/g in one animal, and below the limit of detection (0.1 nmol/g) in two others.

Blood Glucose and Plasma MK-0941 Levels in MK-0941-Treated HFD Mice. Before dosing with MK-0941, all of the HFD mouse treatment groups had mean nonfasted blood glucose levels of approximately 190 mg/dl. At all doses tested (1, 3, 10, and 30 mg/kg), the initial dose elicited a significant reduction in blood glucose and the size of the effect was dose-dependent (Fig. 3, top left plot). The doses of 10 and 30 mg/kg seemed to elicit, at 1 h after dosing, a glucose-lowering effect that approached saturation. At these doses, the mean (\pm S.E.) blood glucose concentrations on day 1 at 1 h after dosing were 91 ± 7 and 90 ± 3 mg/dl, respectively.

During twice-daily dosing with MK-0941 for 14 days (at daily rates of 2, 6, 20, and 60 mg/kg per day), its glucose-lowering efficacy was fully retained (Fig. 3, top plots). Indeed, there seemed to be a trend, especially in the groups given the highest doses, toward lower glucose levels as daily dosing continued. On day 14, the predose mean (\pm S.E.) blood glucose level in the 30 mg/kg-per-day group was 84 ± 6 mg/dl, less than half of what it had been in that same group on day 1. This did not seem to result from PK accumulation of MK-0941, because profiles of plasma MK-0941 from 1 to 12 h after dosing changed little between days 1, 7, and 14 (Fig. 3, bottom plots). Profiles of MK-0941 plasma concentration over time were similar in shape regardless of dose; C_{\max} always occurred 1 h after dosing, and plasma MK-0941 fell rapidly thereafter, with an apparent time to half-fall of approximately 2 h. There seemed to be little or no treatment effect once the plasma concentration of MK-0941 fell below ~ 0.3 μ M. Near-maximal reductions in blood glucose were observed whenever the plasma concentration of MK-0941 exceeded ~ 5 μ M.

Reduction of Blood Glucose by MK-0941 in the *db/db* Diabetic Mouse. Before treatments, *db/db* mice exhibited pronounced hyperglycemia with mean blood glucose levels of approximately 330 mg/dl (Fig. 4). By comparison, mean blood glucose levels in lean *db/+* mice were consistently between 115 and 140 mg/dl. As in HFD mice, treatment of *db/db* mice with single oral doses of 3 or 10 mg/kg MK-0941 resulted in significant reductions in blood glucose. These two doses lowered blood glucose similarly at 1 h after dosing, suggesting that saturation of effect was approached. Over the subsequent 3 h, the reduction in glucose diminished in mice given the lower dose but was stable in mice given the higher dose.

Pharmacokinetics and Pharmacodynamics of MK-0941 in HFD/STZ Mice. Before treatments, HFD/STZ mice also exhibited pronounced hyperglycemia with blood glucose concentrations generally in excess of 300 mg/dl. Blood glucose levels in these mice seemed to be especially sensitive to stress caused by the gavage procedure (Tabata et al., 1998), in that dosing with vehicle [5 ml/kg of 1% (w/v) aqueous

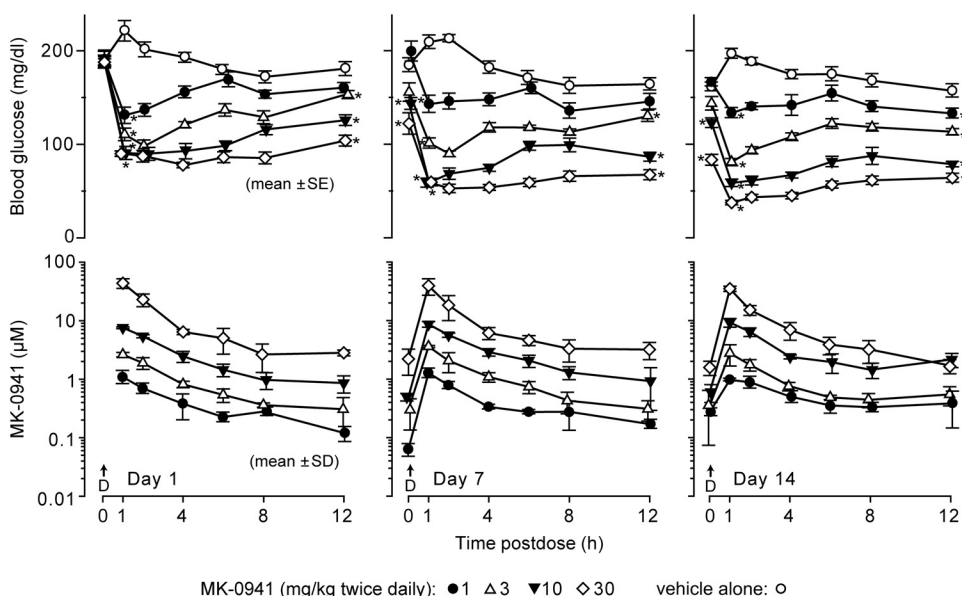


Fig. 3. Mean (\pm S.E.) blood glucose levels ($n = 9$ per group) and mean (\pm S.D.) plasma levels of MK-0941 ($n = 3$ per group) in HFD mice treated twice daily by gavage with vehicle alone (1% aqueous methylcellulose in 5 ml/kg body weight) or vehicle plus 1, 3, 10, or 30 mg/kg MK-0941 (doses of 2–60 mg/kg per day). The glucose and MK-0941 levels were measured immediately before and for 12 h after morning doses (at time D). The first measurements on days 7 and 14 were made approximately 16 h after the last previous dose had been given. After dosing, the animals continued to have access to water but were deprived of food. To improve clarity, statistical significance is indicated only at 1 and 12 h after dosing, and before dosing on days 7 and 14. *, $p < 0.05$ in comparison with the HFD vehicle group.

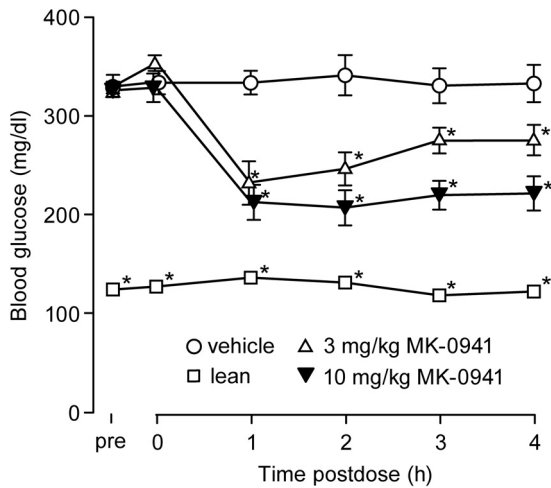


Fig. 4. Mean (\pm S.E.) blood glucose levels in *db/db* mice ($n = 8$ per group) treated with vehicle alone (10 ml/kg 0.5% methylcellulose) or vehicle plus 3 or 10 mg/kg MK-0941 by gavage. The treatments were administered in the morning, approximately 2 h after the lights were turned on. After dosing, the animals continued to have access to water but were deprived of food. Data from lean *db/+* animals are included as a control. *, $p < 0.05$ in comparisons with the *db/db* vehicle group.

methylcellulose] on day 1 was followed by further increase in blood glucose to a mean >400 mg/dl (Fig. 5A).

Administration of 10 mg/kg MK-0941 to HFD/STZ mice was not followed by the increase in blood glucose observed in the vehicle-treated animals. Indeed, blood glucose declined in this group from a mean (\pm S.E.) of 306 ± 12 mg/dl before dosing to 269 ± 28 mg/dl 4 h after dosing. This indicates that the treatment effect more than compensated for the stress artifact. The observed differences in blood glucose between the MK-0941-treated animals and vehicle-treated groups were significant at all three postdose time points (1, 2, and 4 h). Over the 16 days of long-term dosing, this lowering of blood glucose was sustained (Fig. 5B), although the between-groups difference (treated versus vehicle controls) was not significant on day 16.

Before treatments, the animals in the three HFD/STZ groups had similar levels of HbA_{1c}, all between 4 and 5%. At the end of 16 days of treatment HbA_{1c} was significantly lower in the MK-0941-treated group than in the vehicle-treated HFD/STZ controls (Fig. 5C). In the lean control mice, mean HbA_{1c} was $<3\%$ throughout. In the analyses of various liver and plasma biochemical parameters (Table 2), it was observed that total plasma cholesterol was significantly higher in the MK-0941-treated group compared with the vehicle-treated group (285 ± 14 versus 215 ± 20 mg/dl, respectively) and plasma 3-hydroxybutyric acid (3-HBA, a ketone body) was significantly reduced by treatment (67 ± 5 μ M compared with 120 ± 17). No other significant differences were observed. Elevation of plasma ketone body levels is associated with inadequate glycemic control, and thus the mean 3-HBA level in the HFD/STZ-vehicle group was greater than that in the lean control mice. It is noteworthy that 3-HBA in the HFD/STZ-MK-0941-treated group fell to a level considerably lower than that in the lean controls.

The PK analysis in these mice showed similar levels of plasma MK-0941 on treatment days 1 and 16 (Fig. 5A, bottom plot). These plasma drug levels were also similar to those observed in the HFD mice given the 10 mg/kg MK-0941.

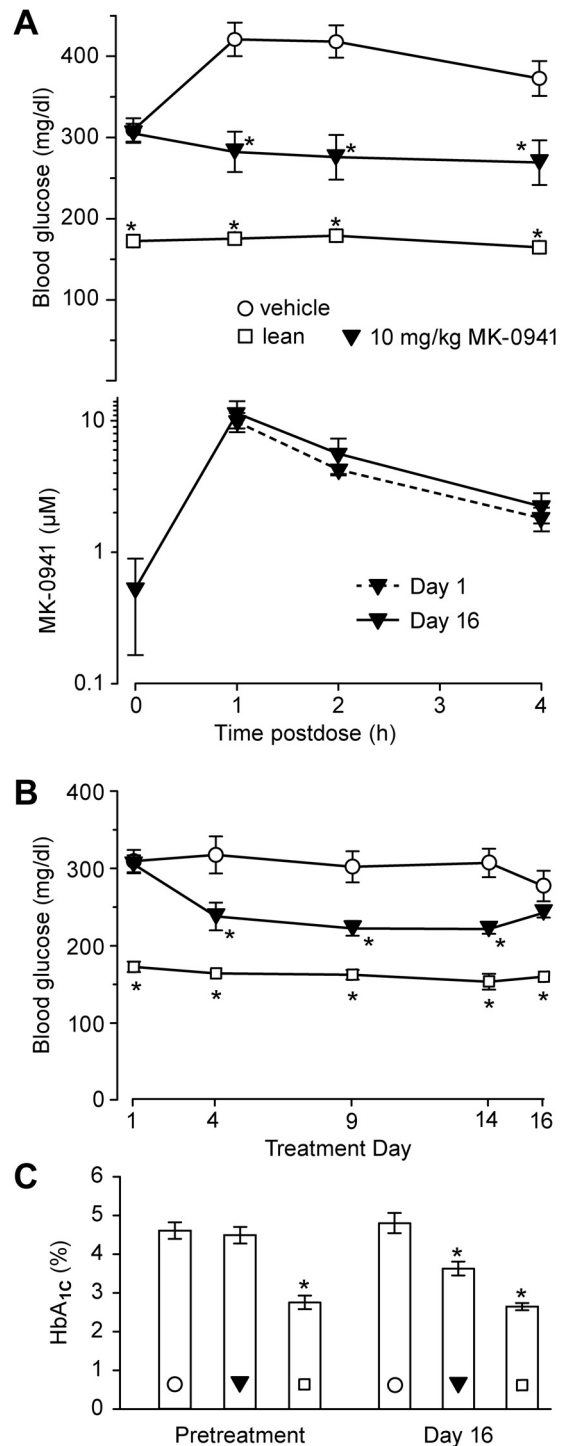


Fig. 5. Mean (\pm S.E.) blood glucose, mean (\pm S.D.) plasma MK-0941, and mean (\pm S.E.) HbA_{1c} concentrations in HFD/STZ mice treated twice daily for 16 days with 10 mg/kg MK-0941 (20 mg/kg per day) or vehicle alone [5 ml/kg 1% (w/v) aqueous methylcellulose]. A, blood glucose before and after dosing (at time 0) on day 1, and plasma MK-0941 concentrations on days 1 and 16. B, predose blood glucose on days 1, 4, 9, 14, and 16. C, HbA_{1c} values before treatment and on day 16. *, $p < 0.05$ in comparisons with HFD/STZ vehicle-treated mice.

Pharmacokinetics and Pharmacodynamics of MK-0941 in the Dog. Single doses of 0.3, 1, and 3 mg/kg MK-0941 produced significant ($p < 0.05$), dose-dependent reductions in plasma glucose concentrations recorded during OGTT in healthy dogs, compared with vehicle alone (Fig. 6A).

TABLE 2
Liver weight and liver/plasma biochemical parameters in HFD/STZ mice treated twice daily for 16 days with either vehicle alone (1% aqueous methylcellulose, 5 ml/kg) or vehicle plus 10 mg/kg MK-0941
The lean control animals were ICR mice maintained on 7012 Teklad LM-485 mouse/rat diet (not a HFD) and not treated with STZ. The data are means ± S.E.

	HFD/STZ, Vehicle (n = 12)	HFD/STZ, MK-0941 (n = 12)	Lean (n = 6)
Liver			
Weight, g	2.3 ± 0.095	2.4 ± 0.14	1.9 ± 0.053
TG, mg/g	105 ± 13	112 ± 18	13 ± 2.2*
TC, mg/g	7.4 ± 1.1	6.4 ± 1.1	2.7 ± 0.10*
Plasma			
FFA, Eq/l	363 ± 26	386 ± 37	187 ± 32*
TG, mg/dl	110 ± 12	83 ± 10	89 ± 16
TC, mg/dl	215 ± 20	285 ± 14*	118 ± 11*
ALT, IU/l	58 ± 6.2	52 ± 5.8	37 ± 14
AST, IU/l	56 ± 3.9	65 ± 4.3	46 ± 8.9
LDH, IU/l	262 ± 32	262 ± 38	104 ± 13*
Lactic acid, mg/dl	37 ± 2.9	40 ± 2.3	28 ± 3.3
3-HBA, μM	120 ± 17	67 ± 5.3*	103 ± 19

HFD/STZ, high-fat diet/low-dose streptozotocin-treated; TG, triglycerides; TC, total cholesterol; FFA, free fatty acids; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase.
* *P* < 0.05 compared with the HFD/STZ vehicle group.

The lowest dose tested (0.1 mg/kg) did not produce a significant effect; the two highest doses (1 and 3 mg/kg) produced similar reductions in glucose, suggesting that a maximal level of response was approached. Calculated over the first 2 h after glucose challenges, the area under the curve for plasma glucose was reduced, relative to vehicle-treated controls, by 16 ± 1.1, 29 ± 6.4, 46 ± 4.2, and 48 ± 3.0% by the doses of 0.1, 0.3, 1, and 3 mg/kg, respectively. The reductions at the doses of 0.3, 1, and 3 mg/kg were significant (*p* < 0.05 versus vehicle). Similar levels of plasma insulin were observed across the five treatment groups (Fig. 6B), despite substantial reductions in plasma glucose.

In the profiles of plasma MK-0941 over time, maximal levels were observed from 1 to 2.5 h after dosing (Fig. 6A, bottom plot). After this, plasma MK-0941 levels declined quickly with an apparent half-life of approximately 2 h (the same as in mice). The mean maximum plasma MK-0941 levels observed were 0.091 ± 0.006, 0.196 ± 0.024, 0.718 ± 0.061, and 3.016 ± 0.288 μM in the groups treated with 0.1, 0.3, 1, and 3 mg/kg MK-0941, respectively. The minimally effective plasma concentration for MK-0941 was approximately 0.2 μM in the dog (elicited by the dose of 0.3 mg/kg) and thus similar to the minimally effective plasma concentration observed in the HFD mouse.

In the multiple-dose study, animals were treated with vehicle alone (*n* = 2) or vehicle plus 1 or 10 mg/kg per day MK-0941 (*n* = 4 and 3, respectively). The dose of 10 mg/kg per day was given to increase the likelihood of maintaining active levels of MK-0941 throughout a 24-h period. On each treatment day, this dose of MK-0941 seemed to elicit a transient increase in plasma insulin (peaking at 30 min after dosing and falling rapidly thereafter; Fig. 7, inset plots in the bottom-most panel), and this was followed by substantial reductions in plasma glucose (Fig. 7, topmost panel). On successive days, mean plasma glucose levels fell to progressively lower values in this treatment group, and thereby followed a pattern similar to that observed in the HFD mouse. On days 1 through 4, the successive minimum values observed for mean (± S.E.) plasma glucose from 0.5 to 2.0 h after dosing were 47.0 ± 7.4, 32.5 ± 1.1, 23.2 ± 2.6, and

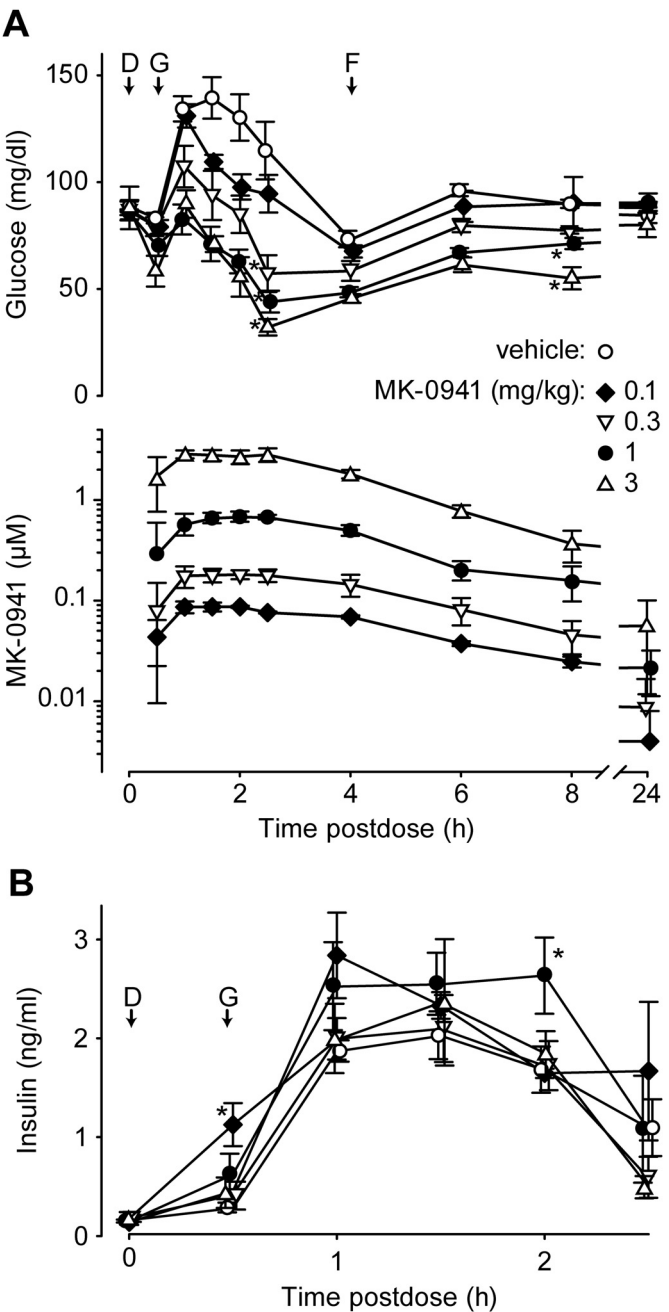


Fig. 6. A, mean (± S.E.) plasma glucose and mean (± S.D.) plasma MK-0941 concentrations from baseline (time = 0, fasted overnight) until 24 h after dosing in healthy dogs after administration by gavage (at time D) of vehicle alone [2 ml/kg 60% (v/v) aqueous polyethylene glycol; *n* = 6] or vehicle plus 0.1, 0.3, 1, or 3 mg/kg MK-0941 (*n* = 3–4) followed 30 min later by an oral glucose challenge by gavage (at time G, 2 g/kg in 10 ml/kg saline). To improve clarity, statistic significance is indicated only at 2.5 and 8 h after dosing. B, mean (± S.E.) plasma insulin in these same animals from baseline to 2.5 h after dosing. *, *p* < 0.05 in comparisons with the vehicle-treated group.

21.9 ± 2.2 mg/dl, respectively. The dogs did not show symptoms of hypoglycemia. As a precaution, nonetheless, they were given supplemental glucose (2 g/kg) at 8 h after dosing on days 3 and 4. Mean body weights in the three animal groups were 11.1, 9.8, and 10.3 kg, respectively, and did not change over the 4 days of study. On the basis of the PK data, the progression to lower levels of plasma glucose on successive days did not seem to be a consequence of drug accumu-

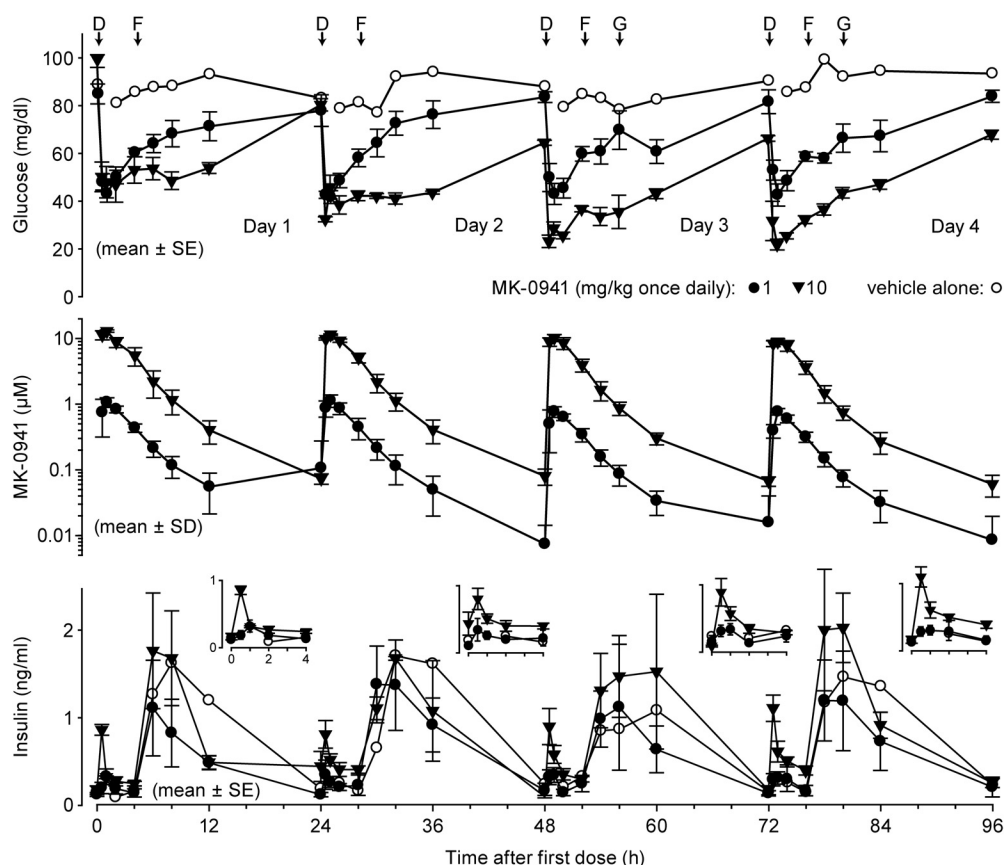


Fig. 7. Mean (\pm S.E.) plasma glucose and mean (\pm S.D.) plasma MK-0941 levels in healthy dogs administered vehicle alone [2 ml/kg 60% (v/v) aqueous polyethylene glycol; $n = 2$] or vehicle plus 1 or 10 mg/kg MK-0941 ($n = 4$ and 3, respectively) once daily for 4 days. Doses were administered by gavage at the times indicated by D. The animals were fed once each day at times F. On days 3 and 4, animals in the 10 mg/kg per day group were given 2 g/kg supplemental glucose by gavage at times G. This was done in response to their low plasma glucose levels; there were no behavioral signs of hypoglycemia. No statistical testing was performed because of the small group sizes.

lation in the plasma. The trough levels for plasma MK-0941 were 0.073 ± 0.013 , 0.080 ± 0.022 , and 0.067 ± 0.011 μ M on days 2 to 4, respectively.

Discussion

Glucokinase is an important target of drug development because this enzyme plays pivotal roles in two key glucoregulatory functions: in the liver, glucokinase controls the first step in glucose metabolism and is therefore important in setting the rate at which glucose is converted into glycogen; in the pancreas, glucokinase plays an essential role in glucose sensing by β cells (Grimsby et al., 2003; Matschinsky et al., 2006). A considerable number of GKAs have been synthesized and tested in animals out of therapeutic interest in enhancing hepatic and pancreatic glucokinase activity in patients with type 2 diabetes (Sarabu et al., 2008, 2011). From among these, MK-0941 and several others have entered into clinical testing.

The present data show that MK-0941 is a potent allosteric activator of human glucokinase. It increases the affinity of glucokinase for glucose by nearly 5-fold and increases by approximately 50% the maximum velocity (V_{max}) at which glucose is phosphorylated by this enzyme. These actions are highly selective for the glucokinase enzyme over its hexokinase isoforms HK I, II, and III, and this is consistent with the fact that these other hexokinases do not bear the allosteric binding site through which GKAs exert their effect on glucokinase (Kamata et al., 2004). Analysis by X-ray crystallography has shown that MK-0941 binds to the same site as other GKAs (K. Kamata, T. Iino, Y. Nagata, and J. Eiki, unpublished data).

Our in vitro data from isolated hepatocytes and islets of Langerhans suggest that MK-0941 has the potential for in vivo effects in both the liver and pancreas. In hepatocytes, saturating doses of MK-0941 increased the rate of glucose phosphorylation by approximately 18-fold; in isolated islets, secretion of insulin was increased approximately 14-fold. The present results also demonstrate powerful glucose-lowering activity of MK-0941 in intact animals, including nondiabetic dogs and three rodent models of diabetes (HFD, *db/db*, and HFD/STZ mice). In the HFD/STZ mouse, reductions in the metabolic markers HbA_{1c} and 3-HBA (a ketone body) provided additional evidence that glycemic control was significantly improved by treatment with MK-0941. Reduction of HbA_{1c} level is an important therapeutic goal in the clinical management of diabetes, because it slowly rises and falls as a lagging index of the average adequacy of glycemic control during the 12 to 18 weeks preceding testing. In the mouse, blood levels of HbA_{1c} adjust to changes in glycemic status much faster than in humans because the average lifespan of red blood cells in the mouse is much shorter (on the order of 18 days) (Akgül et al., 2010). Even so, 16 days (the duration of treatments in the present study) may not have been long enough to allow for full adjustment of HbA_{1c} to the glucose-lowering action of MK-0941.

In a prior study, evidence was presented for liver-selective GKA action due to hepatic conversion of an experimental compound [3-cyclopentyl-*N*-(5-methoxy-thiazolo[5,4-*b*]pyridin-2-yl)-2-[4-(4-methyl-piperazine-1-sulfonyl)-phenyl]-propionamide] to an active metabolite (Bebernitz et al., 2009). Consistent with the interpretation of liver-selective action, treatment of diet-induced obese mice with this agent

reduced plasma insulin levels during OGTT. If a GKA were to lower blood glucose primarily through action in the liver, then one would expect a compensatory reduction in insulin secretion by the pancreas. Such selective action may offer the advantage of reduced risk for hypoglycemia, although perhaps at the expense of increased risk for hepatosteatosis, hepatomegaly, and dyslipidemia.

In the present study, it was observed in the rat that MK-0941 levels were approximately 15-fold higher in the liver than in the pancreas after oral dosing. This finding suggests that MK-0941 may have a liver-preferring action, but this interpretation must be tempered by the following considerations: 1) it is not known what fraction of total liver MK-0941 was free in the cytosol and thus available to activate glucokinase. 2) The concentration of MK-0941 measured in total pancreas may not have accurately reflected the concentration in β cells; the latter make up only a small fraction of total pancreatic mass. 3) Insulin conveyed to the liver via the portal vein may have reduced hepatic gluconeogenesis without appreciably increasing systemic insulin levels.

The physiological findings in the present study suggest that MK-0941 may have a dual but liver-preferring action site of action. During OGTT, there was no consistent change in plasma insulin levels in dogs that had been administered 0.1 to 3 mg/kg MK-0941, and one would expect lowering of plasma insulin if this agent were active solely in the liver. In the multiple-dose experiment in dogs, transient elevations in plasma insulin were observed each day after doses of 10 mg/kg were given. Studies of other GKAs in development have also produced evidence for dual sites of action (Grimsby et al., 2003; Futamura et al., 2006; Fyfe et al., 2007).

Durability of antihyperglycemic efficacy is an important consideration in the development of new treatments for type 2 diabetes because this disease is both chronic and progressive (Kahn et al., 2006; Fahrback et al., 2008). The issue of durability is complex, however, because different processes may act over vastly different periods to alter the efficacy (or apparent efficacy) of antihyperglycemic agents. Over the long term, it is difficult to separate consideration of a drug's durability of efficacy from the question of whether that drug alters the natural progression of type 2 diabetes; consideration over such a period is beyond the scope of the present data. On the other hand, the present data do provide evidence that the action of MK-0941 is not subject to rapid loss of efficacy, such as has been observed during long-term treatments with sulfonylureas. During continuous or rapidly repeated administration, sulfonylureas may lose efficacy quickly (within hours to days) because of compensatory mechanisms that they trigger in pancreatic β cells, mechanisms that desensitize β cells to their insulin secretagogue action (Wählin-Boll et al., 1982; Rustenbeck, 2002). In a prior study, it was observed that long-term administration of the GKA 3-[(1S)-2-hydroxy-1-methylethoxy]-5-[4-(methylsulfonyl)phenoxy]-N-1,3-thiazol-2-yl benzamide (compound B) produced sustained glucose lowering under conditions that elicited rapid desensitization of β cells to the glucose-lowering action of the sulfonylurea glimepiride (Ohshima et al., 2010).

In the present study, the glucose-lowering efficacy of MK-0941 (like compound B) also showed stability during long-term administration. Blood glucose levels did in fact fluctuate widely within each day of dosing, but this was because MK-0941 was rapidly absorbed from the gut and rapidly

removed from the circulation. On successive days of dosing, there was no decrement in the glucose-lowering action of MK-0941; indeed, mean blood glucose levels fell to progressively lower levels on successive days of dosing. It remains unclear why this occurred; it did not seem to be a consequence of accumulation of MK-0941 in the blood. It may have been a consequence of progressive diminution of the strength of complex counter-regulatory mechanisms (such as mobilization of glucagon, epinephrine, and norepinephrine) that mobilize when blood glucose falls to subnormal levels (Cryer, 2008). Centrally, these mechanisms are mediated at least in part by neurons that depend on glucokinase for glucose sensing (Levin et al., 2008). Hence, MK-0941 could, in principle, blunt the counter-regulatory response if it were to reach adequate levels in the brain. In prior studies, however, it was observed in rodents that very little MK-0941 entered the brain (J. Eiki, unpublished observations). Thus, in the present experiments, it is unlikely that MK-0941 interfered with central glucose sensing.

Just as naturally occurring gain-of-function glucokinase mutations produce clinical hypoglycemia (Davis et al., 1999), clinical use of GKAs can be expected to entail some risk for hypoglycemia, and indeed incidences of hypoglycemia have been reported in clinical trials of the GKAs piragliatin (Zhi et al., 2008; Bonadonna et al., 2010) and MK-0941 (Meininger et al., 2011). The present data from rodents and dogs are consistent with the expectation that MK-0941 may be capable of inducing hypoglycemia in humans, especially if given to patients who are not hyperglycemic or not about to eat a meal. In the dog, blood glucose levels in the range of 80 to 120 mg/dl are considered normal (Bilicki et al., 2010), and in the mouse, this range would include 100 to 150 mg/dl (Klueh et al., 2006). In the present study, we observed plasma glucose levels well below these normal ranges (and glucose values in plasma are expected to differ only slightly from values measured in whole blood). It should be borne in mind, however, that the dosing conditions in the present study were (by design) unlike dosing procedures that would likely be followed in a clinical setting in which MK-0941 might eventually be used as a treatment for type 2 diabetes. In our dog experiments, high doses of MK-0941 were administered to animals with normal baseline levels of plasma glucose, and in both our mouse and dog experiments, doses were given without timing to coincide with food ingestion. Given that MK-0941 is rapidly absorbed into the blood and rapidly cleared, as a clinical agent it may be particularly appropriate for use before meals as a means to control postprandial glucose excursions.

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Authorship Contributions

Participated in research design: Eiki, Nagata, Sasaki-Yamamoto, Chiba, and Kadotani.

Conducted experiments: Eiki, Futamura, Sasaki-Yamamoto, Ohshima, Yoshida-Yoshimoto, Fujii, Hosaka, Goto-Shimazaki, Kadotani, and Ohe.

Contributed new reagents or analytic tools: Iino, Nishimura.

Performed data analysis: Sasaki-Yamamoto, Chiba, Fujii, Kadotani, and Lin.

Wrote or contributed to the writing of the manuscript: Eiki, Futamura, Sasaki-Yamamoto, Iino, Goto-Shimazaki, Kadotani, Lin, Langdon, and Berger.

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